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Control and recruitment of hippocampal precursor cells after seizures

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Abstract

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The birth of new neurons, neurogenesis, is sustained throughout life in the mammalian brain including humans. It has been clearly demonstrated in two niches, including the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). Neurogenesis is regulated by a variety of physiological stimuli and affected by a wide range of pathological conditions including epilepsy. Newly-born neurons are believed to be important for certain types of learning and memory as well as mood control. Hippocampal learning increases neurogenesis, and blockade of neurogenesis disrupts learning.

Status epilepticus (SE) transiently enhances neurogenesis; however, patients with chronic epilepsy suffer from learning and cognitive impairments which are associated with a decline in neurogenesis. Kainate is an excitotoxin and often used to model SE and hippocampal damage. Kainate-induced seizures and SE alter hippocampal neurogenesis and coincide with cognitive impairment and mood disorders. Kainate–induced SE is also associated with abnormal morphology and integration of new neurons, which starts immediately after the insult and is long lasting. However, the triggers and the altered proliferation kinetics of precursor cells after SE are poorly understood. To gain further insight into the mechanisms underlying control of neural precursors immediately after SE, we examined the acute effects of Kainate on hippocampal precursor cells *in vitro* and on pre-labelled and un-labelled 'clones' of proliferating hippocampal precursors *in vivo*.

In vitro, we found that Kainate increased symmetric and asymmetric proliferation rate of nestin-positive precursors, via AMPA receptors. It also enhanced the survival of nestin and TuJ1 positive cells with a proportional increase in neurogenesis in a mechanism involving AMPA receptors. Furthermore, Kainate selectively targeted quiescent and highly amplifying precursor cells. Consistently, Kainate/seizures *in vivo* increased cell proliferation of both pre-labelled and un-labelled 'clones' of precursors in the SGZ with increased cell cycle re-entry of the pre-labelled 'clone' and with a tendency to reduce numbers of postmitotic cells. In the granule cell layer (GCL), there was an increased preferential proliferation of the pre-labelled 'clone' without enhancing cell death. The increased proliferation of the pre-labelled 'clone' in the combined SGZ and GCL indicated an enhanced cell cycle re-entry. Furthermore, Kainate/seizures increased doublecortin positive cells in the GCL by 72 h.

We conclude that Kainate/seizure enhances hippocampal precursor proliferation via activation of AMPA receptors without increasing cell death, and that it has a differential effect on the proliferation kinetics and fate choice of precursors in the SGZ and GCL. We also identify proliferating precursor cells at the time of seizure-induction as a possible target to control the generation of abnormal neurons.

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Abbreviations

AMPA	Alpha-amino-3-hydroxy-5methylisoxazole-4-propionic acid
ANOVA	Analysis of variance
AP-ABC	Alkaline phosphatase conjugated Streptavidin-biotin complex
bFGF	Basic fibroblast growth factor
BrdU	5-Bromo-2'-deoxyUridine
BDNF	Brain derived neurotrophic factor
CA	Cornu Ammonios
CNS	Central nervous system
DAB	3,3-Diaminobenzidine
DAPI	4',6-diamidino-2-phenyindole
Dcx	Doublecortin
DG	Dentate gyrus
DIV	Day *in vitro*
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
FJB	Fluoro-Jade B
GABA	Gamma-aminobutyric acid
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GluR	Glutamate receptor subtype
H	Hour
HRP-ABC	Horseradish peroxidase conjugated Streptavidin-biotin complex
ICV	Intra cerebroventricular
IP	Intraperitoneal
Ka	Kainate
KA	Kainate receptor subtype
LI	Labelling index
LTP	Long term potentiation
M-phase	Mitosis phase of the cell cycle

MAM	Methylazoxymethanol
MAP-2	Microtubule-associated protein-2
MI	Mitotic index
Ml	Molecular layer
NBQX	2, 3- Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
NeuN	neuronal nuclei
NMDA	N-methyl-D-aspartate
NR	NMDA receptor subtype
P	Postnatal
PBS	Phosphate buffered saline
PBS-T	PBS containing Triton
PFA	Paraformaldehyde
PI	Propidium iodide
PSA-NCAM	Polysialylated neural cell adhesion molecule
SD	Standard deviation
SEM	Standard error of the mean
SE	Status epilepticus
S-phase	synthesis phase of the cell cycle
SGZ	Subgranular zone
SVZ	Subventricular zone
TBS	Tris-buffered saline
TBS-T	TBS containing Triton
TLE	Temporal lobe epilepsy
TUNEL	TdT-mediated dUTP-biotin nick end labelling

Chapter One

General Introduction

1.1 Overview

"*Once development was ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are sometimes fixed, and immutable: everything may die, and nothing may be regenerated.*" Santiago Ramon Y Cajal (Cajal 1928).

'New neurons are continuously born throughout life'. Indeed, this statement was not acceptable one hundred years ago and is only recently gaining widespread acceptance within the neuroscience community.

Historically the brain was considered as a fully formed organ at birth, with subsequent experiences simply modifying circuits formed by existing neurons within the brain. No replacement of dying/degenerating neurons was believed to occur (Cajal 1928) nor was there any concept of the physiological addition of new neurons to an already "fully formed" brain. It was not until the 1960s that Altman & Das demonstrated the existence of cells within the adult rat brain which are actively dividing and which subsequently produce neurons (Altman and Das 1965). It has been known for decades that, in a variety of mammalian species, specific populations of central nervous system (CNS) neural progenitors normally proliferate well into adulthood (Altman and Das 1965; Kaplan and Hinds 1977; Kaplan and Bell 1984). Indeed, in mammals including humans, it has been revealed that neurons are generated throughout life in restricted neurogenic niches; the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) (Kaplan and Hinds 1977; Gould, Cameron et al. 1992; Kempermann, Kuhn et al. 1997; Eriksson, Perfilieva et al. 1998; Gould, Reeves et al. 1999). In rodents, for example, precursor cells contiguous to the cerebral ventricles give rise to neurons that migrate rostrally to reside within the olfactory bulb (Lois and Alvarez-Buylla 1994). Likewise, a pool of precursor cells within the DG of the hippocampus, a structure important for learning and memory, continues to generate new dentate granule cells all through life (Gould, Tanapat et al. 1998). Moreover, it has been recently reported that stem and/or restricted progenitor cells proliferate and give rise to new cells which then mature into functional neurons in the adult mammalian brain (van Praag, Schinder et al. 2002). The process of neurogenesis is positively and/or negatively

regulated by a wide range of factors that can affect cell proliferation, fate of choice as well as the survival of these newly-born cells (Lowenstein and Parent 1999).

Recent findings indicate that seizures induced by various experimental manipulations such as; kindling (Scott, Wang et al. 1998), pilocarpine (Parent, Yu et al. 1997), and Kainate (Gray and Sundstrom 1998), enhance neurogenesis in the adult rodent DG as well as in the subventricular zone-olfactory bulb pathway (Parent, Valentin et al. 2002). However, in the epileptic brain, the effects of seizure-induced neurogenesis, in terms of either a pathological or reparative role, are only beginning to be understood. Elucidating the underlying mechanisms of neurogenesis has gained great interest in the last decade because of the emerging body of evidence that indicates an important role of adult neurogenesis in hippocampal-dependent learning, spatial memory, and mood control (Gould, Beylin et al. 1999; Elger, Helmstaedter et al. 2004; Shors 2004; Aimone, Wiles et al. 2006; Dranovsky and Hen 2006; Helmstaedter and Kockelmann 2006; Kee, Teixeira et al. 2007; Sahay and Hen 2007; Sisti, Glass et al. 2007; Tashiro, Makino et al. 2007). Interestingly, changes in neurogenesis in chronic epilepsy may alter cognitive function and mood because it affects the generation and/or integration of newly-born neurons within the hippocampal dentate gyrus. An interesting study has revealed that dentate gyrus neurogenesis initially increased after status epilepticus (SE), and then declined in subsequent months during the time of recurrent seizures (Hattiangady, Rao et al. 2004). Importantly, chronic epilepsy is associated with progressive cognitive impairments in patients with TLE (Elger, Helmstaedter et al. 2004; Helmstaedter and Kockelmann 2006) and an increased risk of depression (Mazza, Orsucci et al. 2004). Interestingly, it has been shown that Kainate-induced chronic epilepsy is associated with long-term cognitive impairment in adult rodents, which was reduced by blocking seizures with valporate (Jessberger, Nakashima et al. 2007). Furthermore, it has been demonstrated that seizures may generate cells that are responsible for some abnormal structural plasticity in the epileptic hippocampal formation (Parent, Yu et al. 1997). Seizure-induced neurogenesis also results in the ectopic location of newly-born granule-like neurons in the hilus and inner molecular layer of the DG (Scharfman, Goodman et al. 2000). Ectopic hilar granule cells have some electrophysiological and morphological abnormalities (Ribak, Tran et al. 2000; Scharfman, Sollas et al. 2003). These findings

may suggest that new granule cells may not act to ameliorate seizures, and might even contribute to them. As such, cognitive impairments following seizures might be in part due to circuits that develop between new cells and the host brain (Parent 2002; Scharfman 2004) or a result of declined neurogenesis which may be related to the dispersion of the SGZ into the DG sub regions observed in rodent model of epilepsy (Kralic, Ledergerber et al. 2005).

Kainate-induced seizures transiently increase neurogenesis in the DG of adult rodents (Gray and Sundstrom 1998; Hattiangady, Rao et al. 2004; Jessberger, Romer et al. 2005; Jessberger, Zhao et al. 2007). However, the mechanisms that regulate the acute increase in neurogenesis are not fully understood. In an attempt to investigate the acute and chronic mechanisms Jessberger and Zhao et al 2007 studied the effects of Kainate-induced seizures on the morphology of newly-born neurons over time. They described abnormal morphology of newly-born neurons after seizures which starts immediately and continues even one year after the insult. Importantly, they reported that these abnormal newly-born neurons integrate into the dentate circuitry. In line with this, it has been shown that neuroplastic changes in immature neurons that express the early neuronal marker doublecortin (Dcx) appear one day after pilocarpine-induced seizures (Shapiro and Ribak 2006; Shapiro, Figueroa-Aragon et al. 2007). Other elegant studies have demonstrated that Kainate-induced seizures enhanced the proliferation of astrocyte-like precursor cells (Huttmann, Sadgrove et al. 2003; Jessberger, Romer et al. 2005), and these precursors were described as the source of new neurons in the adult DG (Seri, Garcia-Verdugo et al. 2004). It is therefore likely that abnormal production of new neurons starts at the level of precursor cells and extends through their survival, maturation, and integration.

Together, we conclude that important changes in neurogenesis occur immediately after SE with consequences for cognitive impairments and perhaps epileptogenesis. Therefore, rigorous studies are necessary to understand the mechanisms of altered neurogenesis immediately after SE in an attempt to modify this process for patients benefit.

1.2 Neural stem/progenitor cell niches and neurogenesis

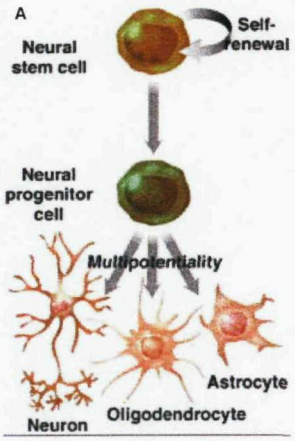
1.2.1 Stem cells

Stem cells can be defined as cells with the ability to divide indefinitely; renewing themselves and generating a variety of cell types (Gage 2000). Totipotent, pluripotent, and multipotent stem cells are the different lineages of stem cells. Totipotent stem cells can give rise to all cells in an organism and present in the earliest stages of embryonic development (Temple 2001). Although pluripotent stem cells are embryonic stem cells, they do not have the capability to produce the trophoplast of the placenta. Multipotent stem cells, neural stem cells being one of them, have more restricted capability of producing cell types (Gage 2000). Fate restriction is the pathway that directs cells with high capability of generating different cell types toward a restricted or finally differentiated cell type.

1.2.2 Neural stem and progenitor cells

Neural stem cells (NSCs) are multipotent stem cells with unlimited capacity for self-renewal by symmetric division and generation of mature NSC types. They also have the tendency of switching into asymmetric division to generate a progenitor cell (Mackowiak, Chocyk et al. 2004) (**Figure 1.1**). In the central nervous system, a single NSC is capable of producing various cell kinds, neurons and glia are included (**Figure 1.1**). On the contrary, progenitors of neurons and glial cells are capable of proliferating, but possessing a limited capacity for self-renewal and are often unipotent (Gage 2000) (**Figure 1.1**).

A



B

Stem cell	An undifferentiated cell which can either produce daughter cells with equal developmental potential or generate cells with more restricted properties
Progenitor/precursor cell	An intermediate cell which is not capable of self-renewal, but can divide and has the capacity to differentiate

Figure 1.1 Neural stem and progenitor cells in the adult brain. A) Demonstrates neural stem cells and their progenitors. B) Displays the differences between stem cells (neural) and progenitor/precursor cells. ((A) *is adopted from (Taupin 2006)*).

1.2.3 Neural stem/progenitor cell niches

While challenging the previous dogma that no new neurons are born in the adult mammalian CNS, the discovery of neural stem and/or progenitor cell niches within the adult CNS holds a great promise for development of new strategies in brain repair. Indeed, recent development of new techniques has resulted in an enormous amount of research demonstrating that neurogenesis, the birth of new neurons, constitutively occurs in two specific niches of the adult mammalian brain (the subventricular zone (SVZ) of the lateral ventricle, and the hippocampal dentate gyrus (DG)), and that there are multipotent neural precursors in many parts of the adult mammalian brain (Altman and Das 1965; Reynolds and Weiss 1992; Gage, Coates et al. 1995; Gage 2000; van der Kooy and Weiss 2000; Alvarez-Buylla, Garcia-Verdugo et al. 2001) (**Figure 1.2**). Furthermore, neurogenesis may also occur in other areas of the adult brain, albeit at lower levels, such as the CA1 area (Rietze, Poulin et al. 2000), the neocortex (Gould, Reeves et al. 1999; Gould, Vail et al. 2001), the striatum (Bedard, Cossette et al. 2002), the amygdale (Bernier, Bedard et al. 2002), the substantia nigra (Zhao, Momma et al. 2003), the third ventricle (Xu, Tamamaki et al. 2005), the subcortical white matter

(Luzzati, De Marchis et al. 2006), and the caudate nucleus (Takemura 2005). However, some of these findings have been a source of debate and controversies and remain to be further investigated and confirmed (Kornack and Rakic 2001; Frielingsdorf, Schwarz et al. 2004).

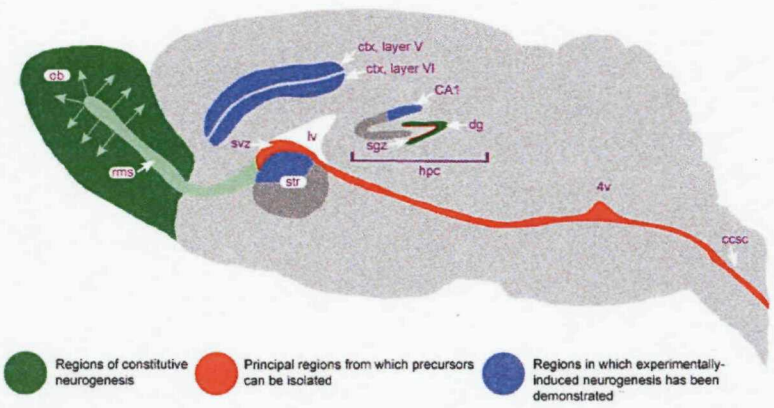


Figure 1.2 Adult neural stem cell niches. Schematic diagram showing the two constitutively neurogenic regions of the adult mammalian CNS (green; SVZ/olfactory bulb and hippocampal dentate gyrus), and some of the principal regions where populations of neural precursors have been identified (red; subgranular zone of the dentate gyrus, and rostro-caudally from the anterior subventricular zone along the neuraxis through the central canal of the spinal cord). Precursors have also been isolated in smaller numbers from several parenchymal regions. The diagram also indicates selected regions in which limited neurogenesis can be induced experimentally (blue). ccsc: central canal of the spinal cord; ctx: cortex; dg: dentate gyrus of the hippocampus; hpc: hippocampal formation; lv: lateral ventricle; ob: olfactory bulb; rms: rostral migratory stream; sgz: subgranular zone (of the dentate gyrus); str: striatum; svz: subventricular zone; 4v: fourth ventricle (Adopted from (Emsley, Mitchell et al. 2005))

1.2.3.1 Subgranular zone stem/progenitor cell niche

Adult neurogenesis comprises the entire set of events of neuronal development beginning with the division of a stem/precursor cell and ending with the presence and survival of a mature, integrated, functioning new neuron. Neurogenesis occurring in the adult hippocampus has been described *in vivo* in rodents (Altman and Das 1965),

monkeys (Gould, Tanapat et al. 1998; Gould, Reeves et al. 1999; Kornack and Rakic 1999), and humans (Eriksson, Perfilieva et al. 1998).

In the adult hippocampus, precursor cells reside in the SGZ, the border between the GCL and the hilus. In 1995, Gage et al isolated neural progenitor cells, positive for the neural progenitor and stem cell marker nestin, from the adult rat hippocampus (Gage, Coates et al. 1995). In a subsequent study, multipotent NSCs were isolated and cultured from the adult hippocampus (Palmer, Takahashi et al. 1997). However, one of the limitations to establish the difference(s) between neural progenitors and stem cells is the lack of NSCs marker in both *in vivo* and *in vitro*. For example, the intermediate filament nestin, a marker of neural progenitor and stem cells (Lendahl, Zimmerman et al. 1990) is also expressed *in vivo* in reactive astrocytes and gliomas (Tohyama, Lee et al. 1992; Clarke, Shetty et al. 1994). Despite these difficulties, researchers used other techniques to identify the origin of newly generated neuronal cells in the adult brain, including [^{3}H]-thymidine autoradiography, immunohistochemistry studies (particularly BrdU-labelling approaches), and retroviral labelling methods (Taupin 2007). A growing body of evidence is supporting the hypothesis that SGZ astrocytes are the primary precursors in the formation of new neurons in the adult hippocampus (Seri, Garcia-Verdugo et al. 2001; Filippov, Kronenberg et al. 2003) (**Figure 1.3**). Indeed, in the previous studies, using retroviral labelling methods, and transgenic mice expressing the green fluorescent protein (GFP) under the nestin promoter and electrophysiology recording, showed that SGZ astrocytes are the primary precursors in the formation of new neurons in the adult hippocampus. Seri et al described a population of small electron-dense cells in the SGZ, type-D cells that are small in size, immuno-positive for the astrocytes marker GFAP, and are dividing. Type-D cells are perhaps transient precursors in the formation of new neurons, as anti-mitotic treatment resulted in the elimination of D cells from the SGZ (Seri, Garcia-Verdugo et al. 2001). It is hypothesized that SGZ astrocytes serve as putative NSCs and give rise to type-D cells, which in turn would give birth to new neurons in the GCL (Seri, Garcia-Verdugo et al. 2001) (**Figure 1.3**). In line with this, Huttmann et al 2003, using GFP transgenic mice under GFAP (astrocytes marker) promoter, have demonstrated the proliferation of SGZ GFAP-like progenitor cells

(Huttmann, Sadgrove et al. 2003) and thus confirming the contribution of this pool of precursors into the adult hippocampal neurogenesis.

Newly-born neurons are generated along the innermost aspect of the granule cell layer (GCL), the sub granular zone (SGZ) of the dentate gyrus (DG) - SGZ stem cell niche-. Then these cells migrate a short distance into the GCL, extend dendrites into the molecular layer of the hippocampus, and send their axons into the CA3 region of the hippocampus (Hastings and Gould 1999; Markakis and Gage 1999). In addition, it has been shown that adult-born hippocampal granule neurons are morphologically indistinguishable from surrounding granule neurons (Kempermann, Gast et al. 2003), and they develop mature electrophysiological properties (van Praag, Schinder et al. 2002; Jessberger and Kempermann 2003; Ge, Goh et al. 2006). Interestingly, the precursor cells were found to mature rapidly and extend their processes into the CA3 region as early as 4 days after division (Hastings and Gould 1999). However, the maturation of newborn cells, from the proliferation of newly-generated cells in the SGZ to the migration and differentiation in neuronal cells of the GCL, takes approximately four weeks (Cameron, Woolley et al. 1993). As many as 9000 new neuronal cells are born per day in the rodent DG, contributing to about 3.3% per month or nearly 0.1% per day of the GCL population (Kempermann, Kuhn et al. 1997; Cameron and McKay 2001), whereas in adult macaque monkey, it is estimated about 0.004% of GCL neurons are generated per day (Kornack and Rakic 1999).

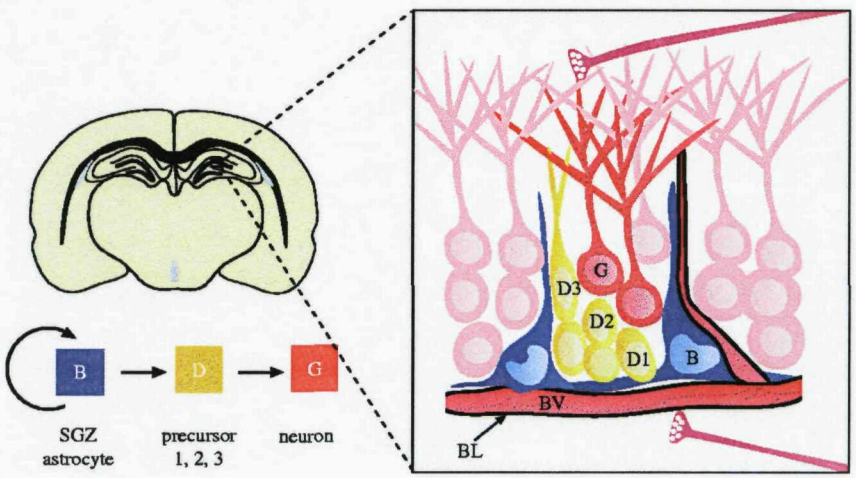


Figure 1.3 Anatomy and precursor cells of the adult SGZ niche. Schema of frontal section of the adult mouse brain showing the SGZ at the interface between the hilus (area below blood vessel) and the granule cell layer (GCL) (light pink cells) of the dentate gyrus. SGZ astrocytes (B, blue) divide to generate intermediate precursors (type D cells; nomenclature according to Seri *et al.* 2004, yellow), which progressively generate more differentiated progeny (type D1 type D2 type D3), which mature into granule neurons (G, red). Neurogenesis occurs in pockets adjacent to blood vessels and although a specialized basal lamina has not yet been described in this region, the vascular basal lamina likely plays an important role in the niche. Afferent axons (pink) from the entorhinal cortex and axons from subcortical regions as well as from local inhibitory interneurons project to the SGZ (Adopted from Riquelme et al 2007).

1.2.3.2 Subventricular zone stem cell niche

Another pool of dividing neuronal progenitor cells, within the adult rodents' brain, is the subventricular zone (SVZ) (Goldman and Luskin 1998; Temple and Alvarez-Buylla 1999). Newly born neurons in the SVZ migrate to the olfactory bulb (OB) through the rostro-migratory stream (RMS), where they differentiate into granule and periglomerular neurons of the OB, in rodents (Altman 1969; Lois and Alvarez-Buylla 1994) and in macaque monkeys (Kornack and Rakic 2001; Pencea, Bingaman et al. 2001). In the SVZ, three main cell types relevant to adult neurogenesis were identified in rodents (Doetsch, Garcia-Verdugo et al. 1997; Garcia-Verdugo, Doetsch et al. 1998) and in

macaque monkeys (Kornack and Rakic 2001). Type-A cells are elongated and smooth cells that do not express GFAP and the intermediate filament vimentine (**Figure 1.4**). These cells are immuno-positive for nestin, polysialylated neural cell adhesion molecule (PSA-NCAM), and the neuroblasts marker ß-tubulin (TuJ1). Type-A cells incorporate [^{3}H]-thymidine and form chains of migrating cells (Doetsch and Alvarez-Buylla 1996). Thus these cells are considered as migrating neuronal precursors of neuroblasts. Type-B cells have irregular shapes (**Figure 1.4**). They also express nestin, GFAP and vimentine but not PSA-NCAM or TuJ1. These cells are detected in the SVZ, where they form a tubular trabecula that ensheathes the chain of type-A cells (Doetsch and Alvarez-Buylla 1996). Type-B cells would correspond to an astrocytic population of relative quiescent cells previously proposed as NSCs in the SVZ (Morshead, Reynolds et al. 1994). Type-C cells are of larger size, have a smooth contour, express nestin but not GFAP, vimentine, PSA-NCAM, or TuJ1 (Doetsch, Garcia-Verdugo et al. 1997) (**Figure 1.4**). These cells are the most actively proliferating cells in the SVZ (50% of [^{3}H]-thymidine-labelled cells were referred to as type-C cells). Type-C cells give birth to both neurons and glia, and correspond to precursors of the type-A cells (for review see (Riquelme, Drapeau et al. 2007)). In this context, a model of neurogenesis in the adult SVZ was proposed: type-B cells that are NSCs, give rise to neural precursors type-C cells in the adult SVZ, that in turn produce type-A cells that migrate in chains through glial tubes formed by type-B cells along the RMS, and will differentiate into interneurons in the OB (Doetsch, Garcia-Verdugo et al. 1997).

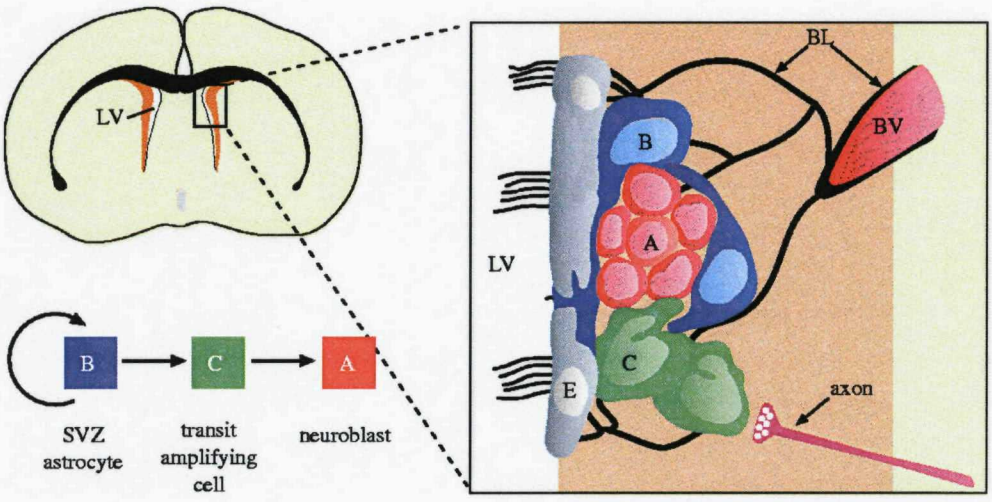


Figure 1.4 Anatomy and precursor cells of the adult SVZ niche. Schema of frontal section of the adult mouse brain showing the SVZ (orange) adjacent to the lateral ventricle (LV). SVZ astrocytes in this region (B, blue) are stem cells which generate migrating neuroblasts (A, red) destined for the olfactory bulb via a rapidly dividing transit-amplifying cell (C, green). Region in box is expanded at right to show the relationship of cells in this region and some elements of the SVZ niche. Multi-ciliated ependymal cells (E, grey) line the walls of the lateral ventricle. Chains of neuroblasts travel through tunnels formed by processes of SVZ astrocytes. Transit-amplifying cells are found in small clusters adjacent to the chains. Signals released from axons (pink) regulate proliferation and survival in this region. A specialized basal lamina (BL, black) extends from perivascular cells and contacts all cell types. Endothelial cells, blood vessels (BV) and the basal lamina are all likely key components of the niche (Adopted from (Riquelme, Drapeau et al. 2007)).

Altogether, these data suggest that a population of astrocyte-like cells correspond to neural precursor cells in the adult SVZ and hippocampus. An accumulating body of evidence supports the glial origin of newly generated neurons in the adult brain (Imura, Kornblum et al. 2003; Morshead, Garcia et al. 2003; Garcia, Doan et al. 2004). However, the origin and identity of newly-born neurons in the adult brain is still the subject to debates and controversies, and yet has to be further investigated and evaluated (Taupin and Gage 2002). It also remains to determine unambiguously the relationship between the precursors of newly generated neuronal cells *in vivo*, and the neural stem/progenitor cells derived from adult brain cultured *in vitro*. An important approach

to understanding stem/progenitor cells in the adult brain includes the isolation and characterization of stem/progenitor cells postnatally and comparison to adult stem/progenitor cells. Since most of the GCL is formed postnatally (Namba, Mochizuki et al. 2005); studying stem/progenitor cells during this transitional stage would help explain the characteristics of these cells in the adult brain.

1.3 Regulators of hippocampal neurogenesis within the stem cell niche

A considerable amount of research was conducted in the last decade to elucidate the mechanisms and the factors that affect precursor cells division, migration, and differentiation into neurons. However, the precise mechanisms that control neuronal fate of choice in the adult nervous system remain elusive. Both precursor cells intrinsic programs as well as extracellular cues including factors delivered by the circulation or secreted by local cells within the stem cell niche play significant roles in regulating neurogenesis. Therefore, we will briefly summarize intrinsic and extrinsic mechanisms of neurogenesis control.

1.3.1 Intrinsic control of neurogenesis

Precursor cells within the stem cell niches use autonomous cues to direct them towards a particular fate. These include self-renewal or mitotic arrest and differentiation. The intrinsic control programs of neurogenesis have been divided into two categories (Abrous, Koehl et al. 2005). Firstly, factors that control cell proliferation which include cell cycle proteins like retinoblastoma (Rb) and its related proteins (p107, p130), necdin as well as E2F protein families (Yoshikawa 2000). Indeed, phosphorylated Rb accumulates in cycling cells during the late G_1 phases and releases E2F, thus allowing S-phase entry (Abrous, Koehl et al. 2005). However, the phosphorylation of Rb is regulated by cyclin-dependent- kinases (CDK) (Weinberg 1995; Abrous, Koehl et al. 2005). Interestingly, the Rb and E2F families are expressed in the proliferative and postmitotic cells within the adults DG and SVZ (Abrous, Koehl et al. 2005). Rb immunoreactivity is high in proliferating neuronal precursors and reduced after terminal

differentiation (Okano, Pfaff et al. 1993). This may suggest that the transient increase in the level of Rb is a key step in the initiation of terminal mitosis in neuronal precursors. E2F1, a member of E2F family, has also been shown to play an important role in regulating hippocampal neurogenesis (Cooper-Kuhn, Vroemen et al. 2002). In this regard, Cooper-Kuhn and colleagues have demonstrated that adult E2F1 knockout mice exhibited a lower level of cell proliferation and reduction in the number of newly-born hippocampal neurons. In contrast to this, Doetsch et al have reported that transgenic mice lacking the protein p27 displayed increased cell proliferation rate of transient amplifying progenitors which was associated with reduction in cell differentiation in the SVZ niche (Doetsch, Verdugo et al. 2002).

Secondly, factors that control precursor cells differentiation such as the basic helix-loop-helix (bHLH) proneural genes also regulate neurogenesis. They include Mash1 (mammalian achaete-scute-homolog), Math1 (mammalian atonal homolog), and Ngns (Neurogenins) which are all expressed early in mitotic neural precursor cells, and differentiation factors involving NeuroD, NeuroD2, and Math2, expressed later in postmitotic cells (Bertrand, Castro et al. 2002). Furthermore, it has been recently demonstrated that these proneural genes are downstream effectors of Pax6, a transcription factor that enhances neurogenesis (Heins, Malatesta et al. 2002; Gotz 2003). Interestingly, the mRNA expression of several bHLH factors was discovered to be present at various degrees within the adult hippocampus and altered after status epilepticus (Elliott, Khademi et al. 2001).

1.3.2 Extrinsic control of neurogenesis

There are many extrinsic factors regulating neurogenesis which include hormones, neurotransmitters and neuroregulators, and growth factors and others. I will briefly shed light on these factors below.

1.3.2.1 Hormones and neurosteroids

Hormones and neurosteriods, which are released into the blood circulation, include adrenal corticosteroids, gonadal hormones (estrogen), and neurosteriods (dehydroxyepiandrosterone (DHEA)) have been determined in the last decade as regulators of neurogenesis. Indeed, hormones are considered as mediators of neurogenesis in the adult mammalian forebrain. In this regard, It has been shown that suppression of corticosterone secretion after bilateral adrenalectomy increased neurogenesis and gliogenesis in the adult DG (Gould, Cameron et al. 1992; Cameron and Gould 1994). Furthermore, it has been demonstrated that the reduction in corticosterone secretion after adrenalectomy was associated with enhanced DG cell proliferation and survival (Rodriguez, Montaron et al. 1998). Interestingly corticosterone could regulate precursor cell proliferation indirectly by increasing glutamate release in the DG (Stein-Behrens, Lin et al. 1994). In this context, it has been found that the effects of either adrenalectomy or high levels of corticosterone on cell proliferation could be blocked by the activation or antagonism of NMDA receptors, respectively (Gould, Tanapat et al. 1997). These studies suggest a significant role for glutamate receptors in regulating hippocampal neurogenesis.

Estrogen also plays a role in neurogenesis. It has been shown that estrogen affects rat dentate gyrus neurogenesis as cell proliferation increased in estrus phase and abolished by ovariectomy (Tanapat, Hastings et al. 1999). Moreover, in female prairie voles, it has been reported that estrus induction, through exposure to males, is associated with increased numbers of dividing cells in the rostral migratory stream (RMS) of the olfactory bulb, possibly via an estrogen-mediated process whereas ovariectomized females exposed to a male did not show an increase in serum estrogen or BrdU labelled cells in the RMS (Smith, Pencea et al. 2001). Furthermore, it has been also proposed that neurogenesis is stimulated in the forebrain subventricular zone of female mice during pregnancy and the effect is mediated by the hormone prolactin (Shingo, Gregg et al. 2003).

Neurosteroids, such as dehydroxyepiandrosterone (DHEA) and pregnenolone (Preg-S), are synthesized in the hippocampus, by glial cells mainly, and affect hippocampal-

mediated functions (Abrous, Koehl et al. 2005). Treatment of adult male rats with DHEA has been shown to enhance hippocampal cell proliferation and survival as indicated by the increase in BrdU incorporated cells (Karishma and Herbert 2002). This effect is believed to be mediated via $GABA_A$ receptors, as $GABA_A$ agonist blocked the proliferation effect that is observed in rats adult DG after Preg-S treatment (Mayo, Lemaire et al. 2005).

1.3.2.2 Neurotransmitters

Neurotransmitters are other factors within the stem cell niches that control precursor cells and their neural progeny cells proliferation and migration (Hagg 2005). One of these neurotransmitters, the amino acid glutamate, plays a significant role in modulating neurogenesis. In this regard, it has been reported that under certain experimental conditions the destruction of the perforant pathway, the main glutamatergic afferent input to the DG arising from the entorhinal cortex, induces hippocampal cell proliferation (Cameron, McEwen et al. 1995). Indeed, Cameron et al have reported that glutamate had inhibitory effects, under these experimental conditions. Glutamate and its four major receptor subtypes; NMDA receptors, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptors, Kainate receptors, and metabotropic receptors have been described in neural development (Cameron, Hazel et al. 1998). For example, it has been demonstrated that NMDA and a low dose of Kainate (<50μM) enhanced the survival of cerebellar granule cells in cultures generated from embryonic rats (Balazs, Jorgensen et al. 1988; Balazs, Hack et al. 1990). Conversely, Gould et al have described that blockade of NMDA receptors in postnatal rats enhanced not only DG cell proliferation but also cell death and thus an overall decrease in cell survival (Gould, Cameron et al. 1994). Furthermore, blockade of NMDA subtype of glutamate receptors by injection of the noncompetitive antagonist (MK-801) increased cell proliferation in the dentate gyrus of adult rats (Cameron, Tanapat et al. 1998). In addition, Gould et al have also reported, in the same study, that administration of NMDA (30mg/kg) decreased DG cell proliferation. Moreover, activation of NMDA receptors by removal of magnesium or by the application of either glycine or glutamate

increased granule cell migration in slices of the developing mouse cerebellum (Komuro and Rakic 1993). They also reported that blockade of NMDA receptors curtailed cell movements. However, glutamate and Kainate have been found to decrease precursor cell proliferation of embryonic cortical explants as indicated by measuring the percentage of cells that incorporated BrdU (LoTurco, Owens et al. 1995). This study has also revealed that the reduction in cell proliferation is most likely to be modulated via AMPA/Kainate receptors. In contrast, it has been shown that glutamate enhances precursors proliferation and neurogenesis in cultures derived from human fetal cortex (Suzuki, Nelson et al. 2006). This effect is modulated via NMDA receptors. The previous studies indicate that the proliferative and survival effects of glutamate, Kainate and NMDA are perhaps dependent on the age of the animals. AMPA/Kainate subtypes of the glutamate receptors have been shown to be expressed in neural progenitors (Gallo, Pende et al. 1995). Furthermore, it has been demonstrated that AMPA/Kainate receptors are expressed in proliferating neuroblasts and postmitotic neurons of cultures derived from embryonic rats (Maric, Liu et al. 2000). Indeed, Maric and colleagues have described that AMPA/Kainate receptors are expressed in BrdU+/TuJ1+ (neuroblasts that incorporated BrdU) cells as well as in BrdU-/TuJ1+ (neuroblasts only) cells. This suggests a role for AMPA/Kainate receptors in neural precursor proliferation. Interestingly, it has been emerged that AMPA enhanced the survival of cerebellar granule cells in cultures derived from postnatal rats (Banaudha and Marini 2000; Wu, Zhu et al. 2004). In this regard, Wu et al have further demonstrated that the survival effects of AMPA were modulated via the neurotrophic factor BDNF. Moreover, administration of AMPA agonist (LY451646) increased hippocampal cell proliferation of adult rats as indicated by the increase of BrdU clusters (Bai, Bergeron et al. 2003). Kainate, an agonist of AMPA/Kainate receptors, has been also reported to enhance hippocampal cell proliferation *in vivo* model of epilepsy (Gray and Sundstrom 1998; Gray, May et al. 2002; Hattiangady, Rao et al. 2008). It also enhances the survival of precursor cells in cultures derived from cerebellar granule cells (Balazs, Hack et al. 1990). In addition, Kainate has both survival and proliferative effects on SVZ precursor cells in cultures derived from postnatal Wistar rats (Brazel, Nunez et al. 2005). However, the mechanisms underlying Kainate effects on precursor cell proliferation and survival are yet to be understood. We will

discuss in **section 1.7** of this chapter the current knowledge of the mechanisms by which Kainate/seizures modulate hippocampal neurogenesis.

Altogether, the results that we have discussed suggest that glutamate, NMDA, AMPA, and Kainate exert a complex influence on overall precursor cell proliferation and cell survival. In addition, the discovery that both glutamate and GABA are cotransmitted at the mossy fibers synapses further suggest that GABA may regulate hippocampal neurogenesis and thus learning and memory (Gutierrez 2003).

Regarding the GABAergic system, several electrophysiological studies have revealed the expression of functional GABA receptors in neural precursor cells and their neuronal progeny cells in the dentate gyrus neurogenic niche (Tozuka, Fukuda et al. 2005; Wang, Kempermann et al. 2005; Ge, Goh et al. 2006). Moreover, GABA initially depolarizes newly-born neurons in the adult brain during the first 2-3 weeks of their neuronal development (Overstreet Wadiche, Bromberg et al. 2005; Tozuka, Fukuda et al. 2005; Ge, Goh et al. 2006). These studies suggest that these newly-born neurons in the adult brain go through the same sequence of developmental milestones as occurs in the embryonic CNS. However, the role of GABA in regulating neurogenesis is complex. For example, GABA reduces the number of proliferating cells in dissociated or organotypic cultures of neocortex (LoTurco, Owens et al. 1995). In contrast, it has been shown that GABA promotes precursor cell proliferation in cultures derived from the postnatal rats' cerebella (Fiszman, Borodinsky et al. 1999). Furthermore, GABA was demonstrated to enhance cell proliferation in the ventricular zone of the embryonic mouse cerebrum in organotypic cultures, however, the revere effect was observed in the subventricular zone (Haydar, Wang et al. 2000). In this regard, Haydar and colleagues have demonstrated that GABA and glutamate enhanced cell proliferation by shortening the cell cycle of precursor cells. On the other hand, GABA also induces migration and motility of acutely dissociated embryonic cortical neurons (Behar, Schaffner et al. 2000). Interestingly, it has emerged that GABA enhances precursor cell proliferation and migration in hippocampal organotypic cultures generated from postnatal mice (Ben-Yaakov and Golan 2003). Moreover, Ben-Yakoov et al 2003 have also reported an increase in nerve growth factor after GABA exposure. Thus, during neurogenesis, GABA emerges as an

important signal for cell proliferation and migration, however, its precise regulation which may depend on the region and cell type affected is still unresolved.

Serotonin (5-hydroxytryptamine) is another neurotransmitter that may play a role in neural plasticity by maintaining the synaptic connections in the hippocampus and thus influence learning and cognition (Mazer, Muneyyirci et al. 1997). Inhibition of serotonin synthesis has been demonstrated to decrease the numbers of newly- born cells and neurons in both the DG and SVZ of adult rats (Brezun and Daszuta 1999). In addition, grafting embryonic 5-HT neurons intrahippocampally reversed the decrease in cell proliferation after damaging serotonin neurons (Brezun and Daszuta 2000) indicating a significant role for serotonin in hippocampal neurogenesis. Furthermore, it has been demonstrated that serotonin receptors subtypes 1A and 2A agonists enhanced the generation of new neurons in the DG and/or olfactory bulb of adult Wistar rats (Banasr, Hery et al. 2004).

1.3.2.3 Trophic and growth factors

There are many trophic factors that have been shown to have mitogenic effects in the adult neurogenic niches (Abrous, Koehl et al. 2005). These trophic factors include basic fibroblast growth factor (FGF-2 or bFGF), epidermal growth factor (EGF), Insulin-like growth factor-I (IGF-I), Brain derived growth factor (BDNF) and many others (Abrous, Koehl et al. 2005). FGF-2 was shown to increase progenitor cell survival and proliferation in cultures derived from the adult rat hippocampi (Gage, Coates et al. 1995). Moreover, it has been reported that FGF-2 is proliferative for precursor cells in the SVZ niche (Kuhn, Winkler et al. 1997). Interestingly, over-expression of FGF-2 by gene transfer in wild-type and FGF-2 deficient mice up-regulates DG precursor cell proliferation, indicating that FGF-2 expression is necessary for increasing cell birth in the hippocampus (Yoshikawa 2000). In addition, it has been demonstrated that intracerebroventricular infusion of FGF-2 enhanced not only DG neurogenesis but also promoted neuronal dendrites growth in middle aged mice (Rai, Hattiangady et al. 2007).

Epidermal growth factor (EGF) is another factor that has been described to have a role in neurogenesis. For example, heparin binding epidermal growth factor (HB-EGF) but not

EGF increases cell proliferation in the dentate gyrus of adult rat brain (Kuhn, Winkler et al. 1997). Furthermore, intracerebroventricular (ICV) infusion of EGF stimulates proliferation in the adult SVZ (Craig, Tropepe et al. 1996; Kuhn, Winkler et al. 1997). The other factor is insulin-like growth factor-I (IGF-I) which is a peptide hormone that is produced in the CNS by both neuronal and glial cells (Anderson, Aberg et al. 2002). It has been shown that IGF-1 induces DG neurogenesis *in vivo* and *in vitro* (for review see (Anderson, Aberg et al. 2002)). In this regard, it has been demonstrated that peripheral administration of IGF-I enhanced precursor cell proliferation in GCL and hilus of the DG (Trejo, Carro et al. 2001). Furthermore, the previous study showed an increase in the number of DG neurons after IGF-I treatment. Interestingly, it has been shown, *in vitro* experiments, that IGF-I acts directly on proliferating precursors from the hippocampus and thus enhances neurogenesis (Aberg, Aberg et al. 2003).

Brain-derived neurotrophic factor (BDNF) is a member of a family of related neurotrophic proteins which enhances the survival of neurons during development (Abrous, Koehl et al. 2005). In the DG, it has been demonstrated that heterozygous BDNF knockout mice exhibit reduction of precursor cell proliferation and survival of BrdU incorporated cells indicating a role for BDNF as a regulator of hippocampal neurogenesis (Lee, Duan et al. 2002). Moreover, in an *in vivo* model ICV infusion of BDNF stimulates the generation, migration, and survival of adult newly-born olfactory bulb neurons (Zigova, Pencea et al. 1998). This research was also supported by demonstrating that BDNF has survival effects on postnatal neurons in the olfactory bulb and dentate gyrus of adult mice (Linnarsson, Willson et al. 2000). Furthermore, a single injection of BDNF carried by adenoviral vector substantially augmented the recruitment of new neurons into both neurogenic and non-neurogenic sites of the adult rat brain (Benraiss, Chmielnicki et al. 2001). However, Scharfman et al have shown that intrahippocampal infusion of BDNF induces spontaneous limbic seizures in approximately 25% of treated adult rats (Scharfman, Goodman et al. 2002). This study suggests that BDNF perhaps has a role in seizure control. Another study on adult male songbird has shown a causal interaction between angiogenesis, an increase in endothelial cell-derived growth factors, and the generation of new neurons in the adult forebrain (Lie, Song et al. 2004). The mechanism by which vascular endothelial growth factors

(VEGF) stimulates dentate gyrus and SVZ neurogenesis in rodents has been described by either induction of precursor cell proliferation (Jin, Zhu et al. 2002), enhancing cell survival (Schanzer, Wachs et al. 2004), or both (Cao, Jiao et al. 2004).

The growth peptide, Neuropeptide Y (NPY), has emerged as a significant regulator of adult neural stem cells in the olfactory epithelium (Hansel, Eipper et al. 2001) and hippocampus (Howell, Scharfman et al. 2003). Indeed, Howell et al have shown that NPY which is released by DG GABAergic interneurons is a potent proliferative factor for hippocampal precursor cells acting through Y1 receptors. He has demonstrated that NPY is promoting the proliferation of neuroblasts (TuJ1 positive cells) and nestin positive neurospheres derived from postnatal Wistar rats (Howell, Scharfman et al. 2003).

1.4 What is the function of hippocampal neurogenesis?

Neurogenesis in the dentate gyrus of adult hippocampus has been extensively studied in the last decade, due at least partially to the tantalizing connection between the hippocampus and the formation of memory (Squire 1982; Moscovitch, Rosenbaum et al. 2005). Of particular interest is the fact that hippocampal neurogenesis can be modulated by many physiological and behavioral events such as environmental enrichment (Kempermann, Kuhn et al. 1997), learning and memory (Gould, Beylin et al. 1999; Kempermann, Gast et al. 2002), exercise (van Praag, Kempermann et al. 1999; Ra, Kim et al. 2002), aging (Kuhn, Dickinson-Anson et al. 1996), and stress (Gould, Tanapat et al. 1998). In addition to pathological events including epilepsy (Gray and Sundstrom 1998), stroke (Liu, Solway et al. 1998), traumatic brain injury (Dash, Mach et al. 2001), and Alzheimer disease (Jin, Peel et al. 2004). We, in the next few sections, will detail the effects of different physiological stimuli on hippocampal neurogenesis.

1.4.1 Learning and memory

The involvement of the hippocampal formation in learning and memory has been recognized for decades (Squire 1982). Rats with hippocampal damage are impaired on

tasks of spatial navigation and spatial memory, such as the Morris water task (Morris, Garrud et al. 1982; Sutherland, Kolb et al. 1982). Moreover, Sutherland et al. have demonstrated that animals with damaged DG show deficits in the Morris water task (Sutherland, Whishaw et al. 1983). These early studies suggest that the hippocampus is of crucial importance to spatial learning and memory. However, despite years of thorough research, the neurobiological basis of these functions is not well understood. Over the past 40 years, a considerable body of evidence has accumulated indicating that the DG of the adult hippocampus generates new neurons in substantial numbers and does so in a broad range of mammalian species, including humans (Altman and Das 1965; Eriksson, Perfilieva et al. 1998; Gould, Reeves et al. 1999). The hippocampus is believed to facilitate learning and memory through its distinctive synaptic plasticity which contributes to the acquisitions and retention of memories (Martin, Grimwood et al. 2000). Altogether, these observations have led to the hypothesis that adult neurogenesis is implicated in hippocampal functions, especially those related to learning and memory (Gould, Tanapat et al. 1999; Kempermann 2002).

Studies on birds provided the first evidence for an association between adult neurogenesis and learning. In this regard, it has been shown that, in the song system of canaries, the generation of new neurons takes place in the high vocal center and is positively related to sex and seasonal differences in song learning (Goldman and Nottebohm 1983; Alvarez-Buylla, Kirn et al. 1990). Similarly, a seasonal fluctuation in adult hippocampal neurogenesis, in black-capped chickadees, is positively related to engaging in spatial learning behaviors, like seed storage and retrieval (Barnea and Nottebohm 1994). Several lines of evidence also suggest a correlation between adult neurogenesis and learning in mammals. In this context, Kempermann et al, using different strains of adult mice, have elegantly demonstrated that mice with few numbers of new neurons performed most poorly during spatial navigation learning in the Morris water maze (Kempermann, Gast et al. 2002). Moreover, training on learning tasks that require the hippocampus has been shown to increase the number of newly-born neurons in the DG. For example, it has been demonstrated that, in adult rats, training with trace eyeblink conditioning, spatial learning in the Morris water maze and conditioned food preference increase the number of newborn cells in the DG (Gould, Beylin et al. 1999;

Olariu, Cleaver et al. 2005). In line with this, it has been shown that learning tasks that do not require the hippocampus, like cues maze training, do not change the number of newly born granule neurons in the DG of the hippocampus (Shapiro, Tanila et al. 1997; Gould, Beylin et al. 1999).

Definitive evidence for a requirement of new neurons can only be established by demonstrating deficits in hippocampal function following depletion of new neurons. In this regard, it has been reported that numerous conditions that decrease neurogenesis in the DG are associated with learning impairments. These include stress (Mirescu and Gould 2006), and aging (Kuhn, Dickinson-Anson et al. 1996; Drapeau, Mayo et al. 2003). Consistently, blocking hippocampal neurogenesis using the antimitotic agent methylazomethanol acetate (MAM) in rats resulted in a substantial reduction in the number of newly-born cells over a 2–weeks period which was associated with an impaired ability to acquire the trace eyeblink conditioning task (Shors, Miesegaes et al. 2001). In this study, the ability to acquire trace memories was restored, when the population of new neurons was allowed to replenish itself. Likewise, a reduction in the number of newborn cells after MAM treatment was accompanied with deficits in hippocampal-dependent a fear memory task (Shors, Townsend et al. 2002).

An emerging body of evidence suggests a critical time period in which the survival of newly-born neurons is enhanced by learning experiences and contributes to acquisition of spatial memory (Kee, Teixeira et al. 2007; Sisti, Glass et al. 2007; Tashiro, Makino et al. 2007). In this regard, Sisti and co-workers have found that learning, in the water maze, enhances the survival of cells that are generated one week before training. Interestingly, their study demonstrated that learning over an extended period of time induces a more persistent memory, which positively relates to the number of cells that reside in the DG. Furthermore, it has been elegantly shown that neurons born after training adult mice in the water maze (hippocampal-dependent task) were recruited into the DG circuits supporting spatial memory acquisition (Kee, Teixeira et al. 2007). In an attempt to elucidate the relation between neurogenesis and learning and memory, Tashiro et al 2007 have described a critical period of time (1-3 weeks after the birth of these neurons) in which they may respond to an external stimuli and thus survive and integrate in the DG circuitry. Furthermore these newly-born neurons are specific to the

learning task as they are reactivated only on recall of the learned task. These studies clearly indicate that the immature stage for the newly-born neurons through adult neurogenesis could be a target by which new neurons may be influenced by variety of hippocampal-dependent learning tasks which in turn exert a long-term influence on the function of the dentate gyrus related to learning and memory.

1.4.2 Environmental enrichment

Environmental enrichment was first described to enhance neurogenesis by Kempermann et al. Using a combination of toys, wheels, tubes, rubber balls, and food supplements, Kempermann and co-workers demonstrated an increased survival of newly born neurons in the adult mice DG (Kempermann, Kuhn et al. 1997). In addition, it has been shown that environmental enrichment not only promotes neurogenesis but also prevents seizures and is neuroprotective (Young, Lawlor et al. 1999). Indeed, Young and colleagues have shown approximately 50% reduction in the GCL apoptotic cell death after enriching the surroundings. Interestingly, this group has also demonstrated that enriched environment protects against Kainate-induced seizures and enhanced the survival of CA1 and CA3 neurons. Moreover, long term environmental enrichment increased hippocampal neurogenesis which was associated with significant improvements of learning parameters (Kempermann, Gast et al. 2002). In addition, Kempermann et al have shown a reduction in lipofuscin load in the dentate gyrus, indicating decreased non-specific age-dependent degeneration. Importantly, housing adult rats in an enriched environment resulted in an enhanced hippocampal neurogenesis which was associated with improved spatial memory (Nilsson, Perfilieva et al. 1999). In an interesting study, Tashiro et al have demonstrated that environmental enrichment during the immature stage enhanced neurogenesis and the survival of mice DG neurons during the adult stage (Tashiro, Makino et al. 2007). They have elegantly shown that more neurons responded to re-exposure to enriched environment in mice that were previously exposed to an enriched environment than control mice. In another study, aged (25 months) Wistar rats housed in an enriched environment showed a significant increase in neurogenesis which was accompanied with an increased hippocampal level

of extracellular glutamate and GABA (Segovia, Yague et al. 2006). This may suggest a role for these neurotransmitters in regulating neurogenesis in aged animals. It has been shown that hippocampal levels of glial cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) increased after enriching the environment (Young, Lawlor et al. 1999). Furthermore, it has been demonstrated that vascular endothelial growth factor (VEGF) increased significantly in animals housed in an enriched environment which was associated with an increased hippocampal neurogenesis and spatial memory performance (Trejo, Carro et al. 2001; Fabel, Fabel et al. 2003; Cao, Jiao et al. 2004). This may suggest an important role for growth and neurotrophic factors in the process of neurogenesis.

1.4.3 Exercise

Various forms of exercises, like voluntary running, forced running, and swimming were demonstrated to increase hippocampal neurogenesis (van Praag, Kempermann et al. 1999; Ra, Kim et al. 2002). Subsequently, in another study, a combination of housing adult mice in enriched environment and voluntary wheel running enhanced hippocampal neurogenesis but not OB neurogenesis (Brown, Cooper-Kuhn et al. 2003). In this regard, it has been demonstrated that insulin-like growth factor I (IGF-I) mediates exercise-induced increases in the number of new neurons in the adult hippocampus (Trejo, Carro et al. 2001). Likewise, vascular endothelial growth factor (VEGF) was found to be up-regulated peripherally in adult mice after voluntary running which was associated with hippocampal neurogenesis (Fabel, Fabel et al. 2003). In this context, Fable and colleagues have abolished running induced-neurogenesis by blocking VEGF.

1.4.4 Aging

The generation of new granule cells in the hippocampal DG takes place throughout life (Altman and Das 1965; Kornack and Rakic 1999). However, the rate of hippocampal neurogenesis wanes by middle age in rodents (Kuhn, Dickinson-Anson et al. 1996; Nacher, Alonso-Llosa et al. 2003; Rao, Hattiangady et al. 2005). This reduction in

neurogenesis may contribute to hippocampal-dependent learning and memory impairments observed during old age (Drapeau, Mayo et al. 2003; Bernal and Peterson 2004;(van Praag, Shubert et al. 2005). Indeed, Drapeau et al have reported that animals with preserved spatial memory, i.e., the aged-unimpaired rats, exhibited a higher level of cell proliferation and a higher number of new neurons in comparison with rats with spatial memory impairments, i.e., the aged-impaired rats (Drapeau, Mayo et al. 2003). Thus the extent of memory dysfunction in aged rats is quantitatively related to hippocampal neurogenesis. Interestingly, it has been observed recently that the reduction in neurogenesis during aging is not attributable to altered number or phenotype of neural progenitor cells (Hattiangady and Shetty 2008). The increased quiescence of neural progenitor cells due to changes in the neural progenitor cell milieu is suggested to be the responsible for the decrease in neurogenesis with age (Hattiangady and Shetty 2008). This study may suggest that recruitment of quiescent cells during old age would contribute into enhancing neurogenesis and thus improve memory impairment. However, the precise reasons for the decrease in hippocampal neurogenesis during old age are not clear. In this regard, it has been reported that a decrease in the concentration of distinct neurotrophic factors and signaling that are known to enhance neural progenitor cells proliferation has an important role in age-related reduction in neurogenesis (Hattiangady, Rao et al. 2005; Shetty, Hattiangady et al. 2005). In this context, Shetty and Hattiangady et al have described a reduction in FGF-2, IGF-1, VEGF, BDNF, and NPY with age and so neurogenesis. These studies may imply the importance of these neurotrophic/neuroproliferative factors in restoring hippocampal neurogenesis in old age and possible target for therapy. Moreover, an increased level of glucocorticoids is associated with aging and reduction in neurogenesis (Cameron and McKay 1999).

1.5 Neurogenesis and Disease

1.5.1 Stroke and traumatic brain injury

An increase in hippocampal neurogenesis has been described after brain ischemia and stroke (Liu, Solway et al. 1998; Jin, Minami et al. 2001; Jin, Wang et al. 2006). Indeed, in the previous studies, global ischemia enhanced progenitor cell proliferation in the SGZ of not only gerbils and rats but also humans. Several other acute and chronic insults to CNS also modulate neurogenesis in the adult brain. In this regard, excitotoxic and mechanical injuries stimulate DG neurogenesis in the adult rats (Gould and Tanapat 1997). Furthermore, it has been demonstrated that traumatic brain injury induces neurogenesis and gliogenesis in the adult mouse brain (Mino, Kamii et al. 2003; Rola, Mizumatsu et al. 2006). In fact, the increase in hippocampal neurogenesis after ischemia and traumatic brain injury enhances the expression of FGF-2, EGF, and BDNF as well as other factors (Yoshimura, Takagi et al. 2001; Yoshimura, Teramoto et al. 2003). Furthermore, it has been observed that antagonizing NMDA receptors during focal ischemia insults increased DG neurogenesis of adult rats (Arvidsson, Kokaia et al. 2001). Although brain ischemia and injuries enhance hippocampal neurogenesis, it is still to be determined if this process contributes to recovery and brain repair after such injuries.

1.5.2 Stress

Many studies have reported that stress decreases the proliferation of precursor cells in the DG of the hippocampus (Mirescu and Gould 2006). Indeed, stress-induced suppression of DG precursor cell proliferation has been demonstrated in the adults of at least four different species (mouse, rat, tree shrew, and marmoset monkeys), suggesting that this phenomenon may be a general character of mammals (Mirescu and Gould 2006). Likewise, different stressors cause similar reduction in DG progenitor cell proliferation. For example, subordination stress, isolation, and resident intruder stress all have similar effects on DG cell proliferation (Gould, McEwen et al. 1997; Gould,

Tanapat et al. 1998; Dong, Goico et al. 2004). Furthermore, it has been demonstrated that stress decreases not only precursor cell proliferation but also neuronal production and thus neurogenesis (Westenbroek, Den Boer et al. 2004). The reduction in hippocampal neurogenesis is associated with an elevated level of glucocorticoids (Mirescu and Gould 2006). In line with this, it has been shown that glucocorticoid receptor agonist dexamethasone inhibits neurogenesis both *in vivo* and *in vitro* of adult rats DG (Kim, Ju et al. 2004).

1.5.3 Mood disorders

Depression is a leading cause of disability worldwide and affects 8-12% of individuals at some point in their lives (Andrade, Caraveo-Anduaga et al. 2003). Hippocampal neurogenesis may have a key role in regulating mood disorders; however, its precise function is not well understood. Interestingly, many factors that are beneficial in treating the behavioral symptoms of depression have been shown to enhance neurogenesis; these factors include electroconvulsive therapy (ECT) (Scott, Wojtowicz et al. 2000; Perera, Coplan et al. 2007), exercise (van Praag, Kempermann et al. 1999; Ernst, Olson et al. 2006), environmental enrichment (Nilsson, Perfilieva et al. 1999) and antidepressant treatments, such as selective serotonin re-uptake inhibitors (SSRIs) (Malberg, Eisch et al. 2000). Indeed, the long timescale for recovery when humans are treated pharmacologically for depression parallels the long timescale of stimulated neurogenesis that is induced by ECT and SSRIs in non-depressed animals (Malberg, Eisch et al. 2000; Scott, Wojtowicz et al. 2000). Moreover, it has recently been shown that SSRIs selectively enhance hippocampal neurogenesis, leaving the ongoing stem-cell proliferation in the SVZ unchanged (Encinas, Vaahtokari et al. 2006). Additionally, in several animal models of depression, disruption of neurogenesis blocks the behavioral effects of SSRIs (Meshi, Drew et al. 2006), whereas the behavioral efficacy of running is correlated with enhanced neurogenesis (Bjornebekk, Mathe et al. 2005). Altogether, the current findings suggest an important role for hippocampal neurogenesis in the pathophysiology of mood disorders. And as such, hippocampal stem/precursor cells may be one of the future targets for treating these disorders.

1.5.4 Epilepsy

It is widely accepted that hippocampal lesions inflicted by Kainate-induced acute seizures lead to epileptogenic structure changes, and cause an increase in the DG cell proliferation (Bengzon, Kokaia et al. 1997; Gray and Sundstrom 1998; Gray, May et al. 2002; Huttmann, Sadgrove et al. 2003; Jessberger, Romer et al. 2005; Scharfman and Gray 2006; Jessberger, Zhao et al. 2007; Shetty and Hattiangady 2007). However, temporal lobe epilepsy (TLE) causes progressive hippocampal dependent memory and cognition impairments and is associated with an increased risk of depression (Helmstaedter and Kockelmann 2006). While Kainate-induced seizures are associated with enhanced neurogenesis, a reduction in hippocampal neurogenesis is observed in chronic temporal lobe epilepsy conditions (Hattiangady, Rao et al. 2004). In this regard, information on whether the role of new neurons is part of the repair or the pathology is incomplete. We will **(in the next sections)** shed light on the role of hippocampal stem/progenitor cell proliferation and death after seizures as well as their contribution into epilepsy.

1.6 Epilepsy and neurogenesis

1.6.1 Epilepsy

Epilepsies are a common, serious heterogeneous group of disorders linked by a propensity to recurrent seizures in affected individuals which have no respect for age, gender, race or social status (Duncan, Sander et al. 2006). Seizures are intermittent, steryotyped disturbances of consciousness, emotions, behavior, motor functions, and/or sensations that are believed to be a result of abnormal neuronal discharge (Hunt, Morrow et al. 2007). The victims of epilepsy include a great many famous individuals in history including Socrates, Alexander the Great, Julius Caesar, Joan of Arc, and Dostoyevsky. In the time of Hippocrates it was believed that epilepsy, often referred as the "falling sickness", only affected individuals that were possessed by evil spirits (Matthew Tremblay 2003). John Hughlings Jackson is credited with being the first

scientist to perform neurobiological analysis of epilepsy in the 1860s. Jackson was the first to describe a partial seizure, as well as the progression of seizures as they spread through the brain. For this reason the spread of partial seizures became known as the "Jacksonian march" (Hunt, Morrow et al. 2007).

Epilepsy occurs in about 1-3% of the human population and is the most common acquired chronic neurological disorder (Shneker and Fountain 2003). The incidence of epilepsy is around 50 per 100000 people per year in developed countries (Forsgren, Beghi et al. 2005). The prevalence of epilepsy is between 4 and 10 per 1000 people (Forsgren, Beghi et al. 2005). Moreover, sudden unexpected death in epilepsy is thought to account for over 500 deaths per annum in the UK (Smithson and Hanna 2002). Approximately 40% of epilepsy patients suffer from temporal lobe epilepsy (TLE) (Shetty and Hattiangady 2007). TLE is characterized by the progressive expansion of spontaneous recurrent motor seizures starting from the limbic system areas, especially the hippocampal formation (French, Williamson et al. 1993; Engel, Wilson et al. 2003). TLE with hippocampal sclerosis is a common type of partial seizure disorder and is manifested by a widespread hippocampal neuronal loss and aberrant mossy fiber sprouting (Buckmaster, Zhang et al. 2002; Mathern, Adelson et al. 2002). Initial precipitating events such as febrile convulsions, trauma, status epilepticus (SE), or encephalitis are often causative of hippocampal damage and sclerotic TLE (Harvey, Berkovic et al. 1997; Fisher, Sperber et al. 1998; Cendes 2004). Antiepileptic drugs suppress seizures in 60-70% of patients without affecting the underlying tendency to generate seizures (Duncan, Sander et al. 2006). Furthermore, approximately 35% of patients with TLE suffer from chronic seizures that are resistant to antiepileptic drugs (Engel 2001; Litt, Esteller et al. 2001; McKeown and McNamara 2001). Importantly, most patients with TLE have learning and memory impairments (Helmstaedter, Brosch et al. 2004; Detour, Schroeder et al. 2005). In addition, TLE is associated with an increased risk of depression and suicide (Mazza, Orsucci et al. 2004). Interestingly, chronic epilepsy that develops following the initial precipitating injury usually occurs after a latent period (French, Williamson et al. 1993; Mathern, Pretorius et al. 1995). Therefore, in order to plan strategies for TLE treatments, we have to understand the molecular mechanisms underlying epileptogenesis shortly after the injury.

1.6.2 Hippocampal neurogenesis and epilepsy

Hippocampal neurogenesis occurs in many species, which includes rodents (Kuhn, Dickinson-Anson et al. 1996; Rao and Shetty 2004), monkeys (Gould, Reeves et al. 1999; Kornack and Rakic 1999), and also humans (Eriksson, Perfilieva et al. 1998). In addition, the majority of proliferating hippocampal cells differentiate into neurons (Rao, Hattiangady et al. 2005; Rao, Hattiangady et al. 2006). It is thought that new neurons are derived from radial glial cells which in turn divide into D-cells (**Section 1.2.4**) that eventually become DG granule cells (Seri, Garcia-Verdugo et al. 2004). Slow proliferation of neural precursor cells generate a population of amplifying cells, which proliferate further and give rise to new neurons and glia (Encinas, Vaahtokari et al. 2006). Moreover, newly-born granule neurons migrate into the GCL, express the mature neuronal marker NeuN, grow dendrites into the molecular layer and send axons into CA3 area (Kempermann, Jessberger et al. 2004; Emsley, Mitchell et al. 2005). Interestingly, immature neurons likely receive gamma-amino butyric acid (GABA) mediated excitatory synaptic inputs at this early stage (Ge, Goh et al. 2006). In contrast, 2-3 weeks are required before major glutamatergic synapses are activated from perforant path afferents (Esposito, Piatti et al. 2005; Ge, Goh et al. 2006). Interestingly, the same period of time (2-3 weeks) coincides with appearance of spines on dendrites of newly generated neurons (Zhao, Teng et al. 2006). Thus, new neurons become functionally integrated after 2 weeks of their birth. Therefore, understanding the control mechanism of newly-born neurons shortly after birth would probably help target therapy at this stage.

Special attention has been paid to hippocampal neurogenesis after seizures because TLE commonly leads to impairments in hippocampal-dependent forms of learning and memory (Helmstaedter 2002). Bengzon and colleagues were the first to show that neuronal depolarization or repetitive discharge-induced either by electrical or pharmacological stimulation-increases neurogenesis in the DG (Bengzon, Kokaia et al. 1997). Thus, induction of seizures (irrespective of the mechanism) induces hippocampal neurogenesis. For example, status epilepticus (SE) induced by intraperitoneal

administration of the chemoconvulsant pilocarpine (Parent, Yu et al. 1997) or unilateral intracerebroventricular infusion of Kainate (Gray and Sundstrom 1998) enhanced bilateral increase in neurogenesis. Moreover, Amygdala-kindling-initiated seizures increased hippocampal neurogenesis (Scott, Wojtowicz et al. 2000). Consistent with the previous studies, it has been demonstrated that neural precursor cells proliferate more in young (<2 years old) patients with TLE (Blumcke, Schewe et al. 2001). Thus, early onset of TLE is probably associated with enhanced DG neurogenesis in pediatric patients. Hippocampal injury and SE induce proliferation of the SGZ precursor cells with the number of newly-born neurons increasing several fold during the first few weeks after the insult (Nakagawa, Aimi et al. 2000; Hattiangady, Rao et al. 2004). However, the mechanisms that caused an acute enhancement of DG neurogenesis are not well understood.

Under normal conditions, most of newly generated neurons in the SGZ migrate into the GCL (Shetty and Hattiangady 2007). However, in SE, a substantial proportion of newly-born neurons migrate away from the GCL into the dentate hilus and molecular layer (Scharfman, Goodman et al. 2000; Parent, Elliott et al. 2006). Similarly, Parent and colleagues have shown that ectopic granule cells were found in the hilus and molecular layer of DG of epileptic humans (Parent, Elliott et al. 2006). In addition, these ectopic newly-born neurons were shown to exhibit several features of normal cells in the GCL, which includes the formation of mossy fiber axons and integration of these axons into the pre-existing hippocampal circuitry (Scharfman, Goodman et al. 2000). However, Scharfman et al have also reported that ectopic granule cells exhibit some features that are inconsistent with normal granule cells in the GCL. In this regard, Scharfman et al and others have reported that ectopic granule cells have an increased proportion of somatic and dendritic asymmetric synapses, enhanced mossy fiber innervations, a distinct pattern of activation during spontaneous seizures, and the occurrence of spontaneous epileptiform bursts (Scharfman, Goodman et al. 2000; Dashtipour, Tran et al. 2001; Pierce, Melton et al. 2005). We conclude from the previous studies that newly-born granule cells that migrate away from the GCL into the hilus and/or molecular layer of the DG after SE most probably contribute to the development of chronic epilepsy. In an attempt to explore the effects of Kainate-induced SE altered neurogenesis, Jessberger

and Zhao et al 2007 have demonstrated that abnormal new neurons appear immediately after Kainate/seizures, integrate and persist permanently in the adult DG.

1.6.3 Neurogenesis in chronic epilepsy

Although hippocampal damage and SE acutely induce DG neurogenesis, in contrast DG neurogenesis declines considerably in chronic epilepsy (Hattiangady, Rao et al. 2004). In this regard, it has been demonstrated that severe seizures during childhood are associated with decreased DG neurogenesis as indicated by the reduction in the numbers of PSA-NCAM in surgically resected hippocampi from children with frequent seizures and in comparison with age-matched autopsy cases (Mathern, Adelson et al. 2002). In line with this, Fahrner et al have recently reported a decrease in the synthesis of mRNA for doublecortin (immature neuronal marker) and absence of Ki-67 positive cells (proliferation marker) in patients with TLE (Fahrner, Kann et al. 2007). Thus, chronic TLE in humans is associated with a decreased neurogenesis. In agreement with this, two studies conducted on rats, where SE was induced by Kainate, showed a significant reduction in DG neurogenesis in chronic epilepsy rats when compared to age-matched control rats (Hattiangady, Rao et al. 2004; Hattiangady, Rao et al. 2008). They also reported that an increased number of spontaneous recurrent motor seizures were associated with more decrease in neurogenesis. A growing body of evidence suggests a role of adult neurogenesis in hippocampal-dependent learning and behavior (Shors 2004; Aimone, Wiles et al. 2006; Dranovsky and Hen 2006), and thus changes in neurogenesis, that have been observed in chronic epilepsy, may alter cognitive function and mood. This is most probably because it changes the generation and/or incorporation of newly-born neurons within the DG. These studies may suggest that a decrease in neurogenesis in chronic epilepsy patients is associated with depression and cognitive impairments. Therefore, enhancing DG neurogenesis in chronic epilepsy conditions might help in restoring memory and cognition.

1.7 Mechanisms of Kainate/seizure-induced neurogenesis

Animal models of epilepsy allow the investigation of the basic molecular and cellular mechanisms of epileptogenesis and its relation to brain damage. Many studies have shown that systemic or intracerebral applications of Kainate cause epileptiform seizures in the CA3 region of the hippocampus (Nadler 1981; Ben-Ari 1985). Interestingly, Kainate-induced seizures propagate from CA3 to other limbic structures and are followed by a pattern of cell loss that resembles that seen in patients with TLE (Ben-Ari 1985). In many studies, it has been shown that Kainate-induced seizures increase hippocampal neurogenesis (Gray and Sundstrom 1998; Covolan, Ribeiro et al. 2000; Nakagawa, Aimi et al. 2000; Gray, May et al. 2002; Hattiangady, Rao et al. 2004; Jessberger, Nakashima et al. 2007; Tokuhara, Sakuma et al. 2007; Hattiangady, Rao et al. 2008). Neurogenesis can be divided into several independent processes: precursor cell proliferation; commitment for a neuronal phenotype; differentiation (development of neuronal features); death of a proportion of newly-born neurons; and maturation of the remaining newly-born neurons into functional neurons. Exploring the mechanisms by which Kainate/seizures stimulate neurogenesis should include studying the processes of neurogenesis mentioned above. The steps of cell proliferation and cell death are independently regulated and thus determine how many neurons are produced. Here, we will shed light on the current knowledge that deals with the effects of Kainate/seizures on precursor cell proliferation and survival as well as the remaining questions that need to be elucidated.

1.7.1 Cell proliferation

Gray et al have elegantly demonstrated that unilateral intracerebroventricular injection of Kainate initiated seizures which in turn enhanced hippocampal neurogenesis in both dentate gyri (ipsilateral and contralateral to Kainate infusion) of adult rats (Gray and Sundstrom 1998). Indeed, they reported an increase in BrdU incorporating cells in the SGZ that were ultimately committed to the neuronal lineage as measured by double-labelling of cells for TuJ1 and BrdU. Although DG cell death was not examined in this study, the enhancement of precursor cell proliferation in the SGZ contralateral to

Kainate treatment perhaps suggests a minimal or indirect role of cell death. Subsequent study has shown that Kainate-induced seizures increased progenitor cell divisions in the DG stem cell niche (Nakagawa, Aimi et al. 2000). In this regard, Nakagawa and colleagues have reported an enhanced precursor cell proliferation, as measured by BrdU immunopositive cells, in the GCL of the DG after 3 days of Kainate treatment which peaked at day 5 and returned back to normal by day 10. Interestingly, this research also demonstrated that pyramidal neuronal degeneration is not necessary for triggering cell proliferation. However, it is not clear if Kainate/seizures affected the length of the cell cycle of proliferating cells and/or drove precursor cells to become postmitotic. In another study, Covolan and colleagues have demonstrated that Kainate-induced SE increased DG cell proliferation (as indicated by BrdU incorporating cells) without affecting cell death as measured by silver staining method (Covolan, Ribeiro et al. 2000). In contrast, pilocarpine-induced SE enhanced cell damage within the GCL with less increase in cell proliferation when compared to Kainate-induced SE in the same previous study. This study may suggest that Kainate-induced SE probably alters the survival of proliferating precursor cells within the SGZ niche, however, the effect of Kainate and/or seizures on the survival of newly-born granule cells remains to be defined. In a pilocarpine-induced seizures model, it has been suggested that an enhanced neurogenesis is associated with a transient reduction in apoptosis as indicated by the initial decrease of caspase-3(Ekdahl, Mohapel et al. 2001). Furthermore, it has been demonstrated that additional re-exposure to seizures at days 6, 7, 33, and 34 following the first electrically-induced SE, neither potentiates cell death mechanisms in the SGZ nor compromises the survival of the newly-born cells (Ekdahl, Zhu et al. 2003). This study may suggest that additional seizures perhaps have minimal or no effect on new neurons and that the initial insult is the relevant one for altering the neurogenic niche.

It is widely accepted that precursor cells reside within the SGZ of the DG throughout life (Gage 2000; Seri, Garcia-Verdugo et al. 2001; Taupin and Gage 2002). These precursor cells are radial glia-like cells that proliferate and give rise to highly amplifying precursor cells that in turn proliferate several times and give birth to new neurons (Seri, Garcia-Verdugo et al. 2001; Seri, Garcia-Verdugo et al. 2004; Encinas, Vaahtokari et al. 2006). In this regard, Huttmann and co-workers have shown that Kainate-initiated seizures

increased the proliferation of GFAP precursor cells in the SGZ of adult transgenic mice (Huttmann, Sadgrove et al. 2003). Furthermore, it has been demonstrated that approximately 20% of doublecortin (Dcx) positive cells (immature neuronal marker) are precursor cells in the cell cycle (Plumpe, Ehninger et al. 2006). Interestingly, in this study, Kainate-induced seizures increased not only BrdU incorporated cells but also it doubled Dcx cell numbers after one week of the treatment. It also dispersed the stem cell niche into the GCL as indicated by the distribution of the Dcx positive cells. Furthermore, Plumpe et al have reported that Kainate/seizures had no effect on the neuronal maturation phase and thus precursor cell proliferation is independent from neuronal maturation. Therefore, exploring the proliferative effects of Kainate/seizures on specific precursor cell types would increase our understating to the mechanisms that control precursor cells contribution in epilepsy.

1.7.2 Cell death

Two patterns of cell death were characterized. Firstly, necrotic cell death was identified and cells were described to have integrity of the cell membrane, organelle damage, and cellular lysis. Secondly, Programmed cell death (apoptosis) involves the systematic disassembly of a cell and can be defined by morphological changes including, cell shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing and apoptotic body formation (Curtin and Cotter 2003). Although apoptosis correlates with physiological cell death; it has been reported to be involved with cell injury induced by a various types of physical and chemical agents (Yakovlev and Faden 2001). Interestingly, accumulating evidence shows an important role for apoptosis in several neurodegenerative diseases, including epilepsy (Marks and Berg 1999; Sastry and Rao 2000).

Studying the mechanisms of apoptotic cell death has revealed different pathways that are responsible for apoptosis initiation and progression. Basically two main pathways can be differentiated by the relative timing of caspase activation and mitochondrial release of cytochrome c **(Figure 1.5)**. In the first, activation of death receptors, e.g. CD95 (APO-1/Fas), trigger caspase-8, which in turn activates caspase-3. In the second, cytochrome c

is released from the mitochondrial intermembrane space prior to caspase activation (**Figure 1.5**). The stressed endoplasmic reticulum (ER) contributes to apoptosis by the unfolded protein response pathway, which induces ER chaperones, and by the ER overload response pathway, which produces cytokines via nuclear factor-kappaB. Multiple other stress-inducible molecules, such as p53, JNK, AP-1, NF-kappaB, PKC/MAPK/ERK, and members of the sphingomyelin pathway have a profound influence on apoptosis (Herr and Debatin 2001).

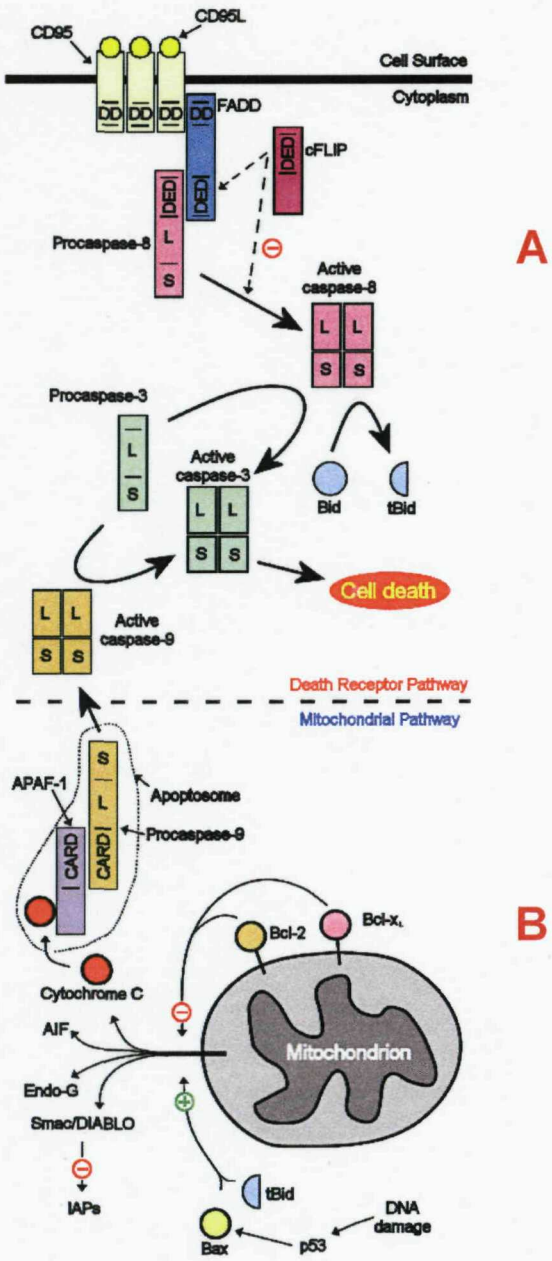


Figure 1.5 Apoptotic cell death pathways. This is a summary diagram of the two main apoptotic pathways ;(A) the death receptor pathway and (B)the mitochondrial pathway (Wang, Yang et al. 2002) .

A large and growing body of evidence suggests that the precise control of neuronal cell death during development is executed at least in part through apoptosis (Yuan, Lipinski et al. 2003). Interestingly, in the DG, approximately 95% of all BrdU-labelled cells in the GCL express neuronal markers within one week of labelling (Cameron and McKay 2001; Brown, Couillard-Despres et al. 2003). In addition, 85% of BrdU labelled cells express mature neuronal markers within four weeks (Brown, Couillard-Despres et al. 2003). On the other hand, it is estimated that around 50% of new granule cells die under

normal conditions (Dayer, Ford et al. 2003). However, Dayer and colleagues have reported that this cell death stops at four weeks after generation of the new granule cell neuron. These interesting results suggest that a substantial number of neuronal cells are probably programmed to die unless they are functionally integrated. Indeed, newly-born neurons need around four weeks to integrate and start functioning (van Praag, Schinder et al. 2002; Jessberger, Romer et al. 2005).

There are numerous studies investigating cell death in epilepsy models. While a substantial and preferential neuronal cell loss was reported in different regions of the hippocampus, the dentate granule cell layer was considered to be relatively preserved (Ben-Ari, Tremblay et al. 1981; Babb, Brown et al. 1984; Babb, Lieb et al. 1984; Cavazos, Das et al. 1994). Subsequently, cell death has been investigated in the hippocampus of adult rats following Kainate induced-seizures (Covolan, Ribeiro et al. 2000; Tooyama, Bellier et al. 2002). In both studies, intraperitoneal (IP) injections of Kainate were used to induce seizures. These two independent studies have demonstrated a significant increase in DG neurogenesis after Kainate-induced seizures. Although Tooyama et al have shown an increased cell death in CA1-CA3 after Kainate treatment, there was no evidence of enhanced cell death in the DG. Likewise, Covolan et al did not show a significant cell death in the DG after Kainate-induced seizures, however, GCL cell death was evident after pilocaprine-induced SE. In line with this, it has recently been shown that although intrahippocampal Kainate-induced seizures enhanced neuronal degeneration in CA1, CA3, and the hilus, there were only a few cells labelled for fluoro jade B (FJB) (a marker of neuronal degeneration) in the SGZ of the DG (Heinrich, Nitta et al. 2006). Interestingly, Heinrich and colleagues have also demonstrated that just a few of the Dcx positive cells in the SGZ were FJB positive and none were TUNEL (terminal deoxynucleotidyl transferease-mediated nick end labelling) positive. In fact, there were no TUNEL labelled cells in the SGZ after Kainate treatment. These results demonstrate that cell death is not necessary to trigger DG precursor cell proliferation after Kainate-induced seizures. The previous studies may suggest that Kainate perhaps alters the survival of vulnerable GCL cells. However, this matter still unresolved and needs further investigation.

1.8 Aims of this project

Hippocampal neurogenesis persists throughout life and is important for learning and memory. It is also altered by acute seizures and chronic epilepsy. Kainate is often used to model temporal lobe epilepsy and hippocampal damage. Kainate/seizures-induced alteration in neurogenesis, initially qualitative and eventually quantitative, is associated with altered hippocampal cognitive function. Moreover the partial inhibition of epileptogenesis, which is associated with reduced aberrant neurogenesis, protects animals from seizure-induced cognitive impairment in a hippocampus-dependent learning task. Jessberger and Zhao et al 2007 have shown that persistently abnormal neurogenesis begins immediately after Kainate-induced status epilepticus and therefore this is the time point to begin examining altered neurogenesis and to modify the process in a clinically relevant manner. As such, we hypothesize that the dynamics of the hippocampal stem cell niche is altered immediately after Kainate application and / or seizures.

Therefore, to obtain further insights into the mechanisms by which status epilepticus alters neurogenesis, I will examine the effects of Kainate on hippocampal precursor cells and their neuronal progeny cells *in vitro*. Furthermore, I will use an *in vivo* model of Kainate-induced seizures to unravel the mechanisms underlying the acute effects of Kainate/seizures on precursor cells proliferation and survival. To achieve this, I will label a 'clone' of proliferating precursor cells before seizures induction and study the effects of Kainate/seizures on the proliferation and survival of this 'clone' of cells as well as postmitotic population.

Identifying the role of precursor cells shortly after seizures may help the appropriate targeting of these cells in the future treatment of either epileptogenesis and / or cognitive impairment in epilepsy.

Chapter Two

Primary Hippocampal Cell Cultures

2.1 Introduction

The aim of this chapter is to formulate a robust general method for *in vitro* cultures of hippocampal stem/progenitor cells. Primary cultures of rat hippocampus are widely used to study stem/progenitor cells *in vitro*. Populations of hippocampal neurons have been consistently isolated and maintained *in vitro* irrespective of the rats age (Brewer 1997). Hippocampal progenitor cells have been generated from postnatal rat pups (Howell, Scharfman et al. 2003) and also from adult rodents (Seaberg and van der Kooy 2002; Brewer and Torricelli 2007). Apart from the hippocampus, neural precursors have also been isolated and cultured from the cerebellum (Zusso, Debetto et al. 2004) and the sub-ventricular zone (SVZ) (Doetsch, Caille et al. 1999). Therefore, short-term culturing of these cells enables us to study the growth, proliferation, survival, and phenotype of stem/progenitor cells and their progeny *in vitro*. These cultures may be studied while alive and examined over extended periods of time with markers of cell viability to study cell death, or may be fixed and immunostained with phenotypic markers to identify cell type and study cell fate (Howell, Scharfman et al. 2003). The above mentioned characteristics place this paradigm as a top priority system to be developed and investigated in order to understand the acute mechanisms of neurogenesis *in vitro*.

The methodologies for generating primary cells have been developed over a number of years. Initial studies were hampered by poor cell viability and low growth rate, making it difficult to study these cells *in vitro*. The discovery and use of mitogenic factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), as well as the generation of serum-free culture medium have been important advances in generating successful cultures of primary mammalian stem/progenitor cells (Reynolds and Weiss 1992; Cameron, Hazel et al. 1998; Gage, Kempermann et al. 1998).

Stem/progenitor cells are abundant within the neurogenic niches in early postnatal life. Consistent with the largely postnatal development of the granule cell layer of the dentate gyrus (Altman and Das 1965), it has been demonstrated that a great number of proliferating progenitor cells are found in the dentate gyrus postnatally (Namba, Mochizuki et al. 2005). Moreover, the postnatal period is an important transition stage between the embryonic and adult life. Therefore, we developed a particular interest in

exploring the control mechanisms of hippocampal stem/progenitor cells and neural precursors at this stage in order to understand adult neurogenesis mechanisms in general and in the subgranular zone in particular.

The philosophy behind investigating hippocampal stem/progenitor cells alongside other cell types *in vitro* is to understand the interaction of different sub-populations of cells, to maximize similarities to a proper stem cell niche, and to investigate stem cell fate choice.

2.2 Neurogenesis labelling methods

2.2.1 Markers of cell proliferation

In order to establish that neurogenesis is occurring, two facts need to be clearly addressed. Firstly, cells have to be identified as dividing. Secondly, the newly born cells, or a fraction of them, have to be recognized as neurons. Investigating neurogenesis therefore requires methods to label newly-born cells and their progeny and to identify their phenotypic fate. Cells that proliferate enter the cell cycle at G1, then progress into S (synthesis) phase, where replication of the DNA takes place, thereafter progress to G2 and finally the M-phase-mitosis, when the cell physically divides. Understanding cell cycle phases is essential to target and label cycling/dividing cells in order to measure the speed of proliferating cells, and the fate of quiescent (Go) cells. Various cell division markers and cellular antigens have been used to measure cell proliferation and differentiation. In the early1960s, monitoring cell division became possible through the introduction of the autoradiography when the radioactive ^{3}H isotope of hydrogen (^{3}H-dT) added to the diets of rats became incorporated into dividing cells (Altman and Das 1965). ^{3}H-dT is incorporated into nuclear DNA during S-phase of the cell cycle and its amount is directly proportional to the number of silver grain overlay (Altman and Das 1965; Altman and Das 1966). The discovery of the thymidine analog: bromodeoxyuridine (BrdU), as an exogenous proliferative marker has been an important contribution to the methods to study cell proliferation. Like ^{3}H-dT, BrdU incorporates in

place of thymidine into DNA during the S-phase of the cell cycle; indicating the number of cells in the S-phase of the cycle over short exposure and survival times (**Figure 2.1**). Because BrdU is detected immunohistochemically, double and triple immunohistochemical labelling of proliferating cells was made possible by introduction (Nowakowski, Lewin et al. 1989), enabling investigators to phenotype and trace dividing cells. Nowadays, BrdU is preferred over ^{3}H-Thymidine because it can be detected in thicker tissue sections, it is not a radiation hazard, and is easier to use (for a review see (Taupin 2007)).

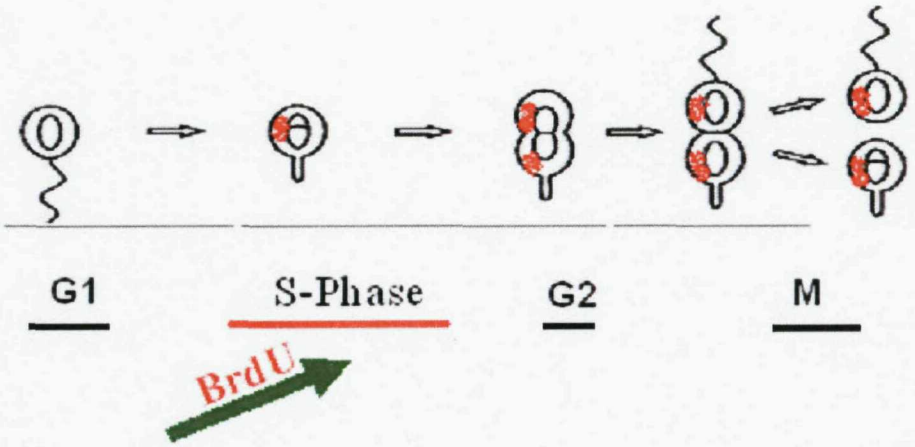


Figure 2.1 BrdU incorporates DNA in S-phase of the cell cycle. This is also passed to daughter cells and can be detected using immunohistochemistry techniques. BrdU = red.

Endogenous, cell cycle phase-specific proteins have been used as alternative methods to investigate cell proliferation. Proliferating cell nuclear antigen (PCNA) is a component of the DNA polymerase δ complex, which is expressed during late G1 phase and early S-phase, and is also detected during G2 and mitosis (M) (**Figure 2.2**) (Takahashi and Caviness 1993; Kawabe, Suganuma et al. 2002). However, the use of PCNA as a proliferative marker has its limitations and drawbacks as it is expressed in cells undergoing DNA repair, and some non-proliferating neurons (Taupin 2007). The discovery of Ki-67 which is a nuclear protein expressed in all phases of the cell cycle except the resting phase Go (**Figure 2.2**) (Scholzen and Gerdes 2000; Kee, Sivalingam

et al. 2002) has added great value to measure cell cycle kinetics and proliferation. Ki-67 is expressed during cell proliferation in all mammalian species from rodents to humans (Endl and Gerdes 2000; Scholzen and Gerdes 2000). Ki-67 also has a very short half life and is not detectable during the DNA repair process (Scholzen and Gerdes 2000; Zacchetti, van Garderen et al. 2003). Unlike BrdU, it has no adverse effects that might be encountered due to BrdU administration and like BrdU; it can be detected using immunohistochemistry. To illustrate, the expression of Ki-67 and BrdU were examined in the proliferative zone of the dentate gyrus of adult Wistar rats using immunohistochemistry techniques in a study by Kee et al. (Kee, Sivalingam et al. 2002). In this study, a quantitative comparison showed 50% higher numbers of Ki-67 positive cells compared to BrdU numbers when examined 24 hours after BrdU injection. This was due to the incorporation of BrdU only in the S-phase while Ki-67 was expressed in all phases of the cell cycle. If the length of time between the exposure to BrdU and cell fixation is less than the cell cycle time, then one can draw useful conclusions from the proportion of dividing (Ki-67+) cells that have entered the S-phase (BrdU+) over this time period. This parameter of cell proliferation is called the Labelling Index (LI) and is expressed as

LI = BrdU+/Ki-67+

Alterations in the LI over time are indicative of the average cell cycle speed within a population of proliferating cells. If cells are progressing through the cell cycle more quickly then a greater proportion of them will have entered the S-phase and therefore the greater will be the LI (Dehay et al 2001). The time taken for the LI = 1 is the average cell cycle time. Co-labelling cells for both Ki-67 and BrdU and measuring the labelling index (BrdU/Ki-67) can be achieved *in vitro* by terminally pulsing cultures with BrdU for a time less than the length of the cell cycle and then measuring the proportion of cells in the S-phase (BrdU incorporated cells) of the total population of cells in the cell cycle (Ki-67 expressing cells).

The fact that Ki-67 is uniquely present during all active cell cycle phases and absent from resting cells makes it an excellent marker for determining what proportion of cells are in the cell cycle or the growth fraction (GF). The GF of a given population of cells is the proportion of cells that express Ki-67 to the total cell counts; for example if DAPI (a nuclear counter stain) is used to measure the total cell counts, the growth fraction is Ki-67/DAPI.

GF = Ki-67+/DAPI

A very important characteristic for Ki-67 is its disappearance once cells exit the cell cycle, which is useful for identifying sub-population of cells that have become post mitotic. This later point is very useful for assessing the survival of stem / precursor cell progeny.

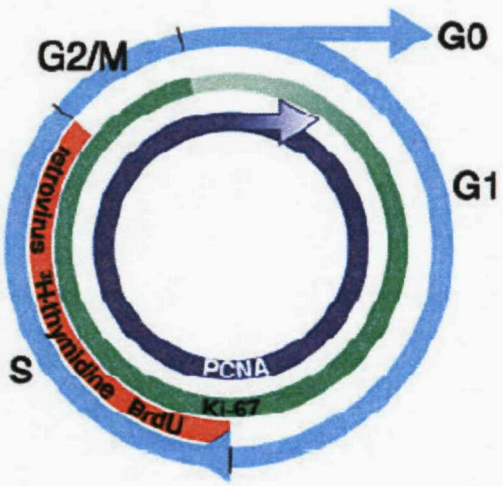


Figure 2.2 Markers of the cell cycle. Exogenous (BrdU) and Endogenous (PCNA and Ki-67) proliferative markers and their existence (Ki-67, PCNA) and ability to incorporate (BrdU) in the cell cycle (Christie and Cameron 2006)

2.3.2 Cell Phenotypes

Studying neurogenesis also requires markers to label the phenotype of proliferating cells and their progeny, not only to quantify neurogenesis but also to study the choice of fate of daughter cells during and after mitosis.

Progress has been made in the last decade to identify numerous markers for stem/progenitor cells, immature neurons, and glia.

Investigating stem/progenitor cells using nestin was a key step in understating the early stages of neurogenesis. The intermediate filament nestin has been reported as a marker of stem/precursor cells in the developing mammalian central nervous system giving rise to both neurons and glia (Lendahl, Zimmerman et al. 1990). Nestin was identified in neuroepithelial stem cells of not only rats (Lendahl, Zimmerman et al. 1990) but also humans (Dahlstrand, Zimmerman et al. 1992). Nestin is defined as a sixth intermediate filament (Liem 1993), characterized by an α-helical central 'rod' domain that contains repeated hydrophobic heptad motifs. It also contains a short N-terminus and an unusually long C-terminus which interacts with other cellular components that are required for nestin expression (for review see (Wiese, Rolletschek et al. 2004)). Immunohistochemistry showed nestin expression in embryonic neuronal precursor cells (Frederiksen and McKay 1988), in the subventricular zone of the adult mammalian brain (Doetsch, Caille et al. 1999), and in the adult mouse dentate gyrus (Fukuda, Kato et al. 2003). Recently, and in agreement with previous work in our lab we have shown that nestin is consistently expressed in primary hippocampal cell cultures from postnatal rats (Howell, Silva et al. 2007).

The investigation of neurogenesis also requires accurate identification of the neuronal phenotype, and thus proteins that are present in the immature neurons are of great interest. Previously, it has been demonstrated that TUC-4 (also called CRMP4 or TOAD); a protein that is expressed after a daughter cell's final division was a good marker of immature neuronal phenotype (Toga, Ambach et al. 1994; Minturn, Geschwind et al. 1995). However, TUC-4 is a difficult antibody to obtain and is no longer available commercially. As an alternative, a number of studies have demonstrated that another marker Doublecortin (Dcx) which is a protein that promotes microtubule

polymerization, and present in migrating neuroblasts and young neurons (Lee, Rebhun et al. 1990; Francis, Koulakoff et al. 1999; Gleeson, Lin et al. 1999). It can label immature neurons for up to 2-3weeks after they become postmitotic (Brown, Couillard-Despres et al. 2003; Rao and Shetty 2004). Importantly, Dcx labels around half of the dividing sub-population of cells in the dentate gyrus, which helps identifying neuronal precursors at the time of cell division (Brown, Couillard-Despres et al. 2003). Other markers that are expressed in neurons at later stages of maturity, include NeuN, neuron specific enolase (NSE), and calbindin-D, are less important when investigating the early mechanisms of neurogenesis *in vitro*. Class III ß-tubulin, also known as TuJ1, is one of the earliest markers of immature neurons, its expression in neurons lasts for about 4 weeks and thus will also label some mature neurons (Fanarraga, Avila et al. 1999). It has been established that class III ß-tubulin is extensively posttranslationally modified during neural development and these modifications are neuron-specific (Lee, Rebhun et al. 1990). Indeed, expression of class III ß-tubulin starts as early as embryonic 8.5 days (Easter, Ross et al. 1993) and can be detected throughout brain development (Menezes and Luskin 1994). With respect to neurogenesis, TuJ1 is used as neuron-specific marker of newly-generated cells in the postnatal and adult brain (Doetsch, Garcia-Verdugo et al. 1997; Parent, Yu et al. 1997; Gray and Sundstrom 1998; Gray, May et al. 2002; Howell, Scharfman et al. 2003; Howell, Doyle et al. 2005; Howell, Silva et al. 2007). We therefore, while studying the mechanisms of neurogenesis *in vitro,* would make use of class III ß-tubulin to immunostain immature neurons and thus study the effect of a factor on newly-born neurons.

Astrocytes are a very important part of the stem cell niche (Zhu and Dahlstrom 2007), are up-regulated in response to brain injury and trauma (Ke and Gibson 2004) and constitute a significant sub-population of cells that might be grown in cultures. As such there is a need to mark these astrocytes in order to investigate their role in neurogenesis. Glial fibrillary acidic protein (GFAP) has been reported as a very reliable astrocytic marker and commonly used in immunohistochemistry to detect astrocytes (Zhu and Dahlstrom 2007). However, substantive evidence now indicates that adult neural stem cells express GFAP and exhibit properties associated with glia *in vivo* (Doetsch, Caille et al. 1999; Seri, Garcia-Verdugo et al. 2001; Garcia, Doan et al. 2004) and *in vitro*

(Laywell, Rakic et al. 2000; Morshead, Garcia et al. 2003). The later feature emphasizes the importance of studying the role of GFAP expressing progenitor cells as well as those progenitors that do not express GFAP and their relation with immature neuronal markers (Song, Stevens et al. 2002).

In an interesting study, it has been shown that there may be two types of mitotic progenitor cells within the SGZ of the dentate gyrus (Encinas, Vaahtokari et al. 2006) (**Figure 2.3**). The first is a sub-population of progenitor cells referred to by these authors as quiescent cells expressing both nestin and GFAP. The other sub-population expresses nestin but not GFAP and are found to be amplifying neural progenitor cells and have the ability to give rise to new neurons. Controversially Encinas and colleagues reported that neuroblasts expressing class III ß-tubulin and Dcx are postmitotic (**Figure 2.3**). However, in an interesting study carried out on primary cultures it has been reported that over 50% of adult CNS cells with neuron-like characteristics retain regenerative and proliferative potential (Brewer 1999). Furthermore, previous studies have demonstrated the presence of an active dividing (nestin positive) sub-population of TuJ1expressing cells which was up regulated after Neuropeptide Y treatment (Howell, Scharfman et al. 2003; Howell, Doyle et al. 2005). Undeniably, we have different populations of progenitor cells in the dentate gyrus therefore, understanding neurogenesis mechanisms requires elucidating the effects of mitogens on these sub-populations of cells in order to understand their role in neurogenesis.

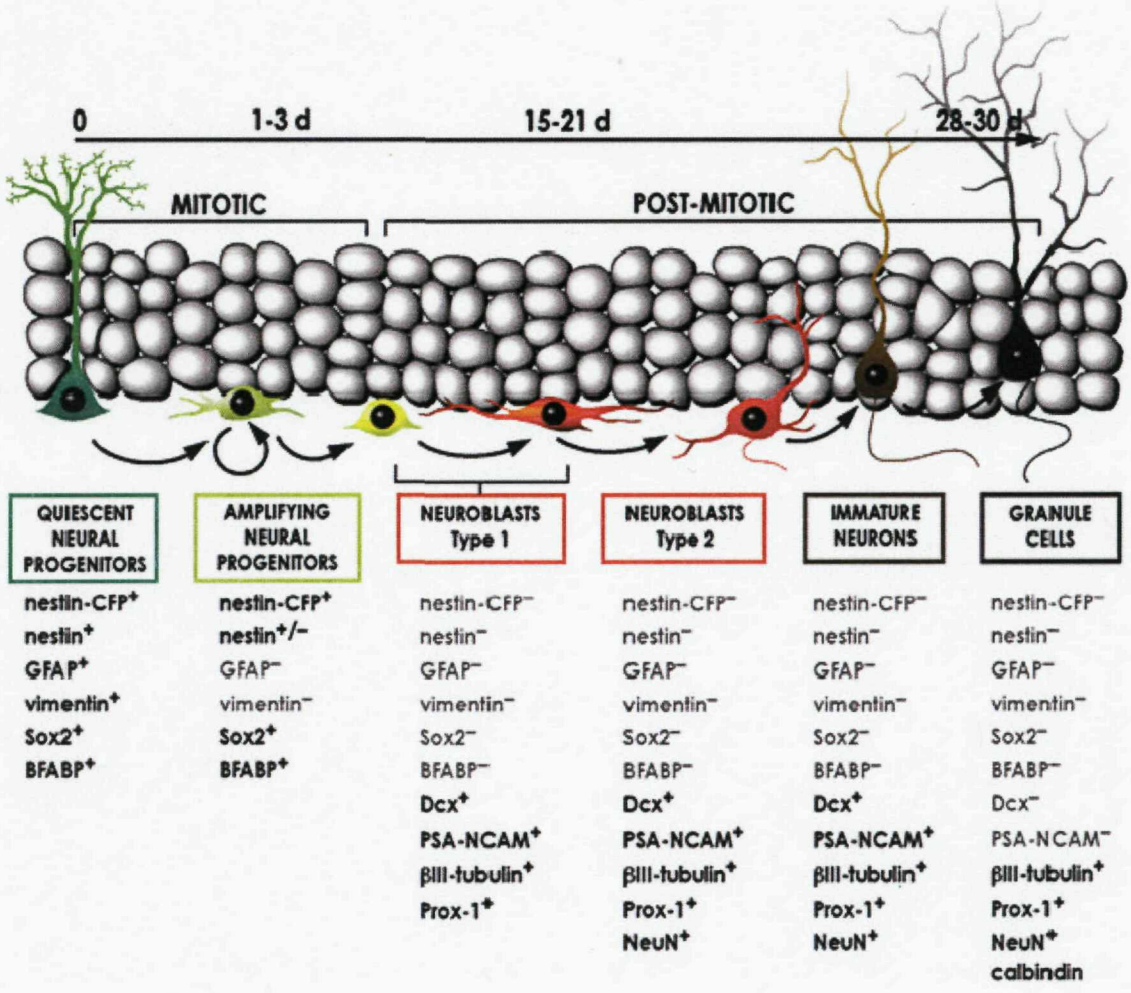


Figure 2.3 A schematic summary of the neuronal differentiation cascade in the dentate gyrus. Quiescent neural progenitors generate, through asymmetric divisions, the amplifying neural progenitors that, after several rounds of symmetric divisions, exit the cell cycle within 1–3 days and become postmitotic neuroblasts type1 cells. Within next 15–21 days, neuroblasts type1 cells mature into neuroblasts type2 and then into immature neurons with apical processes and basal axons and the soma located in the granule cell layer. After an additional 10–15 days, immature neurons acquire the characteristics of mature granule neurons, develop extensive branching, and send long axonal processes, forming the mossy fiber (Encinas, Vaahtokari et al. 2006). Proliferating neuroblasts express nestin and also cells that commite to the neuronal lineage (Howell, Silva et al. 2007)

2.3 Aims

We therefore aim in this chapter to generate, study, and characterize primary hippocampal cells in culture from early postnatal rats.

2.4 Methodology and Materials

2.4.1 Animals

We used Wistar rat pups (7-10 days old). All animal procedures were performed in strict accordance with the Animal Scientific Procedure Act 1986 guidelines and with consent of the University of Southampton Bioethics Committee.

2.4.2 Primary hippocampal cell cultures

Generation of primary hippocampal cultures was based on the method of Howell (Howell, Scharfman et al. 2003) and Brewer (Brewer 1997), with some modifications, as described below.

2.4.2.1 Reagents and preparations:

24-well culture plates (Co-star brand; Fisher Scientific) were used as they are the best in assaying cell division and number in monolayer cultures. A small volume (300μL) of Poly-L Lysine (PLL) (Sigma) was placed into each well for 10 min before being rinsed with growth medium (GM), Neurobasal A (Gibco) supplemented with 2%B27 (Life Technologies) and 0.5mM Glutamine (Sigma, St Louis, MO, USA), and kept in the incubator (5%CO2/air/37 °C) until required. It is important to rinse as excess PLL is toxic to the cells. Pre-warmed papain (22.0U/mg, 2mg/ml; Sigma) was prepared carefully to minimize possible contamination. A two step density gradient of Optiprep (Axa-Shields, Oslo, Norway) was prepared by carefully layering (to ensure minimal

mixing of layers) 1 ml of 10% (density; 1.032g/ml) Optiprep in GM on top of 1 ml 20% (density; 1.064g/ml) optiprep in GM in a 15ml screw top tube and kept aside until needed. Preparing the Gey's buffer solution by supplementing the Gey's balanced salt solution (Life Technologies, Paisley, UK) with 4.5 mg/ml glucose at 4 °C is the last step before starting with the animals.

2.4.2.2 Tissue acquisition:

The animals were sacrificed by atlanto-axial dislocation then decapitated and the whole brain swiftly removed. The hippocampus was dissected under sterile conditions in Gey's buffer solution. Using a pair of spatulas, the corpus callosum was bisected and each cortical hemisphere rolled back. Each hippocampus was separated from the overlying cortical white matter along the natural line of the alveus hippocampus. This procedure was done carefully in order to remove as much white matter as possible, including the subiculum. In spite of this, contamination by loosely adherent subependymal layers of the hippocampal arch and the posterior lateral ventricle could not be ruled out.

2.4.2.3 Tissue processing:

The hippocampi were then placed onto a sterile melinex strip and transverse sections were cut at 400μm using the MacIlwain tissue chopper and placed in GM, Neurobasal A (Gibco) supplemented with 2%B27 (Life Technologies) and 0.5mM Glutamine (Sigma, St Louis, MO, USA), for 5 min. The hippocampi were sliced up to aid the papain digestion of the tissue. Slices were then transferred to pre-warmed Papain (22.0 U/mg, Sigma) solution (2mg/ml) and incubated at 37 °C in small Petri dishes. The Papain was dissolved in GM a minimum of 30 minutes before filter sterilizing (0.22μm pore, Millex-GV, Millipore, USA) and incubation of tissue slices. Enzymatic dissociation aids the release of cells from connective tissue. After 30 minutes, the Papain solution was aspirated and replaced with excess GM (so as to end the papain digestion by effectively diluting with medium) and the tissue was gently triturated with a 1ml white pipette tip to separate clumps. Cells in suspension were aspirated into a separate tube, and the

sediment was suspended in further fresh GM. This procedure was repeated a further 2 times until the cell pellet was fully dissociated, resulting in a cell suspension in 7-9mls GM. Partial purification of cells from debris was achieved by centrifugation through a 2-step density gradient (OptiPrep; Axa-Shields, Oslo, Norway), by spinning for 15mins at 1900rpm. Optiprep is a ready-made sterile solution of Iodixanol. The viable cell fraction was collected, diluted in an excess of GM and spun for 2min at 1100rpm.

2.4.2.4 Cell quantification

The cell pellet, now free of OptiPrep, was resuspended in GM and viable cell counts determined by scoring the number of Trypan blue (Sigma), dye excluding cells (viable cells) within a 1mm by 1mm haemocytometer counting grid. A minimum of 3 grids were quantified per cell preparation. The cell suspension was then diluted in GM to yield ~100,000 viable cells per ml for plating.

2.4.2.5 Cell plating

500µl of cell suspension (100,000cells/ml) in GM was seeded in each well. 2 hours after plating cells were rinsed and replenished with half ml of fresh medium per well. This helps to remove debris that is inhibitory to cell growth. Growth conditions included the addition of the neuropeptide Y (NPY) (Activotec SPP Ltd, University of Southampton), and Kainate (Ka) (Tocris) compared to control conditions of growth medium alone (Neurobasal A/2% B27 and 0.5mM glutamine). All medium included a combined 1% antibiotics/antimycotics (Pen/Strep and Fungizone, Life Tech, USA). Incubator conditions (5%CO2 in air at 37°C) were standard. The culture medium was changed every 3 days replenishing two-thirds of the medium volume with either control medium or medium containing treatment (Kainate). Standard growth conditions are when cells grow in Neurobasal A/ 2% B27/0.5mM Glutamine/ 1% Antibiotics/Antimycotics and incubator (5% CO_2/air/ 37 C^O).

2.4.3 Cultures for con-focal imaging

13 mm glass cover-slips (Fisher Scientific, UK) were sterilized by autoclaving and then placed in 24 well plates. 400 µl of PLL (Poly-L Lysine (Sigma)) were used to coat the cover-slips for 30 min at room temperature before being washed with excess GM and kept in the incubator until required. Following the protocol in **Section 2.4.2** primary hippocampal cell suspensions were prepared and seeded on top of sterile slide glasses.

2.4.4 Bromodeoxyuridine labelling and cell proliferation

Terminal 5-bromo-2-deoxyuridine (BrdU) pulse was used to identify proliferating (S-phase) cells. BrdU is a thymidine analog that incorporates into the DNA of dividing cells in immunohistochemically detectable quantities during the S-phase of cell division. BrdU was added to the growth medium (GM) with/without treatment at a final concentration of 20 µM for 4 or 6 hours (according to the experiment) before cell fixation with 4% paraformaldehyde (PFA) (Sigma).

2.4.5 Cell death detection using propidium iodide (PI)

Cell death was detected using the fluorescent dye propidium iodide (PI) which is a very stable fluorescent dye absorbing blue-green light (493 nm) and emitting red fluorescence (630 nm) (Darzynkiewicz, Bruno et al. 1992). It enters dead and/or dying cells with leaky plasma membranes and binds with nucleic acid to yield a bright red fluorescence. In our model, PI was added to live cultures at a concentration of 5µg/ml and incubated for 40 minutes at 37°C in (5%CO2 in air). Then the whole growth medium was replenished with fresh medium containing DAPI at a concentration of 20µg/ml and incubated for a further 40 minutes at 37°C in (5%CO2 in air). Before imaging and counting live cells (**Sections 2.4.8 & 2.4.10**) (**Figure 2.4**), the final step was to replace the DAPI containing medium with fresh medium. These separate exposures and washings were necessary to reduce background staining. After imaging, cells were

rinsed once in phosphate-buffered saline (PBS) before being fixed with 4% PFA (Sigma) at 4 C° for 30 minutes waiting antibody specific immunostaining.

2.4.6 Quantification of live cells *in vitro*

4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI) is known to form fluorescent complexes with natural double-stranded DNA. Because of this property DAPI is a useful tool in various cytochemical investigations. When DAPI binds to DNA, its fluorescence is strongly enhanced, this has been interpreted in terms of a highly energetic and intercalative type of interaction (Tanious, Veal et al. 1992). DAPI stains nuclei specifically and enters both live and dead/dying cells. Cells that are DAPI positive and PI negative are considered as live cells (**Figure 2.4**). The morphology of the cells' nuclei is observed using an inverted DM IRB fluorescence microscope (Leica Microsystems UK Ltd, Milton Keynes, UK) at excitation wavelength 350 nm. Nuclei are considered to have the normal phenotype when glowing bright and homogenously.

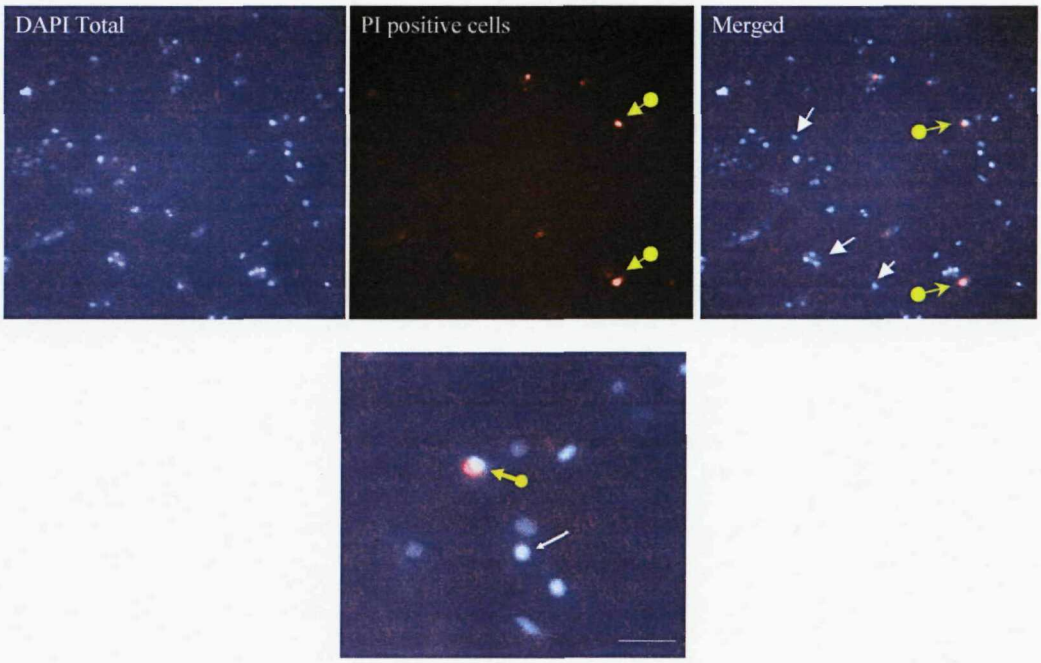


Figure 2.4 Measuring cell death in culture. Cells were grown for 5 days under standard growth conditions (Neurobasal A /B27/and Glutamine). Two-thirds of the growth medium was replenished on day 3. PI and DAPI were applied to cells in culture as detailed in **Section 2.4.6**. Cells were then imaged live where live cells (DAPI positive but PI negative) as well as dead/dying cells (PI positive) were quantified. DAPI in blue and PI in red. White arrows show live cells. Yellow arrows indicate dead/dying cells. Images were captured with 20x objective on an inverted DM IRBE microscope (Leica Microsystems UK Ltd., Milton Keynes, UK). The Open lab capturing system version 2.1 (Improvision Inc., Lexington, USA) was used to do the counting. Scale bar = 50μm.

2.4.7 Immunocytochemistry and Immunofluorescence

The PFA-fixed cell cultures were washed three times in phosphate-buffered saline (PBS) (pH 7.4). For immunohistochemical analysis of BrdU incorporation; cultures were incubated in 2 M HCl for 30 min at 37°C. Cells were then washed three times in PBS. For single, double, or triple immunofluorescence non-specific antibody-binding sites were blocked with PBS containing 0.1% Triton and 5% pre-immune donkey serum (Sigma) at room temperature for 30 min. Cells were then incubated in PBS-0.1% Triton

with one, two or three appropriate primary antibodies **(Table 1.1)**, raised in different species, depending on the experiment and kept overnight at 4°C. Cells were washed once in PBS before the addition of secondary antisera conjugated to cyanin (Cy2), (Cy3) and/or Cy5 (Jackson Immuno-Research, West Grove, PA, USA) at 1:200 or 1:500 in PBS-0.1% Triton for 2 h at room temperature **(Table 1.1)**. All immunostaining procedures were conducted with secondary antibody only controls, which were devoid of primary antibody. There was no detectable immunostaining in the controls. Samples were subsequently counterstained with DAPI; 5μg/mL (Sigma) for 6 min.

Primary Antibody	Species	Clone	Dilution	Source
cleaved anti-caspase-3	Rabbit		1/200	Cell Signaling Technology
anti-ß-III- tubulin	Mouse	TUJ1	1/200	Covance,Berkely,CA,USA
anti-BrdU	Rat	BU1/75	1/200	Oxford Biotechnology,UK
anti-DCX	Guinea pig		1/1000	Chemicon
anti-GFAP	Rabbit		1/400	Sigma
anti-GFAP	Mouse		1/500	Dacco
anti-Ki67	Rabbit		1/500	Novocastra Laboratories, UK
anti-nestin	Mouse		1/500	BD Pharminogen, San Diego,CA,USA
Secondary Antibody				
Cy3 conjugated anti mouse IgG	Donkey		1/500	Jackson immunoresearch, USA
Cy3 conjugated anti rat IgG	Donkey		1/500	Jackson immunoresearch, USA
Cy3 conjugated anti rabbit IgG	Donkey		1/200,1/500	Jackson immunoresearch, USA
Cy3 conjugated anti guinea pig IgG	Donkey		1/500	Jackson immunoresearch, USA
Cy2 conjugated anti mouse IgG	Donkey		1/500	Jackson immunoresearch, USA
Cy2 conjugated anti rat IgG	Donkey		1/500	Jackson immunoresearch, USA
Cy2 conjugated anti rabbit IgG	Donkey		1/500	Jackson immunoresearch, USA
Cy5 conjugated anti rabbit IgG	Donkey		1/500	Jackson immunoresearch, USA

Table 2.1: Primary and secondary antibody's dilutions used in immunocytochemistry in this thesis.

2.4.8 Cell imaging and Quantification

An inverted DM IRBE microscope (Leica Microsystems UK Ltd, Milton Keynes, UK) was used to perform fluorescence cell counting. Six random fields were chosen from each well in control and treatments. The first field was imaged at 11:00 o'clock site and then the rest of the images were taken in a zigzag manner so that six different fields sampling the whole well were imaged using this systematic random sampling paradigm. The same technique was followed in imaging both controls and treated wells. The area of a 20× field was measured using a 25-μm grid graticule slide (Microbrightfield, Williston, VT, USA). Cell counting was performed on the six random 20× fields per well using the Open Lab image-capturing system version 2.1 (Improvision, Lexington, MA, USA) as previously described (Howell et al, 2003, 2005, and 2007). The six fields per well were averaged and cell counts expressed as counts per mm^2 per well. A minimum of 4-10 wells per condition per experiment across at least 2-3 experiments were analyzed for statistically significant effects.

2.4.9 Con-focal microscopy

Samples were cultured on sterile 13mm glass slides (**Section 2.4.3**) and immunostained according to the protocol (**Section 2.4.7**). Cover-slips were washed in PBS twice then once in distilled water to remove salts, and mounted 'face down' in Moviol aqueous mount (Harco, Harlow, UK). A con-focal Zeiss LSM 510 Meta system (Carl Zeiss Ltd, Oberkochen, Germany) was used for the imaging. Sequential scanning capture of each channel using appropriate filters was used before merging and analyzing the images using Zeiss LSM image browser version 3.5.0.376. For negative control cover-slips; the primary antibodies were omitted. 20x and/or 63x oil objectives were used to generate the images according to the experiment and aims.

2.4.10 Statistical analysis

Raw data from the 20× field counts was converted to cells/mm², were averaged and plotted ± SEM (standard error of the mean) and expressed as number of cells per mm² or proportions, based on a sample of four to ten wells per condition per experiment from at least 2 separate experiments. Data points were plotted using Prizm (Graph Pad software Inc., San Diego, CA, USA) and means compared using one-way Anova with Dunnett's or Bonferroni post-hoc tests, two-ways Anova with Bonferroni post-hoc test, or unpaired Student's t-test as appropriate. Statistical significance was set at $p<0.05$.

2.4.11 Phenotype primary hippocampal cells in culture

Cells were grown and maintained *in vitro* for three and five days. Cultures were then fixed on the days three and five, processed for immunocytochemistry against, nestin, GFAP and class III ß-tubulin and counterstained with DAPI as described in (**Section 2.4.7**). Double-labelling of the proliferating sub population of cells was also carried out in a separate series of experiments.

2.4.12 Quantification of Caspase-3 immunoreactive cells in culture

Control and Kainate exposed cultures were grown for 3, and 5 days, processed for immunohistochemistry against either nestin or class III ß-tubulin and counterstained with DAPI. Cells were then stained for active-caspase-3 according to the protocol mentioned in (**Section 2.4.7**). To quantify apoptotic cell death of stem/progenitor and immature neurons in primary hippocampal cultures, nestin or class III ß-tubulin expressing cells were double-labelled with active-caspase-3 and stained cells were counted as mentioned earlier in this chapter (**Section 2.4.9**). Cell death and the role of caspase-3 in primary hippocampal cell cultures will be dealt with in **Chapter 5**.

2.4.13 Labelling Index and growth fraction

Hippocampal cells were grown for 5 days *in vitro* with 4 h, 6 h, 8 h, and 12 h terminal pulses of BrdU. Cells then fixed with 4% PFA for 30 min at 4 C° before being processed and immunostained against BrdU and Ki-67 (**Section 2.4.7**). Labelling index was determined by measuring the proportion of cells that are both BrdU and Ki-67 positive over the total count of Ki-67 cells (BrdU+Ki-67/Ki-67). Growth fraction is the proportion of cells that is in the cell cycle (Ki-67/DAPI) at the time of fixation.

2.5 Results

2.5.1 Growing primary hippocampal cells for 3 and 5 days

To investigate the ability of generating and maintaining primary hippocampal cells, *in vitro* cultures were prepared from Wistar rats P 7-10 as described in (**Section 2.4.2**). For five days experiments two-thirds of growth medium and 1% antibiotic/antimycotic was changed on day three. By the end of both 3 and 5 days cells were stained for PI and DAPI. Live cells were measured by counting cells that were DAPI positive and PI negative according to the protocol (**Sections 2.4.5 and 2.4.6**). Cells were then imaged and analyzed according to sections (**2.4.8 and 2.4.10**) before fixation with 4% PFA.

We found that the total number of live cells increased significantly from 98.13 ± 10.12 cells mm^{-2} on day three to 156.9 ± 5.4 cells mm^{-2} on day five (**Figure 2.5 A**). We also showed a significant decrease in cell death. The proportion of PI positive cells decreased significantly from 19.0 ± 4.0 % on day three to 8.0 ± 1% on day five (**Figure 2.5 B**).

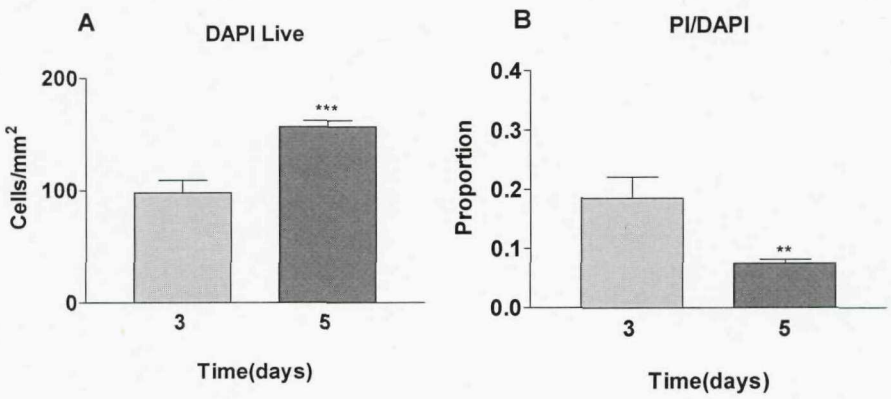


Figure 2.5 Live cell numbers increased between 3 and 5 days in culture. Cells were cultured for 3 and 5 days under standard growth conditions. A) Live cells were measured by counting DAPI positive and PI negative cells and demonstrated a significant increase in the number of live cells in cultures at day 5 when compared to day 3. B) PI/DAPI showed a significant reduction in the proportion of dead/dying cells between 3 and 5 days cultures. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 3 experiments. Data points were plotted using the Graph Pad Prism software and means compared using *simple Student's t-test*. ** $p<0.01$, *** $p<0.001$

2.5.2 Quantifying cell proliferation and survival over time in hippocampal cell cultures

To study the effects of time, hippocampal cells from postnatal day 7-10 Wistar rats were cultured between 4 h and 7 days *in vitro*. In order to assess cell proliferation, cells were pulsed for the terminal 4 h with a final concentration of 20μM BrdU on days 0, 1, 3, 5, and 7. Cells were also stained for PI and DAPI at the end of each time point (4 h, day 1, day 3, day 5, and day 7) to measure the total number of live cells and the proportion of dead/dying cells in time.

This series of experiments demonstrated a significant increase in the total number of cells on days five and seven (**Figure 2.6 A**) accompanied with a significant drop in the proportion of dead/dying cells (PI/DAPI) (**Figure 2.6 C**). We found an increase not only in the numbers of proliferating cells but also in the proportion of proliferating hippocampal cells on days three, five, and seven (**Figure 2.6 B and D**). Interestingly, a significant increase in cell proliferation and a significant decrease in cell death were observed between the days three and five (**Figure 2.6 B, C, and D**).

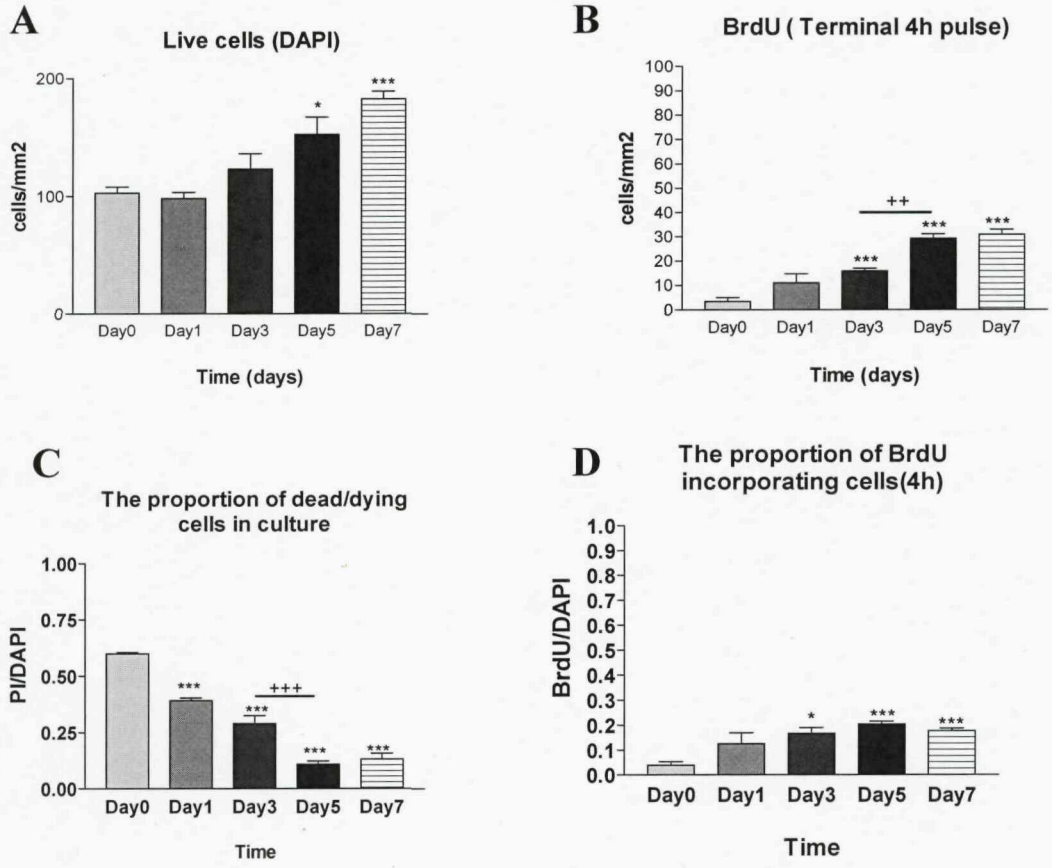


Figure 2.6 Cell viability and proliferation in cultures over 7 days *in vitro*. Cells were cultured for 4 h, 1 day, 3 day, 5 day, and 7 day and maintained *in vitro* under standard growth conditions. Terminal 4 h pulse of a final concentration of 20μM BrdU was applied to all cells. PI and DAPI were also added to cells in culture. We then measured the number of live cells (DAPI positive and PI negative) as well as dead/dying (DAPI positive and PI positive) cells. A) Live cells were measured by counting DAPI positive and PI negative cells at the end of each time point showing an increase in the number of live cells at days 5 and 7 when compared to day 0. B) There was an increase in the numbers of BrdU incorporated cells in the last 4 h before cell fixation at days 3, 5 and 7 when compared to day 0 and between the days 3 and 5. C) PI/DAPI showed the proportion of dead/dying cells with time which decreased significantly over time. D) The proportion of proliferating cells was quantified at each time point examined and demonstrated an increase in the days 3, 5, and 7 when compared to day 0. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least two experiments. Data points were plotted using the Graph Pad Prism software and means compared using One Way Anova with Dunnett's post hoc test .* $p<0.05$,*** $p<0.001$ when comparing each time point with 0 day. ++ $P<0.01$, +++ $P<0.001$ when comparing two time points with each other.

2.5.3 Cell phenotype in primary hippocampal 5 day cultures

To characterize the phenotype of cells that were produced in our hippocampal monolayer cell cultures, we grew cultures under control conditions for 5 days and immunostained them against nestin (stem/progenitor marker), class III ß tubulin or TuJ1, (expressed in immature neurons), and GFAP (astrocytic marker) as detailed in **Section 2.4.7**. Micrographs are shown in **Figure 2.11**.

We report a heterogeneous population of cells in primary hippocampal cultures where 59.1% ± 2.1% of cells expressed nestin (stem/progenitor marker), 23.1% ± 1.9% expressed TuJ1, and 50.2% ± 2.3% were GFAP positive (**Figure 2.7**).

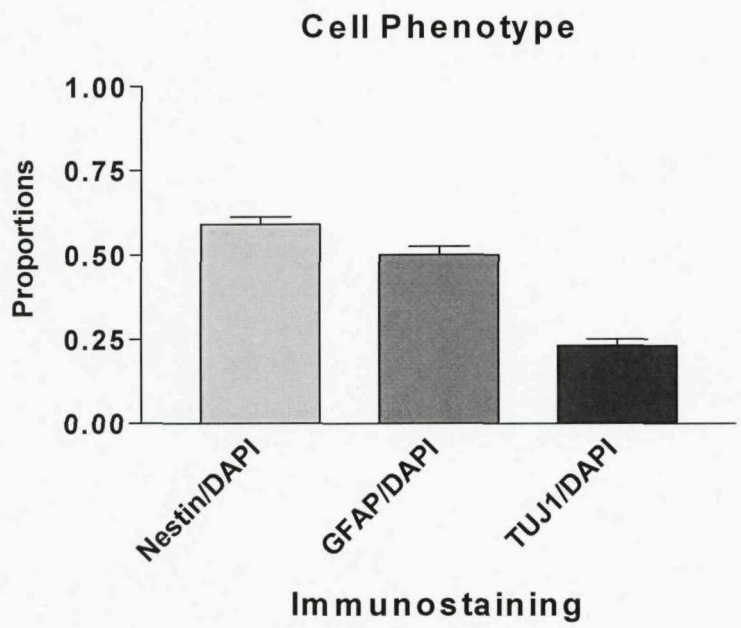


Figure 2.7 Cell phenotypes at 5 days in culture. Cells were cultured for 5 days under standard growth conditions. On day 5 they were fixed and stained against the stem/progenitor marker (nestin), immature neuronal expressing marker (TuJ1), and astrocytic marker (GFAP). Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 3 separate experiments.

2.5.4 The proportions of both nestin and TuJ1 did not change between three and five days in culture

To study progress and fate determination of hippocampal cells in cultures we grew cells for 3 and 5 days under standard growth conditions. For 5 days cultures we replaced two-thirds of the growth medium with fresh medium on day three. At the end of each time point (3 and 5 days) cells were fixed with 4% PFA and immunostained against the stem/progenitor marker (nestin) and the immature neuronal marker TuJ1.

We observed no significant changes in the proportion of stem/progenitor cells and TuJ1 expressing cells between 3 and 5 days under standard growth condition **(Figure 2.8 A and B)**.

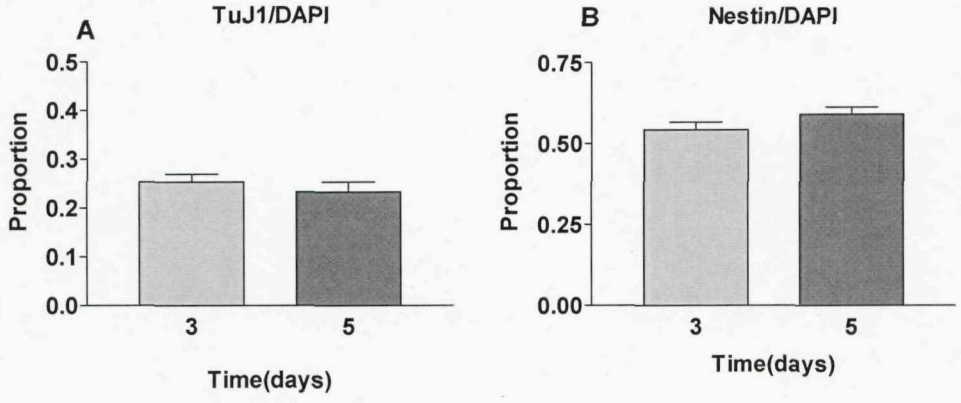


Figure 2.8 The proportions of both nestin positive cells and TuJ1 expressing cells did not change between three and five days in culture. Cells were grown *in vitro* for 3 and 5 days. After cell fixation, cultures were immunostained against either TuJ1 or nestin. We measured the proportion of nestin positive cells and TuJ1 expressing cells at 3 and 5 DIV. A) There was no change in the proportion of TuJ1 expressing cells between days 3 and 5. B) There was no change in the proportion of nestin positive cells between days 3 and 5 *in vitro*. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 3 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using simple Student's t-test.

2.5.5 NPY increased the total number of cells in cultures at concentrations as low as 10pM over 5 days

To further investigate the validity of our primary hippocampal cell cultures model we plated 250 µl of cell suspension (100,000 live cells/ml from P7-10 hippocampus) into 24-well plates. Two hours after plating the cultures were replenished with fresh medium (control conditions) or fresh medium containing 0.01nM NPY, 0.1nM NPY, 100 nM NPY, and 1 µM NPY. Two-thirds of the growth medium with/without enrichment was replaced with fresh medium on day 3. On day 5 cells were fixed with 4% PFA and stained for the nuclear marker DAPI.

We observed a significant increase in the total number of cells under NPY conditions including concentrations as low as 10 pM (**Figure 2.9**).

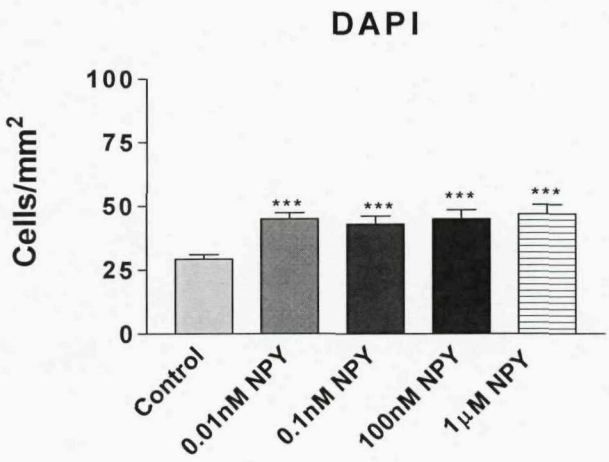


Figure 2.9 NPY increased cell counts at concentrations as low as 10 pM after 5 days in culture. Cells were cultured for 5 days in the presence and absence of 0.01nM NPY, 0.1nM NPY, 100 nM NPY, and 1µM NPY. On day 5 cells were fixed with 4% PFA and stained for DAPI. NPY significantly increased the total number of cells in culture. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment format least two separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using One Way Anova with Dunnett's post hoc test. *** $p<0.001$

2.5.6 FGF-2 increased the total number of cells and cell proliferation

To study the effects of the peptide growth factor FGF-2, hippocampal cells from postnatal 7-10 day Wistar pups were cultured and maintained *in vitro* for three days. Two hours after cell plating, growth medium was replaced with either fresh growth medium for cells growing under standard growth conditions or fresh growth medium enriched with 20 ng/ml FGF-2. Between days 2 and 3 all cells (treated and control) were given a terminal 24 h pulse of a final concentration of 20μM BrdU to validate our methods.

We showed a significant increase in both the total number (**Figure 2.10 A**) and number of BrdU incorporated cells (**Figure 2.10 B**) when comparing control conditions with 20 ng/ml FGF-2 enriched conditions. Moreover, a proportional increase in BrdU incorporated cells was demonstrated as well (**Figure 2.10 C**) implying that FGF-2 increased cell proliferation over three days in culture.

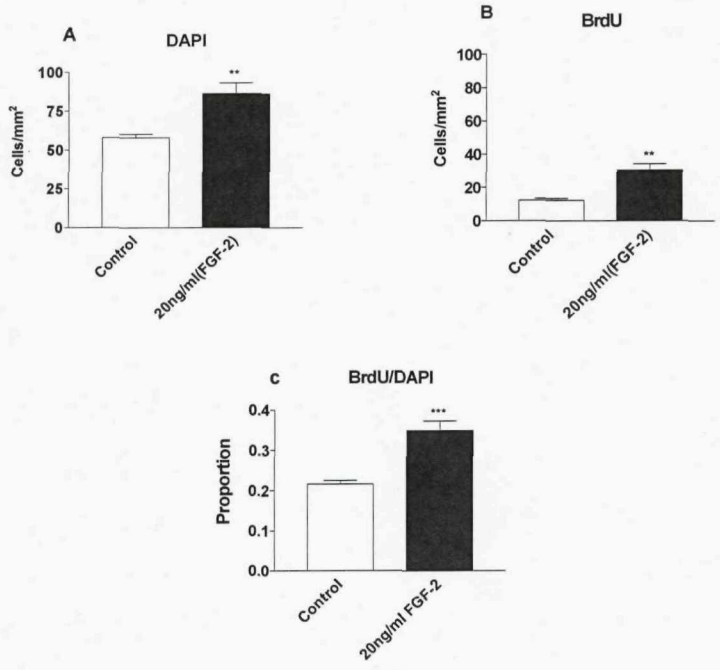


Figure 2.10 FGF-2 increased cell proliferation over three days in culture. Cells were grown for 3 days in the presence and absence of 20ng/ml FGF-2. On day two, a final concentration of 20μM BrdU was applied to all cells for the last 24 h before cell fixation. Cells were then immunostained for BrdU and counterstained for the nuclear marker DAPI. We measured the total number of cells (DAPI), the number of BrdU incorporated cells, and the proportion of BrdU labelled cells. A) There was a significant increase in the total cell counts after 3 DIV of FGF-2 treatments. B) FGF-2 increased the numbers of BrdU incorporated cells in the last 24 h when compared to control conditions. C) There was a proportional increase in cells in the S-phase of the cell cycle under FGF-2 treatment when compared to control conditions. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least two separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using simple Student's *t*-test. ** $p<0.01$, *** $p<0.001$

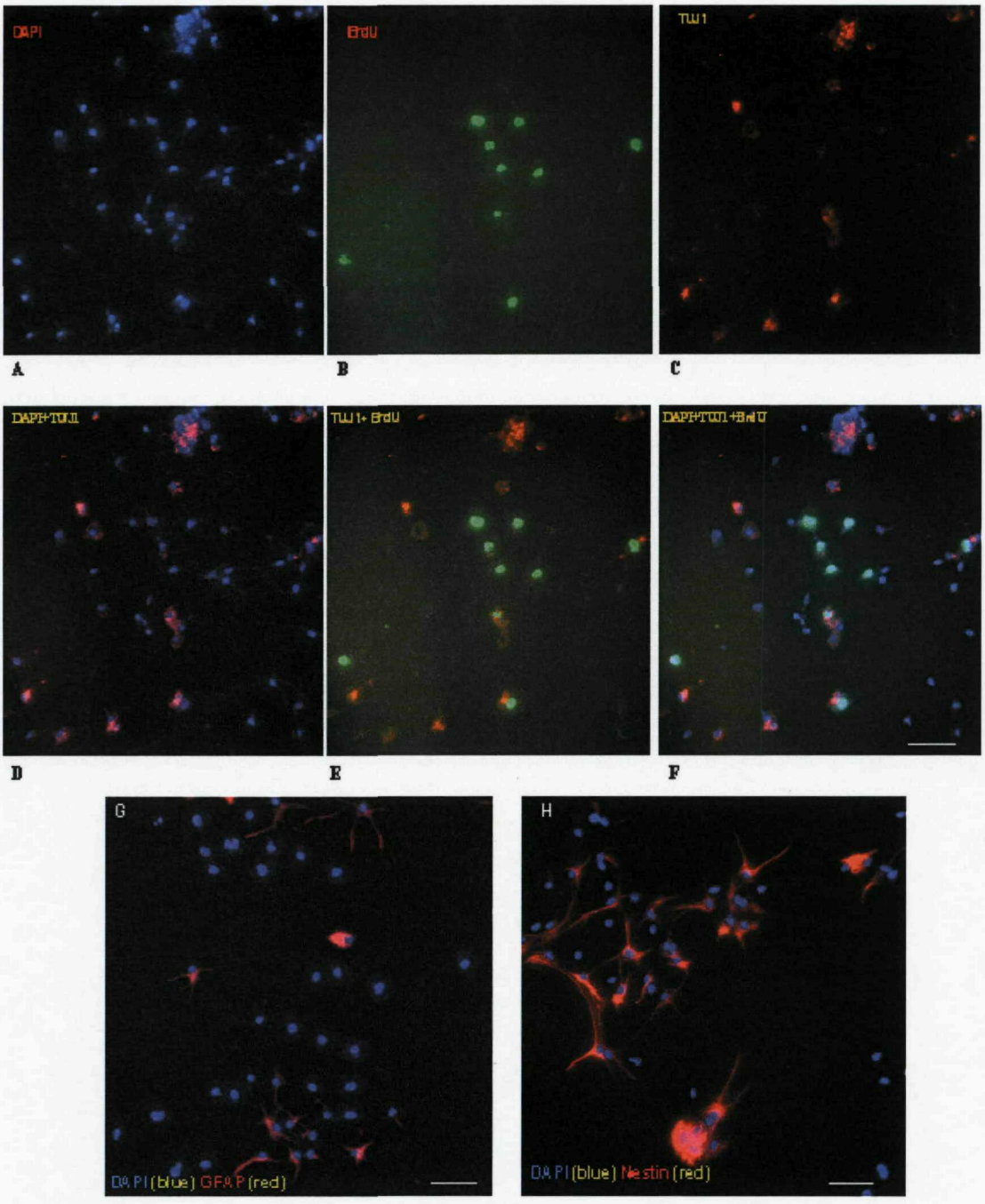


Figure 2.11 Hippocampal cell cultures contain different cell types. Cells were grown for 5 days under standard growth conditions (Neurobasal A/B27/and 0.5mM Glutamine). Two-thirds of the growth medium was replenished with fresh medium on day 3. 4 h terminal pulse of a final concentration of 20μM BrdU was given to all cells in order to evaluate cell proliferation. Fixation for 30 minutes with 4% PFA was done on day 5 before subsequent immunocytochemistry. Primary specific anti-sera against nestin (stem/progenitor marker), neuronal expressing class III ß tubulin (TuJ1), BrdU incorporating cells, and the

astrocytic marker GFAP, were detected with fluorescently linked secondary antibodies. Counterstaining for the nuclear marker DAPI was carried out at the end of immunocytochemistry. Images were captured with 20x objective on an inverted DM IRBE microscope (Leica Microsystems UK Ltd., Milton Keynes, UK) and the Open lab capturing system version 2.1 (Improvision Inc., Lexington, USA). A) This image shows DAPI (blue) which stains the nuclei of all cells in culture. B) BrdU (green) incorporates into the nuclei of cells in S-phase of the cell cycle. C) TuJ1 or class III ß tubulin expressing cells identify a neuronal phenotype. D) Labelling the sub-population of TuJ1 cells (red) among the whole cell count, DAPI (blue), in primary hippocampal cell cultures. E) Proliferating neuroblasts are identified by (BrdU – green-) TuJ1 (red) immunostaining. F) TuJ1 (red), BrdU (green), and DAPI (blue) stained cells in culture. G) GFAP (red), an astrocytic marker, expression in a sub-population of cells. H) This image demonstrates stem/progenitor (nestin-red-) expressing cells.

2.5.7 The effects of washing on fixed cells in culture

In this set of experiments we aimed at investigating the effects of washing on the number of pyknotic DAPI positive cells in culture. Cells were grown for 5 days under the standard growth conditions. Two-thirds of the growth medium was changed on day three. DAPI was applied to cells in culture according to the protocol detailed in (**Section 2.4.5**). This followed by imaging live cells as described in (**Section 2.4.6**). Cells were then fixed and washed in PBS before being stained with DAPI and washed 3 times before being imaged again.

The total number of cells as indicated by DAPI positive cells at five days was 141.8 ± 4.5 cells/mm^2 while cells were still alive. This number of cells decreased significantly to 106.6 ± 2.9 cells/mm^2 after cell fixation and washing (**Figure 2.12 A**). The number of pyknotic DAPI cells (dead) before cell fixation was 31.5 ± 1.7 cells/mm^2. However, only 13.02 ± 1.3 cells/mm^2 were found after cell fixation and washing steps (**Figure 2.12 B**).

Figure 2.13 demonstrates DAPI stained cells with some pyknotic nuclear staining.

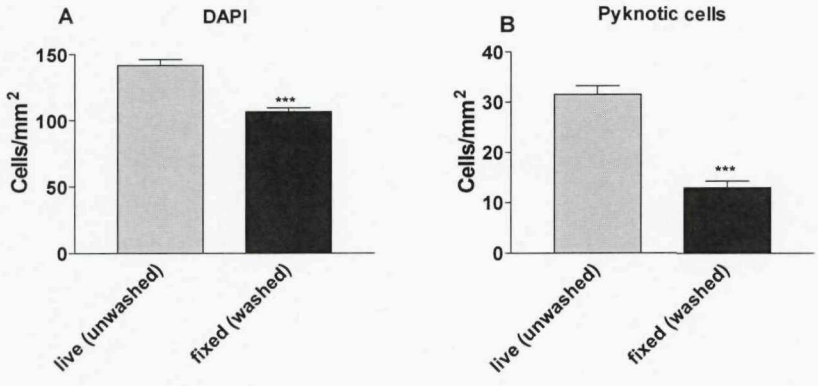


Figure 2.12 Washing decreased the number of pyknotic cells in culture. Cells were grown for 5 days under the standard growth conditions. On day five DAPI was applied to cells followed by subsequent imaging before cell fixation with 4% PFA. We measured the total number of cells live and after cell fixation. We also measured the numbers of pyknotic cells before and after cell fixation and washing. A) There was a significant decrease in the total number of cells (DAPI positive) after cell fixation and washing. B) The number of pyknotic DAPI cells dropped significantly after cell fixation and washing. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least two separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using simple Student's t-test. *** $p<0.001$

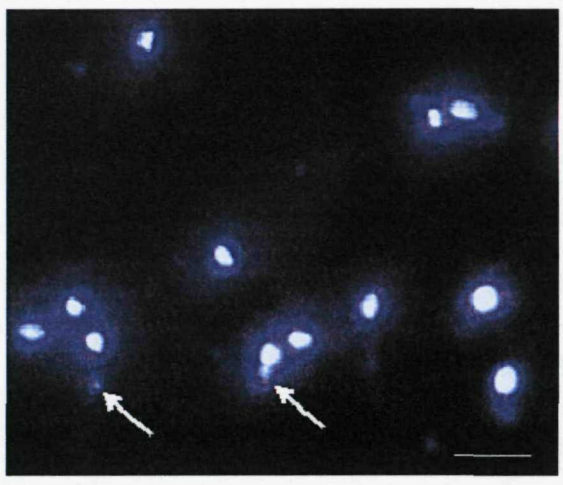


Figure 2.13 DAPI stained cells showing some pyknotic nuclear staining. Cells were cultured for 5 days under the standard growth conditions. Cells were then imaged live for DAPI staining before being fixed, washed and imaged again for DAPI. There was a significant drop in the total number of DAPI cells as well as the number of pyknotic DAPI cells when comparing cells that were imaged live to cells that were imaged after cell fixation and washing. The white arrows show pyknotic DAPI positive cell. Scale bar = 20μm.

2.6 Discussion

2.6.1 Survival and death in primary hippocampal cultures

This series of experiments validated our ability to generate primary hippocampal cultures and study cell survival as well as cell proliferation. The results are consistent with previous work on this culture system (Howell, Scharfman et al. 2003; Howell, Doyle et al. 2005). Our cultures also demonstrate neurogenesis consistent with other studies where new neurons were shown to be successfully cultured from the adult rat hippocampus (Brewer 1997), postnatal hippocampus (Howell, Scharfman et al. 2003; Howell, Doyle et al. 2005; Howell, Silva et al. 2007), sub ventricular zone (SVZ) (Doetsch, Caille et al. 1999; Brazel, Nunez et al. 2005), and even the from the adult cortical cultures (Brewer and Torricelli 2007). We have further demonstrated the proliferation of stem/progenitor cells as well as neuroblasts (Howell, Silva et al. 2007) that underlies neurogenesis.

We report a significant rise in the total number of cells over 5 and 7 days in culture when compared to day 0 (**Section 2.5.2**). This finding is accompanied by a significant drop in the proportion of dead/dying (PI/DAPI) cells in cultures over time (**Section 2.5.2**). In spite of the fact that we managed the initial harvesting and plating of viable cells in less than 2 hours, we found that approximately 59% of our cells are PI positive after 4 h in culture, decreasing significantly to 15% by day 7. This large percentage of PI stained cells at day 0 can be explained by the trauma and injury that cells might have sustained while being processed and cultured. In agreement with our results (**Sections 2.5.1 & 2.5.2**), cell death as measured by PI staining decreased significantly over time in a study done on hippocampal organotypic slices (Pozzo Miller, Mahanty et al. 1994). Ongoing cell death may be of differentiated cells and or cells that have exited the cell cycle from ongoing proliferation. This question will be dealt with in **Chapter five.**

Once studying cell death; investigators tend to label cells after fixation with one of the death markers like caspase-3 (Bauer and Patterson 2005), TUNEL (Shirai, Mizui et al. 2006), and PARP. Although the undeniable usefulness of these methods, their use does not identify dead/dying cells in live cultures which may be washed off during

immunohistochemical processing. Interestingly, in **section 2.5.7** we have determined that cell fixation and washing affect the total number of DAPI positive cells as well as the number of pyknotic DAPI cells. These results imply an effect of washing steps which may underestimate the number of dead cells in culture. Therefore, we have developed a protocol using PI and DAPI to identify those cells that die and those who stay alive in a live *in vitro* model. This method accurately enables us to identify the total number of live cells without underestimating the number of dead cells due to washing steps. In chapter five I have modified this protocol by the introduction of other markers like MitoTrackers green and orange (**see Chapter five**).

2.6.2 Hippocampal cells proliferate in cultures

BrdU immunohistochemistry is considered a powerful tool to study the development of the nervous system, and to prove that neurogenesis occurs in the mammalian brain, including humans. However, the use of BrdU has some pitfalls and drawbacks. DNA repair is a normally occurring process in the life of the cell (Taupin 2007). Because DNA repair involves DNA synthesis and because BrdU is a marker of DNA synthesis as it is incorporated into DNA during the S-phase of the cell cycle, there is a concern that BrdU immunohistochemistry may not only detect proliferating cells in the brain, but also cells undergoing DNA repair. In spite of the fact that DNA repair was reported using immunohistochemistry *in vitro* on irradiated fibroblasts, it requires a 24-48 hours exposure to BrdU (Beisker and Hittelman 1988; Selden, Dolbeare et al. 1993; Selden, Dolbeare et al. 1994). In contrast, Palmer et al 2000 used BrdU to label adult neurogenesis and did not detect radiation-induced DNA repair in fibroblasts in cultures (Palmer, Willhoite et al. 2000). Previous *in vivo* studies reported a decline in newly generated neurons as well as BrdU in neurogenic zones including the ventricular wall and the DG in rodents after fractionated brain irradiation (Parent, Tada et al. 1999; Santarelli, Saxe et al. 2003). These data show that BrdU staining may not reflect DNA repair in most studies. Another important pitfall that we might encounter is whether dying postmitotic neurons could undergo abortive cell cycle reentry and incorporate thymidyne analogs. It has been shown that BrdU was not detected in TUNEL positive

cells in three mice models displaying concomitant stimulation of apoptosis and neurogenic proliferation, olfactory bulbectomy, brain irradiation, and kainic acid induced seizures, suggesting that induction of cell death in experimental models is not efficient in inducing cell cycle reactivation of postmitotic brains (Bauer and Patterson 2005). It has been demonstrated that the cell cycle time for progenitor cells of the young adult rat brain is 25 hours (Cameron and McKay 2001), whilst in mice it was calculated to be 16 hours (Nowakowski, Lewin et al. 1989). Therefore, applying BrdU for the last 4 h (less than the length of 1 cell cycle) to cells in culture will only label cells that enter S-phase of the cell cycle and thus accurately determine cell proliferation.

Previously, growth and trophic factors were used to assist cells to grow in cultures (Lowenstein and Arsenault 1996; Brewer 1997; Brewer, Espinosa et al. 2001); here we show a significant increase in proliferation under standard growth conditions without the use of growth or trophic factors. We have demonstrated a significant rise in proliferating cells by days 3, 5, and 7 when comparing the number and proportion of BrdU incorporating cells with days 0 and 1 (**Section 2.5.2**). We also showed a significant increase between the days 3 and 5 (**Section 2.5.2**). Our data suggest a significant time point, day 3, for maximal cells proliferation *in vitro* under control conditions, which gives us evidence for targeting cells at this time point when investigating the effects of mitotic factors on hippocampal cells.

2.6.3 Cell phenotype in culture

The study of primary cell cultures allows identification of sub-populations of cells that both contribute to and form the neurogenic niche. It has been demonstrated that in cell cultures a mixture of cell populations are generated from the dentate gyrus (Lowenstein and Arsenault 1996), and the whole postnatal hippocampus (Howell, Scharfman et al. 2003; Howell, Doyle et al. 2005). Immunohistochemistry allows identification of not only the proliferating cells but also stem/progenitor cells, immature neurons, and astrocytes.

We stained cells against the stem/progenitor specific protein nestin, neuron specific protein class III ß-tubulin or TuJ1, and the astrocytic marker glial fibrillary acidic

protein (GFAP). We consistently found 50-55 % of our cells expressing the stem/progenitor marker (nestin) after 5 days *in vitro* (**Section 2.5.3**). GFAP stained cells comprise approximately half of the cell counts (**Section 2.5.3**). While we detected 25% of cells to be TuJ1 expressed (**Section 2.5.3**). This is consistent with our previous work (Howell, Scharfman et al. 2003; Howell, Silva et al. 2007). In **Section 2.5.4**, we showed no significant changes in the proportion of TuJ1 and nestin expressing cells between 3 and 5 days which indicates that these sub-populations of cells in culture are growing at the same rate with time. The data in **Section 2.5.3** suggest co–expression of GFAP and nestin in a group of cells and raises questions about the proliferation and fate determination of sub-groups of cells (e.g. nestin positive and GFAP negative) which requires further investigations (**see Chapter three**). In agreement with our results, it has been shown that SGZ atsrocyte-like stem cells express GFAP and give rise to neurons (Seri, Garcia-Verdugo et al. 2004). We therefore, in chapter three, will double-label cells in culture for both GFAP and nestin and study the effects of Kainate on each sub-population of cells.

2.6.4 *In vitro* enrichment for hippocampal cells

In this **Chapter**, we have validated the ability of our cultures to expand in the absence of growth and trophic factors. We, in **Section 2.5.6**, further confirm that hippocampal cells respond to the mitogenic factor FGF-2. We reported a significant increase in the total cell counts and BrdU incorporated cells as well as in the proportion of BrdU incorporated cells after enriching the growth conditions with 20ng/ml FGF-2 consistent with increased cell proliferation. The mitogenic effect of FGF-2 on hippocampal precursor cells *in vitro* has been well described (Palmer, Takahashi et al. 1997). It has been reported to up-regulate dentate neurogenesis *in vitro* (Lowenstein and Arsenault 1996), enhances not only the production of SVZ progenitors, but also their migration to the olfactory bulb (Kuhn, Winkler et al. 1997), and even has been shown to enhance dendrite growth in the dentate gyrus (Rai, Hattiangady et al. 2007).

Neuropeptide Y (NPY) is proliferative for precursor cells. It has been reported to enhance neuronal precursor cell proliferation in the postnatal olfactory epithelium

(Hansel, Eipper et al. 2001). In addition, it also increased the proliferation of hippocampal precursors (Howell, Scharfman et al. 2003) as well as the dentate gyrus precursors (Howell, Doyle et al. 2005). In this **Chapter** we confirmed an increase in the total number of cells under the effects of a very low concentration of Neuropeptide Y (NPY) (0.01 nM) when comparing NPY treated cells with control (**Section 2.5.5**), again validating my cultures and supporting their use to investigate the mechanisms of hippocampal neurogenesis.

2.7 Conclusion

This chapter demonstrates a robust methodology for studying and investigating the mechanisms underlying hippocampal neurogenesis *in vitro*. We have demonstrated diminishing cell death and increasing cell proliferation over the first 7 days in culture and have identified days 3 & 5 *in vitro* as suitable time-points for studying cell proliferation. I have validated my cultures by demonstrating significant effects for NPY and FGF-2 on hippocampal cell proliferation, and demonstrating a heterogeneous population of cells in agreement with previous studies (Lowenstein and Arsenault 1996; Brewer 1997; Howell, Scharfman et al. 2003). In addition, I have developed a new protocol to quantify cell death under live culture conditions allowing a more relevant and accurate estimation of cell death and trophism to be made.

These results and paradigms will enable me to investigate the direct effects of Kainate on stem / precursor cells *in vitro*, which will form the basis of subsequent chapters in this work.

Chapter Three

Examining the proliferative effects of Kainate on hippocampal precursor cells

3.1 Introduction:

Neural stem cells exist not only in the developing mammalian nervous system but also in the adult nervous system of all mammalian organisms, including humans (Gage 2000). Although the number of adult neural stem cell niches is limited, the brain regions to which their progeny migrate in order to differentiate and integrate remain unsettled (Gage 2000). The mechanisms that control endogenous stem cells are poorly understood, both under physiological conditions and after brain injury. A potential role of stem cells in repair includes the activation of endogenous cells to provide "self repair". However, before the full potential of neural stem cells can be realized, we need to understand what regulates their proliferation, as well as the various pathways of differentiation available to their progeny.

It has been demonstrated that neurogenesis is increased after seizures in both the SGZ and rostral SVZ (Bengzon, Kokaia et al. 1997; Parent, Yu et al. 1997; Gray and Sundstrom 1998; Gray, May et al. 2002; Parent 2002). However, the mechanisms whereby stem/precursor cells contribution to seizure-induced neurogenesis are incompletely understood. Kainate is often used to model hippocampal damage associated with status epilepticus (Nadler 1981; Ben-Ari 1985; Coyle 1987; Ben-Ari and Cossart 2000) and to enhance neurogenesis; however, the mechanisms that mediate the effects of Kainate on hippocampal stem / progenitor cells are not clear. As such, there are many questions regarding the mechanisms of proliferation or 'birth' of stem/progenitor cells, the fate choice of their progeny, and their proper functional integration, that have yet to be investigated and addressed after Kainate treatment.

The ability to isolate, culture, and maintain neural stem cells *in vitro* would provide a suitable system to examine the direct effects of Kainate on hippocampal stem cells and thereafter help unveil how stem cells behave in response to a convulsant. Although it has been recently suggested that glutamate, in a model of stroke-induced epilepsy in primary hippocampal neuronal cultures, induced-neuronal injury and provoked spontaneous, recurrent, epileptiform discharges in hippocampal neurons, it is still unclear if stem/progenitor cells and neural precursors seize *in vitro* (DeLorenzo, Sun et al. 2007). Investigating the precise mechanisms underlying the effects of Kainate on different

precursor cell types is only possible if suitable stem/progenitor markers are available. However, in cultures, double labelling of cells against stem/progenitor markers using the intermediate filament protein nestin and GFAP which labels radial glial-like precursor cells (Seri, Garcia-Verdugo et al. 2004) and transiently amplifying precursor cells (nestin + but GFAP-), would pave the way to investigating the different sub-populations of progenitor cells and their response to Kainate treatment. Moreover, in the primary hippocampal cell culture system, many of the cells that are cultured are key parts of the neurogenic niche and the system is therefore of some relevance to the *in vivo* niche and its response to injury.

In the previous chapter we have demonstrated that the primary hippocampal cell culture model is a suitable paradigm for quantifying proliferation using BrdU and Ki-67 immunohistochemistry. We also have demonstrated the ability to measure total cell counts, and the phenotype of cells in culture allowing us to determine effects on net neurogenesis. We, therefore, decided to elucidate neurogenesis mechanisms by investigating thoroughly the effects of Kainate on the birth of different precursor cell types and neuroblasts in primary hippocampal cell cultures.

3.1.1 Kainate, Seizures, and neurogenesis

Kainate, (2-carboxy-4-isopropenyl-pyrrolidin-3-ylacetic acid) is an acidic pyrolidine isolated from seaweed (Coyle 1987). It has been used as an anthelmintic drug for removal of worms in the gut. Kainate is an agonist for a subtype of ionotropic glutamate receptor (AMPA/Kainate) with excitatory actions on neurons in the vertebrate CNS and invertebrate neuromuscular junction (Nadler 1979). Kainate is often used to model temporal lobe epilepsy and neuronal degeneration in rodents (Nadler 1981; Ben-Ari 1985; Coyle 1987; Ben-Ari and Cossart 2000). Systemic or intracerebral injections of Kainate cause epileptiform seizures in the hippocampus. These seizures propagate to other structures in the limbic formation and are followed by a pattern of cell loss similar to that observed in temporal lobe epilepsy patients with hippocampal sclerosis (Nadler 1981; Ben-Ari 1985; Coyle 1987; Ben-Ari and Cossart 2000). Moreover, Kainate administration in rat models is epileptogenic as it induces ongoing convulsions,

degeneration of cornu ammonis (CA) neurons and hyperexcitability of surviving neurons (Nadler 1981; Ben-Ari 2001). Furthermore, *in vivo* studies have demonstrated that status epilepticus (SE) induced-seizure increases hippocampal neurogenesis in adult rats (Parent, Yu et al. 1997; Gray and Sundstrom 1998; Nakagawa, Aimi et al. 2000; Gray, May et al. 2002), and gliogenesis, production of new glia, in the caudal sub ventricular zone (Parent, von dem Bussche et al. 2006). Seizure-induced hippocampal progenitor proliferation in the subgranular zone (SGZ) of the dentate gyrus does not diminish between juvenile and adult rats even though baseline proliferation does (Gray, May et al. 2002), suggesting a latent retention of proliferative potential in response to injury. However, increased dentate gyrus neurogenesis may contribute to the sequels that follows status epilepticus (SE) such as epileptogenesis, as newly born neurons may be further abnormally integrated and contribute to seizure generation (Parent, Elliott et al. 2006; Jessberger, Zhao et al. 2007). Jessberger and colleagues have shown that neurogenesis is morphologically abnormal with aberrant connectivity after SE and remains so for at least one year after the SE (Jessberger, Zhao et al. 2007). They have also shown an association between the prevention of seizure induced neurogenesis by valproate and the generation of the epileptic state with memory impairment (Jessberger, Nakashima et al. 2007), hinting at a role for seizure-induced neurogenesis in epileptogenesis and cognitive impairment associated with hippocampal sclerosis. Shetty and co-workers have reported a chronic reduction in neurogenesis in the dentate gyrus after SE, where precursor cell proliferation is maintained but the proportion that adopt a neuronal phenotype diminishes dramatically (Hattiangady, Rao et al. 2004). The underlying mechanisms of seizures-induced neurogenesis and/or gliogenesis are not fully understood, but they begin acutely after SE and clearly involve early changes in precursor cell proliferation. There is clearly a need to elucidate the mechanisms underlying seizure-induced precursor cell proliferation to understand how these changes occur and to intervene to prevent or reverse them if they indeed have the pathological long-term consequences of epileptogenesis and impaired hippocampal dependant learning.

3.1.2 Neurotransmitters and neurogenesis

Neurotransmitters play a significant role in regulating neurogenesis and mood disorders. The general theory is that neurotransmitters may have a role in controlling precursor cell proliferation and differentiation, as neuronal activity is a major determinant of neurogenesis. Furthermore, positive correlations between the number of new neurons and learning performance would suggest a relationship between neurogenesis and learning (Leuner, Gould et al. 2006). Indeed, certain learning tasks have been reported to be hippocampal-dependent such as a trace conditioning, during which animals learn to associate events that are distant from one another, and allocentric spatial memory; a complex process which involves the creation of an internal map of the surrounding environments (Bangasser, Waxler et al. 2006; Leuner, Gould et al. 2006). It is generally thought that the hippocampus facilitates learning and memory through its unique synaptic plasticity which contributes to the acquisition and retention of memories (Martin, Grimwood et al. 2000). This may indicate that hippocampal neural plasticity may be mediated by neurogenesis; which may be the mechanism underlying learning and memory in hippocampal dependent tasks. In addition, a substantial number of patients with chronic temporal lobe epilepsy, a neurodegenerative disorder which damages the hippocampus, exhibit severe progressive memory and cognitive impairments (Helmstaedter, Kurthen et al. 2003). It is therefore important to identify how neurotransmitters control neurogenesis and how this is disturbed after acute seizures, especially after status epilepticus, as this often predates the development of the epileptic state and is an indicator of future cognitive decline.

The amino acid neurotransmitters, GABA and glutamate, are of particular interest. They are abundantly present not only in the embryonic brain but also in the adult brain. These neurotransmitters can have either primary excitatory or inhibitory roles in adulthood. GABA is excitatory during brain development and in the early postnatal period (Cameron, Hazel et al. 1998) after which it becomes inhibitory. It has been demonstrated that, in the ventricular zone of rats' neocortex, GABA acts on $GABA_A$ receptors (LoTurco, Owens et al. 1995). GABA induces an inward current at resting membrane potentials, presumably owing to a high intracellular Cl^- concentration maintained by

furosemide-sensitive Cl^- transport. GABA through this mechanism depolarizes neurons activating Ca^{2+} channels. Moreover, GABA decreases the number of embryonic cortical cells synthesizing DNA. In addition, depolarization with K^+ similarly decreases DNA synthesis, indicating that neurotransmitters act via membrane depolarization. Indeed, using cortical explant system from the embryonic rats LoTurco et al 1995 have reported that GABA decreased proliferation of cortical progenitors as indicated by BrdU incorporation (LoTurco, Owens et al. 1995). This group also demonstrated that the decrease in proliferation is $GABA_A$ mediated and the effect is likely to be directly on dividing cells. Likewise, in embryonic dissociated cortical culture system, $GABA_A$ was found to have a similar effect (Antonopoulos, Pappas et al. 1997). $GABA_A$ receptors carry primarily Cl^-, and the direction of flow across the membrane largely determines whether GABA depolarizes or hyperpolarizes target cells (Ge, Pradhan et al. 2007). Two major Cl^- transporters that control Cl^- homeostasis in neurons have been identified (Ben-Ari 2002; Owens and Kriegstein 2002). The first one is Na^+-K^+- Cl^- co-transporter (a Cl^- accumulator) which is mainly expressed during embryonic development and down regulated during maturation. The second one is K^+- Cl^- co-transporter (a Cl^- exporter) which is up-regulated during development and neuronal maturation. Interestingly, similar expression patterns of Cl^- co-transporters occur during adult neurogenesis (Ge, Goh et al. 2006). Importantly, in the adult SGZ, radial-glial-like astrocytes serve as neural precursors and give rise to neuroblasts (Alvarez-Buylla and Lim 2004). Electrophysiological studies of retrovirally labelled neural precursor cells and immature neurons in the SGZ have shown that precursor cells, within 2-3 weeks of their birth, are depolarized by GABA because of high Cl^- (Ge, Goh et al. 2006). Indeed Ge et al have demonstrated that immature cells exhibit a depolarizing tonic response to GABA. This may suggest that ambient GABA could come from local interneurons. Indeed, in an elegant study, Walker et al have shown that synaptic signalling is mediated via the release of GABA from mossy fibers terminals (Walker, Ruiz et al. 2001). However, the role of GABA activation in regulating SGZ precursor cell proliferation and/or survival remains to be determined.

Interestingly, a range of neuropeptides including Neuropeptide Y, somatostatin, and vasoactive intestinal polypeptide were reported to be expressed on GABAregic

interneurons of the adult mouse hippocampus (Jinno and Kosaka 2003). In our lab, it has been shown that NPY is proliferative for postnatal hippocampal precursor cells and their neuronal progeny cells, and the neuroproliferative effect is mediated via Y1 receptor subtype (Howell, Scharfman et al. 2003). In line with this, NPY receptor knock-out mice have significantly reduced cell proliferation, specifically in the DG (Howell, Doyle et al. 2005). In addition, it has been demonstrated that NPY has an effect on DG precursor cells after status epilepticus (Howell, Silva et al. 2007), and also NPY-responsive precursors were identified in the caudal subventricular zone (cSVZ) and subcallosal zone (SCZ), where seizures modulate glial precursors. These studies imply a significant role for NPY, likely released from GABA interneurons, in regulating hippocampal neurogenesis.

There have been a number of studies unfolding the role of dopaminergeic, adrenergic, serotonergic, and cholinergic systems on development and neurogenesis (for review see (Cameron, Hazel et al. 1998; Abrous, Koehl et al. 2005). For example, the chronic use of antidepressant drugs that act as serotonin (5-hydroxytryptamine) reuptake inhibitors increase cell proliferation in the rodent dentate gyrus which implies a role for this transmitter in controlling neurogenesis (Malberg, Eisch et al. 2000). It was also shown that dentate gyrus and SVZ proliferation was blocked when either serotonin synthesis was inhibited or serotonin neurotoxin was administered (Brezun and Daszuta 1999). Importantly, Santarelli pointed out that antidepressant–induced dentate gyrus neurogenesis in mice plays a behavioural role, and disrupting antidepressant-induced neurogenesis blocks behavioural responses to antidepressants which suggest a functional role for neurogenesis in the behavioural effects of antidepressants (Santarelli, Saxe et al. 2003). Nicotine was also reported to decrease proliferation in embryonic and postnatal cortical explants (for review see (Cameron, Hazel et al. 1998)).

Glutamate receptors convey most of the excitatory synaptic transmission in the mammalian central nervous system (Steinhauser and Gallo 1996). Many studies investigated the role of glutamate and its four major receptor types NMDA (N-methyl-D-aspartate) receptors, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid) receptors, Kainate receptors, and metabotropic receptors were conducted in the last decade. A survival effect of NMDA was described on cultures generated form 7-8 old

day rat cerebellum (Balazs, Jorgensen et al. 1988) but the mechanism is elusive. However, another study demonstrated no proliferative effects of NMDA on the embryonic cortical explant system cells (LoTurco, Owens et al. 1995). It has also been shown that blockade of NMDA receptors enhances proliferation in the postnatal dentate gyrus (Gould, Cameron et al. 1994). Activation of NMDA receptors in the adult dentate gyrus down regulates proliferation and the NMDA antagonist dizocilpine maleate (MK-801) up-regulates cell proliferation (Cameron, McEwen et al. 1995). Interestingly, neurogenesis is suppressed by NMDA activation, and adrenal steroids which suggests a common pathway of action, since MK-801 reverses the corticosterone-induced reduction in the adult rodent dentate gyrus proliferation, and NMDA receptor activation inhibits the adrenalectomy–induced increase in cell proliferation (Cameron, Tanapat et al. 1998). The role of AMPA/Kainate receptors is even less well understood. This might be because of the poor availability of specific agonists and/or antagonists to differentiate between the effects of both receptors. Interestingly, it has been demonstrated, in embryonic occipital cortex of monkeys, that high levels of Kainate receptors are observed but not other glutamate receptor sub-types (Lidow and Rakic 1995). This may suggest a significant role for Kainate receptors in regulating cell proliferation in embryonic primates. However, some studies investigating the role of glutamate on cell proliferation, reported it to be down regulated via AMPA/Kainate receptors activation (LoTurco, Owens et al. 1995; Steinhauser and Gallo 1996). It has also been demonstrated that Kainate mediates cerebellar cell survival (Balazs, Hack et al. 1990) while glutamate has direct inhibitory effect on cell proliferative from embryonic cortical explants (LoTurco, Owens et al. 1995). Despite studies examining the effects of Kainate *in vivo* and in organotypic explant cultures, little is known regarding the direct effects of Kainate on precursor cells in culture. On the other hand, it has been shown that glutamate (300μM), acting via AMPA/Kainate receptor subtypes, decreased cell proliferation in E16 and E18 rats' cortical explants (LoTurco, Owens et al. 1995).

There is thus strong evidence that glutamate regulates neurogenesis in the embryonic, postnatal and adult brains. However, the mechanisms of these effects of glutamate on stem/progenitor cells in the postnatal and adult life and their role in neurogenesis are not clearly understood. Therefore, it is of great value to investigate the role of glutamate or

its analogs, like Kainate, on primary hippocampal cell culture system and describe its effects on stem/progenitor cells and immature neurons which will help understanding the mechanisms of neurogenesis and development of neuronal control of neurogenesis.

Table 3.1 details other neurotransmitters that might be of interest and their role in controlling cell proliferation in different part of the central nervous system.

Factor	Effect	System
Glutamate		
Non-NMDAR agonist (Kainate)	↓ Proliferation	E16/E18 rat CTX explants
NMDAR agonist (NMDA)	↓ Proliferation	Adult rat DG
NMDAR antagonist (MK 801)	↑Proliferation	P2-5 and Adult rat DG
GABA		
GABAR agonist (GABA)	↓ Proliferation	E16/E18 rat CTX explants
$GABA_A$ agonist (GABA, muscimol)	↓ Proliferation	E16 dissociated rat CTX
Biogenic amines		
↓ Monoamines (αMPT, reserpine)	↓ Proliferation	P11 SVZ
Neuroleptics	↓ Proliferation	P11 SVZ
Decreased serotonin	↓ Proliferation	E8-12 SC and HP
Cholinergic agonists (nicotine)	↓ Proliferation	Embryonic or postnatal CTX
Opioids		
Met^5-enkephalin	↓ Proliferation	P6 rat CB
Opioid R antagonist (naltrexone)	↑Proliferation	P6 rat CB, DG, SVZ
VIP/PACAP		
PACAP R agonist (PACAP, μM VIP)	↓ Proliferation	E13.5 rat dissociated CTX
PACAP R agonist (μM VIP)	↑Proliferation	E15.5 rat dissociated SCG
VIP antagonist	↓ Proliferation	E9 mouse proencephalon

Table 3.1 Summary of Neurotransmitter effects on proliferation in the central nervous system. ↑ = increased; ↓ = decreased; CB = Cerebellum; CTX = Cortex; DG = Dentate gyrus; HP = Hippocampus; SCG = Superior cervical ganglion; SVZ = Sub ventricular zone; E = Embryonic; P = postnatal. VIP = Vasoactive intestinal peptide; PACAP = Pituitary Adenylate Cyclase-Activating Peptide. (Cameron, Hazel et al. 1998; Abrous, Koehl et al. 2005).

3.2 Chapter aims

As demonstrated in **Chapter 2**, the primary hippocampal cell culture paradigm is a relatively reliable controlled system that gives us the opportunity to explore the effects of different factors on specific target cells over a range of time points and periods. Using the methods detailed in **Chapter 2** we will determine and quantify the effects of Kainate on the proliferation of different stem/progenitor cell phenotypes as well as the phenotypic fate choices of their progeny. The goal of this chapter is to study the proliferative effects that Kainate might have on hippocampal precursor cells and to begin to elucidate the underlying mechanisms of hippocampal neurogenesis.

3.3 Experimental Methodology

3.3.1 Experimental Aims

We prepared our primary cell cultures following the protocols described in **Chapter 2**; however, we will, in this part, describe modifications that we have done for these particular experiments. Then we will show and discuss a diverse range of experiment's results.

3.3.2 Determining Kainate dose-response curve experiments in live hippocampal cell cultures

Primary hippocampal cell cultures were prepared as detailed in **Chapter 2 (Section 2.4.2)**. Two hours after plating, the cultures were replenished with standard growth medium or standard growth medium enriched with 0.5μM, 5μM, and 50μM Kainate (Tocris) and maintained *in vitro* for 5 days. Counts of live cells were measured following the methods described in **Chapter 2 (Sections 2.4.5 and 2.4.6)**. Then Cultures were imaged and data analyzed using the techniques mentioned in **Chapter 2 (Sections 2.4.8 and 2.4.10)**.

3.3.3 Defining Kainate time-course experiments

In order to examine the effects of Kainate on hippocampal cells over time, cells were grown for **4 h, 1 day, 3 days, 5 days and 7 days** under control and 5μM Kainate enriched conditions (**Figure 3.1**). 5μM Kainate was identified in our Kainate dose-response experiments as the lowest proliferative concentration and thus it will be used to investigate cell proliferation. Four hours terminal pulse of a final concentration of 20μM BrdU was applied to all conditions. At the end of each time point propidium iodide (PI) and DAPI were added to cells in culture to measure cell death and quantify the counts of live cells (**Sections 2.4.5 and 2.4.6**). According to the procedures in **Sections 2.4.8 and**

2.4.10, cultures were then imaged before being fixed with 4% PFA for 30 min and stained for BrdU using immunocytochemistry methods detailed in **Chapter 2** (**Section 2.4.7**).

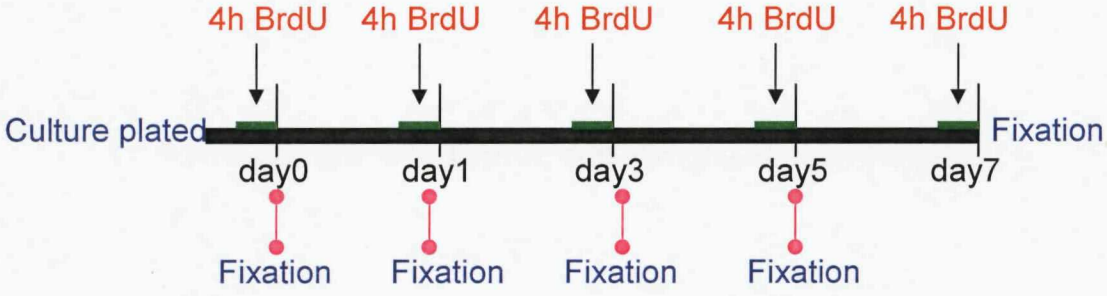


Figure 3.1 **Kainate time-course experiments paradigm**

3.3.4 Investigating the effects of short terminal pulses of Kainate and 5-bromo-2-deoxyuridine (BrdU) on cell proliferation and cell death in culture

After 3 and 5 days of culturing cells under the standard growth conditions; a group of cells were exposed to a final concentration of 20μM BrdU for the last 6 h, while the second group was exposed to 20μM BrdU and 5μM Kainate for the last 6 h. All groups were stained for PI and DAPI before cell fixation **Chapter 2** (**Sections 2.4.5 & 2.4.6**). After imaging live cells, they were fixed with 4% PFA for 30 min before being immunostained for BrdU as described in **Chapter 2** (**Section 2.4.7**) and detected with the CY2 fluorophor. In another set of experiments cells were immunostained for nestin and TuJ1 (as described below). In addition, double labelling hippocampal cells for TuJ1 and nestin was carried out and the proportion of cells that co-labelled for both markers determined.

3.3.5 Assessment of cell proliferation

In our primary cell culture system, we used BrdU as a marker of S-phase entry. We

applied BrdU directly to cells for the last 6-8 hours to a final concentration of 20μM before cells were rinsed once in PBS and fixed with 4% PFA for 30 minutes at 4C°. Double labelling of the proliferating cells with various cell classes specific markers is essential to identify phenotypic sub-populations of proliferating cells. To investigate which cell specific phenotype Kainate drives to proliferate we used terminal short pulses (as indicated) of BrdU. Then we used immunocytochemistry methods that detailed in **Chapter 2** (**Section 2.4.7**) and stained cells for BrdU and either nestin, as a marker of progenitor cells, or TuJ1, as a neuroblasts marker. We then calculated the proportion of cells that co-labelled for BrdU for each specific cell phenotype generating a Mitotic Index (MI) for each phenotype. An increase in this proportion indicates an effect of Kainate on the proliferation of the population of that particular cell phenotype. The "proliferative" effect could be due to either an increased recruitment of cells into the cell cycle i.e. an increase in the Growth Fraction (GF) or an increase in the average speed of the cell cycle that can be measured using the labelling index (LI) (see below), or a combination of both. The size of the proliferating population was measured by immunostaining for the cell cycle phase-specific protein Ki-67, which is expressed in all phases of the cell cycle except for the resting phase (G_O). This allowed us to estimate the GF which is expressed as the proportion of cycling precursors (Ki-67 positive) with respect to the total number of cells (DAPI positive) or a subset of cells based on a specific phenotypic marker. An increase in the growth fraction indicates a rise in the number of the dividing cells through the recruitment of quiescent cells to divide.

Short pulse BrdU incorporation identifies the fraction of cycling precursors (Ki-67 positive) in the S-phase and determines the Labelling Index (LI) (Dehay, Savatier et al. 2001). Because the S-phase duration is usually invariant, variations of LI reflect changes in the cell cycle duration (Schmahl 1983). An increase in the labelling index indicates that the factor facilities the transitions of cells from G_1 phase of the cell cycle to the S-phase and/or it shortens G_1 phase of the cell cycle (increased proliferation rate).

Finally, BrdU also has the characteristic of long-term retention in incorporated cells and its passage to their daughter cells before being diluted below the limits of detection (usually 4 cell cycles). This characteristic enables cell lineage tracing and quantification of the survival of the progeny of the initially proliferating cohort of cells.

3.3.6 Fluorescence Immunocytochemistry, Cell imaging, Counting and Statistical analysis

The PFA-fixed cell cultures were immunostained for the required marker following the procedures in **Chapter 2** (**Sections 2.4.7**) and using the appropriate primary and secondary antibodies (**see Chapter 2 Table 2.1**).

Cell imaging was performed either on an inverted Leica DM IRB microscope (Leica Microsystems UK Ltd) or on laser scanning con-focal microscope with 3D projections reconstructed using the Zeiss LSM software as detailed in **Chapter 2** (**Sections 2.4.8 & 2.6.9**). Cell counting and statistical analysis were also carried out as described in **Chapter 2** (**Section 2.4.8 and 2.4.10**).

3.4 Results

3.4.1 Three days exposure to Kainate enhanced hippocampal cell proliferation at a concentration as low as 5μM

To study the effects of different concentrations of Kainate on the total cell number, cell proliferation, and cell cycle kinetics; monolayer cultures were grown either under control conditions or in 0.5μM, 5μM, 50μM, and 100μM Kainate and maintained for 3 days as detailed in **Chapter 2** (**Section 2.4.2**). Cells were pulsed with a final concentration of 20μM BrdU for the terminal 4 hours before being fixed and stained against BrdU and Ki-67 and counterstained for the nuclear marker DAPI (**Section 2.4.7**). Imaging, counting and statistical analysis were carried out as mentioned in **Chapter 2** (**Sections 2.4.8 and 2.4.10**).

We found a significant increase in the total number of cells (**DAPI**) under Kainate (**0.5-100μM**) conditions (**Figure 3.2 A**). For example, DAPI counts increased dramatically from 210.8 ± 13.1 cells/mm² under control maintained conditions to 364.5 ± 9.8 cells/mm² under 5μM Kainate treated conditions (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$). In our experiments, BrdU incorporated cells in the last 4 hours increased significantly under 5μM, 50μM, and 100μM Kainate when comparing these conditions with controls (**Figure 3.2 B**). The mitotic index (MI) was measured by calculating the proportion of BrdU cells that entered S-phase of the cell cycle in the last 4 hours (BrdU/DAPI). A significant increase in the MI was observed under the influence of both 5μM and 50μM Kainate only, in comparison with control conditions (**Figure 3.2 D**). While we found that 24 ± 2 % of cell incorporated BrdU under standard growth conditions, this proportion increased significantly to 39 ± 4 % in response to 5μM Kainate treatment (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 3.2 D**). The counts of Ki-67 expressing cells was 103.2 ± 10.32 cells/mm² under control conditions and increased significantly to 178.0 ± 11.7 cells/mm² under 5μM Kainate (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 3.2 C**). A similar significant increase was observed

in 50μM, and 100μM Kainate in comparison with control cultured cells **(Figure 3.2 C)**. An increase in the growth fraction was seen only after enrichment of the conditions with 50μM Kainate **(Figure 3.2 E)**. Interestingly, the labelling index increased significantly under 0.5-100μM Kainate treatment when compared to control maintained cells **(Figure 3.2 F)**.

Our results suggest that 3 days exposure to 5μM Kainate increased the proliferation of cells as indicated by a significant rise in (BrdU/DAPI). Moreover, the same Kainate exposure increased the speed of the average cell cycle as indicated by a significant rise in the labelling index (BrdU/Ki-67) but had no effect on the GF (Ki-67/DAPI), indicating no recruitment of quiescent cells into the cell cycle. Therefore 5μM Kainate demonstrated a pure effect on increasing cell cycle speed, and therefore was used as the working concentration to investigate the possible proliferative effects of Kainate on hippocampal progenitor cells and neuronal precursors.

Although there was no significant drop in the total number of cells under 100μM Kainate, we observed no proportional increase in BrdU incorporated cells or growth fraction. While 0.5μM Kainate had no effect on the total number of cells in the cell cycle as indicated by Ki-67 immuno-positive cells, there was a significant drop in the growth fraction (Ki-67/DAPI). This is most likely due to the increase in the total cell counts (DAPI) under 0.5μM Kainate which was not accompanied by an increase in Ki-67 expressing cells, suggesting a pure survival effect.

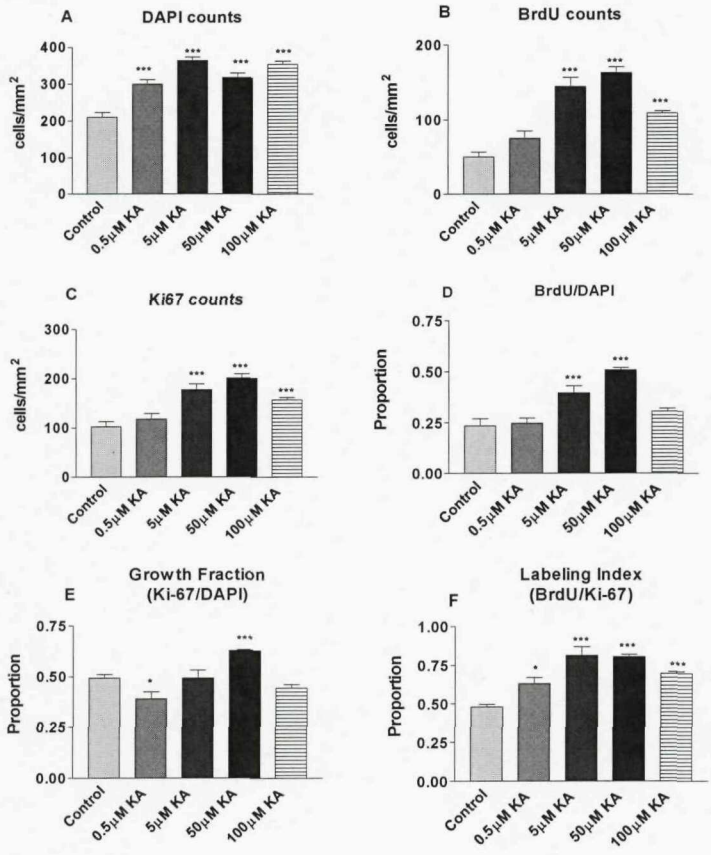


Figure 3.2 5μM Kainate is proliferative for hippocampal cells in culture. Cells were grown for 3 days in the presence and absence of 0.5μM, 5μM, 50μM, and 100μM Kainate. We found that 5μM Kainate consistently increased cell proliferation (BrdU/DAPI) and the speed of the cell cycle (BrdU/Ki-67). A) The total cells were measured by counting DAPI stained cells and indicated a significant increase in response to 0.5μM, 5μM, and 50μM, and 100μM Kainate when compared to control conditions. B) The counts of BrdU + cells increased significantly in, 5μM, 50μM, and 100μM Kainate treatments compared to control conditions. C) Ki-67 counts measures the number of cells in the cell cycle and we found a significant increase in Ki-67 counts under 5μM, 50μM, and 100μM Kainate treated cultures in comparison with control maintained cells. D) The proportion of BrdU cells, the mitotic index, increased significantly when cells were treated with 5μM, 50μM Kainate compared to control conditions. E) The growth fraction (Ki-67/DAPI) increased only under 50μM Kainate enriched conditions. F) The labelling index, which is the proportion of dividing cells that entered the S-phase of the cell cycle, increased under 0.5μM, 5μM, 50μM, and 100μM Kainate treatments in comparison with control maintained cells. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *One way Anova with Dunnett's Multiple Comparison Test* .* $p<0.05$, *** $p<0.001$

3.4.2 Three days exposure to 5μM Kainate is proliferative for cells in culture at day 3

In the previous set of experiments we found that 5μM Kainate is proliferative for cells in culture. But this was measured after 3 days of Kainate exposure. It is therefore important to investigate the effects of Kainate over time. Time-course experiments were performed to achieve this goal and to investigate the effects of Kainate exposure on the total number of live cells (DAPI positive and PI negative cells), proliferation (BrdU incorporated S-phase of the cell cycle in the last 4 h of their life), and cell death (PI stained cells).

Dissociated hippocampal cell cultures were generated as described in **Chapter 2** (**Section 2.4.2**). Cells were grown for (4 h, 1 day, 3 days, 5 days and 7 days) under control and 5μM Kainate enriched condition and 4 h terminal pulse of BrdU to a final concentration of 20μM was applied to both control and Kainate treated cells (**Section 3.3.3**). At the end of each time point propidium iodide (PI) was added to cells at a concentration of 5μg/ml and incubated for 40 minutes at 37°C in (5%CO2/ air). After that, the whole growth medium was replaced with fresh medium containing DAPI at a concentration of 20μg/ml and incubated for 40 minutes at 37°C in (5%CO2/air). Cultures were then imaged (**Section 2.4.8**) before being fixed with 4% PFA for 30 min and immunostained for BrdU using the techniques mentioned previously (**Section 2.4.7**).

We found **59%** of cells were PI positive (dead/dying) within the first 4 h after plating under control conditions (**Figure 3.3 D**), while after 4 h of Kainate treatment the proportion of PI positive cells was found to be **46%** with no statistical difference between control and Kainate conditions (Two ways Anova with Bonferroni's multiple comparison test). While cell death as indicated by PI staining dropped to **13%** after 7 days under control conditions, it dropped to **5%** after 7 days of 5μM Kainate exposure but this difference was not statistically significant when compared to control conditions at day 7 (Two ways Anova with Bonferroni's multiple comparison test) (**Figure 3.3 D**).

In **Figure 3.3 B,** we demonstrated a significant increase in BrdU counts after 1 day exposure of Kainate and in comparison with day 0. Moreover, we found a significant increase in BrdU counts under Kainate conditions at 3, 5, and 7 days when compared to

control maintained cells (**Figure 3.3 B**). Interestingly, a proportional increase in BrdU incorporation was reported only on day three when comparing Kainate treated cells to control maintained cultures (**Figure 3.3 C**). Although there is a trend towards an increase in the counts of cells that are DAPI positive and PI negative (Live cells) after 3 days of Kainate exposures we found a significant increase in live cell counts on days five and seven under Kainate enriched conditions when compared to control conditions (**Figure 3.3 A**).

These results suggest that 3 days of Kainate exposure enhanced the rate of hippocampal cell proliferation as indicated by BrdU/DAPI. Although, we did not find a significant increase in the proliferation rate (BrdU/DAPI) at day 5, we found a net increase in the counts of DAPI positive and PI negative cells (Live cells) at this particular time point. This increase in the number of live cells at day 5 may have been secondary to the increased proliferation seen by day 3. It could also suggest that Kainate might have a trophic or survival effect in addition to a proliferative one. Therefore, in the next series of experiments we will explore the effects of Kainate on the proliferation of hippocampal progenitor cells and neuronal precursors at days 3 and 5. We will also apply short pulses of Kainate and investigate direct effects on proliferation. We will further examine a possible trophic effect of Kainate on hippocampal cells in **Chapter 5**.

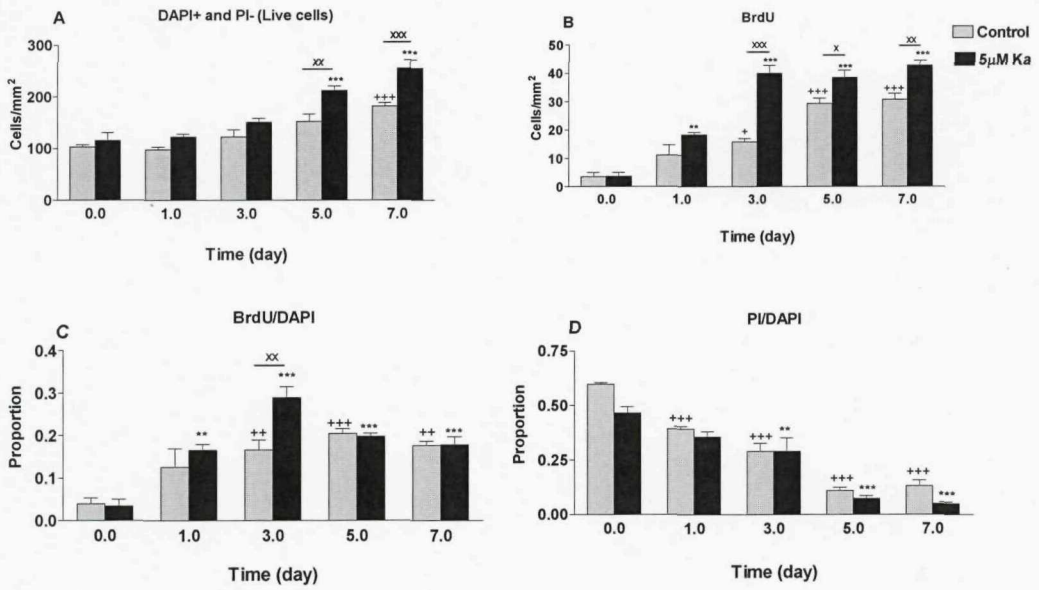


Figure 3.3 5μM Kainate increased cell counts over time and is proliferative after 3 DIV. Kainate increased the total number of cells and BrdU-incorporated cells but has no significant effect on cell death. Hippocampal cell cultures were grown for the indicated number of days under standard control conditions or in the presence of 5μM Ka. BrdU was added for the terminal 4 h of each time point. (A) The number of live cells (DAPI positive and PI negative) increased significantly on days 5 and 7 under Kainate conditions when compared to control conditions. (B) An increase in the counts of BrdU incorporated cells was detected after 1, 3, 5, and 7 days of Kainate exposure when compared to 4 h exposed Kainate at day 0 and increased relative to control at each of days 3, 5 and 7. (C) An increase in the rate of cell proliferation was observed after 3 days of Kainate treatment in comparison with control conditions as indicated by the rise in BrdU/DAPI. (D) Although we observed a drop in PI positive cells (dead/dying) over time (59% at day 0 to 13% at day 7) under control conditions and (46% at day 0 to 5% at day 7) under Kainate exposure, there was no significant difference between Kainate enriched conditions and control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Comparison was done by applying *two ways Anova with Bonferroni's multiple comparison test.** $p<0.05$, ** $p<0.01$, *** $p<0.001$ when comparing Kainate conditions across time. + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$ when comparing control conditions across time. X $p<0.05$, XX $p<0.01$ when comparing Kainate condition with the control at each time point.

3.4.3.1 Three days exposure to Kainate is proliferative for Nestin-expressing hippocampal progenitor cells at 3 DIV

The question we addressed next was to determine which cell population Kainate was proliferative in culture. We grew cells for 3 days under standard growth conditions (control) and under 5μM Kainate **Chapter 2** (**Section 2.4.2**). Cells were terminally pulsed for 4 hours with a final concentration of 20μM BrdU. Imaged live for DAPI and then fixed and stained against the proliferative marker BrdU and the stem/progenitor marker nestin.

Although we did not find a significant increase in the total number of live cells after 3 days under Kainate conditions and in comparison with control (**Figure 3.4 A**), we found an increase in the counts of BrdU incorporated cells; as 17.04 ± 1.9 cells/mm² were BrdU positive under control conditions and increased to 36.7 ± 2.7 cells/mm² after 3 days of Kainate exposure (unpaired simple Student's t-test) (**Figure 3.4 B**). In addition, a significant proportional increase in cells that incorporated BrdU was also found (**Figure 3.4 C**). This finding is consistent with previous results (**Section 3.4.2**). We further demonstrated a significant increase in the counts of nestin cells after 3 days of Kainate treatment in comparison with control conditions (**Figure 3.4 D).** Moreover, we observed a significant increase in the proportion of both nestin positive cells (**Figure 3.4 F**) and BrdU labelled cells (**Figure 3.4 B).** Interestingly, while 15.9 ± 1.9 cells/mm² were nestin positive and BrdU positive in control maintained cultures, 33.52 ± 3.2 cells/mm² were co-labelled for both nestin and BrdU after 3 days of Kainate exposure (unpaired simple Student's t-test) (**Figure 3.4 E**). Significantly, we found 24.1 ± 3.3 % of stem/progenitor (nestin) cells entered the S-phase within the terminal 4 h under control conditions and this proportion significantly increased to 42 ± 3.7 % under 5μM Kainate (unpaired simple Student's t-test), demonstrating that Kainate increased the proliferation of the nestin positive cell population (**Figure 3.4 G**).

These results suggest that Kainate enhances cell proliferation and the proliferation of nestin positive cells over three days in culture. It also suggests either a survival effect on proliferating nestin cells and or a switch towards symmetric cell division of these nestin positive cells, given that we observed a proportional increase in nestin positive cells.

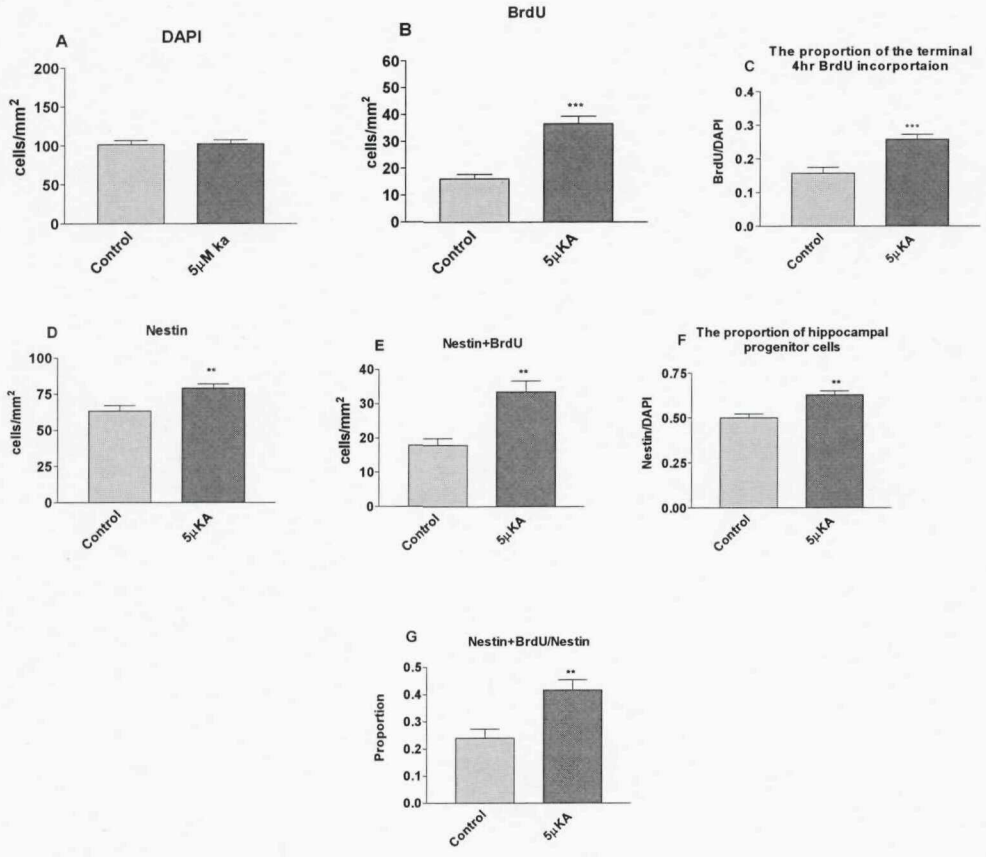


Figure 3.4 Kainate increased the proliferation of Nestin positive cells after 3 days. Three days of 5μM Kainate exposure increased BrdU incorporated cells, nestin (+), and BrdU (+) nestin (+) counts with proportional increase in the rate of cell proliferation and proliferating nestin cells. Cultures were grown for 3 days in the absence and presence of 5μM Kainate. On day 3 they were pulsed with a final concentration of 20μM BrdU for the last 4 h. After fixation cells were stained against nestin, BrdU, and counterstained for the nuclear marker DAPI. A) No increase in the total cell numbers as indicated by counting DAPI stained cells. B) BrdU incorporated cells in the last 4 h increased after 3 days of Kainate treatment. C) The proportion of the proliferating cells in culture (BrdU/DAPI) significantly increased in response to Kainate when compared to control. D) An increase in the counts of stem/progenitor (nestin) cells was observed after 3 days of Kainate exposure. E) The counts of the proliferating progenitor (nestin) cells in hippocampal monolayer cultures increased significantly under Kainate treatment and in comparison with control conditions. F) There was a significant increase in the proportion of stem/progenitor (nestin) cells in response to 3 days of Kainate exposure. G) The proportion of proliferating progenitor hippocampal cells increased significantly after 3 days of Kainate treatment when compared to control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 3 experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t test.*** $p<0.01$, *** $p<0.001$

3.4.3.2 Short pulse of Kainate increased the labelling index but did not affect the growth fraction at 3 DIV

We have demonstrated (**Sections 3.4.2 and 3.4.3.1**) that 3 days of Kainate exposure yielded a significant increase in total cell proliferation (BrdU/DAPI) and in the proliferation of nestin positive cells. However, the mechanism of the proliferative effect is not yet clear, i.e. does Kainate increase the growth fraction, the proliferation rate or both? In order to understand the mechanisms that resulted in net increase in cell proliferation after 3 days of Kainate exposure, we will study the effects of a short (6 h) Kainate exposure on cell counts, S-phase BrdU incorporation and cell cycle kinetics at day three.

We generated primary cultures from Wistar rat pups hippocampi (7-10 days) following the protocol in **Chapter 2** (**Section 2.4.2**). At day 3 a terminal 6 h pulse of 5μM Kainate was given to cultures undergoing treatment. Another group of cells was maintained under standard growth conditions and considered our control group. All conditions (control and treated cultures) were terminally (6 h) pulsed to a final concentration of 20μM BrdU. Cells were fixed with 4% PFA and immunostained against the proliferative marker BrdU and the cell cycle marker Ki-67 as detailed in **Chapter 2** (**section 2.4.7**). Finally, imaging and analysis were carried out as detailed in (**Section 2.4.8 and 2.4.10**) and data displayed in (**Figures 3.5**).

We found a significant increase not only in BrdU incorporated cells **(Figure 3.5 A)** but also in the proportion of BrdU incorporated cells after 6 h of Kainate treatment in comparison with control conditions (**Figure 3.15 C**). The counts of BrdU significantly increased from 11.1 ± 1.7 cells/mm² under control conditions to 21.4 ± 1.9 cells/mm² under Kainate conditions (unpaired simple Student's t-test, $p<0.001$). While, 11.5 ± 1.1 % of cells incorporated BrdU in control maintained conditions, 20.9 ± 1.9 % of cells incorporated BrdU after 6 h of Kainate exposure. This increase was statistically significant (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.15 C**). This suggests a significant increase in the rate of cell proliferation. There was no significant increase in either the number of Ki-67 immuno-positive cells or the growth fraction (**Ki67/DAPI**) (**Figure 3.15 B and D**). Importantly, terminal 6 h of 5μM Kainate enrichment

significantly increased the labelling index (BrdU and Ki-67 double labelled cells with respect to the total Ki-67 cells). The labelling index increased from 15.3 ± 1.6 % under control conditions to 25.9 ± 1.6 % after 6 h of Kainate treatment (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.15 E**).

These results show that short exposure of Kainate increased cell proliferation (BrdU/DAPI) by increasing the speed of the cell cycle (Labelling index). There was no increase in the growth fraction (Ki-67/DAPI) which implies that Kainate did not recruit quiescent precursor cells.

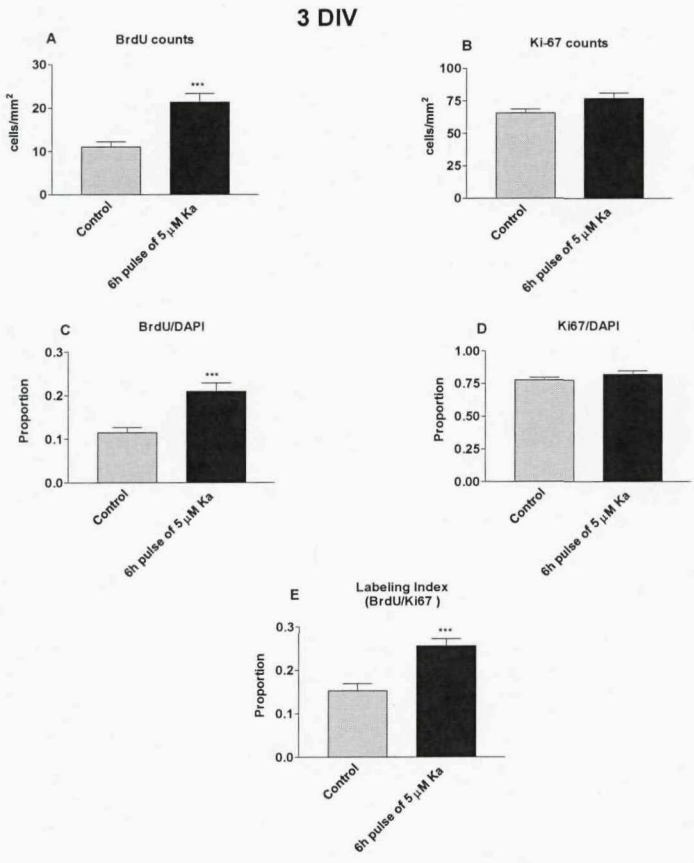


Figure 3.5 6 h of Kainate exposure at 3 DIV increased cell proliferation by increasing the cell cycle speed. Monolayer primary hippocampal cell cultures were generated from postnatal rat pups (7-10 days) and maintained under standard growth conditions for 3 days. 6 h terminal pulse of 5μM Kainate was pulsed to a group of cells, while another group of cells was maintained under control conditions. Terminal 6 h pulse of a final concentration of 20μM BrdU was applied to all conditions (treated cells and control) to investigate this short pulse of treatment on growth fraction and labelling index. Labelling index (BrdU/Ki-67) and BrdU/DAPI increased significantly under Kainate treatment in comparison with control. A) There was a significant increase in the number of BrdU incorporated cells (S-phase entry) after 6 h of Kainate exposure. B) Ki-67 expressing cells in 3 days cultures and terminal 6 h of Kainate treatments showed no significant rise in cell counts in comparison with control conditions. C) The proportion of proliferating cells increased significantly after 6 h Kainate exposure when compared to control conditions. D) The growth fraction did not change which means there was no recruitment of quiescent cells into the cell cycle. E) The labelling index after terminal 6 h of Kainate exposure increased significantly in treated conditions when compared to control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired Student's t-test*. *** $p<0.001$

3.4.3.3 Six hours pulse of Kainate is proliferative for hippocampal stem/progenitor cells at day 3 *in vitro*

We have demonstrated (**Section 3.4.3.1**) that 3 days of Kainate exposure is proliferative for nestin expressing cells. Our results in **Section 3.4.3.2** revealed a significant proliferative effect of 6 h Kainate exposure on hippocampal cells after three days under control culture conditions. We in this section are going to examine the effects of short term Kainate exposure on the proliferation of nestin expressing cells. Using our primary hippocampal cell culture system we prepared cells as detailed in **Chapter 2** (**Section 2.4.2**). On day three 6 h terminal pulse of a final concentration of 20μM BrdU was applied to all wells but whereas 5μM Kainate was given to half of the wells, while the rest of the wells were kept under standard control conditions (untreated). Cells were fixed with 4% PFA and immunostained against the proliferative marker BrdU and the stem/progenitor marker nestin **Chapter 2** (**Section 2.4.7**). Finally, cells were imaged and data analyzed as mentioned on **Chapter 2** (**Sections 2.4.8 and 2.4.10**).

Another set of experiments was carried out in which cells were terminally pulsed for the last 8 hours with BrdU (a final concentration of 20μM) whereas 5μM Kainate was applied to cells undergoing treatment. After cell fixation; they were probed for nestin, Ki-67 and BrdU (triple immunohistochemistry) in order to measure the labelling index of nestin sub-population of cells and thus the speed of the cell cycle for nestin expressing cells.

Our results again revealed a significant increase in nestin immuno-positive cell counts (**Figure 3.17 A**). While 63.7 ± 3.3 cells/mm² expressed nestin under control conditions, this number increased significantly to 79.5 ± 2.4 cells/mm² after 6 h of Kainate exposure (unpaired simple Student's t-test, $p<0.01$). In addition, we described a significant proportional increase in nestin sub-population of cells in response to 6 h Kainate treatment when compared to control condition (**Figure 3.6 B**). This increase was a result of an increase in nestin immuno-positive cells but not in the total cell counts (DAPI) (**Figure 3.6 G**). We further demonstrated a significant rise in the counts and proportion of BrdU incorporated cells under Kainate treated conditions when compared to control conditions (**Figure 3.6 C and D**), consistent with a proliferative effect on cells. 17.9 ±

1.9 cells/mm² of nestin immuno-positive cells entered S-phase in the last 6 h under control conditions, while 6 h of Kainate exposure increased those cells significantly to 33.5 ± 3.2 cells/mm² (unpaired simple Student's t-test, $p<0.01$) (**Figure 3.6 F**). Interestingly, we found that 26.2 ± 2.1 % of nestin expressing cells incorporated BrdU under standard growth conditions which increased significantly to 40.8 ± 1.8 % after 6 h of Kainate exposure (unpaired simple Student's t-test, $p<0.01$) (**Figure 3.6 E**), demonstrating that 6 h of Kainate exposure has direct proliferative effects on nestin-positive hippocampal progenitor cells.

Furthermore, triple immunohistochemistry for BrdU, Ki-67 and nestin was carried out. Con-focal images were taken and 100 nestin immuno-positive cells were counted. Co-localization of nestin expressing cells with BrdU and Ki-67 was also measured. All nestin positive cells were Ki-67 immuno-positive (**Table 3.2**). This indicates that all hippocampal precursor cells are in the cell cycle. There was a significant proliferative effect of Kainate on nestin sub-population of cells. The labelling index for nestin cells increased from 20% under control conditions to 43% after 8 hours of Kainate treatment (**Table 3.2**). Degrees of freedom: 1, Chi-square = 12.3 and $p <0.001$.

The increase in the number and proportion of nestin immuno-positive cells after short pulse of Kainate might be due to a synergistic proliferative and survival effects. We have outlined a significant proliferative effect of Kainate on nestin sub-population and the survival effect will be dealt with in **Chapter 5**.

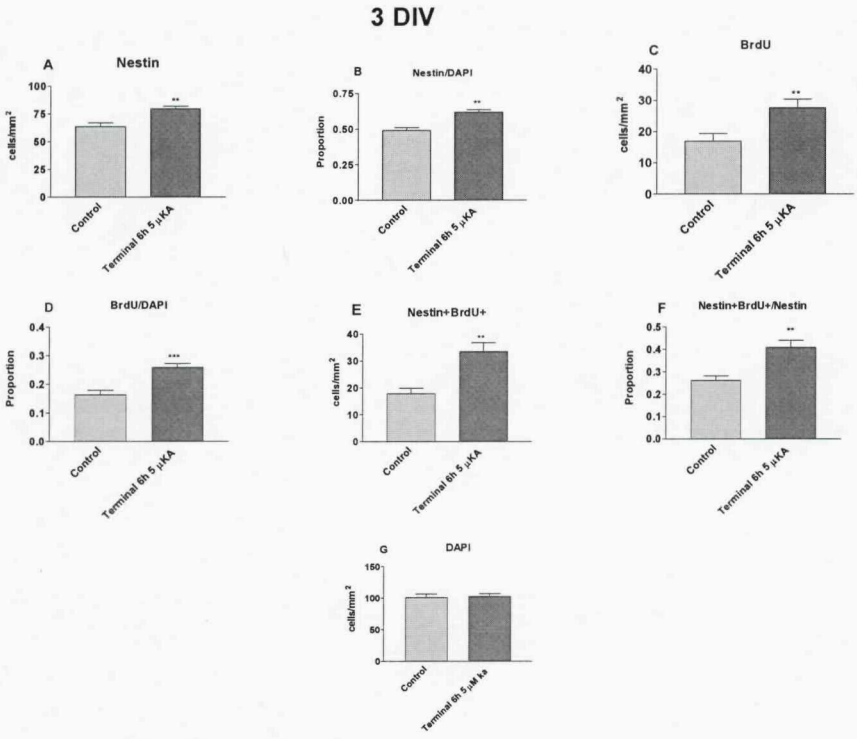


Figure 3.6 6 h of Kainate treatment enhanced progenitor cell proliferation at day 3. Cells were cultured for 3 days under standard growth conditions. On day three, a 6 h terminal pulse of 5µM Ka was pulsed to one group of cultures, while the other group remained untreated as a control. 20µM BrdU was given to all conditions (treated cells and control) for the last 6 h. Cells were fixed and immunostained against nestin and BrdU. Kainate treatment increased the counts of nestin immuno-positive cells, the proportion of nestin expressing cells in culture, the number of BrdU incorporated cells in the last 6 h and the proportion of nestin expressing cells that entered S-phase of the cell cycle in the last 6 h. A) Nestin expressing cell counts increased after a 6 h pulse of Kainate. B) The proportion of nestin sub-population of cells in culture increased significantly after 6 h of Kainate exposure in comparison with controls. C) An increase in the counts of BrdU positive cells was also demonstrated after 6 h of Kainate treatment. D) The proportion of S-phase BrdU incorporating cells in primary hippocampal cultures increased after a 6 h pulse of Kainate. E) The number of proliferating stem/progenitor cell in the S-phase of the cell cycle increased after 6 h of Kainate exposure compared to controls. F) The proportion of proliferating nestin expressing cells in the S-phase of the cell cycle increased significantly after 6 h of Kainate in comparison with control maintained cultures, confirming a proliferative effect on nestin –positive cells. G) There was no significant effect of Kainate on the total cell number measured by DAPI. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t-test*. ** $p<0.01$, *** $p<0.001$

	3DIV Hippocampal cells in culture	
	Nestin+ and Ki-67+ but BrdU-	Nestin+, Ki-67+ and BrdU+
Control	80	20
8 hours Kainate	57	43

Table 3.2 Kainate enhanced the proliferation rate of nestin immuno-positive cells. Hippocampal cells were grown for 3 days before being pulsed with 5μM Kainate and BrdU for the last 8 hours. Triple immunohistochemistry for BrdU, Ki-67 and nestin was carried out. Con-focal images were taken and 100 nestin immuno-positive cells were counted and co-localization of nestin expressing cells with BrdU and Ki-67 was also measured. All nestin positive cells were Ki-67 immuno-positive. There was a significant proliferative effect of Kainate on nestin sub-population of cells. The labelling index for nestin cells increased from 20% under control conditions to 43% after 8 hours of Kainate. Degrees of freedom: 1, Chi-square = 12.3 and $p < 0.001$

3.4.3.4 Three days of 5μM Kainate enhanced neuronal precursor cell proliferation at 3 DIV

To assess the effect of Kainate on the proliferation of neuroblasts, which are the proliferative progeny of more immature precursors in culture, single cell suspension was generated as described in **Chapter 2** (**Section 2.4.2**). Cells were terminally pulsed for 4 hours with a final concentration of 20μM BrdU. After fixation with 4% PFA, cells were processed for BrdU incorporation and TuJ1 expression. Numbers and proportions of proliferating TuJ1 cells (TuJ1 cells that incorporated BrdU) were determined under control and after 3 days of 5μM Kainate exposure.

Consistently we found a significant increase in BrdU cell counts and proportion of BrdU incorporated cells when comparing 3 days Kainate treated conditions with control conditions (**Figures 3.8 B&C**). TuJ1 cell counts increased significantly under 5μM Kainate (60.8 ± 1.4 cells/mm^2 versus 36.3 ± 1.9 cells/mm^2 under control conditions) (unpaired simple Student's t-test, $p<0.001$) (**Figures 3.8 D**). Moreover, a significant proportional increase in TuJ1 sub-population of cells was observed after 3 days of Kainate exposures in comparison with control conditions (**Figures 3.8 E**) demonstrating that Kainate increased net neurogenesis in culture over three days. Interestingly, the number of TuJ1 cells that entered S-phase of the cell cycle (incorporated BrdU) increased significantly from 2.4 ± 0.7 cells/mm^2 under control conditions to 16.1 ± 1.2 cells/mm^2 under Kainate treatment (unpaired simple Student's t-test, $p<0.001$) (**Figures 3.8 F**). In parallel with this 5-fold increase in proliferating TuJ1 expressing cells we found that the proportion of TuJ1 cells that incorporated BrdU increased significantly from 5.6 ± 1.2 % under control conditions to 25.9 ± 1.6 % after 3 days of Kainate exposure (unpaired simple Student's t-test, $p<0.001$) (**Figures 3.8 F**).

Figure 3.7 shows the pattern of proliferating neuronal precursors in cultures. We observed that these neuroblasts are round in shape and have no processes, with a punctate TuJ1 staining pattern, consistent with an immature phenotype and the reported literature (Palmer et al 1997; Howell et al 2007).

These results demonstrated a significant proliferative effect of Kainate on hippocampal

neuroblasts in culture. They also showed that Kainate exposure yields a significant net increase in the TuJ1 sub-population and thus neurogenesis over time. The next step was to determine the mechanism of the proliferative effect of Kainate on neuroblasts.

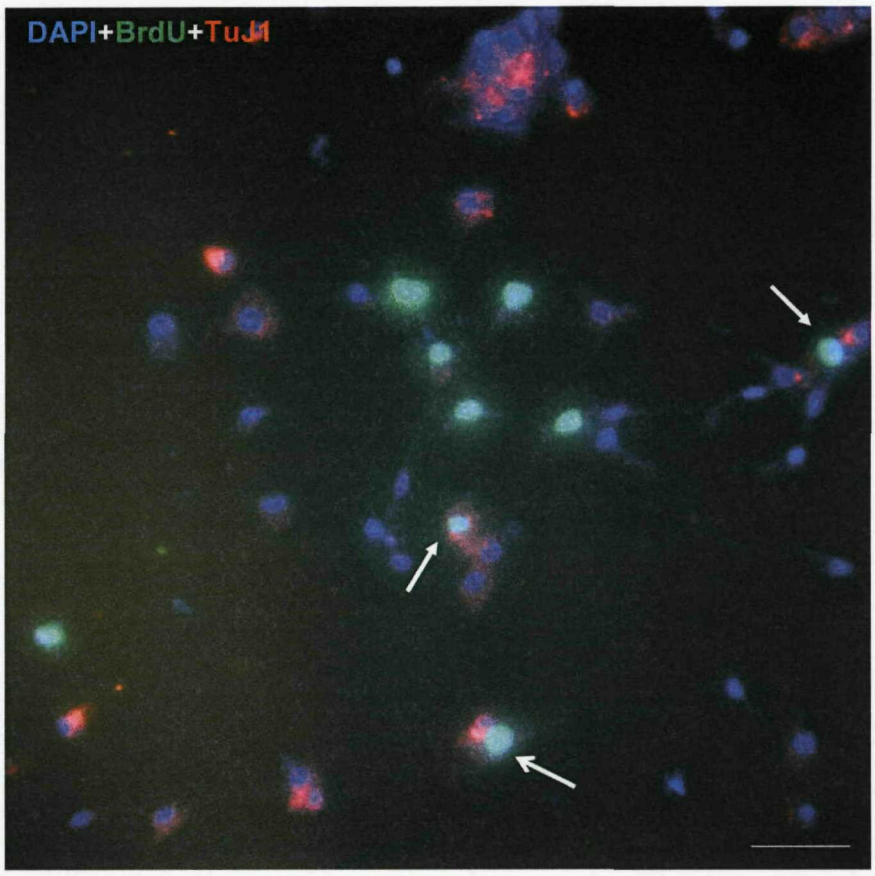


Figure 3.7 Kainate enhanced the proliferation of neuronal precursor cells in hippocampal cultures. Cells were cultured in the presence and absence of 5μM Kainate and maintained for 3 days. They were terminally pulsed with BrdU for 4 hours. After fixation with 4% PFA, cells were probed with antibodies to BrdU (green), TuJ1 (red), and counterstained for the nuclear marker DAPI (blue). We showed a significant increase in TuJ1 and the proliferating neuronal precursor cells under Kainate treatment. White arrows show sample of the proliferating neuronal precursor cells. Imaging was performed on an inverted Leica DM IRB microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). Open Lab image capturing system version 2.1 (Improvision, Lexington, USA) was used to count cells. Control experiments with the primary antibody omitted were devoid of staining. We observed punctate pattern of TuJ1 staining (red). Scale bar = 40μm.

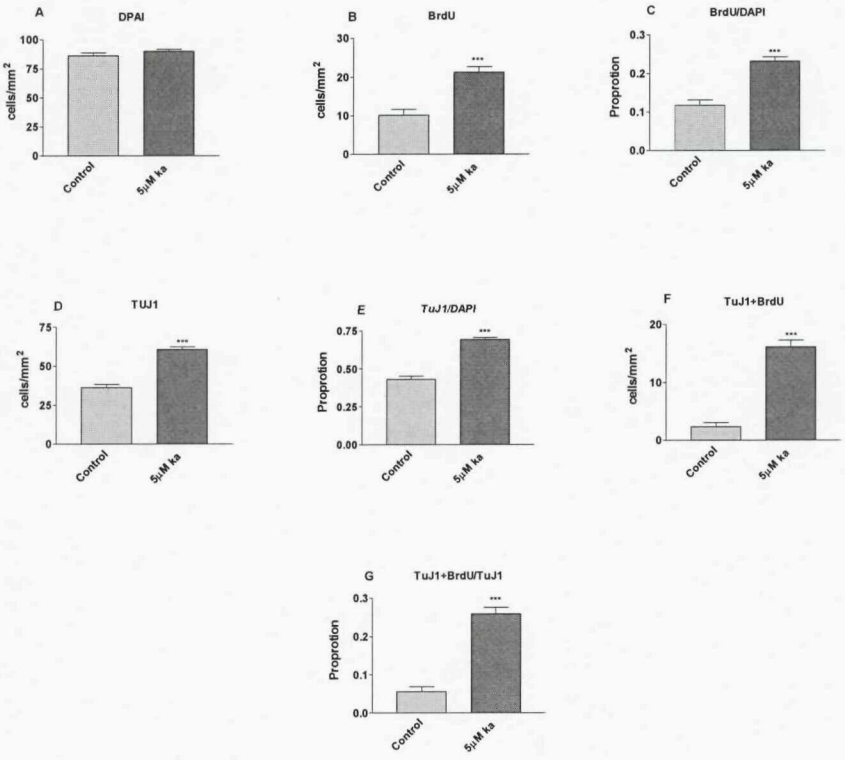


Figure 3.8 Three days of 5µM Kainate enhanced neuronal cell precursor proliferation at 3 DIV. Cells were cultured for 3 days in the absence and presence of 5µM Ka. On day 3 they were pulsed with a final concentration of 20µM BrdU for the last 4 h. After fixation cells were stained against TuJ1, BrdU, and counterstained for the nuclear marker DAPI. We found a significant proliferative effect of Kainate on TuJ1 cells with a net rise in TuJ1 sub-population of cells. A) The total cell counts was measured by counting DAPI stained cells in live model and indicated no significant changes. B) The counts of BrdU incorporated cells increased significantly in the last 4 h under Kainate conditions compared to control. C) In parallel with the rise in BrdU cell counts, the proportion of the proliferating cells in culture increased significantly in response to 3 days of Kainate exposure. D) A significant increase was observed in the counts of TuJ1 expressing cells after three days under Kainate conditions. E) The proportion of neuroblasts in culture increased significantly in Kainate treated cells when compared to control maintained cells. F) There was a significant increase in counts of proliferating neuroblasts (TuJ1+BrdU) in hippocampal monolayer cultures after 3 days of Kainate treatment compared to controls. G) The proportion of proliferating TuJ1 expressing hippocampal cells (TuJ1 expressing cells that entered S-phase in the last 4 h) increased significantly under Kainate exposure and in comparison with control (untreated) conditions, demonstrating that three days of Kainate increased the proliferation of neuroblasts. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t test*. *** $p<0.001$

3.4.3.5 Exposure to a 6 hour pulse of Kainate did not enhance neuronal precursor cell proliferation at 3 DIV

We reported significant effects of Kainate on the proliferation of neuroblasts in 3 DIV maintained cultures (**Section 3.4.3.4**) and a proportional increase in TuJ1 sub-population of cells in 3 DIV (**Sections 3.4.3.4**). In this section we investigated the mechanism of the proliferative effect of Kainate on proliferating neuronal precursor cells using short pulse exposures.

We generated monolayer cell cultures as described in **Chapter 2** (**Section 2.4.2**). On day three 6 hours terminal pulse of a final concentration of 20μM BrdU was applied to all wells whereas 5μM Kainate was given to half of the wells, while the rest of the wells were kept under standard control conditions (untreated). Cells were fixed with 4% PFA and immunostained against the proliferative marker BrdU and the neuroblasts marker TuJ1 **Chapter 2** (**Section 2.4.7**).

The numbers and proportion of TuJ1 expressing cells were measured in control and after 6 h of Kainate exposure. We did not observe significant effects of 6 h Kainate treatment on TuJ1 expressing cells (**Figure 3.9 A & B**). BrdU incorporated cell in the last 6 h increased significantly from 11.0 ± 1.6 cell/mm² under control conditions to 21.6 ± 1.5 cell/mm² after 6 h of Kainate exposure (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.9 C**) as previously demonstrated. In parallel we found a consistent significant increase in the proportion of cells that entered S-phase of the cell cycle after 6 h of Kainate treatment when compared to control conditions (**Figure 3.9 D**). The addition of 5μM Kainate onto cells in culture for 6 h did not increase the number or proportion of TuJ1 positive cells that incorporated BrdU (entered S-phase of the cell cycle) (**Figure 3.9 E and F**).

Although 6 h of Kainate exposure is proliferative for hippocampal cells and progenitor cells (**Section 3.4.3.3**), there is no proliferative effects for this short term Kainate exposure on TuJ1 positive hippocampal neuronal precursors in culture.

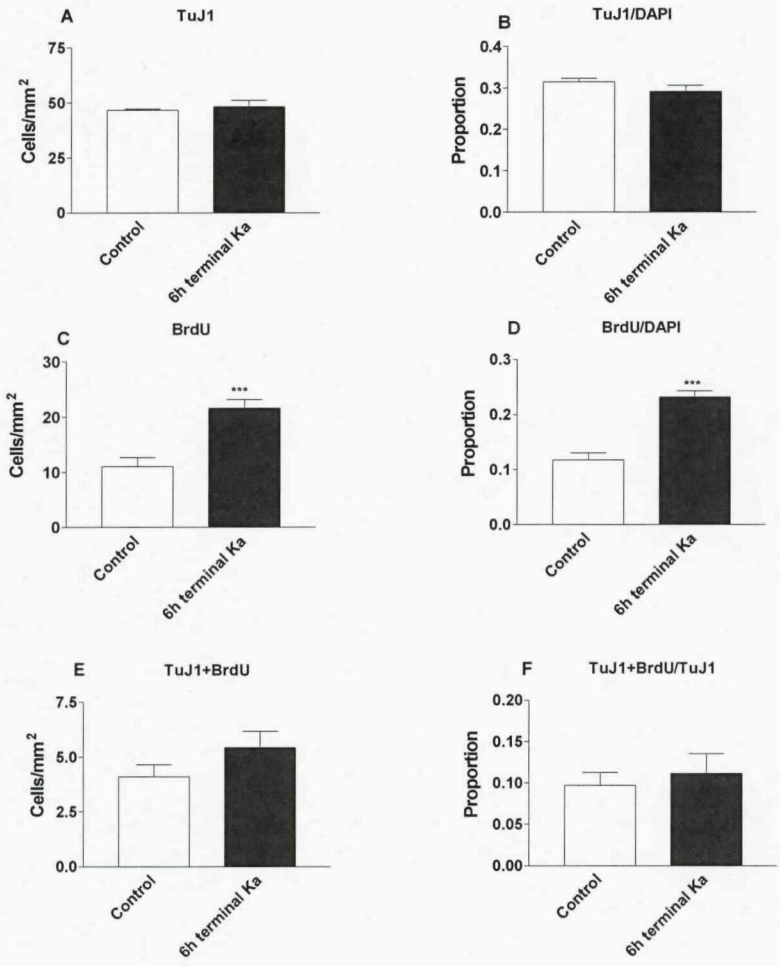


Figure 3.9 Short 6 hours pulse of Kainate had no effect on neuronal precursors at day 3. Cells were cultured for 3 days under the standard growth conditions. On day three 6 h terminal pulse of 5μM Ka was given to one group of cultures, while the other group of cultures maintained as control. 6 h terminal pulse of a final concentration of 20μM BrdU was applied to all conditions (treated cells and control). Cultures were fixed and immunostained against TuJ1and BrdU. There was no significant proliferative effect of 6 h Kainate treatment on neuronal precursors. A) TuJ1expressing cell counts did not change over 6 h of Kainate treatment. B) The proportion TuJ1 positive cells was the same under control and 6 h Kainate conditions. C) There was a significant increase in BrdU incorporated cells after 6 h of Kainate exposure when compared to control conditions. D) The proportion of cells that entered S-phase of the cell cycle (BrdU positive) increased significantly under Kainate conditions in comparison with controls. E), F) There was no significant effect of 6 h Kainate treatment on the number and proportion of TuJ1 cells that incorporated BrdU. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t-test*. *** $p<0.001$

3.4.3.6 Kainate treatment did not affect co-expression of Nestin positive and TuJ1 positive cells in 3 DIV

We reported earlier in this **Chapter** that Kainate increased the proliferation of TuJ1 expressing cells and nestin positive cells after 3 DIV (**Sections 3.4.3.2 & 3.4.3.4**). We also found significant proportional increase in the TuJ1 sub-population of cells as well as nestin expressing cells after 3 DIV (**Sections 3.4.3.2 & 3.4.3.4**). Although the behaviour of these cell populations appears similar over three days exposure, we also found significant differences in the effects of short exposure, with Kainate being proliferative for nestin positive cells but not TuJ1 positive cells. Therefore, we decided in this series of experiments to double label cells that co-express both nestin and TuJ1 to address any significant differentiation effect that Kainate might have on progenitor cells. Primary hippocampal cell cultures were prepared following the protocol in **Chapter 2** (**Section 2.4.2**) and maintained under control conditions or in the additional presence of 5μM Kainate for three DIV. Cells were fixed with 4% PFA and double labelled for the stem/progenitor marker nestin and the neuroblasts marker TuJ1. The proportion of cells that co-express both nestin and TuJ1 in culture was measured under both Kainate and control conditions. To assess differentiation, the proportion of nestin positive cells that express TuJ1 was determined.

There were no significant changes in the proportion of cells that were co-labelled for both nestin and TuJ1 in culture when comparing Kainate exposed cells to control maintained cells (**Figure 3.10 A**). We found 10.3 ± 1.4 % of cells co-express both nestin and TuJ1 in culture and this proportion did not change in response to 3 days of Kainate exposure. The proportion of nestin cells that expressed TuJ1 was 21.4 ± 3.1 % under control conditions and did not change after 3 days of Kainate treatment (**Figure 3.10 B**). Likewise, there was no significant effect for Kainate on TuJ1 cells that expressed nestin (**Figure 3.10 C**). Interestingly, there was a significant increase in the numbers of cells that were nestin immuno-positive but TuJ1 immuno-negative under Kainate conditions (Kainate, 76.4 ± 4.6 cells/mm^2, vs. control, 57.6 ± 4.1 cells/mm^2) (unpaired simple Student's t-test, $p<0.05$) (**Figure 3.10 D**).

These results suggest that Kainate does not have significant differentiation effects on

progenitor cells after 3 DIV and its effect is mainly on nestin precursor cells.

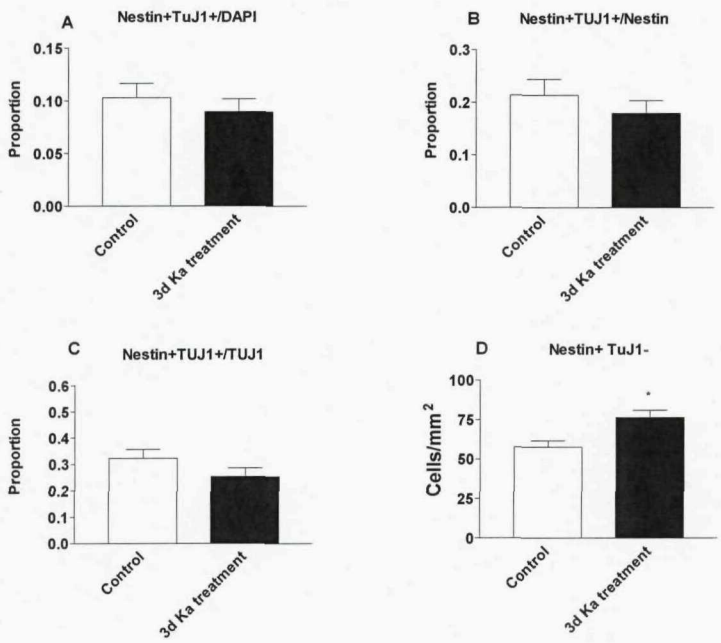


Figure 3.10 Kainate had no effect on co-expression of TuJ1 positive and Nestin positive cells in 3 DIV. Cells were cultured for 3 days in the absence and presence of 5μM Kainate. After fixation cells were stained against nestin and TuJ1. 3 days of Kainate exposure did not have an effect on progenitor cell differentiation. A) There was no significant change in the proportion of cells that co-express both nestin and TuJ1 when comparing Kainate treated conditions with control conditions. B) The proportion of nestin positive cells that expressed TuJ1 did not change after 3 days of Kainate treatment in comparison with controls. C) The proportion of TuJ1 positive cells that expressed nestin did not change after 3 days of Kainate treatment. D) There was a significant increase in the number of nestin immuno-positive cells that were TuJ1 negative after 3 days of Kainate treatment. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 experiments. Ka = Kainate. 3d = 3 days. Data points were plotted using the Graph Pad Prism software and means compared using unpaired *simple Student's t test.* * $p<0.05$

3.4.4.1 Kainate is proliferative for Nestin expressing hippocampal progenitor cells after 5 DIV

We have demonstrated that 3 days of Kainate treatment enhanced the proliferation of hippocampal cells, progenitor cells (nestin positive cells), and TuJ1 expressing cells in culture (**Sections 3.4.2, 3.4.3.1 and 3.4.3.4**). In our time-course set of experiments we found that 5 days of Kainate treatment resulted in a significant increase in hippocampal cells in culture and also in cells that incorporated BrdU. But no proportional increase in cell proliferation (BrdU/DAPI) was found (**Section 3.4.2**). Therefore, to understand the mechanism by which cell counts increased by day five, we explored the proliferative effects of 5 days of Kainate treatment on progenitor cells (nestin positive) and neuronal precursor cells (TuJ1 expressing cells). We will also address the effects of short Kainate exposure on cell cycle kinetics, progenitor cells, and neuronal precursors to examine any direct proliferative effects that Kainate might have on hippocampal cells and / or cell phenotype.

We grew cells for 5 days under standard growth conditions (control) and under 5μM Kainate as detailed in **Chapter 2** (**Section 2.4.2**). Cells were terminally pulsed for 4 hours with a final concentration of 20μM BrdU. Then cells were fixed and stained against the proliferative marker BrdU and the stem/progenitor marker nestin. Thereafter, cells were imaged and analyzed as mentioned in methodology (**Sections 2.4.8 & 2.4.10**). We measured total cell counts (DAPI positive cells), numbers, and proportions of BrdU incorporated cells. Moreover, we also determined the number and proportion of proliferating nestin positive cells (nestin expressing cells that entered S-phase in the last 4 h) under control and Kainate conditions.

The total number of cells increased significantly under 5μM Kainate (182.2 ± 3.9 cells/mm^2 versus 130.8 ± 3.0 cells/mm^2 under control conditions) (unpaired simple Student's t-test) (**Figure 3.11 A**). Cells that entered S-phase of the cell cycle within that terminal 4 hours prior to fixation (BrdU incorporated cells) increased significantly from 17.7 ± 1.7 cells/mm^2 under control condition to 29.3 ± 1.7 cells/mm^2 after 5 days of Kainate treatment (unpaired simple Student's t-test) (**Figure 3.11 B**). However, the proportion of BrdU incorporated cells did not increase under Kainate conditions when

compared to control conditions (**Figure 3.11 C**). The increase in the total cell counts under Kainate treatment was paralleled with a significant rise in cells that expressed nestin (46.7 ± 1.7 cells/mm^2 under control condition increased to 92.2 ± 2.7 cells/mm^2 under Kainate conditions) (unpaired simple Student's t-test) (**Figure 3.11 D**). Although the number of nestin immuno-positive cells has doubled after 5 days of Kainate treatment, there was no significant proportional increase in this sub-population of cells (**Figure 3.11 E**). Moreover, 5 days of 5μM Kainate exposure significantly increased the numbers of nestin positive cells that have incorporated BrdU from 5.5 ± 0.7 cells/mm^2 under control condition to 17.5 ± 1.8 cells/mm^2 under Kainate conditions (unpaired simple Student's t-test) (**Figure 3.11 F**). Interestingly, this significant rise in nestin positive cells that incorporated BrdU (nestin+ and BrdU+) resulted in 2-fold increase in the proportion of nestin positive cells that have incorporated BrdU after 4 hours exposure to BrdU and 5 days of Kainate treatment, in comparison with control conditions (**Figure 3.11 G**). **Figure 3.12** demonstrates the pattern of nestin expressing cells and the proliferation of this sub-population of cells.

Our results suggest that 5 days of Kainate treatment is proliferative for hippocampal progenitor cells. However, it is unclear whether Kainate has a short direct effect on progenitor cells as it does at day 3. Therefore, we will investigate the proliferative effects that Kainate might have on hippocampal cells in general and progenitor cells in particular after a short (6 h) exposure.

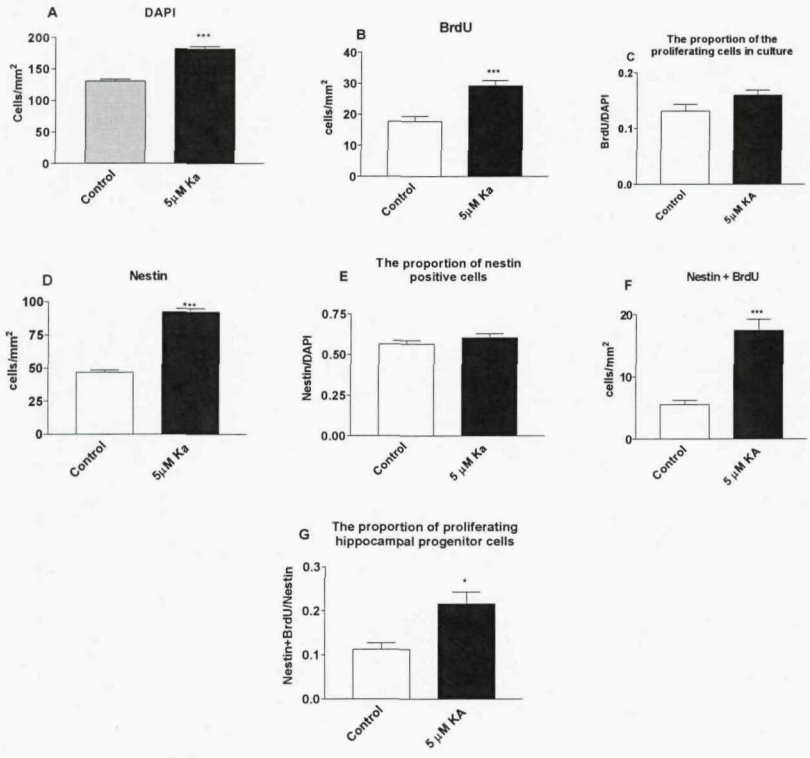


Figure 3.11 Kainate is proliferative for Nestin expressing cells after 5 DIV. Cells were cultured for 5 days in the absence and presence of 5μM Ka. On day five they were pulsed with a final concentration of 20μ M BrdU for the last 4 h. After fixation cells were stained against nestin and BrdU. 5 days of Kainate treatment increased the total cell counts (DAPI), the counts of BrdU incorporated cells, nestin positive cells, and the proportion of proliferating nestin positive cells. A) A significant increase in the total cell counts (DAPI) under Kainate treatment was seen when compared to controls. B) BrdU incorporating cells in the last 4 h increased significantly after 5 days of Kainate treatment. C) There was no change in the proportion of BrdU incorporating cells in culture. D) The counts of stem/progenitor (nestin positive) cells increased significantly after 5 days of Kainate exposure in comparison with control conditions. E) There was no change in the proportion of stem/progenitor (nestin positive cells) cells in culture under Kainate conditions when compared to controls. F) The counts of the proliferating progenitor (nestin positive that incorporated BrdU) cells increased significantly after 5 days of Kainate exposure when compared to control conditions. G) The proportion of proliferating progenitor (nestin+) cells increased significantly after 5 days *in vitro* when comparing Kainate conditions with control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 3 experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t-test.** $p<0.05$, *** $p<0.001$

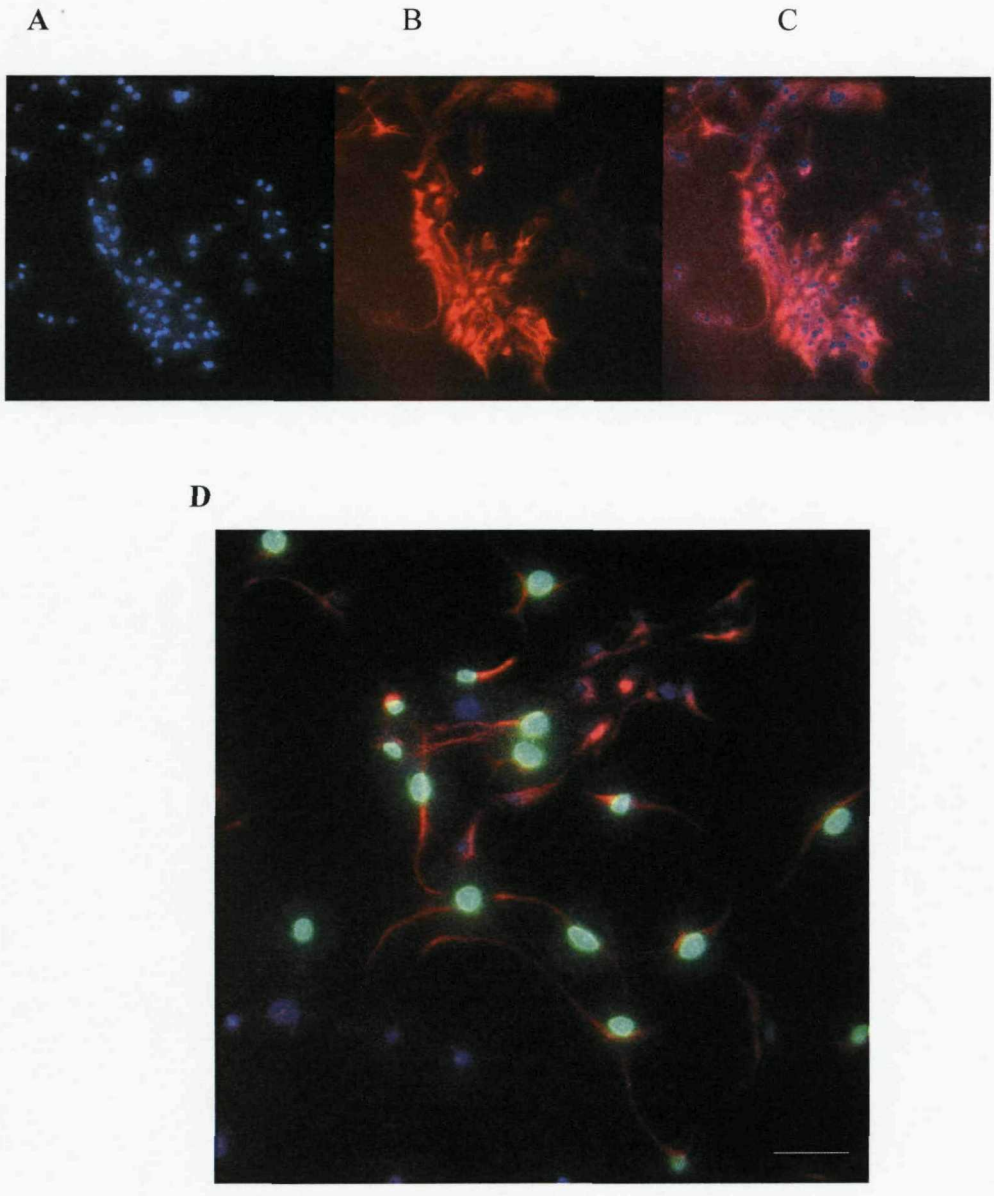


Figure 3.12 Kainate enhanced the proliferation of Nestin-positive cells in hippocampal cultures. Cells were cultured in the presence and absence of 5μM kainate and maintained for 5 days. They were terminally pulsed with BrdU for 4 hours. After fixation with 4% PFA, cells were probed with antibodies to BrdU (green) (D), nestin (red) (B C & D), and counterstained for the nuclear marker DAPI (blue) (A). We showed a significant increase in the total number of nestin positive cells and the proportion of nestin cells that were in the S-phase of the cell cycle under Kainate treatment. Imaging was performed on an inverted Leica DM IRB microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). Open Lab image capturing system version 2.1 (Improvision, Lexington, USA) was used to count cells. Control experiments with the primary antibody omitted were devoid of staining Scale bar = 40μm.

3.4.4.2 A short 8 hours pulse of Kainate increased the number of proliferating cells but not the proportion or the speed of the cell cycle

We have demonstrated (**Section 3.4.3.2**) that short exposure of Kainate at day 3 yielded a significant increase in cell proliferation (BrdU/DAPI) and in the rate of the cell cycle (labelling index - BrdU/Ki-67). In the previous section (**Section 3.4.4.1**) we reported a significant increase in total cell counts and in the number of BrdU incorporated cells after 5 days of Kainate treatment. However, there was no proportional increase in the number of BrdU incorporated cells compared to the total number of cells. In order to understand the mechanisms that resulted in a net increase in cell counts after 5 days of Kainate exposure and whether Kainate has direct proliferative effects on progenitor cells, we will study the effects of short (8 h) Kainate exposure on cell counts, BrdU incorporated cells and cell cycle kinetics at day five.

Cultures were generated as before (**Section 2.4.2**). A terminal 8 h pulse of 5μM Kainate was given to cultures undergoing treatment. Another group of cells was maintained under standard growth conditions and considered our control group. All conditions (control and treated cultures) were pulsed with a final concentration of 20μM BrdU for the last 8 h. Cells were fixed with 4% PFA and immunostained against the proliferative marker BrdU and the cell cycle marker Ki-67 as detailed in **Chapter 2 (section 2.4.7)**.

Surprisingly, we found that 8 h of Kainate exposure significantly increased the total hippocampal cell count in comparison with control conditions (182.6 ± 11.0 cells/mm^2 under control conditions versus 253.6 ± 7.0 cells/mm^2 under Kainate treatment) (unpaired simple Student's t-test, $p< 0.001$) (**Figure 3.13 A**). We found that 57.0 ± 5.3 cells/mm^2 incorporated BrdU under control conditions and 82.8 ± 7.5 cells/mm^2 incorporated BrdU after 8 h Kainate exposure with a significant difference between the two conditions (unpaired simple Student's t-test, $p<0.05$) (**Figure 3.13 B**). We also found that the number of proliferating cells (Ki-67 positive cells) increased from 165.7 ± 11.0 cells/mm^2 under control conditions to 237.4 ± 7.7 cells/mm^2 under Kainate treatment (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.13 C**). However, we did not find a proportional increase in BrdU incorporated cells after 8 h of Kainate treatment and in comparison with control conditions (**Figure 3.16 D**) which we had expected to

find since the number of proliferating cells had increased. The growth fraction (Ki-67/DAPI) and the labelling index (BrdU/Ki67) did not change when comparing Kainate treated cells with control conditions (**Figure 3.16 E & F**).

These results demonstrate that an 8 hour pulse of Kainate increased the total cell counts and cells that entered S-phase after short exposure but without increasing the general cell proliferation (BrdU/DAPI) or the speed of the average cell cycle (BrdU/Ki-67). It also implies that Kainate did not recruit quiescent cells, as there was no increase in the growth fraction. This must mean that Kainate had an additional trophic effect on cycling cells and this point is further explored in **Chapter 5**.

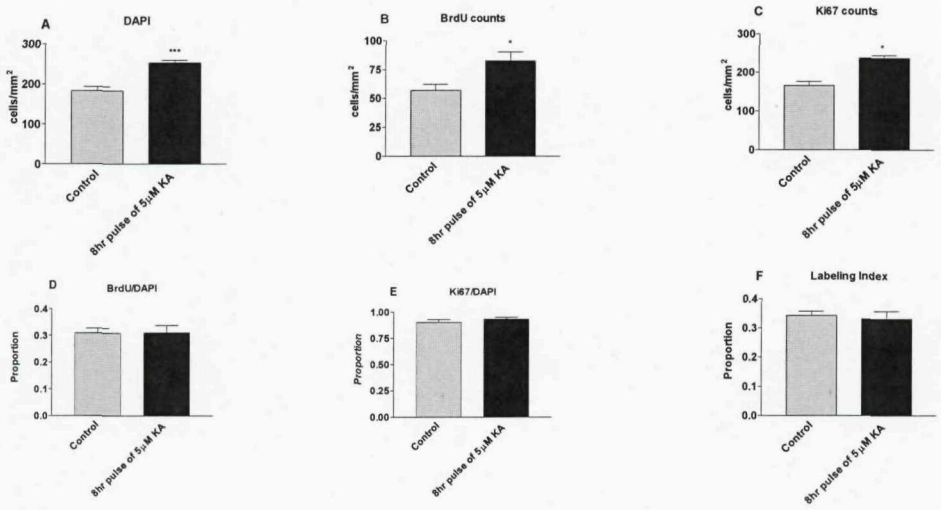


Figure 3.13 Terminal 8 h of Kainate exposure at day 5 increased BrdU incorporated cells but not the growth fraction or labelling index. Monolayer primary hippocampal cell cultures were generated from postnatal rat pups (7-10 days) and maintained under standard growth conditions for 5 days. 8 h terminal pulse of 5μM Kainate was applied to a group of cells, while another group of cultures was maintained under control conditions. Terminal 8 h of a final concentration of 20μM BrdU was given to all conditions. Cells were fixed and immunostained against both BrdU and Ki-67. We found a significant increase in DAPI counts, BrdU incorporated cells, and Ki-67 positive cells after 8 h pulse of 5μM Kainate. A) There was a significant increase in total cell counts (DAPI) after 8 h of Kainate exposure when compared to control conditions. B) BrdU incorporated cells in the last 8 h in culture increased significantly under Kainate conditions. C) There was a significant increase in Ki-67 expressing cells in 5 days cultures and terminal 8 h of Kainate treatments. C) There was no change in the proportion of proliferating cells (BrdU/DAPI) when comparing Kainate conditions with control conditions. D) The growth fraction (Ki-67/DAPI) was the same under control and treated conditions. E) No difference in the labelling index after terminal 8 h of both BrdU and Kainate when comparing Kainate conditions with control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 3 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t-test*.* $p<0.05$, *** $p<0.001$.

3.4.4.3 Terminal short pulse of Kainate is proliferative for nestin positive hippocampal progenitor cells

We have demonstrated (**Section 3.4.4.1**) that 5 days of Kainate treatment is proliferative for nestin expressing cells. Our results (**Section 3.4.4.2**) revealed a significant increase in the numbers of proliferating and cycling cells without a proportional increase after short term of Kainate exposure to hippocampal cells. Here we examine the effects of short term of Kainate treatment on the proliferation of nestin expressing cells. Using our Primary hippocampal cell culture system we prepared cells as detailed in **Chapter 2** (**Section 2.4.2**). On day five 6 h terminal pulse of a final concentration of 20μM BrdU was applied to all wells but whereas 5μM Kainate was given to half of the wells, while the rest of the wells were kept under standard control conditions (untreated). Cells were fixed with 4% PFA and immunostained against the proliferative marker BrdU and the stem/progenitor marker nestin **Chapter 2** (**Section 2.4.7**). Finally, cells were imaged and data analyzed as detailed in **Chapter 2** (**Sections 2.4.8 and 2.4.10**).

Another set of experiments was carried out in which cells were terminally pulsed for the last 8 hours with BrdU (a final concentration of 20μM) whereas 5μM Kainate was applied to cells undergoing treatment. After cell fixation; they were probed for nestin, Ki-67 and BrdU (triple immunohistochemistry) in order to measure the labelling index of nestin sub-population of cells and thus the speed of the cell cycle for nestin expressing cells.

Again our results revealed a significant increase in the number of nestin immuno-positive cell counts (**Figure 3.14 A**) after a 6 h of Kainate exposure. 48.6 ± 1.5 cells/mm^2 expressed nestin under control conditions and increased significantly to 73.4 ± 3.9 cells/mm^2 after 6 h of Kainate exposure (unpaired simple Student's t-test, $p<0.01$). There was no proportional increase in nestin sub-population of cells after Kainate treatment at day 5 (**Figure 3.14 B**). We demonstrated a significant rise in the raw counts, but not the proportion, of BrdU incorporated cells (of total cells) under Kainate treated conditions when compared to control conditions (**Figure 3.14 C and D**). Interestingly, there was a significant increase in both the number and proportion of nestin positive cells that entered the S-phase of the cell cycle within the last 6 hours under Kainate

conditions compared to controls (Kainate, 22.1 ± 2.3 cells/mm² and 29.6 ± 2.0 %, vs. controls, 6.1 ± 1.2 cells/mm² and11.5 ± 2.6 % numbers and proportions, respectively) (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.6 E and F**).

Furthermore, triple immunohistochemistry for BrdU, Ki-67 and nestin was carried out. Con-focal images were taken and 100 nestin immuno-positive cells were counted. Co-localization of nestin expressing cells with BrdU and Ki-67 was also measured. All nestin positive cells were Ki-67 immuno-positive (**Table 3.3**). There was a significant proliferative effect of Kainate on nestin sub-population of cells. The labelling index for nestin cells increased from 10% under control conditions to 25% after 8 hours of Kainate (**Table 3.3**). Degrees of freedom: 1, Chi-square = 7.8 and $p <0.01$. Sample images are shown in **Figure 3.15.**

Our results demonstrate that 6 h or 8 h of Kainate exposure has direct proliferative effects on hippocampal nestin positive progenitor cells at day 5 in cultures.

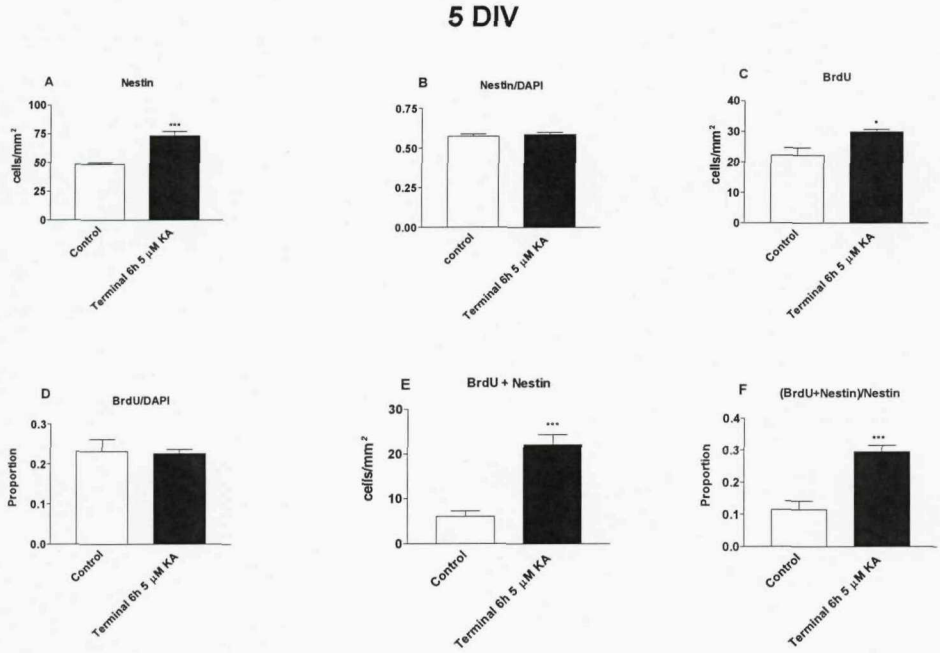


Figure 3.14 Short treatment of Kainate is proliferative for Nestin positive cells at day 5 *in vitro*. Cells were cultured for 5 days under standard growth conditions. On day five, a 6 h terminal pulse of 5µM Ka was given to one group of cells, while the other group of cultures was maintained as control. BrdU was applied to all conditions for the terminal 6 h. Then cell fixation and immunostaining against nestin and BrdU were carried out. 6 h of Kainate exposure increased the counts and proportion of nestin positive cells that entered S-phase of the cell cycle in the last 6 h. A) Nestin expressing cell counts increased significantly after a short pulse of Kainate. B) The proportion of nestin sub-population of cells in culture was the same under control and Kainate conditions. C) The counts of BrdU positive cells increased significantly after 6 h of Kainate exposure. D) There was no change in the proportion of proliferating cells in primary hippocampal cultures in response to 6 h Kainate treatment. E) Nestin and BrdU positive proliferating stem/progenitor cell counts increased under Kainate conditions when compared to control conditions. F) The proportion of proliferating nestin expressing cells increased significantly after 6 h Kainate treatment at day 5. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t-test.** $p<0.05$, *** $p<0.001$.

	5DIV Hippocampal cells in culture	
	Nestin+ and Ki-67+ but BrdU-	Nestin+, Ki-67+ and BrdU+
Control	90	10
8 hours Kainate	75	25

Table 3.3 Kainate enhanced the proliferation rate of nestin immuno-positive cells at day 5. Hippocampal cells were grown for 5 days before being pulsed with 5μM Kainate and BrdU for the last 8 hours. Triple immunohistochemistry for BrdU, Ki-67 and nestin was carried out. Con-focal images were taken and 100 nestin immuno-positive cells were counted and co-localization of nestin expressing cells with BrdU and Ki-67 was also measured. All nestin positive cells were Ki-67 immuno-positive. There was a significant proliferative effect of Kainate on nestin sub-population of cells. The labelling index for nestin cells increased from 10% under control conditions to 25% after 8 hours of Kainate treatment. Degrees of freedom: 1, Chi-square = 7.8 and $p < 0.01$

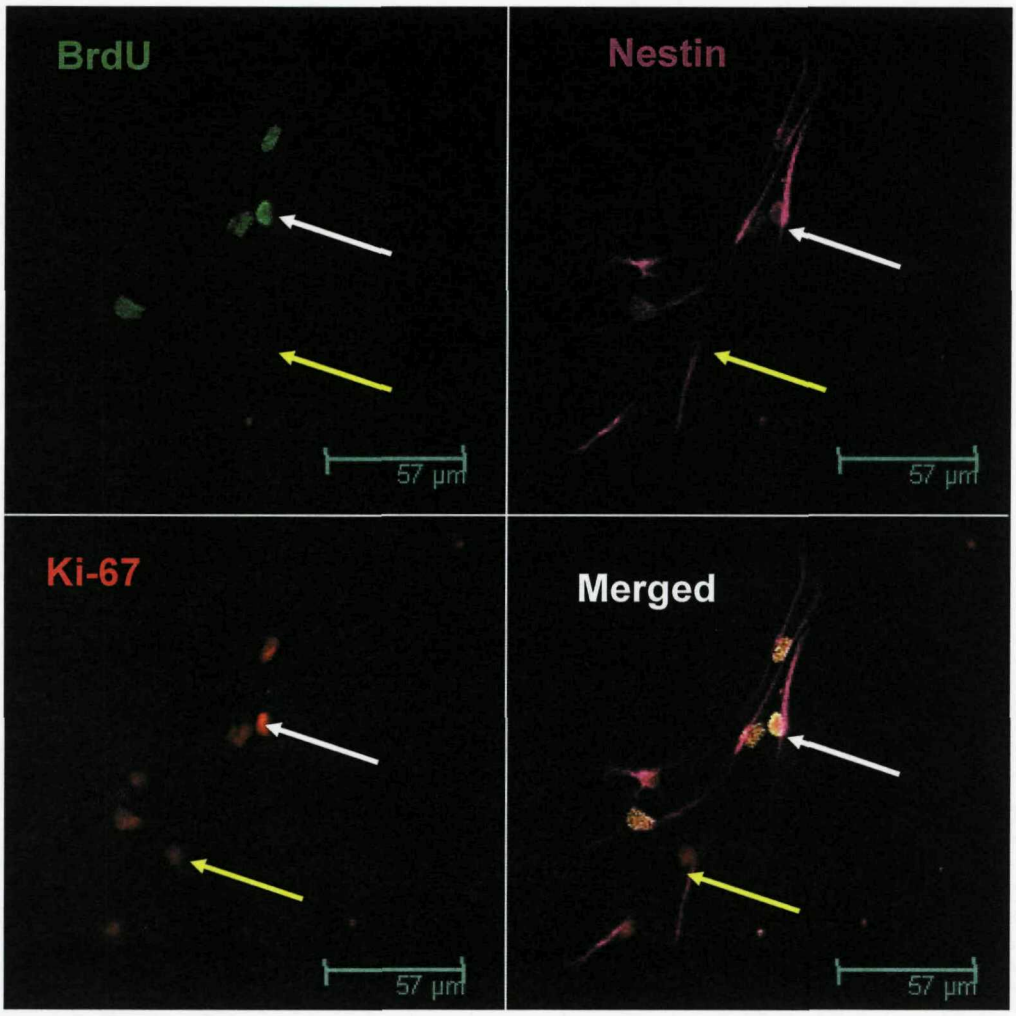


Figure 3.15 Micrograph of proliferating stem/progenitor cells. We grew primary hippocampal cell for 5 days under standard conditions and 5µM Kainate. Two-thirds of the growth medium with/without 5µM Kainate was replaced on day three. 8 hours terminal pulse of BrdU and Kainate was applied to cultures. Fixation for 30 minutes with 4% PFA was done on day 5 before consequent immunocytochemistry. Primary specific anti-sera against nestin (stem/progenitor marker), BrdU, and Ki-67 were detected with fluorescently linked secondary antibodies. The staining shows BrdU in green, nestin in purple color, and Ki-67 in red. White arrows show samples of nestin positive cell that incorporated BrdU and also expressing Ki-67. Yellow arrows demonstrate samples of nestin positive cell that expressing Ki-67 but not BrdU (cycling nestin cells). Images were captured with 40x oil objective on a laser scanning con-focal microscope and 3D projections reconstructed using Leica Microsystems LAS AF lite software. Control experiments with the primary antibody omitted were devoid of staining. Scale bar = 57µm.

3.4.4.4 Kainate increased the proportion of TuJ1 positive cells in 5 DIV

We have demonstrated that 5 days of Kainate treatment increases the proliferation of progenitor (nestin positive) cells (**Section 3.4.4.1**) and also increases the counts of proliferating (BrdU incorporated) cells and the total cell counts (DAPI). In order to have an increase in neurogenesis there should be a consequent increase in the number of neurons. Therefore, we will address the effects of 5 days exposure to Kainate on neuronal precursors and their proliferation.

To assess the effect of Kainate on neuroblasts' counts and proliferation in culture, single cell suspension was generated from hippocampi of postnatal rat pups 7-10 days. Cells were grown either under control conditions or under 5μM Kainate enriched conditions and maintained for 5 DIV. Two-thirds of the growth medium with/without enrichments was changed on day three. Cells were terminally pulsed for 4 hours with a final concentration of 20μM BrdU. After fixation with 4% PFA, cells were immunostained against both the neuronal marker, TuJ1, and the proliferation marker, BrdU, **Chapter 2** (**Section 2.4.7**). We measured the total cell counts (DAPI), TuJ1 expressing cell counts and proportion, the counts of BrdU incorporated cells, and neuroblasts that entered S-phase of the cell cycle in the last 4 hours.

The total cell counts increased significantly from 122.8 ± 2.1 cells/mm^2 under control conditions to 171.7 ± 3.7 cells/mm^2 under Kainate conditions (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.16 A**). TuJ1 expressing cells increased significantly after 5 days of Kainate exposure when compared to control maintained cells (44.53 ± 4.4 cells/mm^2 under control versus 74.1 ± 3.7 cells/mm^2 under Kainate conditions) (unpaired simple Student's t-test, $p<0.01$) (**Figure 3.16 B**). Moreover, the proportion of TuJ1 immuno-positive cells significantly increased from 34.3 ± 3.5 % under control conditions to 45.3 ± 2.6 % after 5 days of Kainate treatment (unpaired simple Student's t-test, $p<0.05$) (**Figure 3.16 C**). Consistently, the number of BrdU incorporated cells increased significantly under Kainate conditions when compared to cells under control conditions (**Figure 3.16 D**). However, there was no change in the proportion of BrdU incorporated cells (of total cells) after 5 days of Kainate exposure in comparison with control conditions (**Figure 3.16 E**). Although the numbers of proliferating neuroblasts

(TuJ1 positive cells that entered S-phase of the cell cycle) increased significantly form 7 ± 1.9 cells/mm² under control to 16.9 ± 1.3 cells/mm² under Kainate conditions (unpaired simple Student's t-test, $p<0.01$) (**Figure 3.16 F**), there was no proportional increase in BrdU incorporation among TuJ1 positive cells (**Figure 3.16 G**), although there was a trend towards this.

These results suggest that 5 days of Kainate treatment yielded a net rise in neurogenesis. The next step is to investigate the mechanisms of this effect, and specifically whether a short (6 h) exposure of Kainate has direct effect on neuroblasts as it does on hippocampal progenitor (nestin positive) cells.

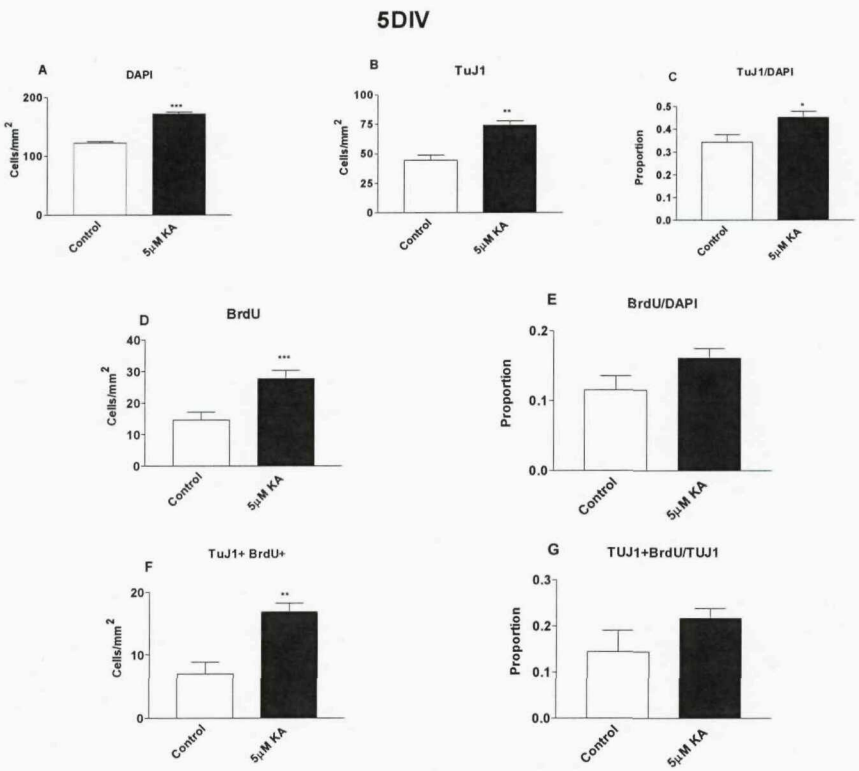


Figure 3.16 5 days of Kainate treatment increased the proportion of TuJ1 sub-population of cells. Cells were cultured for 5 days in the absence and presence of 5μM Ka. Two-thirds of the growth medium with/without treatment was replaced on day three. On day 5 all conditions were pulsed with a final concentration of 20μM BrdU for the last 4 h. After fixation cells were stained against TuJ1, BrdU, and counterstained for the nuclear marker DAPI. We found that Kainate treatment increased the total cell counts (DAPI), TuJ1 positive cells, the proportion of TuJ1 cells in culture, BrdU incorporated cells, and the TuJ1 positive cells that entered S-phase of the cell cycle in the last 4 h. A) The total cells were measured by counting DAPI stained cells and increased significantly after Kainate treatment. B) There was a significant increase in the counts of TuJ1 expressing cells under Kainate conditions compared to control conditions. C) The proportion of TuJ1 cells increased significantly after 5 days of Kainate exposure when compared to control conditions. D) The counts of BrdU incorporated cells increased significantly in response to Kainate treatment. E) There was no change in the proportion of proliferating cells incorporating BrdU over the terminal 4 hours, between control and Kainate conditions in culture. F) The counts of proliferating neuroblasts increased significantly after Kainate treatment in comparison with control conditions. G) There was no change in proportion of proliferating TuJ1 expressing hippocampal cells when comparing Kainate with control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using unpaired *simple Student's t-test*.* $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.4.4.5 Terminal short pulse of Kainate increased the proportion of TuJ1 positive cells at day 5

We reported, in the previous section, that 5 days of Kainate treatment increased the proportion of TuJ1 sub-population of cells. In this part we aim at investigating short term (6 h) effects of Kainate on proliferating neuroblasts in order to establish the possibility of direct proliferative effects for Kainate on neuronal precursors in cultures.

Primary hippocampal cells were prepared as mentioned in **Chapter 2** (**Section 2.4.2**). 6 hours terminal pulse of a final concentration of 20μM BrdU was applied to all wells but whereas 5μM Kainate was given to half of the wells, while the rest of the wells were maintained under standard control conditions (untreated). Cells were fixed with 4% PFA and immunostained against the proliferative marker BrdU and the neuroblasts marker TuJ1 **Chapter 2** (**Section 2.4.7**).

Consistent with previous data in **Section 3.4.4.2** there was a significant increase in the total cell number as indicated by DAPI counts (**Figure 3.17 A**). Importantly, at day 5 we again observed a significant rise in both the counts (29.0 ± 1.7 cells/mm^2 under control conditions versus 40.6 ± 1.8 cells/mm^2 after 6 h of Kainate exposure (unpaired simple Student's t-test, $p<0.001$)) and the proportion of TuJ1 sub-population of cells (**Figures 3.17 B & C**). Although the proliferating TuJ1 cells (TuJ1+BrdU+) were 6.2 ± 0.8 cells/mm^2 under control conditions and increased significantly to 15.5 ± 1.7 cells/mm^2 after 6 h of Kainate exposure (unpaired simple Student's t-test, $p<0.01$) (**Figure 3.17 D**), we again did not find a significant increase (there was a trend towards an increase) in the proportion of these proliferating neuronal precursors in response to 6 h Kainate treatment (unpaired simple Student's t-test, $p = 0.2$) (**Figures 3.17 E**).

Therefore, the proportional increase in TuJ1 sub-population of cells cannot be explained by a short proliferative effect of Kainate on cells only and suggests that another mechanism of action, possibly trophic or a directive effect towards neuronal commitment might be involved. If Kainate does have such a synergistic survival role explaining the net rise in the proportion of TuJ1 expressing cells even after 6 h of Kainate treatment, then there must be significant death of proliferating neuroblasts in culture, and this will be the focus of **Chapter 5**.

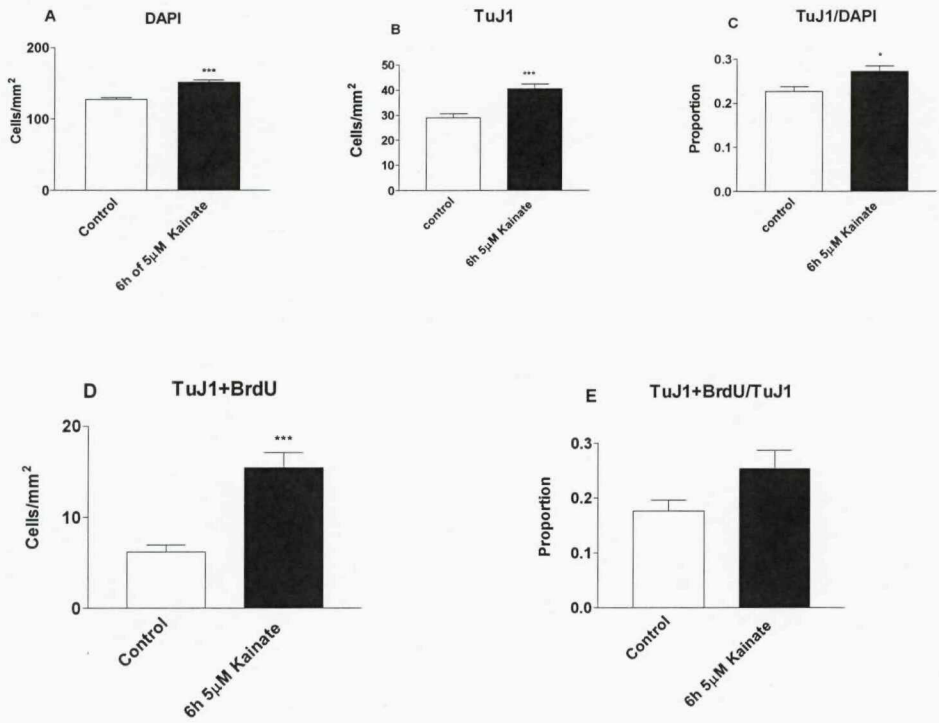


Figure 3.17 6h of Kainate treatment increased the proportion of TUJ1 positive cells at day 5. Cells were cultured for 5 days under standard growth conditions. Two-thirds of the growth medium was changed on day 3. On day five 6 h terminal pulse of 5μM Kainate was given to a group of cells, while the other group of cultures were maintained in control conditions. 6 h terminal pulse of a final concentration of 20μM BrdU was applied to all conditions. Then cell were fixed and immunostained against TuJ1 and BrdU. We found a significant increase in the number and proportion of TuJ1 positive cells. A) TuJ1expressing cell counts increased significantly after 6 h of Kainate treatment. B) The proportion of TuJ1 positive cells increased in response to Kainate. C) The counts of the proliferating TuJ1expressing cells increased significantly when comparing Kainate with control conditions. D) There was no change in the proportion of the proliferating TuJ1 positive cells after 6 h of Kainate exposure in comparison with control conditions. E) There was no significant increase in the proportion of proliferating neuroblasts. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 3 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t-test*.* $p<0.05$, *** $p<0.001$

3.4.4.6 Kainate enhanced differentiation of hippocampal progenitor cells towards a neuronal lineage by 5 DIV

We reported earlier in this **Chapter** that Kainate is proliferative for TuJ1 expressing cells at 3 but not day 5 DIV (**Sections 3.4.3.4 and 3.4.4.4**), but we also observed a significant proportional increase in TuJ1 sub-population of cells in 5 DIV (**Sections 3.4.4.4**). In addition there was an increase in nestin immuno-positive cell number but not their proportion after 5 days under Kainate treatment. Therefore, we decided in this series of experiments to double label cells that co-express nestin and TuJ1 to address any significant differentiation effect that Kainate might have on progenitor cells. If there is a differentiation effects after 5 days of Kainate treatment, this would explain the net increase in neurogenesis we observed after 5 days in culture under Kainate conditions. Primary hippocampal cell cultures were prepared following the protocol in **Chapter 2** (**Section 2.4.2**). On day five cells were fixed with 4% PFA and double labelled for the stem/progenitor marker nestin and the neuroblasts marker TuJ1. Total cell counts were assessed by DAPI stained cell nuclei.

Consistent with previous findings there was no proportional increase in nestin sub-population of cells after 5 days of Kainate treatment when compared to control conditions ((**Figure 3.18 A**). In line with what we have found before, there was a significant increase in the proportion of TuJ1 expressing cells after 5 days of Kainate treatment and in comparison with controls (Kainate, 29 ± 1.0%, vs. control 21± 1.1%) (unpaired simple Student's t-test, $p<0.05$) (**Figure 3.18 B**). Interestingly, 5 μM Kainate increased co-expression of both nestin immuno-positive cells and TuJ1 positive cells. While, under control conditions we found 7.8 ± 0.9 cells/mm^2 expressing both nestin and TuJ1, this number significantly increased to 19.6 ± 0.7 cells/mm^2 after 5 days of Kainate exposure (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.18 C**). Moreover, the proportion of cells that expressed both nestin and TuJ1 increased significantly in culture when comparing Kainate to control conditions (6.0 ± 0.6 % under control conditions versus 12.5 ± 0.6 % under Kainate treatment) (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.18 D**). Importantly, we observed a proportional increase in nestin expressing sub-population of cells that expressed TuJ1. This proportion significantly increased from

10.6 ± 0.8 % under control conditions to 22.9 ± 0.8 % under Kainate treatment (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.18 E**). In line with this, there was a significant drop in nestin immuno-positive cells that did not express TuJ1 after 5 days of Kainate treatment when compared to controls (Kainate, 80.7 ± 2.0 %, vs. control, 89.5± 0.8 %). In addition, the proportion of TuJ1 cells that expressed nestin increased significantly after 5 days of Kainate treatment when compared to control conditions (Kainate, 47.6 ± 2.7%, vs. controls, and 26.5 ± 2.2%) (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.18 G**). Sample images are displayed in (**Figure 3.19**). These findings suggest a significant combination of proliferation and differentiation from 5 days of Kainate exposure drove precursor cells towards a neuronal lineage, as a simple differentiation effect would have lowered the proportion of TuJ1 cells expressing nestin.

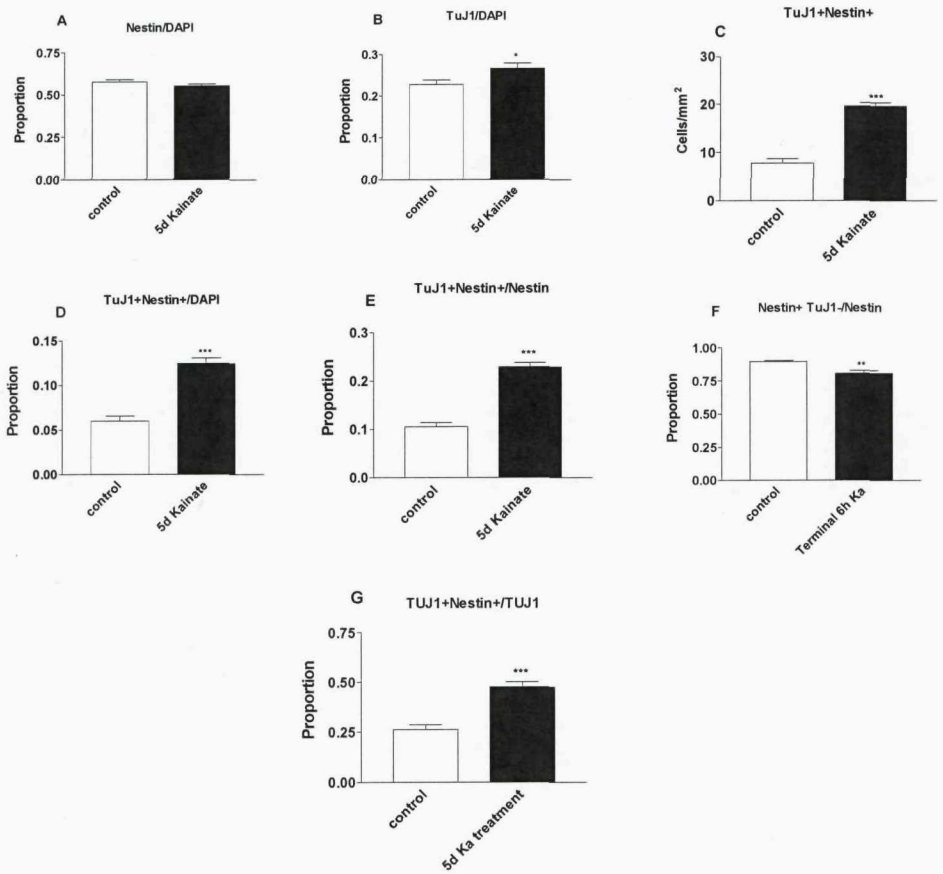


Figure 3.18 Kainate enhanced progenitor cells differentiation after 5 DIV. Cells were cultured for 5 days in the absence and presence of 5μM Kainate. On day five cells were fixed and stained against nestin and TuJ1. Kainate increased the cell counts and the proportion of cells that were immuno-positive for both nestin and TuJ1 (double-labelled for TuJ1 and nestin). It also increased the proportion of nestin positive cells that expressed TuJ1 which implies a differentiation effect. A) There was no significant proportional increase in nestin immuno-positive cells after 5 days of Kainate treatment. B) There was a significant proportional increase in TuJ1 sub-population of cells under Kainate conditions. C) There was an increase in nestin positive and TuJ1 positive cells after 5 days of Kainate treatment. D) The proportion of cells that co-express both nestin and TuJ1 increased significantly in culture when comparing Kainate to control conditions. E) The proportion of stem/progenitor (nestin) cells that expressed TuJ1 increased after 5 days of Kainate exposure in comparison with control conditions. F) The proportion of nestin only cells (that did not express TuJ1) decreased under Kainate conditions. G) There was a significant increase in TuJ1 positive cells that expressing nestin after 5 days of Kainate treatment. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t test.* * $p<0.05$, ** $p<0.01$, *** $p<0.001$

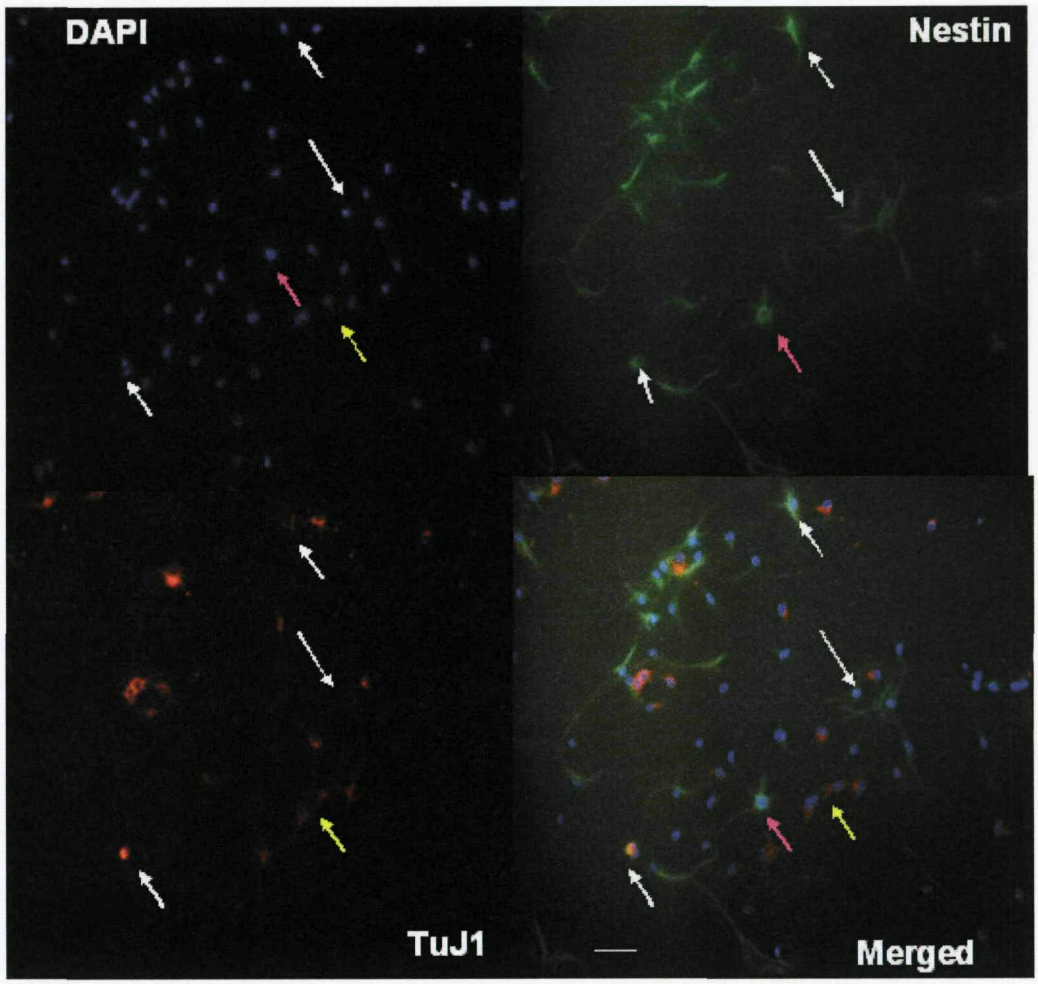


Figure 3.19 Micrograph of stem/progenitor (Nestin) cells that co-expressing TuJ1 cells. We grew primary hippocampal cells for 5 days under standard growth conditions and under 5μM Kainate. Cells were fixed for 30 minutes with 4% PFA on day 5 before consequent immunocytochemistry. Primary specific anti-sera against nestin (stem/progenitor marker) and the neuronal marker TuJ1 were detected with fluorescently linked secondary antibodies. Counterstaining for the nuclear marker DAPI was carried out at the end of immunocytochemistry. The micrograph shows DAPI in blue, nestin in green, and TuJ1 in red. White arrows show sample of nestin positive and TuJ1 positive cells. Pink arrows show sample of cells that express nestin only. Yellow arrows show sample of TuJ1 expressing cells only. Images were captured with 20x objective on an inverted Leica DM IRB microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). Volocity image capturing system version 4.0 (Improvision, Lexington, USA) was used to count cells. Control experiments with the primary antibody omitted were devoid of staining. Scale bar = 20μm.

3.4.4.7 Kainate increased the number but not proportion of GFAP positive cells in culture

We have demonstrated significant effects of Kainate on nestin positive cells as well as TuJ1 expressing cells. In this part we will address the effects of 5 days of 5μM Kainate treatment on hippocampal astrocytes or astrocyte-like GFAP expressing cells.

We cultured hippocampal cells for 5 days in the absence and presence of 5μM Kainate as described in **Chapter 2** (**Section 2.4.2**). After cell fixation, they were processed for the expression of GFAP **Chapter 2** (**Section 2.4.7**). We measured the total cell counts by counting DAPI positive cells. We assessed GFAP expressing cell counts and the proportion of GFAP immuno-positive cells in culture.

We consistently found that DAPI cell counts increased from 156.2 ± 4.9 cells/mm^2 under control conditions to 230.0 ± 9.6 cells/mm^2 under Kainate treatment. This increase is statistically significant (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.20 A**). While 79.5 ± 5.2 cells/mm^2 expressed GFAP under control maintained conditions, 128.7 ± 9.6 cells/mm^2 expressed GFAP after 5 days of Kainate exposure. This rise in GFAP immuno-positive cells was significant (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.20 B**). However, there was no change in the proportion of GFAP expressing cells in culture when comparing Kainate with control conditions (**Figure 3.20 C**).

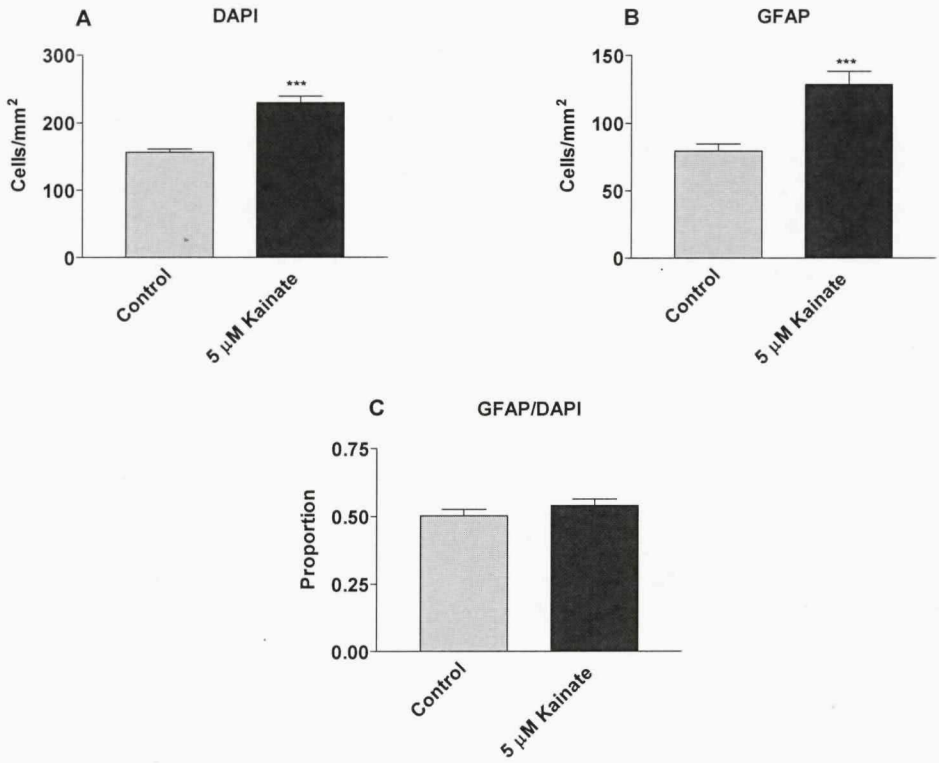


Figure 3.20 Kainate increased the number but not proportion of GFAP immuno-positive cells after 5 DIV. Cells were cultured for 5 days in the absence and presence of 5µM Kainate. On day five cells were fixed and stained against GFAP and counterstained for DAPI. Kainate increased GFAP expressing cell counts after 5 days *in vitro* (DIV). A) There was a significant increase in the total cell counts (DAPI) after 5 days of Kainate treatment. B) GFAP expressing cells increased significantly in culture when comparing Kainate with control conditions. C) There was no change in the proportion of GFAP cells in culture when comparing Kainate with control conditions. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t test.* *** $p<0.001$

3.4.4.8 Kainate increased numbers of Nestin positive and GFAP positive as well as Nestin positive and GFAP negative expressing cells

In (**Sections 3.4.4.1 and 3.4.4.7**) we have described a significant increase in the total cell counts (DAPI), nestin immuno-positive cells, and the counts of GFAP expressing

cells after 5 days of Kainate treatment. In this set of experiments we will demonstrate the effects of 5 days Kainate exposure on cells that co-express both nestin and GFAP. Primary hippocampal cells from postnatal (d7-10) Wistar rats were prepared following the protocol in **Chapter 2** (**Section 2.4.2**) and cultured on glass slides for 5 DIV under control conditions or in the additional presence of 5μM Kainate. Cells were double labelled for the stem/progenitor marker nestin and the astrocytic marker GFAP.

Cells that co-express both nestin and GFAP increased from 56.8 ± 3.8 cells/mm² under control conditions to 94.2 ± 8.0 cells/mm² under Kainate treatment. This increase was statistically significant (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.21 A**). There was no change in the proportion of nestin immuno-positive cells that expressed GFAP when comparing Kainate to control conditions (**Figure 3.21 B**). We did not find significant effects for 5 days of Kainate treatment on the proportion of GFAP cells that expressed nestin (**Figure 3.21 B**). Interestingly, 24.0 ± 2.7 cells/mm² were nestin positive and GFAP negative under control conditions and significantly increased to 45.9 ± 3.6 cells/mm² after 5 days of Kainate exposure (unpaired simple Student's t-test, $p<0.001$) (**Figure 3.21 D**). Although we found a significant increase in the counts of nestin expressing cells only, there was no proportional change in this sub-population of cells in response to 5 days of Kainate treatment (**Figure 3.21 E**). There was approximately 5% decrease in the proportion of GFAP cells only under Kainate conditions when compared to controls. However, this decrease was statistically insignificant (**Figure 3.21 F**).

These results suggest that the increase in GFAP immuno-positive cells that we demonstrated in (**Section 3.4.4.7**) is due to an increase in a population of cells that co-express both nestin and GFAP. Although the numbers of both nestin expressed cells but GFAP unexpressed cells and the numbers of cells that co-express both nestin and GFAP increased significantly under Kainate treatment, there was no proportional change in both sub-populations of cells. This is due to the fact that the rate of increase in both sub-populations of cells is paralleled with an increase in the total cell counts (**Sections 3.4.4.1 and 3.4.4.7**). There may be also a synergistic survival effect on these sub-population of cells.

Sample of the images that were counted and analyzed is displayed in (**Figure 3.22**) demonstrating all different sub-populations of cells.

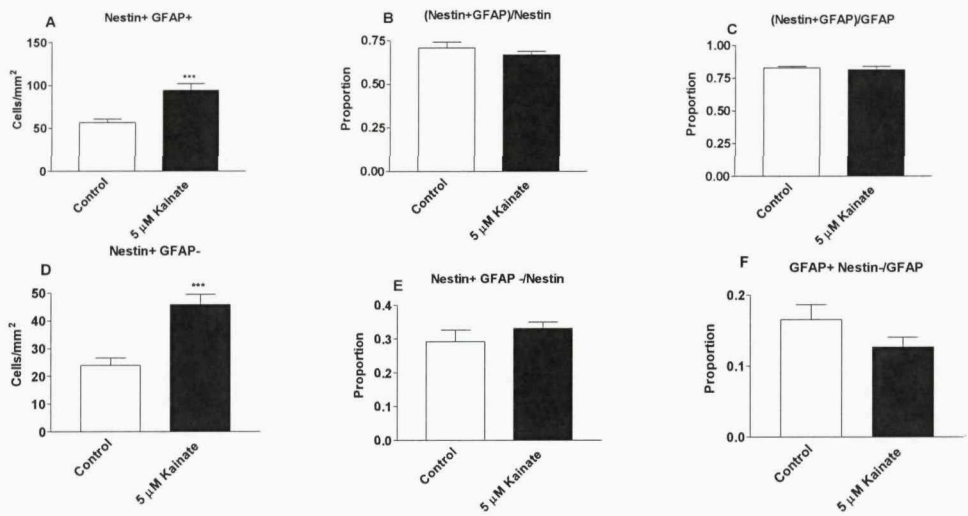


Figure 3.21 Kainate increased Nestin positive and GFAP positive as well as Nestin positive and GFAP negative expressing cells after 5 DIV. Cells were cultured for 5 days in the absence and presence of 5μM Ka. After fixation cells were double labelled for nestin and GFAP. 5 days of Kainate exposure increased the counts of nestin positive and GFAP positive as well as nestin positive and GFAP negative expressing cells in culture. A) The counts of both nestin and GFAP co-expressing sub-population increased significantly after 5 days of Kainate treatment in comparison with control conditions. B) There was no change in the proportion of nestin positive cells that expressed GFAP after 5 days of Kainate exposure when compared to control conditions. C) The proportion of GFAP positive cells that expressed nestin did not change as well. D) GFAP negative and nestin positive sub-population of cells increased under Kainate when compared to control conditions. E) The proportion of nestin positive but GFAP negative cells in hippocampal cultures did not change after Kainate exposure. F) There was no change in the proportion of GFAP positive but nestin negative cells after 5 days of Kainate treatment. Values represent mean ± SEM based on a sample of 4-8 glass slides per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *unpaired simple Student's t test.* *** $p<0.001$

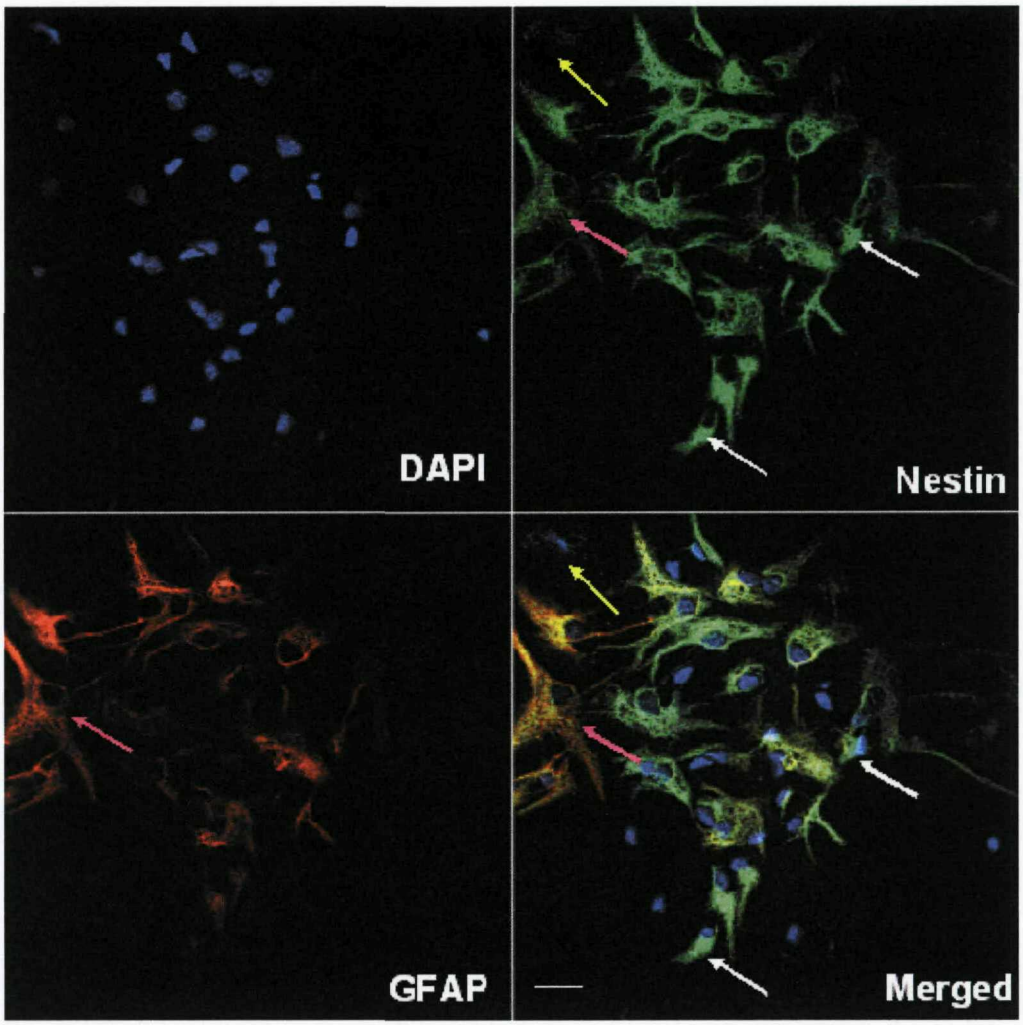


Figure 3.22 Micrograph of stem/progenitor cells with astrocyte-like properties. We grew primary hippocampal cell for 5 days under standard conditions and under 5μM kainate. Two-thirds of the growth medium with/without 5μM Kainate was replaced on day 3. Fixation for 30 minutes with 4% PFA was done on day 5 before consequent immunocytochemistry. Primary specific anti-sera against nestin (stem/progenitor marker) and the astrocytic marker GFAP were detected with fluorescently linked secondary antibodies. Counterstaining for the nuclear marker DAPI was carried out at the end of immunocytochemistry. The staining shows DAPI in blue, nestin in green, and GFAP in red. White arrows show samples of nestin positive but GFAP negative cells. Pink arrows show samples of cells that expressing both nestin and GFAP. Yellow arrows show sample of weak nestin staining in cells that do not express GFAP. Images were captured with 20x objective on a laser scanning con-focal microscope and 3D projections reconstructed using the Zeiss LSM software. Control experiments with the primary antibody omitted were devoid of staining. Scale bar = 20μm.

3.5 Discussion

Summary of Results:

Using primary hippocampal culture system we have demonstrated that Kainate increases overall cell numbers and the number of proliferating hippocampal cells in culture. We also identified 3 days in culture as a target time point for Kainate to enhance cell proliferation. We have found that 5μM Kainate enhances cell proliferation as indicated by the increase in the mitotic index (BrdU/DAPI) and the speed of the cell cycle (labelling index) at 3 DIV. We also addressed the effects of 3 days of Kainate treatment on progenitor cells (nestin positive) and neuroblasts (TuJ1 expressing cells). While we describe that 3 days of Kainate treatment and 6 /8 h of Kainate exposure at day three are proliferative for hippocampal nestin-expressing cells as indicated by the increase in both the mitotic index and the labelling index of nestin immuno-positive cells, only 3 days of Kainate treatment enhances the proliferation of neuronal precursor cells (TuJ1 positive). Moreover, our results have demonstrated an increase in the total cell counts as well as a net rise in TuJ1 sub-population of cells after five days in culture. In order to study the mechanism by which there was a net increase in neurogenesis by day five, we investigated the proliferative effects of Kainate on hippocampal progenitor (nestin positive) cells, and neuronal precursor (TuJ1 positive) cells after 5 days of Kainate treatment and after short (6 h or 8 h) Kainate exposure at day five. We have shown that either 5 days of Kainate treatment or 6 h/8 h of Kainate exposure at day five are proliferative for nestin immuno-positive cells as indicated by the increase in the proportion of proliferating nestin positive cells (BrdU+ nestin+ /nestin+). We further demonstrate that 8 hours pulse of Kainate increases significantly the speed of the cell cycle of nestin positive cells as measured by the labelling index (BrdU+ nestin+ Ki-67+/nestin+ Ki-67+). While five days of Kainate treatment increases the number of proliferating TuJ1 sub-population of cells, 6 h of Kainate exposure at day five does not affect the proliferation of TuJ1 positive cells in culture. Since primary hippocampal cell culture yields a heterogeneous population of cells it was necessary to phenotype these cells and thus we report an increase in nestin, TuJ1, and GFAP in response to 5 days of Kainate treatment with proportional rise in TuJ1 sub-population of cells only.

Interestingly, co-localizations between nestin and GFAP and nestin and TuJ1 were also identified under both control conditions and Kainate conditions. We show that 5 days of Kainate treatment has a combination of proliferation and differentiation effects on progenitor (nestin positive) cells towards a neuronal lineage, which is not detected after 3 DIV. Moreover, 5 days of Kainate treatment causes a significant increase in the number of cells that express nestin and GFAP as well as cells that express nestin only.

3.5.1 Kainate proliferative effects on hippocampal cells in culture are dose and time dependent

Characterising cell proliferation:

The proliferation of a population of cells can be characterised by two parameters; the speed of the average cell cycle (Labelling Index) and the proportion of that population of cells that are within the cell cycle (Growth Fraction) (Nowakowski, Lewin et al. 1989; Dehay, Savatier et al. 2001). We adopted two basic strategies for examining cell proliferation *in vitro*: Firstly; we treated cultures, Kainate and control, with a terminal pulse of the exogenous S-phase marker BrdU. Secondly; we detected Ki-67 antigen which is an endogenous protein expressed in mitotically active cells. These markers are detected using immunocytochemistry techniques. According to our paradigm; we applied BrdU to cells (control and Kainate exposed) for the last 4 or 6 hours (or as indicated) before cell fixation. This BrdU use gives us the ability to quantify cells that are in or have entered the S-phase of the cell cycle in the last few hours prior to fixation. Ki-67 is a very useful tool to obtain a "snap shot" of the mitotic activity at the time of cell fixation. A combined use of the two markers will measure the speed of cell cycle (BrdU+Ki-67/Ki-67) and this was achieved by identifying the proportion of cells in the S-phase of the cell cycle (BrdU incorporated) with respect to the population of actively dividing/cycling cells (Ki-67 positive) and this proportion is referred to as the labelling index. The greater the speed of the cell cycle the greater will be the proportion of BrdU incorporated cells over Ki-67 positive cells (labelling index) within a time frame of one cell cycle.

Measuring the proportion of cells that entered the S-phase of the cell cycle of all cells in culture (The Mitotic Index = BrdU/DAPI) identifies an effect on the population of proliferating cells. However, it does not differentiate between an increase in the speed of the cell cycle in proliferating cells (Labelling Index) and increases in the recruitment of quiescent cells into the cell cycle (Growth Fraction). Using terminal BrdU pulse and calculating the proportion of BrdU incorporating cells as well as the labelling index are recognized methods to study proliferation and differentiate it from trophism (Lu, Black et al. 1996). Moreover, identifying cells that are Ki-67 positive with respect to the total cell counts at the time of cell fixation allows us to calculate the growth fraction (Ki-67/DAPI). An increase in the growth fraction implies recruitment of quiescent cells to become mitotically active.

These methods for identifying cell proliferation using terminal BrdU pulses and measuring the rate of cell proliferation have been successfully used in primary hippocampal cultures from the postnatal rats and *in vivo* of adult rodents (Nowakowski, Lewin et al. 1989; Lu, Black et al. 1996; Hayes and Nowakowski 2002; Kee, Sivalingam et al. 2002; Dayer, Ford et al. 2003; Howell, Scharfman et al. 2003; Howell, Doyle et al. 2005; Namba, Mochizuki et al. 2005; Howell, Silva et al. 2007).

Dose effects:

We have investigated the proliferative effects of 0.5μM, 5μM, 50μM, and 100μM Kainate on hippocampal cells in culture. We report that Kainate enhances hippocampal cell proliferation at a concentration as low as 5μM (**Sections 3.4.1 and 3.4.2**) as the proportion of BrdU incorporated cells and the labelling index increased after 3 days of 5μM Kainate treatment. Although our results demonstrate a significant increase in the counts of mitotic cells (Ki-67) under 5μM Kainate treatment, there was no increase in the growth fraction (Ki-67/DAPI) and thus no increased recruitment of quiescent cells. This implies that the proliferative effect of three days of Kainate is mediated solely by an increase in the speed of the cell cycle. Although we show that 0.5μM Kainate increases the total cell number (DAPI), there is no increase in both the number of BrdU-incorporated cells and the mitotic index (BrdU/DAPI) under 0.5μM Kainate. In contrast to 0.5μM, higher concentration of Kainate (50μM) increases the total cell number

(DAPI), mitotic index (BrdU/DAPI), labelling index (BrdU+Ki-67/Ki-67) and growth fraction (Ki-67/DAPI) of overall hippocampal cells in culture. However, higher dose of Kainate (100μM) decreases the proportion of BrdU-incorporated cells and the growth fraction in comparison with 5μM and/or 50μM Kainate. This decrease might suggest a toxic effect of high dose of Kainate on proliferating cells. Although, our data demonstrates significant proliferative effects of 50μM Kainate on overall hippocampal cells in culture, we have chosen the lowest concentration (5μM) identified to increase both the labelling index and cell cycle S-phase entry to be used in our investigation to unveil the proliferation control mechanisms in culture.

Time course:

Our findings suggest a significant time point (day 3) where cells in culture respond maximally to Kainate. In contrast, we show that 5 days of Kainate treatment (**Section 3.4.2**) increases the total cell counts as well as the numbers of BrdU incorporated cells but not the general cell proliferation (BrdU/DAPI). In order to explore the mechanisms by which altered cell proliferation (BrdU/DAPI) and the speed of the cell cycle (BrdU/Ki-67) increase at day three but not at day five we have tested the effects of short term Kainate exposure on cell proliferation at days 3 and 5 where terminal 6 h or 8 h pulse of both Kainate and BrdU were applied to cells. We found that 6 h Kainate exposure at day 3 significantly increases both general cell proliferation (BrdU/DAPI) and the labelling index (BrdU/Ki-67) (**Section 3.4.3.2**). In contrast, although 8 h of Kainate treatment at day five increases the counts of BrdU incorporated cells as well as Ki-67 positive cells, there is no change in overall cell proliferation (BrdU/DAPI) or the labelling index (BrdU/Ki-67) (**Section 3.4.4.2**).

These results suggest that Kainate exposure is directly proliferative for hippocampal cells at day three. Furthermore, at either time points (days 3 or 5) there is again no recruitment of quiescent cells as indicated by an unaltered growth fraction (Ki-67/DAPI) after Kainate treatment. The lack of a general increase in cell proliferation after 5 days of Kainate treatment suggests the involvement of another mechanism of action that might be related to survival effects on proliferating cells. Another possibility is that the increase in the overall cell numbers (DAPI), BrdU incorporated cell counts and Ki-67

expressing cells after 5 days of Kainate treatment is a result of the enhanced cell proliferation at day 3. In agreement with our findings Brazel and colleagues, investigating the effects of glutamate on SVZ stem/progenitor cells from immature brains, have demonstrated significant proliferative effects for glutamate and Kainate on cultures from postnatal sub ventricular zone (Brazel, Nunez et al. 2005). After 6 h of the treatment they applied ^{3}H thymidine to cells for 40 hours before cell fixation and obtaining tritium counts to measure cell proliferation. As we discussed earlier, we used both BrdU and Ki-67 markers to measure cell proliferation which gives accurate figure of the proliferating cells at different time points. Furthermore, Brazel and co-workers have shown that 3μM Kainate has the same survival effect on cells in culture as glutamate when applied in combination with the non-competitive NMDA antagonist KYNA (7-chloro-kynurenic acid). Although Brazel et al 2005 reported a significant proliferative and survival effect for glutamate on neurospheres from the SVZ, his study failed to show an increase in the different sub-population of cells in response to glutamate *in vitro*. Our results from the hippocampal stem cell niches, agrees to some extent with what this group has shown. In this context, Brazel et al have shown that Kainate and/or glutamate enhance cell proliferation in SVZ cultures. However, SVZ and the hippocampus are both stem cell niches (Taupin and Gage 2002). And thus stem cells might be affected by Kainate and/or glutamate in a similar way in both niches. The basal CSF (Cerebro-spinal fluid) glutamate level was found to be 20μM in postnatal rats and has been hypothesised to play an important role in controlling progenitor cell proliferation and survival (Vannucci, Brucklacher et al. 1999). This endogenous glutamate activates AMPA/Kainate receptors at some stage and might be related to the control of SVZ proliferation process under normal physiological conditions and after seizures (Parent, Valentin et al. 2002). Short term proliferative effects of Kainate on hippocampal cells strongly suggests direct enhancement of proliferation which might involve activation/deactivation of specific receptors of the glutamate family which will be addressed in **Chapter 4**.

These data suggest that the maximum proliferation under Kainate conditions is at day 3 and there is a corresponding increase in cell numbers by day 5. However, no significant increase in either overall cell proliferation or the speed of the average cell cycle was

detected at day 5. The increase in the counts of BrdU-incorporated cells, Ki-67 positive cells, as well as the total cell counts after 5 DIV of Kainate treatment can be explained by the proliferative expansion of a precursor cell population at day three whose rate of proliferation thereafter was unchanged by Kainate treatment explaining the lack of a significant proportional increase in cell proliferation (BrdU/DAPI) at day 5 *in vitro*. Since primary hippocampal cell cultures contain mixed sub-populations of cells, Kainate might act differently on each sub-population. And as such, we studied the proliferative effects of Kainate on cell phenotype (nestin and TuJ1 immunopositive cells) and this will be discussed in the next section. Another possibility is that we have a sub-population of proliferating cells that show increased survival after Kainate. Therefore, the survival role of Kainate should be explored and this will be the focus of **Chapter 5**. The down regulation of hippocampal cell proliferation after day 3 strongly agrees with the published decline in the proliferative characteristics of dentate and hippocampal progenitor cells in the early postnatal rats with time (Namba, Mochizuki et al. 2005).

3.5.2 Long term Kainate treatment has both proliferative and differentiation effects on hippocampal precursors towards a neuronal lineage

In the previous section, we have demonstrated that Kainate has overall proliferative effects on hippocampal cells in culture. We also identified days 3 and 5 as possible time points to be a target for Kainate. However, the hippocampus includes not only precursor cells but also other cell phenotype like immature neurons (Namba, Mochizuki et al. 2005; Encinas, Vaahtokari et al. 2006; Namba, Mochizuki et al. 2007). In order to elucidate the mechanisms underlying the increase in hippocampal cell proliferation *in vitro*, we examined the proliferative effects of Kainate on nestin precursor cells and TuJ1 immunopositive neurons after 3 and 5 DIV. The proliferation of stem/progenitor cells was examined by co-labelling nestin immunopositive cells with BrdU and or Ki-67 under both Kainate and control conditions. Our results reveal that 3 days of Kainate treatment enhances the proliferation of hippocampal stem/progenitor (nestin positive) cells as indicated by the increase in the proportion of nestin cells that entered S-phase of

the cell cycle (incorporated BrdU) with respect to overall nestin sub-population (nestin+ and BrdU+/nestin+) (**Section 3.4.3.1**). Likewise, we find a significant proportional increase in TuJ1 (immature neuronal marker) cells that incorporated BrdU after 3 days of Kainate treatment (**Section 3.4.3.4**). Interestingly, 3 days of Kainate treatment increased the proportion of nestin positive cells and TuJ1 expressing cells but not overall DAPI cell counts. At first glance, these results seem to contradict each other. However, it has been demonstrated that a proportion of nestin positive cells co-localizes with TuJ1 cells in the DG of paediatric patients (Blumcke, Schewe et al. 2001) and in cultures from postnatal rats' hippocampi (Howell, Silva et al. 2007). In agreement with this, we report that approximately 20% of nestin positive cells expresses TuJ1 and around 25% of TuJ1 positive cells express nestin (**Section 3.4.3.6**). Cells that co-express both markers (nestin and TuJ1) are precursor cells that are already committed to a neuronal fate. Furthermore, once we look at the percentage of increase in each sub-population of cells we find that there is just under 10% increase in nestin sub-population of cells after 3 DIV of Kainate. Similarly there is a less than 20% increase in TuJ1 sub-population of cells and due to the overlaps between nestin and TuJ1; we failed to show a significant increase in the total cell counts by day 3. Another possibility may be that other cell phenotypes, perhaps GFAP cells, are committing to a neuronal lineage and thus we observe a net rise in TuJ1 sub-population of cells.

Importantly, 5 days of Kainate treatment increases the total number of cells in culture as well as the proliferation of nestin precursor cells as indicated by the increase in the proportion of nestin positive cells that incorporated BrdU (**Section 3.4.4.1**). However, there is no proportional increase in nestin sub-population of cells by day 5. On the other hand, we find a significant proportional increase in TuJ1 sub-population of cells by day 5, and thus net neurogenesis, but not in the proliferation of TuJ1 expressing cells (**Section 3.4.4.4**). In this regard, it has been shown that the proliferative properties of DG neural precursor cells decrease with time in the postnatal hippocampus (Namba, Mochizuki et al. 2005; Namba, Mochizuki et al. 2007). Thus we are expecting a reduction in the proliferation of immature neurons once they become older. Another possibility is that neuroblasts have become resistant to Kainate after 5 days of exposure; therefore, these neuroblasts stopped proliferating after 5 days of the treatment. Again, a

proportion of nestin positive cells co-localizes with TuJ1 positive cells and thus we report an increase in both nestin positive cells that are expressing TuJ1 and TuJ1 positive cells that are expressing nestin (**Section 3.4.4.6**). Furthermore, the proportion of cells that co-expresses both nestin and TuJ1 with respect to overall cells has doubled. This suggests that more nestin positive cells are committing to a neuronal lineage after 5 days of Kainate treatment. Another interpretation is that more nestin precursor cells are dividing both symmetrically and asymmetrically. And thus we observe an increase in the proportion of cells that co-expresses nestin and TuJ1. In addition, the proportion of nestin-only positive cells (nestin+ and TuJ1-/nestin+) decreased after 5 days of Kainate treatment. Altogether, these results demonstrate that Kainate have both proliferation and differentiation effects on hippocampal precursor cells towards a neuronal lineage. The fact that there is an increase in the raw numbers of proliferating TuJ1 cells but not in their proportion may be perhaps due to a trophic effect of Kainate on these proliferating cells and this will be addressed in **Chapter 5**.

In summary, we report significant proliferative effects of Kainate on precursor cells and neuroblasts. While Kainate is proliferative for precursor cells after 3 and 5 days in culture, it enhances the proliferation of neuroblasts after 3 days only. The next question is whether Kainate has direct proliferative effects on precursor cells and neuroblasts and thus on net neurogenesis. This will be the focus of the next section.

3.5.3 Kainate has direct proliferative effect on hippocampal precursor cells but not on immature neurons

Our results show that short (6/8 h) exposure to Kainate has an overall proliferative effects on hippocampal cells at day three (**Section 3.4.3.2**) but not day 5 (**Section 3.4.4.2**) in culture. Surprisingly, there is an overall increase in the total cell numbers after short pulse of Kainate at day 5 but not day 3. In order to explore the effects of Kainate on the proliferation of both nestin positive cells and TuJ1 expressing cells, we double-labelled each of these markers (nestin and TuJ1) with BrdU after short exposure to Kainate at days 3 and 5. Although there is a significant proliferative effects after 6

hours of Kainate exposure at day 3 on overall cell proliferation (in line with our results in **Section 3.4.3.2**), we find that this short Kainate exposure has no effect on TuJ1 sub-population of cells (**Section 3.4.3.5**). This finding implies that Kainate has no direct proliferative effect on neuroblasts at day 3 in culture. In contrast, 6 h of Kainate exposure at day 3 increases the proliferation of nestin positive cells which results in a small proportional increase in nestin sub-population of cells (**Section 3.4.3.3**). Moreover, we report that Kainate increases not only the mitotic index of nestin positive cells (nestin+ and BrdU+/nestin+) but also the speed of the cells cycle as measured by the labelling index of nestin positive cells after 8 hours of Kainate treatment. Indeed we find that the labelling index of nestin positive cells increases from 20% under control conditions to 43% after 8 hours of Kainate exposure at day 3 (**Section 3.4.3.3**). These results suggest that Kainate increases the speed of cell cycle of nestin precursor cells.

Interestingly, a short pulse of Kainate at day 5 increases the total cell number as indicated by DAPI cell counts (**Sections 3.4.4.2, 3.4.4.3, and 3.4.4.5**). We also report a significant increase in the number of nestin positive cells as well as more importantly the proportion of proliferating nestin expressing cells as indicated by the increase in the mitotic index (nestin+ and BrdU+/nestin+) of this sub-population of cells (**Section 3.4.4.3**). Moreover, we find that 8 hours of Kainate exposure at day 5 increases the speed of the cell cycle of precursor cells as measured by the labelling index of nestin positive cells (nestin+ BrdU+ and Ki-67+/nestin+ and Ki-67+). Herein, we show while 25% of nestin cells undergo S-phase entry in 8 h under Kainate conditions, only 10% do so under control conditions. Although we show that all nestin positive cells are expressing Ki-67 (cycling), these series of experiments further confirm that the speed of the cell cycle of nestin positive cells decreases with time. For example, while 20% of nestin positive cells are in S-phase at day 3 under control conditions, only 10% are in S-phase at day 5. Interestingly, in line with our results, Namba and co-workers have demonstrated that the proliferative characteristics of precursor cells decrease with age in the postnatal hippocampus (Namba, Mochizuki et al. 2005; Namba, Mochizuki et al. 2007). However, we are the first to show that the speed of the cell cycle of precursor cells decrease with age.

Surprisingly, we find a significant increase in TuJ1 sub-population of cells and also a

small (<10%) increase in the proportion of TuJ1 cells after 6 hours exposure to Kainate at day 5 (**Section 3.4.4.5**). Although there is an increase in the number of proliferating neuroblasts (TuJ1+ and BrdU+), there is no proportional increase in the proliferation of TuJ1 sub-population. This may suggest that the increase in the numbers of proliferating TuJ1 positive cells is due to both a proliferative and neuronal lineage commitment effect on a more primitive proliferating nestin positive precursor. In addition, there may be a synergistic survival effect on these cells, which will be examined in **Chapter 5**.

These data suggest that Kainate is proliferative for nestin expressing progenitor cells after short term Kainate treatment at days 3 and 5. It has been reported that glutamate acts directly on the dividing cells in cultures and plays a role in regulating cell proliferation (LoTurco, Owens et al. 1995; Steinhauser and Gallo 1996). Furthermore, functional glutamate receptors were identified in cultures (Barres, Koroshetz et al. 1990). The receptor mediation of the effects of Kainate will be addressed in **Chapter 4**. These studies strongly support our findings where we have shown direct proliferative effects of Kainate on stem/progenitor cells. Interestingly, it has been demonstrated that glutamate increases precursor cell proliferation in the rat embryonic ventricular zone, while decreasing proliferation in the embryonic SVZ (Haydar, Wang et al. 2000). Postnatally, stem/precursor cells reside in the SVZ and are hypothesized to be direct descendents of the embryonic ventricular zone (Marshall and Goldman 2002). Thus, postnatal SVZ stem/precursor cells may behave like embryonic ventricular zone stem cells. In agreement with this, Brazel et al 2005 reported that glutamate is proliferative for SVZ precursor cells that were cultured from postnatal Wistar rats. In this context, perhaps DG stem/precursor cells share this characteristics with SVZ stem cells and proliferate in the presence of Kainate. In addition, Neuropeptide Y has been demonstrated to have direct effects on the proliferation of both nestin and TuJ1 expressing cells in cultures form the postnatal hippocampi (Howell, Scharfman et al. 2003; Howell, Doyle et al. 2005). Thus Kainate, like NPY, enhances proliferation of progenitor cells but, unlike NPY, it does not enhance proliferation of neuroblasts directly.

3.5.4 Kainate is selectively affecting precursor cells and not astrocytes in the hippocampal stem cell niche

Hippocampal neurogenesis generates new granule cell neurons that integrate into the dentate gyrus throughout adult mammalian life. Extensive studies have described several aspects of embryonic and adult neurogenesis. However, few attempts have been made to explore the postnatal, a transition stage between embryonic and adult, neurogenesis and its control mechanisms (Namba, Mochizuki et al. 2005). It is broadly accepted that the adult dentate gyrus contains different sub-populations of stem/progenitor cells (Gage 2000; Taupin and Gage 2002; Encinas, Vaahtokari et al. 2006; Encinas and Enikolopov 2008). A key aspect for understanding neurogenesis is unveiling the role of these stem/progenitor cells, their ability to give rise to neurons, and interactions with other sub-types of cells in the neurogenic niche. We describe that, in our primary hippocampal cultures, approximately half of the cells expresses the stem/progenitor marker nestin (**Section 3.4.4.1**). GFAP sub-population of cells also represents around half of cells in culture (**Section 3.4.4.7**), while nearly one-third of cells *in vitro* express the neuronal maker TuJ1 (**Section 3.4.4.4**). Here we demonstrate that 5 days of Kainate exposure increases the counts of nestin, GFAP, and TuJ1 in cultures. Importantly, we report a consistent proportional increase in TuJ1 sub-population of cells in 3 and 5 DIV (**Section 3.4.3.4 & 3.4.4.4**) and, thus, a resultant net rise in neurogenesis. Moreover, we find that 5 days of Kainate treatment increases the numbers of GFAP positive cells but not their proportion (**Section 3.4.47**). However, GFAP labels both astrocytes and astrocyte-like stem cells (Seri et al 2001, 2004). Therefore, we carried out double-labelling immunohistochemistry for GFAP and nestin in order to determine the target of Kainate effects and whether Kainate is acting on precursor cells or astrocytes. In **Section 3.4.4.8** of this **Chapter** we have identified a sub-population of cells expressing both nestin and GFAP. This sub-population of cells was highlighted in a study conducted on adult mice and they were identified to be quiescent neural progenitors with the ability to divide asymmetrically to give rise to an amplifying neural progenitor cells (Encinas, Vaahtokari et al. 2006). Herein, although we did not transfect our cells with retroviral vectors, we report that 40% ± 2% of cells in primary hippocampal cultures are expressing both nestin and GFAP. In addition, 100% of nestin cells are Ki-67 positive.

This may suggest that cells expressing both nestin and GFAP are quiescent neural progenitors. Consistent with this, our proposed quiescent neural progenitors have triangular somas with single or double processes, in agreement with what Encinas et al have shown *in vivo*, and co-express nestin and GFAP (**Figure 3.25 white arrows**). Although we describe a significant increase in the numbers of cells that co-express nestin and GFAP after 5 days of Kainate treatment, there is no proportional rise in this sub-population of cells. This suggests that Kainate does not increase the symmetric division of these more primitive precursor cells but rather affects a nestin positive transiently amplifying GFAP negative cell population. Indeed an amplifying neural progenitor sub-population of cells (defined by (Encinas, Vaahtokari et al. 2006)) was also observed in our culture system. These cells express nestin but do not express GFAP and have small somas around 10μM oval or round in shape (**Figure 3.23 pink arrows**). We report that 17% ± 1% of cells in culture and 30% ± 3% of the nestin sub-population are expressing nestin but do not express GFAP and could be classified as amplifying neural progenitors according to Encinas classification. Kainate increased the counts of the amplifying progenitor cells (nestin positive but GFAP negative) but without a significant proportional increase. However, we observe a slight insignificant increase in this sub-population of cells (nestin+ and GFAP-/nestin+) and also a small insignificant decrease in nestin cells that express GFAP (nestin+ and GFAP+/ nestin+). These results may suggest an effect of Kainate on amplifying progenitor cells. However, a combination of proliferation and differentiation might be involved as well. We further describe a sub-population of GFAP positive cells that does not express nestin and have big star shape-like somas (**Figure 3.23 yellow arrows**). These cells, considered mature astrocytes, comprise 17% ± 2% of the GFAP sub-population and 8% ± 0.8% of the total cells grown in primary hippocampal cultures. There is a slight decrease (5%) in this sub-population of cells after 5 days of Kainate treatment that was statistically insignificant (**Section 3.4.4.8**).

An important study investigated stem/progenitor cells with astrocyte-like properties describing these progenitors as the source for intermediate neural precursors D-cells which give rise to neurons in the adult DG (Seri et al 2004). Herein, we demonstrate that Kainate is enhancing the proliferation of nestin precursor cells and around 70% of these

cells are astrocyte-like precursor cells. Our results, in agreement with (Encinas, Vaahtokari et al. 2006), suggest that quiescent neural progenitors may divide asymmetrically to give neurons in response to Kainate treatment and also astrocytes-like progenitor cells give rise to neurons (Garcia, Doan et al. 2004; Merkle, Tramontin et al. 2004; Seri, Garcia-Verdugo et al. 2004). However, symmetric division of a fraction of these precursors can not be excluded. Indeed, in agreement with this there is no decrease in the proportion of nestin only positive cells indicating that symmetric cell division is taking place as well. In addition, 5 days of Kainate treatment drives a fraction of progenitor cells (nestin positive cells) to commit a neuronal fate. All of these explanations will support the net increase in neuronal lineage under Kainate treatment and thus neurogenesis.

Our results demonstrate that Kainate is selectively affecting precursor cells and their neuronal progeny cells. Furthermore, we show that our *in vitro* system contains the key cell types that are the main part of the neurogenic niche and therefore, exploring neurogenesis mechanisms *in vitro* would be very much help explain neurogenesis *in vivo*.

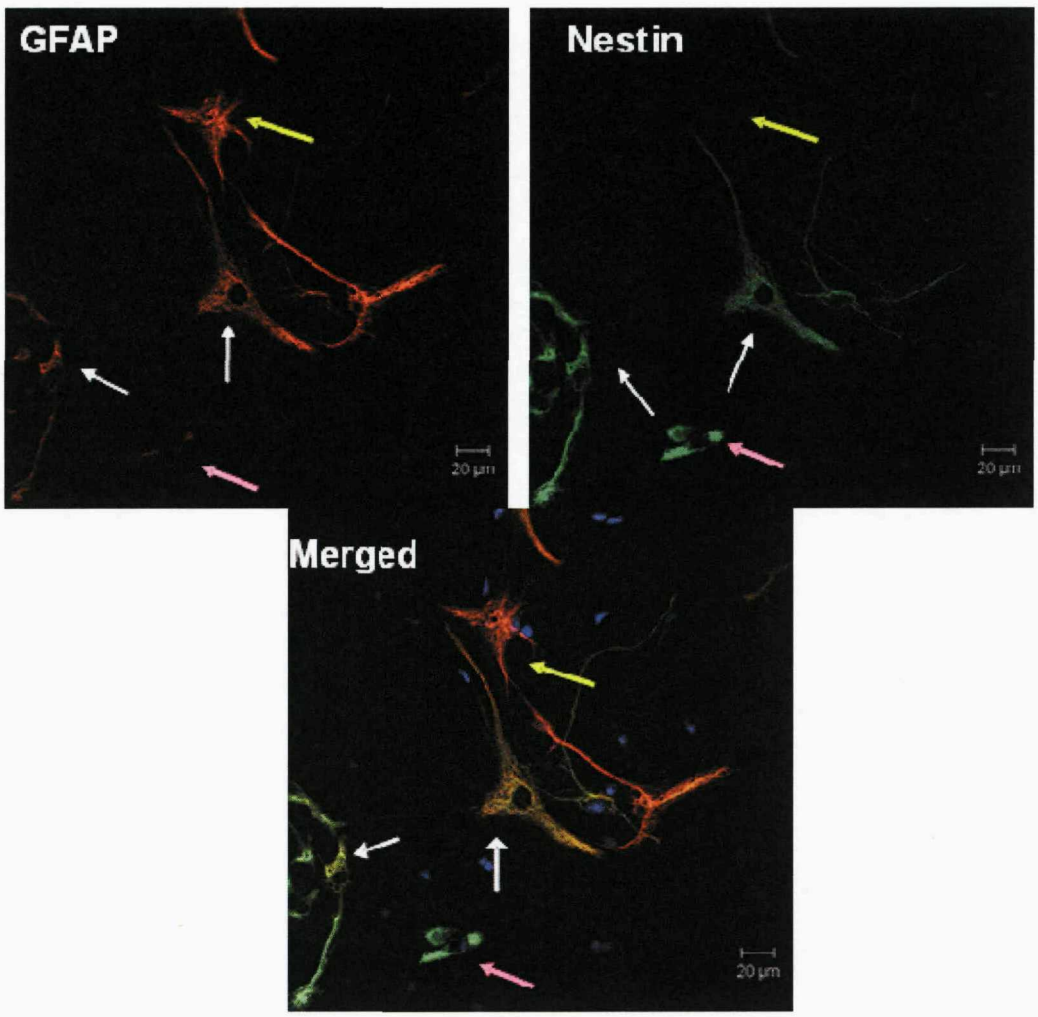


Figure 3.23 Quiescent neural progenitor cells in primary hippocampal cultures. This figure shows stem/progenitor cells in cultures with astrocyte-like properties. We grew primary hippocampal cells for 5 days under standard conditions and under 5µM Kainate. Two-thirds of the growth medium with/without treatment was replaced on day 3. Fixation for 30 minutes with PFA 4% was done on day 5 before consequent immunocytochemistry. Primary specific anti-sera against nestin (stem/progenitor marker) and the astrocytic marker GFAP were detected with fluorescently linked secondary antibodies. Counterstaining for the nuclear marker DAPI was carried out at the end of immunocytochemistry. Images were captured with 20x objective on a laser scanning con-focal microscope and 3D projections reconstructed using the Zeiss LSM software. A, B, and C images show the same population of cells stained against nestin (green), GFAP (red) and counterstained for DAPI (blue). A) The yellow arrows show an example of GFAP positive and nestin negative cell. B) The white arrows demonstrate nestin positive and GFAP negative cells and pink arrows show nestin only positive cells. C) Merged image for DAPI (blue), nestin (green), and GFAP (red). Scale bar = 20µm.

3.6 Conclusion

A remarkable proliferative role for Kainate was demonstrated in this chapter. We have shown significant effects of Kainate upon stem/progenitor (nestin positive) cells and TuJ1 positive cells (immature neurons). We found that 3 and 5 days of Kainate treatment enhanced the proliferation of hippocampal precursor cells. However, Kainate was proliferative for neuroblasts after 3 days in culture. We further show that Kainate treatment for either 3 or 5 days resulted in a net increase in TuJ1 sub-population and thus neurogenesis. 5 days of Kainate treatment had also a combination of proliferation and differentiation effects on hippocampal precursor cells towards a neuronal lineage. Interestingly, short exposure to Kainate enhanced the proliferation of nestin precursor cells but not TuJ1 positive cells at either 3 or 5 days indicating a direct proliferative effect of Kainate on precursor cells. Furthermore, we show that Kainate selectively affected precursor cells and highly amplifying progenitor cells divided symmetrically and asymmetrically to give rise to new neurons. The presence of a mixture of cell phenotypes in primary hippocampal cultures proposes a novel *in vitro* stem cell niche that can be studied to unravel the control mechanisms of stem cells and helps understand their behaviour *in vivo*.

Chapter Four

Kainate modulates Hippocampal Neurogenesis via AMPA receptors

4.1 Introduction

Using a primary hippocampal cell culture paradigm, we have shown (**Chapter 3**) that Kainate has proliferative effects on hippocampal precursor cells and differentiation and possibly trophic effects on their neuronal progeny cells. In the CNS the effects of Kainate have been shown to be mediated via both AMPA as well as Kainate receptor subtypes (Bleakman and Lodge 1998). Kainate receptor subtypes represent a category of the L-glutamate ionotropic receptors; which also extends to involve α-amino-3-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA), and N-methyl-D-aspartate (NMDA) receptor subtypes (Dingledine, Borges et al. 1999).

In this chapter, I will outline the pharmacology of Kainate and AMPA receptors in mediating the effects of Kainate on hippocampal cell proliferation. We will also seek to characterize receptor modulation of Kainate effects on both nestin progenitor cells and their neuronal precursors. In addition, I will describe a preliminarily study of NMDA-receptor subtypes involvement in hippocampal cell proliferation *in vitro*.

4.1.1 Kainate binding site distribution in the CNS

Kainate receptors are ubiquitously distributed in the CNS. In situ hybridization studies have revealed a prominent expression of Kainate receptor subtypes throughout the CNS including the hippocampus (Bettler, Boulter et al. 1990; Paternain, Herrera et al. 2000). Likewise, using RT-PCR techniques, Ruano and colleagues have demonstrated that Kainate receptor mRNAs are expressed in cultured hippocampal neurons (Ruano, Lambolez et al. 1995). In agreement with their observations, Kainate receptors have also been identified in cultured hippocampal neurons from embryonic rats using patch-clamp electrophysiology techniques (Lerma, Paternain et al. 1993). Furthermore, using real-time PCR and western blot methods, AMPA and Kainate receptors have been shown to be expressed in primary neuronal cultures from the embryonic Wistar rats' hippocampi (Janssens and Lesage 2001). It is, therefore, well established that Kainate receptors are involved in synaptic transmission in many regions of the nervous system. However, little

is known about the physiological role of Kainate receptors as it has not been possible to fully distinguish them from AMPA receptors using pharmacological tools (Lerma 2006).

Kainate receptor subunits were originally described as being postsynaptic and found in the principal cells and interneurons of the hippocampus and other regions of the CNS (Huettner 2003; Huttmann, Sadgrove et al. 2003). However, a growing body of evidence indicates that Kainate receptors are also significantly localized in presynaptic terminals, where they modulate neurotransmitter release (Huettner 2003; Huttmann, Sadgrove et al. 2003; Lerma 2003). The first direct evidence of Kainate receptors' involvement in synaptic transmission demonstrated a presynaptic reduction in transmitter release by Schaffer collaterals onto CA1 neurons upon the activation of Kainate receptors in acute hippocampal slices (Chittajallu, Vignes et al. 1996). It has been shown that presynaptic Kainate receptors down-regulate transmission evoked from hippocampal GABAergic interneurons in the rat hippocampus (Rodriguez-Moreno, Herreras et al. 1997). In addition to presynaptic Kainate receptor subtypes that may modulate transmitter release, some cells also express postsynaptic Kainate receptor subtypes that can directly mediate excitatory transmission. Indeed, activation of postsynaptic Kainate receptors at mossy fiber synapses onto CA3 neurons has been described in the rat hippocampus (Vignes, Bleakman et al. 1997). Moreover, it has been reported that postsynaptic Kainate receptors contribute to thalamocortical transmission in slices obtained from postnatal rats which indicates that Kainate receptors are present at these synapses in early development (Kidd and Isaac 1999). Moreover, synaptic transmission by Kainate receptors has been reported in the basolateral amygdala (Li, Calejesan et al. 1998), and it also mediates transmission between cone photoreceptors and specific classes of "off" bipolar cells in squirrel retina (DeVries and Schwartz 1999).

Like Kainate receptors, AMPA receptors are also widely distributed in the CNS. They have been found throughout the brain, with high levels of expression in the hippocampus (Blackstone, Moss et al. 1992). Indeed, using a variety of techniques including radioligand binding ^{3}H-AMPA (Monaghan, Yao et al. 1984), single-cell real time PCR, in situ hybridization histochemistry (Geiger, Melcher et al. 1995), and immunohistochemistry (Petralia and Wenthold 1992), AMPA receptor subtypes and their corresponding mRNAs have been shown to have a widespread distribution in the

CNS with a particular abundance in the hippocampus (Blackstone, Levey et al. 1992). Moreover, AMPA/Kainate receptor subtypes have been described on neuroblasts from postnatal mice (P25) and have an important role in neurogenesis (Platel, Lacar et al. 2007). This group has also shown that glutamate receptors are expressed on stem cells with astrocytes-like properties.

There is substantial evidence that Kainate receptors contribute to the well-known ability of Kainate to elicit seizures (Ben-Ari and Cossart 2000) and cause excitotoxic cell death (Nadler 1979). It has been found that GluR5 selective antagonists can block the induction of seizures induced by pilocarpine or electrical stimulation and suppress pre-established seizure activity (Smolders, Bortolotto et al. 2002). However, the mechanisms underlying the exact contribution of Kainate receptors compared to AMPA receptors in the mediation of seizure activity remain to be established.

4.1.2 Molecular biology and pharmacology of Kainate/AMPA receptors

Kainate/AMPA (**Figure 4.1 B & C**) receptors belong to the subfamily of the non-NMDA receptors. For many years, AMPA (**Figure 4.1 C**) and Kainate (**Figure 4.1 B**) receptors have been considered as one functional unit because of pharmacological difficulties in differentiating them. On these bases, they have been termed as AMPA/Kainate receptors. Studying the role of Kainate and/or AMPA receptors in synaptic transmission has become possible after the discovery of selective AMPA receptors antagonists (Huettner 2003). As such, it should now be possible to differentiate whether Kainate effects on hippocampal cells are mediated via AMPA receptors, Kainate receptors, the both of them, or neither of them.

Glutamate (**Figure 4.1 A**) operates via ionotropic and metabotropic receptors (Johansen, Greenwood et al. 2003). While the ionotropic glutamate receptors, which are ligand-gated ion channels, are classified into three heterogeneous receptor subtypes: NMDA, AMPA, and Kainate receptors (**Figure 4.1 D, C, and B respectively**), the metabotropic glutamate receptors belong to the family of G-protein-coupled receptors and comprise metabotropic glutamate receptors 1-8 (mGluR1-8) (Johansen, Greenwood et al. 2003).

Interestingly, glutamate receptors were identified not only throughout the CNS of animal models but also in postmortem human CNS tissue (Blackstone, Levey et al. 1992).

There are five different subunits that contribute to Kainate receptors: Kainate receptors-1 (KA1), KA2, glutamate receptor-5 (GluR5), GluR6, and GluR7 (**Figure 4.1 B**) (Hollmann and Heinemann 1994). They are subdivided into two families, based on their amino acids sequence homology and agonist binding properties; GluR5, GluR6, and GluR7 are approximately 70% identical (Bettler, Boulter et al. 1990; Bettler, Egebjerg et al. 1992) whereas KA1 and KA2 subunits are also 70% identical (Sakimura, Morita et al. 1992). However, Kainate receptor subtypes (KA1 and KA2) display only 40% identity with GluR5, GluR6, and GluR7. Four subunits of AMPA receptor have been cloned, and termed Glutamate receptors 1-4 (GluR1-4) (**Figure 4.1 C**) (Hollmann, O'Shea-Greenfield et al. 1989; Boulter, Hollmann et al. 1990; Keinanen, Wisden et al. 1990). Both subdivisions of Kainate receptors subunits share weaker identity with subunits of AMPA (30-35%) and NMDA receptors (10-20%) (for review see (Huettner 2003)).

While there is a considerable amount of information on NMDA and AMPA receptors and their functions, Kainate receptors have been less tractable, primarily because, until recently, there was a lack of pharmacological tools to differentiate the activation of Kainate and AMPA receptors, which display cross-activation by their defining agonists. Many of the pharmacological agonists and antagonists active at Kainate receptors also interact with AMPA receptors (Lerma, Paternain et al. 2001). This lack of pharmacological specificity has hindered the understanding of Kainate receptors functions for several years. However, investigating the effects that might be mediated via Kainate receptors is possible after the discovery that 2, 3-benzodiazepines, particularly GYKI53655 and the more commercially available GYKI52466 antagonize AMPA receptors, but not Kainate receptors (Paternain, Morales et al. 1995). In addition to blocking AMPA receptor subtypes, the application of compounds that antagonize both AMPA and Kainate receptors has further contributed in exploring Kainate receptors' specific effects (Wilding and Huettner 1996). In this interesting study on hippocampal neurons, Wilding and colleagues have demonstrated that native glutamate receptors of the Kainate subtypes are affected poorly or not at all by compounds of the

benzodiazepines family which have been proven to be very effective in blocking responses induced at AMPA receptors. The non-competitive AMPA receptors antagonist, GYKI52466, potently inhibited the response to AMPA as well as the steady response by Kainate at AMPA receptors ($IC_{50} = 9.8 \pm 1.0$ μM) (Paternain, Morales et al. 1995). However, it has been shown that AMPA and Kainate receptors were blocked by applying a concentration of ≥30μM GYKI52466 (Bleakman, Ballyk et al. 1996) which may indicate that using a low concentration of GYKI52466 (10μM) would abolish AMPA actions only. In this regard, the introduction of selective AMPA receptors antagonists has paved the way to increase the understating for Kainate receptors' role in neurogenesis.

2, 3- Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) is an analog of the quinoxalinedione antagonists to the non-N-methyl-D-aspartate (non-NMDA) glutamate receptor (Sheardown, Nielsen et al. 1990). NBQX is a potent and selective inhibitor of binding to the AMPA and Kainate subtypes of the glutamate receptor (Wilding and Huettner 1996) with no activity at the NMDA receptors (Sheardown, Nielsen et al. 1990). It has been demonstrated that NBQX is neuroprotective against global ischemia when administered 2 hours after an ischemic challenge (Sheardown, Nielsen et al. 1990). Wilding and co-workers have examined the effect of a fixed concentration of NBQX on the concentration-response relation to Kainate. In this regard, they have reported that in the presence of this antagonist, Kainate currents in cortical neurons were reduced by 50% at a concentration of 160μM. The dose response related to Kainate was shifted towards 1300μM by the inclusion of just 2μM NBQX.

In our study we will use GYKI52466 (10μM) and NBQX (30μM) to examine the receptor mediation of effects of Kainate on postnatal hippocampal progenitor cells *in vitro*.

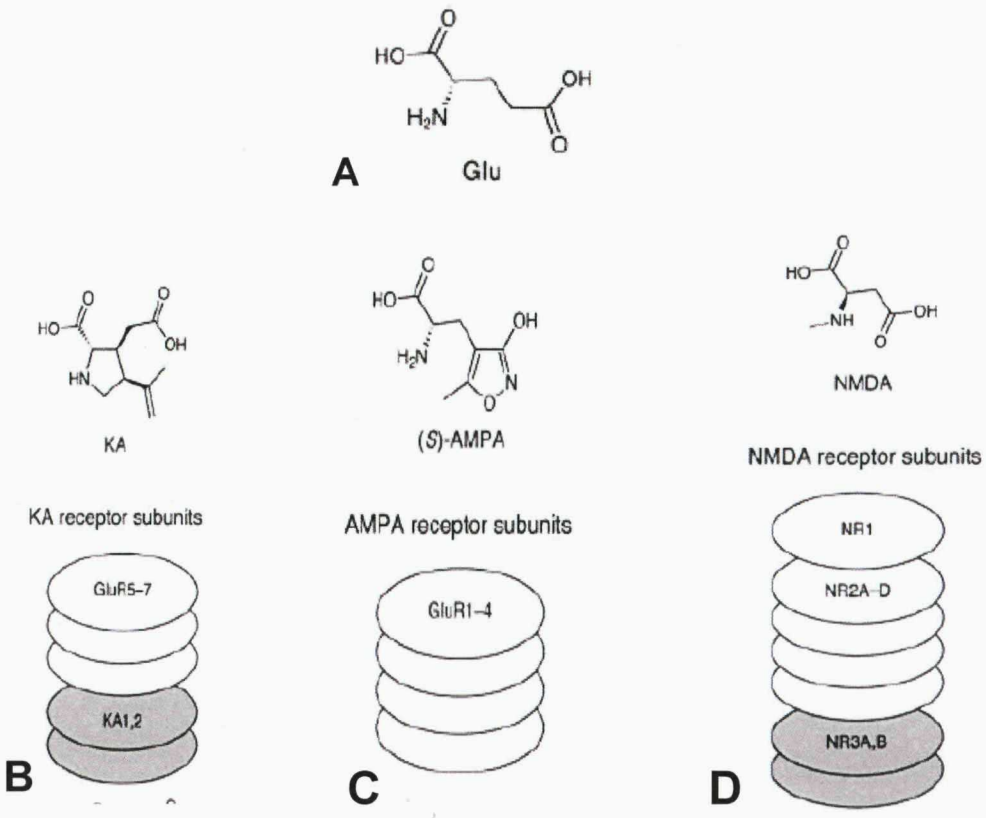


Figure 4.1 The structure and subunits of ionotropic glutamate receptors. A) This figure shows the chemical structure for glutamate (Glu). B) This is the chemical structure of Kainate (Ka) and its five receptor subunits (GluR5-7, KA1, 2). C) This figure demonstrates the chemical structure of (S)-AMPA and its four receptor subtypes (GluR1-4). D) This is the chemical structure of NMDA and its seven receptor subunits (adopted from (Johansen, Greenwood et al. 2003))

4.1.3 Functional importance of Kainate/AMPA receptors in neurogenesis

While Kainate has been shown to mediate excitatory effects in the CNS and model temporal lobe epilepsy (Ben-Ari 1985; Ben-Ari and Cossart 2000), it is an agonist for glutamate receptor subtypes, which are involved in excitatory neurotransmission in the mammalian CNS. In fact, these receptors are regulators of different physiological

processes including learning and memory. Indeed, glutamate is involved in mediating basal excitatory synaptic transmission and many forms of synaptic plasticity such as long-term potentiation and long term depression, which are thought to be essential for learning and memory (Bliss and Collingridge 1993; Lees 2000). Glutamergic neurotransmission is also involved in some important developmental functions including migration, survival, differentiation, and neuritogenesis of new neurons (Mattson and Kater 1987; Simon, Prusky et al. 1992; Rossi and Slater 1993; Rakic and Komuro 1995). In addition, glutamergic transmission also regulates neuronal maturation and synaptogenesis (Rodriguez-Moreno and Sihra 2007). As such, glutamate plays a significant role in regulating neuronal activity.

Excessive release of endogenous glutamate has been found in association with a wide range of acute and chronic neurodegenerative disorders including cerebral ischemia, epilepsy, Parkinson's, and Alzheimer's disease (Catarzi, Colotta et al. 2007). Using a microdialysis electrode, it has been demonstrated that glutamate levels have increased after epileptic seizures (Walker, Galley et al. 1995). In spite of the fact that Kainate is not endogenously present within the CNS, Kainate induced-seizures increase extra-cellular glutamate levels in the CNS (Liu, Stafstrom et al. 1997). Indeed, this group has reported that glutamate levels increased at the same time as seizures started. Similarly, Kainate is a potent excitant of CA3 neurons in the hippocampus (Westbrook and Lothman 1983). Importantly, seizures induced by Kainate have been shown to increase hippocampal neurogenesis (Gray and Sundstrom 1998). An elegant study, on GLuR6 knockout mice, has indicated a role of the GluR6 Kainate receptor in the epileptogenic effects of systemic administration of Kainate (Mulle, Sailer et al. 1998). Indeed, Mulle and colleagues have demonstrated a reduction in seizures in GluR6 knockout mice. Interestingly, it has been demonstrated that glutamate induces epilepsy-like effects in an *in vitro* model of primary hippocampal neuronal cultures (DeLorenzo, Sun et al. 2007). However, the mechanisms controlling glutamatetergic neurotransmission-induced epileptogenesis are not well understood. It has been reported that an increased synaptic release of glutamate is present during periods of global and focal ischemia (Boris-Moller and Wieloch 1998; Caragine, Park et al. 1998). Importantly, dentate gyrus neurogenesis and synaptogenesis have been described to be enhanced after global ischemia (Bernabeu

and Sharp 2000). Moreover, an increased neurogenesis has been found after treating human neural progenitor cells with glutamate (Suzuki, Nelson et al. 2006). Furthermore, glutamate and its analog Kainate have been demonstrated to increase the proliferation of neural progenitor cells derived from SVZ of postnatal Wistar rats (Brazel, Nunez et al. 2005). Although Kainate-induced glutamate release is associated with epileptogenesis, it also enhances neurogenesis in these models as well. This may suggest that glutamergic neurotransmission is involved in the regulation of neurogenesis. Therefore, glutamate receptors subtypes may represent potential therapeutic targets for many neurological diseases.

While these studies clearly show that glutamate and/or its analog Kainate through Kainate/AMPA receptors are important for hippocampal neurogenesis, the exact receptor involvement is far from complete. Therefore, we will, in this chapter, study whether the proliferative effects of Kainate on hippocampal cells are mediated via AMPA and/or Kainate receptor subtypes.

4.1.4 NMDA and neurogenesis

The N-methyl-D-aspartate subtype of glutamate receptor (NMDA-R) plays an important role in physiological and pathological processes in the central nervous system, including neuronal development, plasticity and neurodegeneration (Chatterton, Awobuluyi et al. 2002). NMDA receptors are divided into seven receptor subunits including NR1, NR2A-D, and NR3A and B receptor subunits (**Figure 4.1 D**). The functional release is manily made up of NR1/3 subunits (Chatterton, Awobuluyi et al. 2002).

The production and maturation of granule neurons are influenced by NMDA receptor manipulations. While treating adult rats with NMDA decreased both the rate of cell proliferation in the subgranular zone of the dentate gyrus as well as the number of newly born granule neurons (Cameron, McEwen et al. 1995), a range of NMDA receptor antagonists has been shown to increase neurogenesis and proliferation in this hippocampal region (Cameron, McEwen et al. 1995; Okuyama, Takagi et al. 2004). Furthermore, NMDA receptor antagonist has been demonstrated to enhance

hippocampal neurogenesis in aged rats (Nacher, Alonso-Llosa et al. 2003). NR1 and NR2 subunits of NMDA receptors were reported to be expressed intensely not only in the dentate gyrus of adult rodents (Petralia, Wang et al. 1994) but also in the dentate granule cell layer at birth and during early postnatal life (Monyer, Burnashev et al. 1994). However, the mechanisms by which NMDA receptors influence dentate neurogenesis are yet to be elucidated. Therefore, we will preliminarily investigate the effects of MK-801 (non-competitive antagonist of NMDA receptors) on the proliferation of hippocampal cells in order to explore whether MK-801 treatments *in vitro* has the same role as *in vivo*.

4.2 Aims

We aim in this chapter to use agonists and antagonists of both AMPA and Kainate receptors in order to explore the contribution of each one of them to the general proliferative effects of Kainate in postnatal hippocampal cell cultures and the Kainate-induced increase in the numbers of progenitor cells and their neuronal precursors. We will also apply MK-801, a non-competitive antagonist of NMDA, to hippocampal cells in culture to investigate whether it has the same proliferative effects as *in vivo*.

4.3 Experimental methodology

We generated hippocampal dissociated cultures according to the protocol that detailed in **Chapter 2 (Section 2.4.2)**. We will, in this section, outline briefly specific experiments' design.

4.3.1 Examining the effects of NBQX and GYKI52466 on hippocampal cell proliferation

In these series of experiments, primary hippocampal cultures were generated from 2-8 Wistar rats of P7-10 as detailed in **Chapter 2 (Section 2.4.2)**. Cultures were maintained

under standard growth conditions for 3 days. On day three, cells were divided into 6 groups. A group of cells was maintained as control, while each one of the other five groups included the addition of one of the following treatments; 30μM NBQX, 10μM GYKI52466, 5μM Kainate, 5μM Kainate and 30μM NBQX, 5μM Kainate and 10μM GYKI52466 for the terminal 6 h before cell fixation. BrdU was also applied to a final concentration of 20μM to all groups (treated and controls) for the last 6 h. Cell were then fixed with 4% PFA and processed for BrdU and Ki-67 immunocytochemistry as described in **Chapter 2 (Section 2.4.7)**. Imaging, cell counting and statistical analysis were carried out as mentioned in **Chapter 2 (Sections 2.4.8 and 2.4.10)**

4.3.2 Investigating the effects of NBQX and GYKI52466 on hippocampal progenitor cells and neuroblasts

We reported (**Chapter 3**) that Kainate increased progenitor cells and neuronal precursors in culture. To investigate if AMPA and/or Kainate receptors mediate Kainate effects on hippocampal nestin expressing cells and TuJ1 immuno-positive cells, primary hippocampal cell cultures were generated from postnatal Wistar rat pups P7-10 as described in **Chapter 2 (Section 2.4.2)**. Cultures were maintained under the standard growth conditions or in the presence of 30μM NBQX, 10μM GYKI52466, 5μM Kainate, 5μM Kainate and 30μM NBQX, 5μM Kainate and 10μM GYKI52466 for 5 days. Cell were then fixed and immunostained against the progenitor cell marker, nestin or the neuronal cell marker TuJ1 as detailed in **Chapter 2 (Section 2.4.7)**.

4.3.3 Determining AMPA dose-response curve on hippocampal cells in culture

To address the question whether the AMPA receptor agonist, (S)-AMPA, has significant effects on primary hippocampal cell cultures and/or these effects mimic Kainate effects or not, we prepared single cell suspension from postnatal (P7-10) Wistar rats and cultured them into 24-well plates as detailed in **Chapter 2 (Section 2.4.2)**. Cultures

were maintained under standard growth conditions or in the presence of 0.1μM AMPA, 0.5μM AMPA, 5μM AMPA, 10μM AMPA, and 20μM for 5 days. Cells were then fixed with 4% PFA and stained for the nuclear stain DAPI as detailed in **Chapter 2 (Section 2.4.6)**.

4.3.4 Examining the proliferative effects of MK801 on hippocampal cells in 3 and 5 DIV cultures

In order to study the possible effects that the non-competitive NMDA receptor antagonist; MK801, might have on the proliferation of hippocampal cells *in vitro*, we prepared primary hippocampal cell cultures from postnatal rats of P7-10 as detailed in **Chapter 2 (Section 2.4.2)**. Cultures were maintained in the presence and absence of 1μM MK801, 10μM MK801, and 100μM MK801 for either 3 or 5 days. For five days cultures, two-thirds of the growth medium with/without treatments was changed on day three. At the indicated time (days three or five), a terminal 6 h BrdU pulse to a final concentration of 20μM was applied to all cells. Cells were then fixed and processed for BrdU and Ki-67 immunocytochemistry as described in **Chapter 2 (Section 2.4.7)** and counterstained for the nuclear stain DAPI to measure the total cell numbers. Imaging, cell counting and statistical analysis were carried out as mentioned in **Chapter 2 (Sections 2.4.8 and 2.4.10)**.

4.4 Results

4.4.1 Kainate enhanced the proliferation of hippocampal cells via AMPA receptors

We have shown (in **Chapter 3** (**Section 3.4.3.2**)) that a terminal (6 h) pulse of 5μM Kainate is proliferative for hippocampal cells in culture. However, the receptors mediating this effect are not clear. Therefore, in these series of experiments and after growing hippocampal cells for 3 days we used NBQX as competitive antagonist of both AMPA and Kainate receptors and the non-competitive AMPA receptors antagonists GYKI52466 to differentiate the receptor underlying the effects of Kainate on hippocampal cells proliferation. The experiments were designed as described in (**Section 4.3.1**).

As expected, BrdU incorporated cells in the last 6 h increased significantly from 7.5 ± 0.7 cells/mm^2 under control conditions to 18.5 ± 1.2 cells/mm^2 under Kainate treatment (One way Anova with Dunnetts multiple comparison post-hoc test. $p<0.001$). However, treating cells under Kainate conditions with either NBQX or GYKI52466 abolished the effects of Kainate (**Figure 4.2 B**). While Ki-67 positive cell counts increased significantly after 6 h of Kainate exposure compared to control conditions, there were no significant changes when enriching the conditions with either NBQX or GYKI52466 (**Figure 4.2 C**). The mitotic index as indicated by the proportion of BrdU incorporated cells with respect to the total cell counts (BrdU/DAPI) significantly increased under Kainate conditions (14.8 ± 0.8 % versus 6.3 ± 0.5% under control conditions) (One way Anova with Dunnetts multiple comparison post-hoc test. $p<0.001$) (**Figure 4.2 D**). However, the combined addition of GYKI52466 or NBQX and Kainate to cells abolished the effects of Kainate on the proportions of cells that incorporated BrdU as measured by the proportion of BrdU cells with respect to the total number of cells (BrdU/DAPI) (**Figure 4.2 D**). As we found before, there was no change in the growth fraction (Ki-67/DAPI) under Kainate treatments. Similarly, we did not observe changes in Ki-67/DAPI after enriching the conditions (control and Kainate) with either NBQX or

GYKI52466 (**Figure 4.2 E**). The speed of the cell cycle as indicated by the proportion of BrdU incorporated cells to the total cell numbers in the cell cycle (labelling index) (BrdU/Ki-67) increased significantly from 10.2 ± 2.1 % under control conditions to 19.6 ± 1.1 % after 6 h of Kainate exposure (One way Anova with Dunnetts multiple comparison post-hoc test. $p<0.001$). However, this Kainate effect on the rate of cell proliferation was not evident after the cells were treated with either NBQX or GYKI52466 (**Figure 4.2 F**). Likewise, neither NBQX nor GYKI52466 had effects when applied to hippocampal cells on their own.

Our results (as indicated by the increase in mitotic index (BrdU/DAPI)) confirm that 5μM Kainate has direct proliferative effects on hippocampal cells in culture and on the speed of the cell cycle (BrdU/Ki-67). Interestingly, this effect was abolished when applying 30μM NBQX (antagonist of both AMPA and Kainate receptors) to the 5μM Kainate conditions. This suggests that Kainate effects are mediated via AMPA/Kainate receptors. Furthermore, and to differentiate whether Kainate effects are on AMPA or Kainate receptors, we added 10μM GYKI52466 (antagonist of AMPA receptors) to Kainate treatment. There was no change in hippocampal cell proliferation after blocking AMPA receptors. This implies that the effects of Kainate on hippocampal cell proliferation are AMPA mediated. In order to test any significant effects for blocking AMPA and/or Kainate receptors in the absence of Kainate we added either NBQX or GYKI52466 to control conditions and found no significant changes.

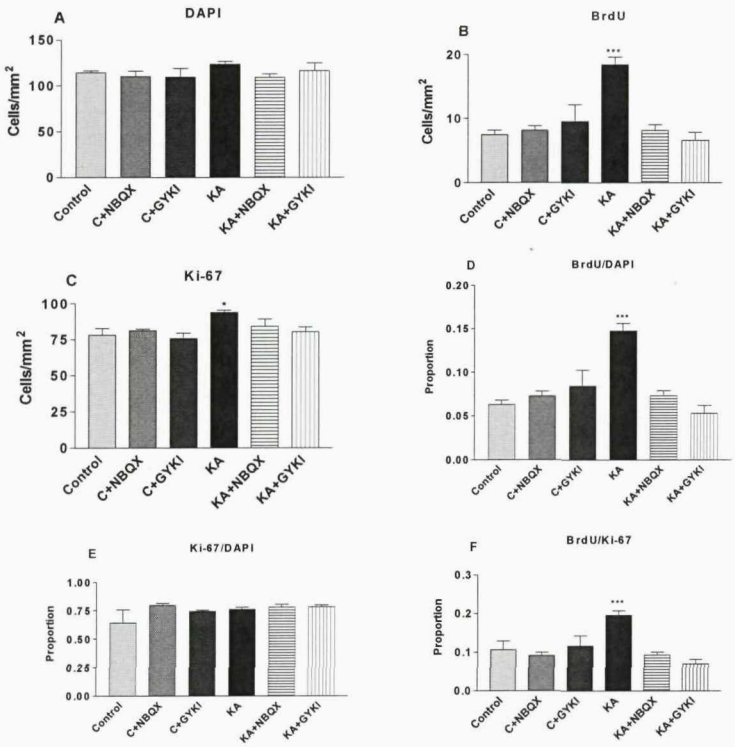


Figure 4.2 Kainate enhanced hippocampal cell proliferation via AMPA receptors. Cells were grown for 3 days under control conditions. The different conditions include 6 h terminal pulse of 30μM NBQX, 10μM GYKI52466, 5μM Kainate, 5μM Kainate plus 30μM NBQX, 5μM Kainate and 10μM GYKI52466. A final concentration of 20μM BrdU was applied to all conditions for the last 6 h as well. Cultures were fixed and immunostained for BrdU and Ki-67. 5μM Kainate enhanced cell proliferation via AMPA receptors. A) There was no increase in the total number of cells (DAPI stained nuclei) in the different conditions and in comparison to control conditions. B) Kainate increased BrdU incorporated cells which was abolished by blocking AMPA receptors by GYKI52466. C) Ki-67 immuno-positive cell counts increased after 6 h of Kainate treatment and abolished in the other treated conditions when compared to control conditions. D) The mitotic index as measured by BrdU/DAPI that increased in response to 6 h Kainate was abolished when blocking AMPA receptors. E) There were no significant changes in the growth fraction under Kainate conditions or in the presence of either NBQX or GYKI52466 and in comparison with control conditions. F) The labelling index increased after 6 h Kainate exposure but this rise was abolished in the presence of either NBQX or GYKI52466 implying a significant role for AMPA receptors. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using One way Anova with Dunnetts multiple comparison post-hoc test.* $p<0.05$, *** $p<0.001$

4.4.2 Kainate treatment increased hippocampal precursor cells via AMPA receptors

In this set of experiments, we addressed whether the increase in nestin expressing cells under Kainate treatments is mediated via AMPA and/or Kainate receptors. Cells from the hippocampi of postnatal rats were grown either under the standard growth conditions or in the presence of 30μM NBQX, 10μM GYKI52466, 5μM Kainate, 5μM Kainate and 30μM NBQX, 5μM Kainate and 10μM GYKI52466 for 5 days. Cells were then immunostained for the progenitor cell marker nestin and counterstained for the nuclear marker DAPI.

As we found before, the total cell counts increased significantly under 5μM Kainate (152.4 ± 11.4 cells/mm² versus 103.1 ± 0.7 cells/mm² under control conditions) (One way Anova with Dunnetts multiple comparison post-hoc test. $p<0.001$). However, the addition of the antagonists (NBQX or GYKI52466) to either control or Kainate conditions did not affect the total cell number after 5 days in culture (**Figure 4.3 A**). Consistently, nestin expressing cells increased significantly from 56.7 ± 7.1 cells/mm² in control conditions to 94.5 ± 11.4 cells/mm² after 5 days of Kainate treatment (One way Anova with Dunnetts multiple comparison post-hoc test. $p<0.001$). However, the combined treatment of Kainate with either NBQX or GYKI52466 abolished the increase in nestin immuno-positive cells (**Figure 4.3 B**).

Our data shows that Kainate increases the counts of nestin immuno-positive cells in culture by acting on AMPA receptors.

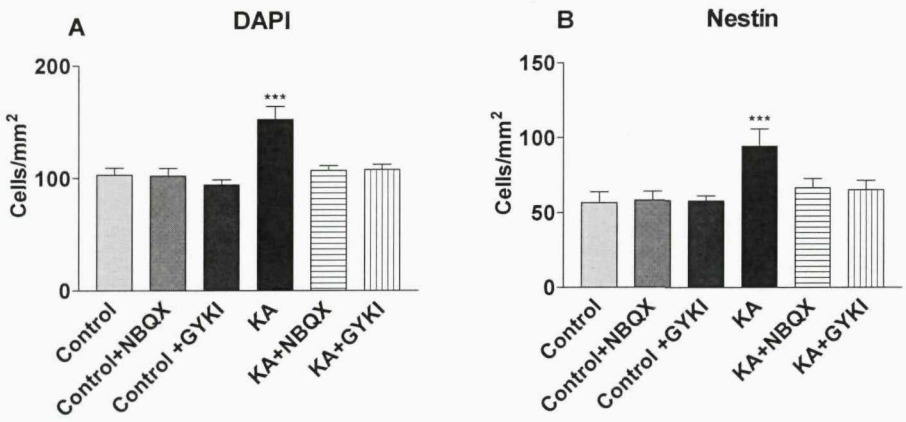


Figure 4.3 The Kainate effect of increasing the numbers of progenitor cells in culture is mediated by AMPA receptors. Cells were grown for 5 days under standard growth conditions or in the additional presence of 30μM NBQX, 10μM GYKI52466, 5μM Kainate, 5μM Kainate plus 30μM NBQX, 5μM Kainate and 10μM GYKI52466. Cultures were fixed and immunostained for nestin and counterstained for DAPI. Kainate increased nestin immuno-positive cells via AMPA receptors. A) There was a significant increase in the total number of cells (DAPI stained nuclei) under Kainate conditions which was abolished by blocking AMPA receptors. B) Nestin expressing cells increased significantly under Kainate conditions when compared to control conditions but the addition of either NBQX or GYKI52466 to Kainate conditions abolished this increase. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using One way Anova with Dunnetts multiple comparison post-hoc test. *** $p<0.001$

4.4.3 Kainate treatment increased the proportion of TuJ1 positive cells via AMPA receptors

In a further set of experiments, cells dissociated from postnatal hippocampi were grown for 5 days under control conditions or under control plus 30μM NBQX, 10μM GYKI52466, 5μM Kainate, 5μM Kainate and 30μM NBQX, or 5μM Kainate and 10μM GYKI52466. Cells were fixed with 4% PFA and stained for the neuronal marker TuJ1 and counterstained for the nuclear marker DAPI. Counts of TuJ1 immuno-positive cells and their proportions with respect to the total number of cells were worked out.

Our results demonstrated a significant rise in the total numbers of cells (DAPI) after 5 days of Kainate exposure which was abolished by the addition of either NBQX or GYKI52466 to Kainate treated cells (**Figure 4.4 A**). As described (in **Chapter 3**), in this set of experiments, Kainate significantly increased the numbers of TuJ1 expressing cells (80.7 ± 2.1 cells/mm^2 versus 44.7 ± 3.1 cells/mm^2 under control conditions) (One way Anova with Dunnetts multiple comparison post-hoc test. $p<0.001$). However, co-treatment of cultured hippocampal cells with either NBQX or GYKI52466 and Kainate abolished the effects of Kainate (**Figure 4.4 B**). Importantly, the addition of NBQX or GYKI52466 with Kainate to hippocampal cells abolished the Kainate-enhanced proportional increase in TuJ1 immuno-positive cells (**Figure 4.4 C**). **Figure 4.5** represents a sample of the images that were analyzed.

Our results suggest that AMPA receptors have an important role in mediating the effects of Kainate on postnatal hippocampal neuroblasts.

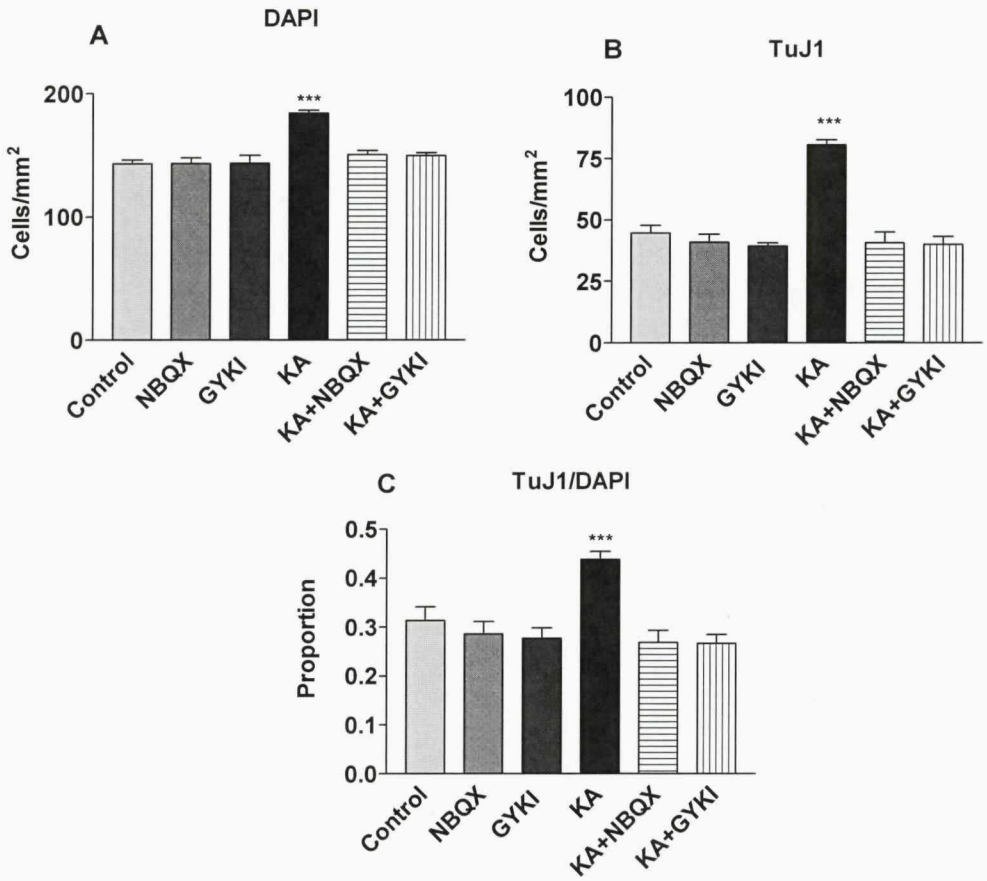


Figure 4.4 Kainate increased the proportion of TuJ1 immuno-positive cells in culture via AMPA receptors. Cells were grown on glass slides for 5 days under standard growth conditions or in the additional presence of 30μM NBQX, 10μM GYKI52466, 5μM Kainate, 5μM Kainate plus 30μM NBQX, 5μM Kainate and 10μM GYKI52466. Cultures were fixed and stained for TuJ1 and counterstained for DAPI. Kainate increased the proportion of TuJ1immuno-positive cells via AMPA receptors. A) There was a significant increase in the total number of cells (DAPI stained nuclei) under Kainate conditions which was abolished by blocking AMPA receptors. B) TuJ1 expressing cells increased significantly under Kainate conditions when compared to control conditions but the addition of either NBQX or GYKI52466 to Kainate conditions abolished this increase. C) The proportion of TuJ1 expressing cells increased significantly under Kainate conditions when compared to control conditions but the addition of either NBQX or GYKI52466 to Kainate treated cells abolished this increase. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using One way Anova with Dunnetts multiple comparison post-hoc test. *** $p<0.001$

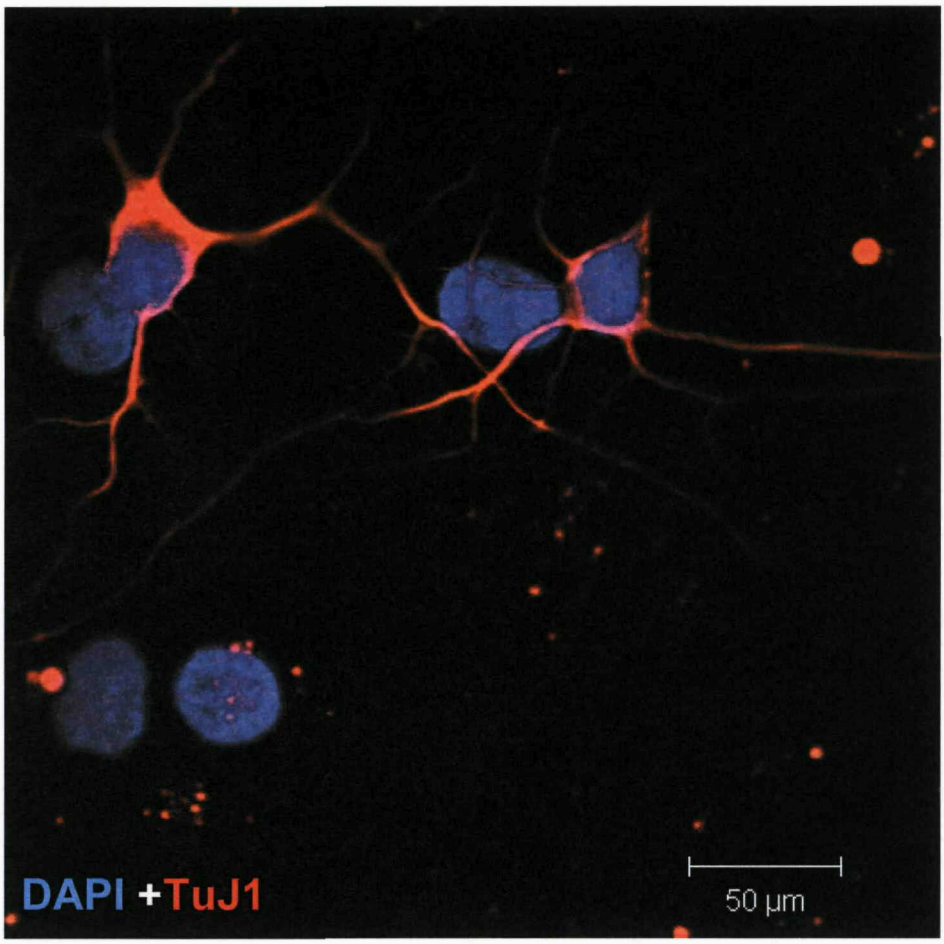


Figure 4.5 Micrograph of TuJ1 cells in culture. We grew primary hippocampal cell for 5 days under standard growth conditions and 5µM Kainate. Two-thirds of the growth medium with/without 5µM Kainate was replaced on day 3. Fixation for 30 minutes with 4% PFA was done on day 5 before consequent immunocytochemistry. Primary specific anti-sera against TuJ1 (neuronal marker) was detected with fluorescently linked secondary antibodies. Counterstaining for the nuclear marker DAPI was carried out at the end of immunocytochemistry. The staining shows DAPI in blue and TuJ1 in red. Images were captured with 63x oil objective on a laser scanning confocal microscope and 3D projections reconstructed using the Zeiss LSM software. Scale bar = 50µm.

4.4.4 AMPA increased hippocampal cell counts in culture

In the previous **Sections,** we have demonstrated that the effects of Kainate are mediated via AMPA receptor subtypes. Here, we will investigate whether AMPA, as an agonist for AMPA receptors, has an effect on the total number of cells in culture. Dissociated cells were prepared from postnatal rats hippocampi and were grown for 5 days in the presence and absence of 0.1μM AMPA, 0.5μM AMPA, 5μM AMPA, 10μM AMPA, or 20μM AMPA (**Section 4.3.3**). Two-thirds of the growth medium with/without treatments was changed on day three. After cell fixation, cells were stained for the nuclear stain DAPI. Imaging, counting and statistical analysis were carried out as described in **Chapter 2 (Sections 2.4.8 and 2.4.10)**.

Although 0.1μM AMPA increased the total number of cells as measured by DAPI to 152.1 ± 2.1 cells/mm² versus 127.9 ± 4.9 cells/mm² under control conditions, this rise was not statistically significant (One way Anova with Dunnett's Multiple Comparison Test) (**Figure 4.6**). The same insignificant rise in cell counts was observed in 0.5 μM AMPA enriched conditions. Interestingly, 5μM AMPA increased significantly the total cell numbers to 168.9 ± 10.8 cells/mm² versus 127.9 ± 4.9 cells/mm² under control conditions (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 4.6**). The application of higher concentrations of AMPA (10μM and 20μM) did not have an effect on the total cell counts.

Our data show that AMPA increases the total cell numbers as indicated by an increase in DAPI and this increase is dose dependent.

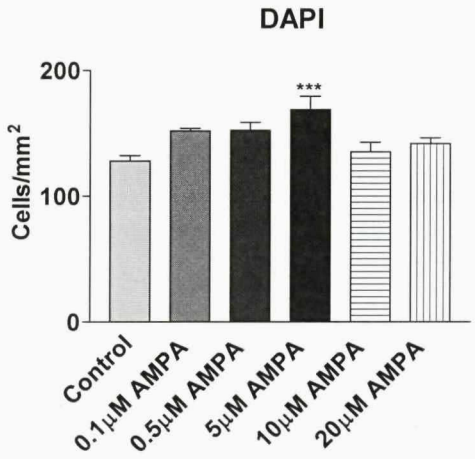


Figure 4.6 5μM (S)-AMPA increased the total hippocampal cell counts in culture. Cells were grown for 5 days under standard growth conditions or in the additional presence of 0.1μM AMPA, 0.5μM AMPA, 5μM AMPA, 10μM AMPA, or 20μM AMPA. Cultures were fixed and stained for the nuclear marker DAPI. AMPA increased the total number of hippocampal cells in culture. A) There was a significant increase in the total number of cells (DAPI stained nuclei) under 5μM AMPA conditions and in comparison wuth control conditions. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using One way Anova with Dunnetts multiple comparison post-hoc test. *** $p<0.001$

4.4.5.1 MK-801 increased the counts of BrdU incorporated cells, Ki-67 expressing cells and the total hippocampal cells in 3 DIV

To study the effects of different concentrations of MK-801 on the total cell numbers, cell proliferation, and cell cycle kinetics, monolayer cultures were grown either under control conditions or under 1μM, 10μM, or 100μM MK-801 and maintained for 3 days as detailed in (**Section 4.3.4**). Cells were pulsed with a final concentration of 20μM BrdU for the terminal 4 hours before being fixed and stained against BrdU and Ki-67 and counterstained for the nuclear marker DAPI (**Section 2.4.7**). Imaging, counting and statistical analysis were carried out as mentioned in **Chapter 2** (**Sections 2.4.8 and 2.4.10**).

We found a significant increase in the total number of cells (**DAPI**) under 1μM and 10μM MK-801 (**Figure 4.7 A**). DAPI cell counts increased significantly from 85.9 ± 3.5 cells/mm² under control conditions to 130.7 ± 10.0 cells/mm² under 1μM MK801 treated condition (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 4.7 A**). We also found a significant increase in the total cell counts under 10μM MK801 when compared to control conditions. However, 100μM MK-801 decreased significantly the total cell counts to 62.1 ± 3.8 cells/mm² (One way Anova with Dunnett's Multiple Comparison Test, $p<0.05$) (**Figure 4.7 A**). Although BrdU incorporated cells increased from 47.78 ± 2.5 cells/mm² under control conditions to 83.8 ± 6.6 cells/mm² after 3 days of 1μM MK-801 treatment (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 4.7 B**), there was no significant increase in the mitotic index as indicated by (BrdU/DAPI) except under 100μM MK-801 enriched conditions (**Figure 4.7 D**). In parallel with the increase in BrdU incorporated cells, we found that both 1μM MK-801 and 10μM MK-801 significantly increased Ki-67 expressing cells in culture (**Figure 4.7 C**). The numbers of Ki-67 immuno-positive cells increased from 56.7 ± 3.2 cells/mm² under control conditions to 92.6 ± 7.9 cells/mm² after 3 days of 1μM MK-801 exposure (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 4.7 C**). In spite of the significant rise in Ki-67 expressing cells after 3 days of 1μM or 10μM MK-801, there was no significant effect for these MK-801 concentrations on the growth fraction (Ki-67/DAPI); however, 100μM MK-801 increased the growth fraction significantly when compared to control conditions and thus it recruited quiescent cells (**Figure 4.7 E**). The speed of the cell cycle as indicated by the labelling index (BrdU/Ki-67) increased from 82.3 ± 3.8 % under control conditions to 90.8 ± 1.2 % under 1μM MK-801(**Figure 4.7 F**). This rise was statistically significant (One way Anova with Dunnett's Multiple Comparison Test, $p<0.05$). Similar significant increase was observed under 100μM MK-801 treated conditions in comparison with control conditions.

Our results suggest that the increase in the total cell counts, BrdU incorporated cells and Ki-67 positive cells after 3 days of MK-801exposure is dose dependent. We also report that although high dose of MK-801 (100μM) decreases the total cell numbers, it is affecting the proliferating cells as it increases the mitotic index (BrdU/DAPI) and the

speed of the cell cycle (BrdU/Ki-67) and also recruits quiescent cells to proliferate. This might also indicate that using 100μM MK-801 is toxic for non-dividing hippocampal cells.

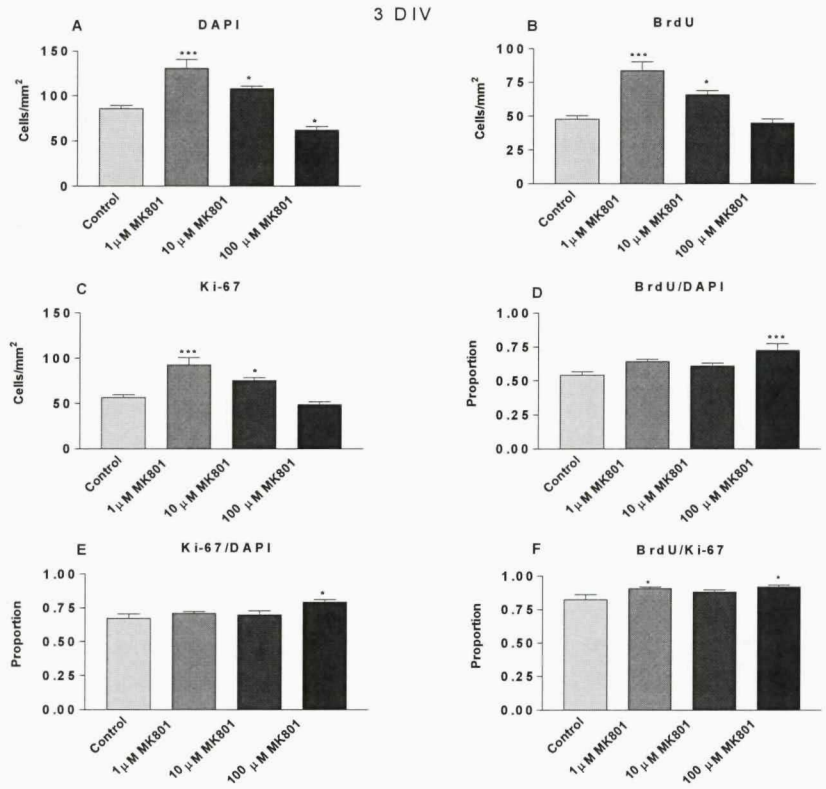


Figure 4.7 MK-801 increased hippocampal cell counts and proliferation in 3 DIV. Primary hippocampal cells were generated and incubated for 3 days in the standard growth conditions or in the presence of 1μM MK-801, 10μM MK-801, or 100μM MK-801. Four hours terminal pulse of BrdU to a final concentration of 20μM was applied to all conditions. Cultures were then fixed and double labelled for BrdU and Ki67. MK-801 increased the total cell counts, BrdU-incorporated cells, and Ki-67 positive cells. A) 1μM MK-801 and 10μM MK-801 increased the total cell counts significantly when compared to control conditions, whereas 100μM MK-801 showed the opposite. B) The number of BrdU incorporating cells after terminal 4 hours BrdU pulse increased significantly under 1μM MK-801 and 10μM MK-801 enriched conditions. C) Ki-67 expressing cells in 3 days cultures increased under 1μM MK-801 and 10μM MK-801 and in comparison to control. D) The proportion of proliferating (BrdU labelled) cells in primary hippocampal cultures increased after 3 days of 100μM MK-801 treatment. E) 100μM MK-801 significantly increased Ki-67/DAPI (growth fraction). F) The speed of the cell cycle as indicated by BrdU/Ki-67 increased significantly after 3 days of either 1μM MK-801 or 100μM MK-801 exposure. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using One way Anova with Dunnetts multiple comparison post-hoc test.* $p<0.05$, *** $p<0.001$

4.4.5.2 MK-801 is proliferative for hippocampal cells in 5 DIV cultures

We investigated the proliferative effects of MK801 on hippocampal cells in 5 days cultures. Cultures generated from postnatal rats' hippocampi were grown either under control conditions or in the additional presence of 1μM, 10μM, or 100μM MK-801 and maintained for 5 days as described in (**Section 4.3.4**). Cells were pulsed with a final concentration of 20μM BrdU for the terminal 4 hours before being fixed and stained against BrdU and Ki-67 and counterstained for the nuclear marker DAPI (**Section 2.4.7**). Imaging, counting and statistical analysis were carried out as mentioned in **Chapter 2** (**Sections 2.4.8 and 2.4.10**).

We found a significant increase in the total number of cells (**DAPI**) under 1μM and 10μM MK-801 (**Figure 4.8 A**). DAPI cell counts increased significantly from 251.8 ± 21.0 cells/mm² under control conditions to 373.5 ± 25.7 cells/mm² under 1μM MK-801 treated condition (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 4.8 A**). Significant rise in DAPI cell counts was also observed under 10μM MK-801 and in comparison with control conditions. However, there was no change in the total cell numbers under 100μM MK801 conditions (**Figure 4.8 A**). BrdU incorporated cells increased from 45.6 ± 2.9 cells/mm² under control conditions to 154.6 ± 18.9 cells/mm² under 1μM MK-801 conditions (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 4.8 B**). Similar significant increases were observed under 10μM MK-801 and 100μM MK-801conditions (**Figure 4.8 B**). Interestingly, the mitotic index as indicated by (BrdU/DAPI) increased significantly under 1μM MK-801 exposed cells (41.1 ± 3.3 % versus 17.7 ± 1.2 % under control conditions) (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 4.8 D**). We also found a significant rise in this proportion of cells that incorporated BrdU under 10μM MK-801 and 100μM MK-801 conditions when compared to control conditions (**Figure 4.8 D**). In parallel with the increase in BrdU incorporated cells, we found that 1μM MK-801, 10μM MK-801, and 100μM MK-801 significantly increased Ki-67 expressing cells in culture (**Figure 4.8 C**). The numbers of Ki-67 immuno-positive cells increased from 61.8 ± 5.9 cells/mm² under control conditions to 192.7 ± 24.2 cells/mm² after 5 days of 1μM MK-801 treatment (One way

Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 4.8 C**). Interestingly, the growth fraction (Ki-67/DAPI) increased under the three MK-801 concentrations (**Figure 4.8 E**). This may imply recruitment for quiescent cells to proliferate. The speed of the cell cycle as indicated by the labelling index (BrdU/Ki-67) increased from 68.0 ± 2.9 % under control conditions to 80.3 ± 3.1 % under 1μM MK-801(**Figure 4.8 F**). This rise was statistically significant (One way Anova with Dunnett's Multiple Comparison Test, $p<0.05$). Similar significant increases were observed in 10μM MK-801 and 100μM MK-801 treated conditions and in comparison with control conditions.

Samples of the images that were counted and analyzed are shown in (**Figure 4.9**).

These results show that MK-801 is proliferative for hippocampal cells *in vitro* at a concentration as low as 1μM. In spite of the fact that 100μM MK-801 does not have an effect on the total cell numbers, it significantly increases the mitotic index (BrdU/DAPI), the speed of the cell cycle (BrdU/Ki-67), and the growth fraction (Ki-67/DAPI). This suggests that this concentration of MK-801 has a pivotal effect on hippocampal cells; while it might target the proliferating cells at one side, it is toxic for others.

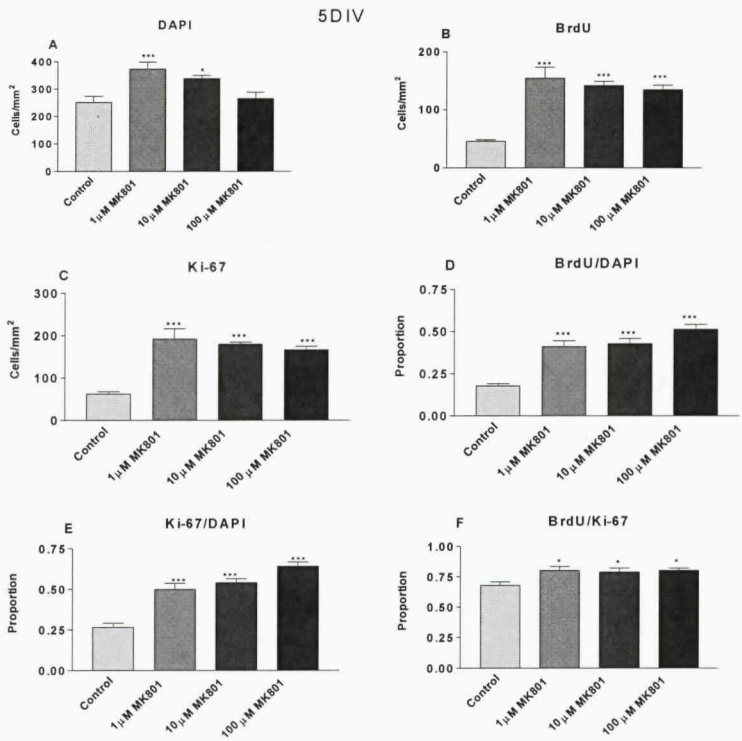


Figure 4.8 MK-801 increased hippocampal cell counts in 5 DIV. Primary hippocampal cells were generated and incubated for 5 days under the standard growth conditions or in the additional presence of 1μM MK-801, 10μM MK-801, or 100μM MK-801. On day 3 two-thirds of the growth medium with/without enrichment was changed. A final concentration of 20μM BrdU was given to all conditions for the terminal 4 hours. Cultures were then fixed and double labelled for BrdU and Ki-67. MK-801 increased the total cell counts, the mitotic index (BrdU/DAPI), the speed of the cell cycle (BrdU/Ki-67), and also recruits quiescent cells to proliferate as indicated by an increase in the growth fraction (Ki-67/DAPI). A) 1μM MK-801 and 10μM MK-801 increased the total cell counts significantly when compared to control conditions, but 100μM MK-801 showed no significant effect. B) The numbers of BrdU incorporated cells after terminal 4 hours BrdU pulse increased significantly under 1μM MK-801, 10μM MK-801 and 100μM MK-801 enriched conditions. C) Ki-67 expressing cells in 5 days cultures increased in 1μM MK-80, 10μM MK-801 and 100μM MK-801 and in comparison with control. D) The proportions of proliferating (BrdU labelled) cells in primary hippocampal cultures increased after 5 days of MK-801 treatment. E) Different concentrations of MK-801 significantly increased Ki-67/DAPI (growth fraction). F) The speed of the cell cycle as indicated by BrdU/Ki-67 increased significantly after 5 days of a wide range of MK-801 doses exposure. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using One way Anova with Dunnetts multiple comparison post-hoc test.* $p<0.05$, *** $p<0.001$

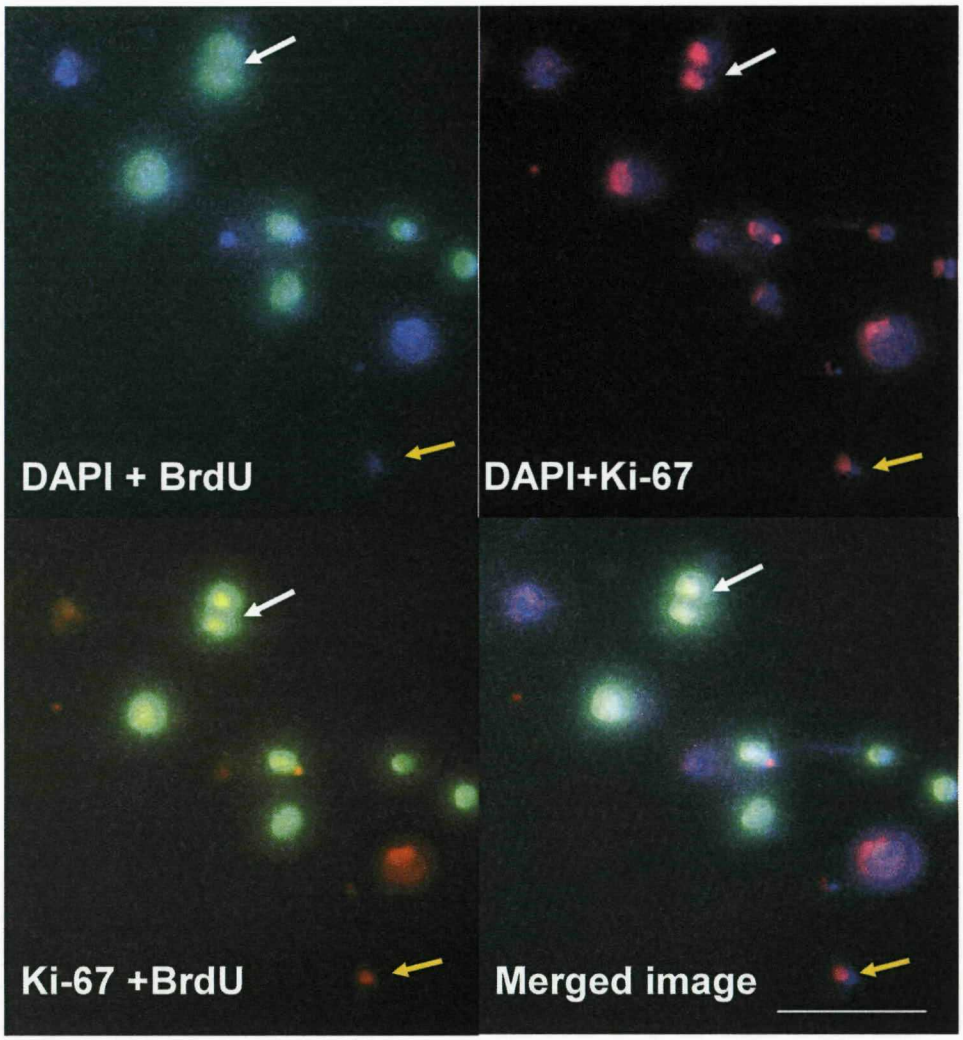


Figure 4.9 MK-801 enhanced the proliferation of hippocampal cells in cultures. Cells were cultured in the presence and absence of 1μM MK-801 for 5 days. They were terminally pulsed with BrdU for 4 hours. After fixation with 4% PFA, cells were probed with antibodies to BrdU (green), Ki-67 positive cells (red), and counterstained for the nuclear stain DAPI (blue). We showed a significant increase in total cell numbers (DAPI), in the numbers and proportions for BrdU incorporated cells, and Ki-67 positive cells and growth fraction, as well as in the speed of the cell cycle as indicated by BrdU/Ki-67. White arrows show sample of Ki-67 immuno-positive cells that incorporated BrdU. Yellow arrows demonstrate Ki-67 positive cells only. Imaging was performed on an inverted Leica DM IRB microscope with 20x objectives (Leica Microsystems UK Ltd, Milton Keynes, UK). Open Lab image capturing system version 2.1 (Improvision, Lexington, USA) was used to count cells. Scale bar = 40μm.

4.4.5.3 MK-801 increased the proportion of hippocampal progenitor cells in culture

In this set of experiments, we studied the effects of MK-801 on hippocampal progenitor cells in 3 days cultures. Primary cells generated from postnatal rats' hippocampi were grown either under control conditions or in the presence of 1μM, 10μM, or 100μM MK-801 and maintained for 3 days as described in **Section 4.3.4**. Cells were fixed and immunostained for the stem/progenitor marker nestin and counterstained for the nuclear stain DAPI (**Section 2.4.7**). Imaging, counting and statistical analysis were carried out as mentioned in **Chapter 2 (Sections 2.4.8 and 2.4.10)**.

We consistently found that the total number of cells (DAPI) increased significantly under 1μM and 10μM MK-801 conditions when compared to control conditions and 100μM MK-801 had the opposite effect (**Figure 4.10 A**). In (**Figure 4.10 B)** we demonstrated that nestin immuno-positive cells increased from 39.3 ± 3.6 cells/mm² under control conditions to 73.1 ± 3.2 cells/mm² under 1μM MK-801. This increase was statistically significant (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$). A similar significant rise was observed under 10μM MK-801 conditions, but not under 100μM MK-801. Interestingly, 3 days exposure to 1μM MK-801 increased significantly the proportion of nestin immuno-positive cells (61.2 ± 2.0 % versus 46.13 ± 0.8 % under control conditions) (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 4.10 C**). Importantly, 100μM MK-801 increased the proportion of nestin expressing to 66.5 ± 1.5 %. This rise was statistically significant (One way Anova with Dunnett's Multiple Comparison Test, $p<0.001$) (**Figure 4.10 C**).

These results demonstrate that 1μM MK-801 increases the proportion of hippocampal progenitor cells in culture and also show that 100μM MK-801 has a significant effect on nestin sub-population of cells.

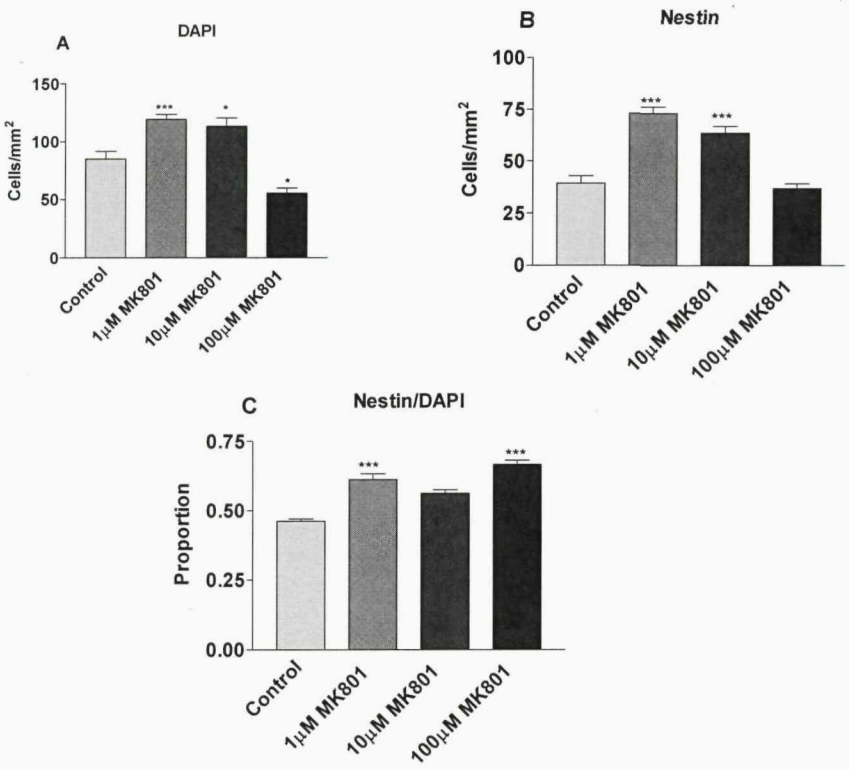


Figure 4.10 MK-801 increased the proportion of hippocampal progenitor cells in culture. Primary hippocampal cells were generated and incubated for 3 days under the standard growth conditions or in the additional presence of 1μM MK-801, 10μM MK-801, or 100μM MK-801. Cultures were then fixed and immunostained for nestin. MK-801 increased the total cell counts, the numbers of nestin immuno-positive cells, and the proportion of this sub-population of cells. A) 1μM MK-80 and 10μM MK-801 increased the total cell counts significantly when compared to control conditions, but 100μM MK-801 showed a significant decrease in the total number of cells. B) The numbers of nestin expressing cells increased significantly under 1μM MK-801 and 10μM MK-801 enriched conditions. C) The proportion of progenitor cells in culture increased significantly under 1μM MK-80 and 100μM MK-801 and in comparison with control. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using One way Anova with Dunnetts multiple comparison post-hoc test.* $p<0.05$, *** $p<0.001$

4.5 Discussion

We have demonstrated (**Chapter 3**) that Kainate, which is an agonist for AMPA/Kainate receptors, enhances hippocampal progenitor cell proliferation and increases the proportion of neuroblasts in culture. Kainate and AMPA receptors are widely distributed throughout the CNS, particularly in the hippocampus (Huettner 2003; Catarzi, Colotta et al. 2007). Therefore, the effects of Kainate on hippocampal cells might involve an action that is mediated via Kainate receptors, AMPA receptors, both of them, or neither of them. To test this hypothesis, we used the non-selective AMPA and Kainate receptor antagonist NBQX (Wilding and Huettner 1996) and the non-competitive antagonist of AMPA receptors GYKI52466 (Paternain, Morales et al. 1995). NBQX is a competitive antagonist for both AMPA and Kainate receptors when applied at high dose (Wilding and Huettner 1996). Therefore, we applied 30μM NBQX to block the effects mediated by both receptors. GYKI52466 is a non-competitive antagonist of AMPA receptors (IC_{50} = 9.8 ± 1.0 μM) (Paternain, Morales et al. 1995), and as such, we used 10μM GYKI52466 to block AMPA receptors mediated effects only. We have applied 30μM NBQX to control conditions and to 5μM Kainate treated cells and 10μM GYKI52466 to control conditions and Kainate exposed cells as well. We have demonstrated that neither NBQX nor GYKI52466 affects hippocampal precursor cells under control conditions. However, the addition of either NBQX or GYKI52466 in conjunction with Kainate abolished the proliferative effects of Kainate on cultured hippocampal cells. Moreover, the combined application of either GYKI52466 or NBQX with Kainate on hippocampal cell cultures abolished Kainate-induced increases in both nestin and TuJ1 immuno-positive cells. Finally, we found that, like Kainate, (S)-AMPA, the AMPA receptor selective agonist, increases the total numbers of hippocampal cells in culture.

NMDA plays a significant role in hippocampal neurogenesis (Nacher and McEwen 2006). Indeed, it has been demonstrated that NMDA treatments decreased the rate of cell proliferation as well as the generation of new neurons in the adult rat dentate gyrus (Cameron, McEwen et al. 1995). In agreement, in the same study it has been shown that MK-801 (non-competitive NMDA receptor antagonists) enhanced dentate gyrus neurogenesis. These findings taken together strongly implicate NMDA in the regulation

of hippocampal neurogenesis, yet, the mechanisms are not well-characterized. Therefore, we investigated the effects of MK-801 on hippocampal progenitor cells and hippocampal cell proliferation. Consistent with Cameron et al findings, we show a significant proliferative effect of MK-801 on postnatal hippocampal cells in an effect that depends on the dose of MK-801 and the age of cell cultures. We further demonstrate a significant proportional increase in nestin-expressing hippocampal progenitor cells after 3 days of MK-801 treatments.

4.5.1 AMPA receptors are involved in regulating hippocampal neurogenesis

In **Section 4.4.1**, and in agreement with what we have found before (**Chapter 3**), we have shown that short terminal pulse of 5μM Kainate increases the numbers of cells that incorporated BrdU. It also increases mitotic index as indicated by the significant rise in the proportion of cells in the S-phase of the cell cycle with respect to the total cell counts (BrdU/DAPI). We demonstrate an increase in the labelling index under Kainate conditions as indicated by a rise in the proportion of cells that incorporated BrdU with respect to total cycling Ki-67 immuno-positive cells (BrdU+ & Ki-67+/Ki-67). This implies that Kainate increases the speed of the cell cycle at 3 DIV. Interestingly, the addition of either NBQX or GYKI52466 to Kainate conditions abolished the proliferative effects of Kainate treatment. In order to understand how either NBQX or GYKI52466 abolishes Kainate effects we will analyze the effects of each compound on cells under Kainate treatment on its own. NBQX is an antagonist for both AMPA and Kainate receptors, which completely abolished the proliferative effects of Kainate. We conclude that blocking both AMPA and Kainate receptors has abolished the effects of Kainate. This suggests that these effects are mediated via either AMPA or Kainate receptors or both of them. GYKI52466 is a selective antagonist of AMPA receptors. The addition of GYKI52466 abolished the effects of Kainate on hippocampal cells and their proliferation. This supports the hypothesis that Kainate effects *in vitro* are mediated selectively via AMPA receptors and not via Kainate receptors. Treating hippocampal

cells under control conditions with either NBQX or GYKI52466 does not have an effect on the total hippocampal cell counts or proliferation. This suggests that either AMPA and/or Kainate receptors are either not expressed (unlikely) or are not activated under standard growth conditions in our culture system. In **Section 4.4.2**, we demonstrated that the effect of Kainate on progenitor (nestin immuno-positive) cells was abolished in the presence of either NBQX or GYKI52466. This also implies that the increase in hippocampal progenitor cells is AMPA receptor mediated.

In **Chapter 3**, we showed that Kainate enhanced hippocampal neurogenesis *in vitro* as indicated by the proportional increase in TuJ1 immuno-positive subpopulation of cells. Therefore, in this chapter I investigated the effects of Kainate on TuJ1 sub-population of cells after applying either NBQX or GYKI52466 to Kainate enriched conditions and to control conditions (**Section 4.4.3**). Again, we show that there are no significant effects of Kainate on TuJ1 expressing cells or their proportions in culture in the presence of either NBQX or GYKI52466. This result indicates that blocking AMPA receptors abolished the increase in neurogenesis due to Kainate exposure.

In order to understand the mechanisms underlying AMPA receptors mediation of Kainate effects on hippocampal cells in cultures, we applied different concentration of (S)-AMPA, the active form of AMPA receptors agonists, on hippocampal cells *in vitro*. Although 0.1μM (S)-AMPA increases the total number of cells (as indicated by DAPI cell counts); this rise is not statistically significant. However, 5μM (S)-AMPA significantly increases the total number of hippocampal cells in culture. We also show that higher concentrations of (S)-AMPA (10μM and 20μM) do not have an effect on the total cell numbers. The decrease in hippocampal cell numbers under 10μM and 20μM(S)-AMPA might be due to the activation of NMDA receptor subtypes as it has been shown that a mixed subunits of NMDA/AMPA receptor subtypes are present in the hippocampus (Barnard et al 1997). High dose of AMPA *in vitro* might act on NMDA receptor subtypes and activation of NMDA receptors has been demonstrated to down regulate hippocampal neurogenesis (Cameron et al 1995). Investigating the interaction between AMPA and NMDA receptor subtypes would be an interesting avenue for future research. The other likely possibility is that higher concentration of AMPA (10μM and 20μM) enhances cell death in cultures. This would be a good filed to be explored in the

future. In agreement with our results that AMPA receptor subtypes modulate the proliferative effects of Kainate, it has been demonstrated that both acute and chronic treatments of adult rats with LY451646 (AMPA receptor potentiator) enhance the proliferation of DG cells as indicated by the increase in the clusters of BrdU incorporated cells after 24 hours and 21 days of LY451646 treatment (Bai, Bergeron et al. 2003). Thus, AMPA receptor subtypes regulate, at least in part, the proliferation of hippocampal precursor cells. Moreover, in an elegant study, Xu et al 2005 have demonstrated that intracerebroventricular administration of (S)-AMPA enhanced not only the proliferation of SVZ precursor cells (as indicated by the increase in Ki-67+ cells) but also increased Dcx expressing cells in postnatal rats (Xu, Ong et al. 2005). Therefore, AMPA is enhancing precursor cells proliferation and neurogenesis in postnatal rats' pups.

Taken together, our results demonstrate that blocking AMPA receptors abolished not only the proliferative effects of Kainate but also the differentiation effects on precursor cells towards a neuronal lineage. Indeed, we have shown in **Chapter 3** that the net increase in neuroblasts by day 5 is in part due to differentiation effects of Kainate. This effect is blocked by AMPA receptors antagonist, and as such, we conclude that AMPA receptor subunits modulate hippocampal precursor cells differentiation as well. Furthermore, we show in **Chapter 5** that Kainate has a survival effects as well. Therefore, our results demonstrate that Kainate modulate hippocampal neurogenesis through a common mechanism that involves AMPA receptors. We conclude that AMPA receptors have a role in regulating hippocampal neurogenesis by modulating the effects of Kainate on progenitor cells and neuronal precursors.

It has been shown, using patch clamp and real time PCR, that hippocampal progenitor (nestin immuno-positive) cells and TuJ1 immuno-positive cells express AMPA receptor subtypes in cultures generated from E18 rat hippocampi (Hagimura, Tsuzuki et al. 2004). The expression of AMPA receptor subtypes on both hippocampal progenitor cells and neuronal precursor cells *in vitro* suggests a role for AMPA receptors in hippocampal neurogenesis and supports our work that AMPA receptor subtypes are modulating hippocampal neurogenesis. Furthermore, it has been demonstrated in both *in vivo* and *in vitro* studies that CNS neurons express much higher density of AMPA receptors than

Kainate receptors (Spruston, Jaffe et al. 1994; Wong, Mayer et al. 1994; Paternain, Morales et al. 1995). This may suggest a significant role of AMPA receptors in the control of neuronal growth mechanisms. In line with the direct effects of Kainate on AMPA receptors, it has been shown that glutamate acts directly on dividing cells cultured from rat embryonic neocortex (LoTurco, Owens et al. 1995) and functional glutamate cells are present in cultures (Barres, Koroshetz et al. 1990). These studies support the direct proliferative effects that Kainate have via AMPA receptor subtypes on hippocampal cells. AMPA receptors are a subdivision of glutamate receptors and are highly likely that these receptors are expressed on cells in our cultures (as demonstrated by using NBQX and GYKI52466), and thus, we observed a significant effect for Kainate on them. Although Brazel et al 2005 have reported a significant proliferative and survival effect of 3μM Kainate on progenitor cells cultured from the SVZ, she found no evidence that this effect was mediated via AMPA receptor subtypes, suggesting that Kainate acts on SVZ progenitor cells perhaps via different mechanisms. We, in this project, demonstrate significant proliferative effects of 5μM Kainate on hippocampal progenitor cells and further show that this effect is AMPA receptor subtypes mediated. Furthermore, it has been shown that glutamate enhances the proliferation and neurogenesis in human neural progenitor cells cultured from the fetal cortex (Suzuki, Nelson et al. 2006). Interestingly, this group has also demonstrated the expression of AMPA/Kainate receptors by progenitor cells. This suggests a significant role for AMPA/Kainate receptors in neurogenesis in humans. In addition, Kainate induced-seizures have been found to down regulate Kainate receptor subtypes in the immature hippocampus (Tandon, Yang et al. 2002). This might suggest that Kainate treatment on cultures generated from postnatal hippocampus down regulates Kainate receptor subtypes and thus the effect of Kainate is mediated via another receptor subtypes which are AMPA receptor in this case. In addition, it has been shown that GluR2/3 receptor subtypes were up-regulated in the dentate gyrus after Kainate-induced seizures (Friedman, Pellegrini-Giampietro et al. 1994). Likewise, the expressions of AMPA receptor subtypes (GluR2/3) were found to be increased on the dentate granule cells and hilus of humans with TLE (de Lanerolle, Eid et al. 1998). Although neurogenesis was not the aim of the previous studies, the increase in the expressions of AMPA receptor

subtypes in epilepsy may suggest their involvement in regulating hippocampal neurogenesis.

In addition, it is widely known that anti-depressant drugs enhance neurogenesis (Encinas, Vaahtokari et al. 2006). It has been described that AMPA receptors play a significant role in mood disorders with the possibility of being up regulated after the use of anti-depressant drugs but the mechanisms are not well understood (Alt, Nisenbaum et al. 2006). Our results suggest that AMPA receptors play an important role in regulating hippocampal neurogenesis at least in the early postnatal period. This may also suggest that AMPA receptors play a role in the mechanisms by which anti-depressants like fluoxetine up regulate hippocampal neurogenesis. Thus, AMPA receptors represent potential targets for therapeutic intervention in many neurological diseases.

4.5.2 MK-801 enhances hippocampal cell proliferation and increases progenitor cells *in vitro*

We have shown in **Section 4.4.6.1** that MK-801 increases the total cell numbers in culture as well as the numbers of BrdU incorporated cells and Ki-67 immuno-positive cells in 3 DIV. However, this increase is dose dependent. We demonstrate that 1μM MK-801 has an overall significant effect on hippocampal cells. However, there are no significant effects for 1μM or 10μM MK-801 on the speed of the cell cycle as indicated by (BrdU/Ki-67), the proportion of proliferating cells (BrdU/DAPI), and the growth fraction (Ki-67/DAPI). This is most likely because these cells are proliferating and expanding in numbers at the same rate. A synergistic survival role might be also implicated and form a good avenue for future investigations. In **Section 4.4.6.2,** we demonstrated that either 1μM or 10μM MK-801 enhances the mitotic index as indicated by the increase in (BrdU/DAPI), the speed of the cell cycle as measured by (BrdU/Ki-67), and the growth fraction (Ki-67/DAPI) in 5 DIV. These data indicate that 5 days exposure to MK-801 is essential to initiate the proliferative characteristics. Importantly, we show that after 5 days in culture 1μM and 10μM MK-801 recruit quiescent cells to start dividing as shown by the increase in the growth fraction (Ki-67/DAPI).

Interestingly, we observe a proportional increase in progenitor (nestin immuno-positive) cells under 1μM MK-801 enriched conditions (**Section 4.8.6.3**). This implies a significant effect of NMDA receptor activation on hippocampal progenitor cells. These results also suggest that the proliferative characteristics of MK-801 depend on the age of the cultures as well as the concentration of the treatment.

In spite of the fact that 100μM MK-801 decreases the total cell numbers as we have shown in **Section 4.4.6.1**, this MK-801 concentration is significantly increasing the mitotic index (BrdU/DAPI), the labelling index-BrdU/Ki-67 (speed of the cell cycle), and the growth fraction (Ki-67/DAPI). This suggests that 100μM MK-801 is having a pivotal effect on hippocampal cells. While it is recruiting quiescent cells, acting on dividing cells and is proliferative, it might enhance cell death as well. Dead/dying cells, however, might be washed away during immunocytochemistry and this causes a significant decrease in the total cell counts (DAPI). This effect of 100μM MK-801 is supported by 5 DIV data in **Section 4.8.6.2** as there is no change in the total cell numbers at this time point (day 5) which suggests that the proliferative characteristics of 100μM MK-801 is compensating the cell loss we observe in 3 DIV. In line with these results, 100μM MK-801 enhances hippocampal cell proliferation, recruits quiescent cells to be actively dividing and increase the speed of the cell cycle after 5 days in culture. The significant rise in all of the previously mentioned parameters suggests that 100μM MK-801 is mitogenic for hippocampal cells irrespective of the cultures age. Although, there is no significant increase in the numbers of nestin immuno-positive cells, most of the cells in culture express nestin under 100μM MK-801. Indeed, 66.5 ± 1.5 % of cells under 100μM MK-801 are nestin immuno-positive cells which is significantly higher than control conditions. This important finding supports the hypothesis that 100μM MK-801 is not only proliferative but also targeting hippocampal progenitor cells, or a proportion of them, and may also be toxic to other sub-populations. In a study conducted on neurospheres generated from embryonic (E18) rats it has been shown that long exposure of neurospheres to 30μM MK-801 decreased the numbers and the size of these spheres and also NMDA did not have an effect on the spheres, but no other concentration of MK-801 was investigated (Mochizuki, Takagi et al. 2007). Exploring the mechanisms by which high dose of MK-801 regulates hippocampal

neurogenesis might be a target for future studies. It has also been demonstrated that 30μM MK-801 prevents delayed cell death due to crush injury in slice cultures, but does not have an acute survival effects (Bendel, Langmoen et al. 2004). Previous studies, in line with what we have found, suggest that MK-801 effects are dose and time dependent.

It has been demonstrated that NMDA decreases neurogenesis as indicated by a decrease in the rate of proliferation and the numbers of newly born neurons in adult rats (Cameron, McEwen et al. 1995). In the same study, the addition of MK-801 significantly increased dentate gyrus cells in the S-phase of the cell cycle. In agreement with a study of Cameron et al 1995, we show that MK-801 is proliferative for hippocampal cells *in vitro*. MK-801 might act directly on cells in culture or activate other factors that enhance cell proliferation. Interestingly, it has been reported that NMDA receptors are expressed in some proliferating cells of the adult subgranular zone (Nacher, Varea et al. 2007). This might suggest that NMDA receptors are expressed in cultures and their blockade by MK-801 mediates the enhancement of proliferation we observe. Moreover, it has been shown that MK-801 enhances neurogenesis in the dentate gyrus of adult tree shrew (Gould, McEwen et al. 1997) and adult rats (Okuyama, Takagi et al. 2004). However, the mechanisms underlying NMDA role in neurogenesis are elusive.

Our preliminary results demonstrate that MK-801 has proliferative characteristics that are dose and age dependent and thus implies a significant role for NMDA receptors in controlling neurogenesis. MK-801 might have a survival role as well. The effects that we observed are not only on hippocampal proliferating cells but also on progenitor cells.

Altogether, these findings suggest that glutamate has a potential role in modulating the proliferation and survival of hippocampal precursor cells *in vitro*. They also suggest that glutamate is perhaps released from cells in culture under standard growth conditions. Because blocking NMDA receptors have complex effects on different sub-population of cells. Studying the combined effects of NMDA, AMPA and Kainate receptors on the proliferation and survival of precursor cells might be helpful in elucidating the differential effects of NMDA receptors.

4.6 Summary

Kainate is most likely to enhance hippocampal cell proliferation and neurogenesis by activating or acting on AMPA receptors. The rise in hippocampal cell proliferation causes a proportional increase in neuroblasts and thus neurogenesis. Therefore, AMPA receptors are important for hippocampal neurogenesis and for the effects of Kainate on hippocampal cells in culture and as such AMPA receptors modulate hippocampal neurogenesis *in vitro*. NMDA receptors have also a significant role in hippocampal neurogenesis. MK-801 increases hippocampal progenitor cells and enhances cell proliferation *in vitro*.

Chapter Five

Investigating the survival role of Kainate on hippocampal cells in culture

5.1 Introduction

In **Chapter 3,** we have detailed the proliferation of stem/progenitor cells and their neuronal precursors in hippocampal cell cultures both under control conditions and after Kainate treatment. Our results showed that cultured hippocampal cells (both progenitor cells and neuroblasts) proliferate *in vitro* under Kainate conditions but the increase in cells numbers, especially after short term of Kainate exposure, was not fully explained by the proliferative effects of Kainate alone. However, proliferation is not the only process occurring in the stem cell niche, cell death is also present (Kuan, Roth et al. 2000; Yuan and Yankner 2000; Dayer, Ford et al. 2003). Cell death and/or survival might counterbalance or enhance the survival of newly-born hippocampal cells and thus, in our model there is a necessity to measure cell death in addition to cell proliferation. The effect of a treatment on hippocampal cells through these mechanisms (cell proliferation and cell death) might increase, decrease, or have no change in cell numbers. Some of these changes act synergistically on the overall population i.e. increased cell proliferation and decreased cell death would both act to increase the overall population. Alternatively, other changes could cancel each other's effect i.e. increased proliferation and increased cell death, which might tend to produce no change in the overall population.

At 3 DIV, the increase in the numbers of hippocampal cells and their progenitors is mainly due to a proliferative effect of Kainate as indicated by the increase in the mitotic index and the speed of the cell cycle (labelling index). However, we could not rule out the involvement of cell death. At 5 DIV we found an increase in the numbers of hippocampal cells and their progenitors but there was no effect of Kainate on the proportion of proliferating cells (mitotic index) or on the speed of the cell cycle (labelling index). Therefore, we hypothesize that Kainate may have a survival effect on hippocampal cells, progenitor cells, and their neuronal precursors. Here, we will generally study the mechanisms of cell death in hippocampal cultures and more specifically the death mechanisms of progenitor cells and neuronal precursors under normal (control) and Kainate conditions.

5.1.1 Mechanisms of neuronal cell death

It has been demonstrated that about half of the neurons generated during neurogenesis die before completion of maturation of the central nervous system (Lossi, Cantile et al. 2005). A large and growing body of evidence suggests that neurons die through apoptosis and neuronal cell death seen during development is executed at least in part through apoptosis (Yuan, Lipinski et al. 2003). Neuronal cell death occurs under various physiological (Srinivasan, Roth et al. 1998) and also pathological conditions including stroke and neurodegenerative diseases (Lipton 1999). The best understood mechanism of neuronal cell death is apoptosis (Yuan, Lipinski et al. 2003). Apoptosis is regulated by an evolutionarily conserved cellular pathway that consists of the caspase family, B-cell lymphoma 2 (Bcl-2) family, and the adaptor protein Apaf-1 (Yuan, Lipinski et al. 2003). Apoptosis research in the last decade has provided deep mechanistic insights into the role of apoptosis in regulating neuronal cell death. Emerging evidence supports the inhibition of apoptosis as a promising strategic approach for the treatment of at least a subset of neurodegenerative disorders (Yuan, Lipinski et al. 2003). However, developing anti-apoptotic drugs is still a big challenge to recent science and technology. Although neuronal cell death mechanisms have been extensively investigated, the control and triggering mechanisms of stem/progenitor cell death are not fully understood.

5.1.2 Neuronal survival and neurogenesis

New neurons continue to be generated in the dentate gyrus from late embryonic to adult stage in all mammals (Namba, Mochizuki et al. 2005). However, in the rat dentate gyrus, granule cell neurons are largely generated during the postnatal period (Namba, Mochizuki et al. 2005). However, while a substantial number of cells in the granule cell layer has been found to die within 1-2 months after their birth, those ones which survive this period of time and differentiate into neurons have been shown to grow to adulthood and perhaps play a role in memory and learning (Dayer, Ford et al. 2003; Kempermann, Gast et al. 2003; Prickaerts, Koopmans et al. 2004). Interestingly, during this period of rapid cell death the survival of immature neurons has dramatically increased as a

response to hippocampal dependent behavioral tasks (Gould, Beylin et al. 1999; Gould, Tanapat et al. 1999; Ambrogini, Cuppini et al. 2000). Furthermore, neurotrophic factors enhance neuronal cell survival in the developing mammalian brains (Segal and Greenberg 1996; Kwon 1997). In this context, growth factors such as FGF-2 (basic fibroblast growth factors), BDNF (brain derived neurotrophic factor), and EGF (epidermal growth factor) have been demonstrated to increase the survival of newly-born dentate gyrus cells and thus contribute to hippocampal neurogenesis (Reynolds and Weiss 1992; Gage, Coates et al. 1995; Lowenstein and Arsenault 1996). For example, FGF-2 has been reported to promote the survival of newly-born neurons in hippocampal cultures generated from Wistar rats' embryos (Creuzet, Loeb et al. 1995). Importantly, exposure to an enriched environment dramatically increased hippocampal neurogenesis, along with a substantial improvement in behavioral performance (Kempermann, Gast et al. 2002). This effect is believed to involve both an increase in cell proliferation as well as the survival of at least a proportion of these proliferating cells (Kempermann 2002; Kempermann, Gast et al. 2002; Tashiro, Makino et al. 2007). Indeed, it has been elegantly shown that environmental enrichment enhanced hippocampal neurogenesis and reduced spontaneous apoptotic cell death by 45% in immature Wistar rats' hippocampi (Young, Lawlor et al. 1999). Moreover, Young et al have demonstrated that environmental enrichment induced the expression of glial-derived neurotrophic factor and brain derived neurotrophic factor indicating their meditation for enhancement of cell survival. Interestingly, they have also found that neurogenesis and cell survival that were associated with enriched conditions protected the animals against Kainate-induced seizures and excitotoxic injury (Young, Lawlor et al. 1999). Likewise, hippocampal neurogenesis has been found to be increased in environmentally enriched conditions in aged Wistar rats (Segovia, Yague et al. 2006). We conclude from these studies that a combined role of both cell survival and increased proliferation regulate hippocampal neurogenesis and its function.

The hippocampal formation is important for learning and memory (Gould, Beylin et al. 1999). In this regard, hippocampal neurogenesis has been reported to be essential for certain types of hippocampal dependent memory formation (Shors, Miesegaes et al. 2001). Furthermore, it has been shown that learning over time enhances the survival of

newly-generated hippocampal neurons (Sisti, Glass et al. 2007). While Kainate-induced seizures in the postnatal rats enhanced dentate gyrus neurogenesis, it increased CA3 subfield apoptosis (Dong, Csernansky et al. 2003). In addition, although Kainate-induced temporal lobe epilepsy acutely increased neurogenesis, chronic effects result in a significant decrease in hippocampal neurogenesis (Hattiangady, Rao et al. 2004). Furthermore, chronic temporal lobe epilepsy is associated with learning and memory impairments in humans and animal models of TLE (Mikati, Tarif et al. 2001; Alessio, Damasceno et al. 2004). However, it is unclear whether Kainate has an acute apoptotic effect on dentate gyrus granule precursor cells. Thus the effects of Kainate on cell death have yet to be explored. Although it has been shown that Kainate-induced status epilepticus did not enhance cell death in the GCL as indicated by fluoro-jade B (FJB) staining at day 3 after the insult (Sloviter, Zappone et al. 2003), it remains unsettled whether Kainate/seizures alter the survival of GCL precursor cells.

These studies suggest that apoptosis along with proliferation regulates hippocampal neurogenesis which may have a functional importance. Therefore, investigating the role of apoptotic cell death shortly after Kainate treatments *in vitro* and Kainate induced status epilepticus *in vivo,* is essential to understand seizure-induced hippocampal neurogenesis and its initial contributions to the sequels of chronic TLE.

5.1.3 Quantifying cell death

Cell death plays a critical role in the control mechanisms of neurogenesis. Propidium iodide (PI) has extensively been used to study cell death in organotypic hippocampal slice culture system prepared from postnatal rats (Best, Sundstrom et al. 1996; Pringle, Benham et al. 1996; Pringle, Sundstrom et al. 1996; Pringle, Iannotti et al. 1997; Cater, Benham et al. 2001; Lipski, Wan et al. 2007). This dye is usually added to culture medium during and after the insult, where it incorporates into the nucleus of cells whose membranes have become compromised (Wilde, Sundstrom et al. 1994). Likewise, PI has been also used to investigate cell death in primary hippocampal neuronal cultures (Masuda, Monahan et al. 2005; Xu and Zheng 2007). In addition to PI, other cell markers have been used, such as 4',6-diamidino-2-phenyindole (DAPI) and

bisbenzimide (Hoescht33342), these markers stain the nucleus of all cells (live and dead) and dead cells can then be identified by their morphology, the nuclei being shrunken and condensed (pyknotic) or fragmented (blebbing) (Howell, Scharfman et al. 2003). However, the use of DAPI is not an accurate method to quantify cell death as some of the dead cells are washed away during immunostaining. Another marker that has been used to quantify cell survival is MitoTracker; a dye which specifically gets incorporated by the mitochondria of respiring cells and emits green or red fluorescent (**see Section 5.3.3**), according to the dye, that can be detected thereafter (de la Monte, Neely et al. 2001; Waters and Smith 2003). Thus the number of cells that are MitoTracker positive indicates the number of viable (alive) cells in culture. MitoTrackers are synthesized in different colors (Red, Orange, and Green) and also contain mildly thiol-reactive chloromethyl moiety that appears to be responsible for keeping the dye associated with mitochondria after fixation. Therefore, MitoTrackers and in combination with DAPI and PI can be used to investigate hippocampal cell survival in cultures before cell fixation.

On the other hand, TdT-mediated dUTP- biotin nick end labelling (TUNEL) method has been used both *in vitro* and *in vivo* to study cell death. This method involves labelling the breaks in the DNA strands that form when cell undergo apoptotic death (Nijhawan, Honarpour et al. 2000). However, TUNEL stain identifies strand breaks in DNA and BrdU incorporation leads to a weakening of the DNA, which may lead to fragmentation and thus gives false positive staining. Furthermore, TUNEL may not detect cells that are instructed to die through apoptosis and this may result in underestimating apoptotic cell death.

Alternatively, caspases have been used to measure cell death in a variety of models. Caspases are a number of proteins that are up regulated or activated in the signaling cascade of cell death (Earnshaw, Martins et al. 1999). These markers (e.g. activated caspase-3 (**see section 5.1.4**)) have been used as an alternative method to measure apoptotic cells death in many studies (**see Section 5.1.4**).

The other method which has gained a lot of interest recently is Time-lapse microscopy. Cultured cells can be videoed and tracked for a specified period of time. This method has been used to investigate the effects of astrocytes on neurogenesis by tracking co-

cultured stem cells and astrocytes *in vitro* (Kornyei, Szlavik et al. 2005). Moreover, time-lapse video microscopy has been recently used to track neural progenitor cells in the rostral migratory stream, in immature transgenic mice, that carry the reporter gene GFP under the nestin promoter (Zhao and Nam 2007). In this regard, Zhao and colleagues, using time-lapse of brain slices, have described the behavior of nestin progenitor cells after ischemia in these transgenic mice. Interestingly, Coskun and coworkers have used time-lapse method to study SVZ neuronal progenitor divisions (Coskun, Falls et al. 2007). Using this technique Coskun and colleagues generated SVZ cells from postnatal rat pups and videoed them for a few days where they described cytokinesis and cell cycle of these dividing progenitors to be between 14-17 h. Therefore, time-lapse method can be used to video proliferating cells, track them and follow their survival. Furthermore, performing immunohistochemistry of cultures videoed under time-lapse will reveal the phenotype of dividing progenitor cells and whether they commit a neuronal lineage.

5.1.4 Immunoreactive Caspase-3 as a marker of cell death

The discovery of a family of at least 14 cystein proteases called caspases has eased the exploration of the apoptotic cell death studies. Caspases or, cystein proteases, (cysteinyl aspartate-specific proteases) are enzymes that cleave specific proteins at aspartate residues (Degterev, Boyce et al. 2003). They contain cysteine residues in their active sites and many caspase isoforms promote apoptosis. Many factors such as DNA damage, oxidative stress, and death receptor ligands activate through one of the two main pathways: the death receptor pathway and the mitochondrial pathway leads to the active form of caspases (Herr and Debatin 2001; Joza, Kroemer et al. 2002). Caspase isoforms are broadly categorised into initiators, effectors and inflammatory caspases (Kaufmann and Hengartner 2001). Initiator caspases such as caspase-8 and 9 cleave and activate effector caspases, such as caspase-3, leading to cell death by apoptosis (Kaufmann and Hengartner 2001). Caspase activity leads to a proteolytic cascade in which one caspase can activate other caspases. This in its turn amplifies the apoptotic signalling pathway.

Recent studies have suggested an important role for neuronal apoptosis in cell loss following acute brain injuries as well as chronic neurodegenerative diseases (Yakovlev and Faden 2001). Casapses have been found to contribute significantly to the morphological and biochemical manifestations of apoptotic cell death. Interestingly, a key role for caspase-3 appears to be the major effector of neuronal apoptosis induced by a variety of stimuli (Yakovlev and Faden 2001). In addition, a key role for caspase-3 in injury induced neuronal loss has been established using semispecific peptide caspase inhibitors. Similarly, deprivation of growth factors was shown to induce apoptotic cell death and activate caspase-3 (**Figure 5.1**) (Yuan and Yankner 2000). We outlined, earlier in this chapter, apoptotic neuronal cell death as the main mechanism of cell death. Generally speaking, caspase expression and/or activities are commonly used to detect cell death *in vivo* and *in vitro*. Cerebral ischemia studies in rats using in situ hybridization showed that transient forebrain ischemia leading to delayed apoptotic death of CA1 pyramidal neurons, results in a prolonged expression of caspase-3 mRNA in these neurons (Ni, Wu et al. 1998). Ni and colleagues also observed that while the up-regulation of caspase-3 mRNA in CA1 pyramidal neurons is prominent 24 hours after transient global ischemia, expression is maintained at high levels for at least 72 hours after ischemia. However, by 96 hours after ischemia, Ni reported a marked decrease in caspase-3 mRNA expression in CA1 pyramidal neurons showing severe degenerative changes (e.g., nuclear condensation). This suggests early expression of caspase-3 precedes the morphological degeneration of cells dying by apoptosis which raises up the possibility that, after injury, we might find normal cellular appearance followed by degeneration of cells. Interesting research was also conducted on humans and showed that neuronal cell death in human brain infarcts has some of the early biochemical features of programmed cell death with up-regulation of caspase-3 and rapid disappearance of DNA-PKCS and PARP (Love, Barber et al. 2000). Interestingly, they also observed that the morphological changes are not those of apoptosis, the DNA cleavage occurs relatively late, and some of the TUNEL-staining is probably mediated by the release of endogenous endonucleases during protease or microwave pretreatment of the damaged tissue. Furthermore, it has been demonstrated that activation of caspase-

3 is essentially involved in the apoptotic cell death cascade following spinal cord injury (Springer, Azbill et al. 1999).

In an interesting study, cell death was measured in a Kainate model of hippocampal degeneration of sensitive (FVB/N) and resistant (129/SvEMS) strains of mice (Faherty, Xanthoudakis et al. 1999). This study has revealed the activation of caspase-3 30 h following Kainate treatments in the sensitive FVB/N strains but not in the resistant one (129/SvEMS). Furthermore, caspase-3 activation has been reported to take place 18 h before detection of pyknosis of TUNEL labelling. They also observed continuous expression of caspase-3 up to 4 days post Kainate injection with no activation of caspase-3 in the resistant, 129/SvEMS strain, neither was there evidence of pyknosis or TUNEL staining (Faherty, Xanthoudakis et al. 1999). This study highlights the importance of the activation of caspase-3 and its necessity as a key component of Kainate-induced cell death. Therefore, measuring activated caspase-3 would be a reliable method of determining apoptotic cell death under Kainate treatments. Another important study was carried out on primary hippocampal cultures from postnatal mice, where active caspase-3 and TUNEL co-localization was measured after the cells were exposed to glutamate (Brecht, Gelderblom et al. 2001). They found that about only 2% of the cells were co-labelled for TUNEL and the active caspase-3 at the different time points of the treatment. This research suggests that while active caspase-3 in the absence of TUNEL indicates a dynamic degenerative process, TUNEL marks the end stage of severe irreversible cell damage regardless of the origin of the cell. Therefore, detection of caspase-3 would be more accurate for studying apoptotic cell death than TUNEL. It has also been found that caspase inhibitors increase short-term survival of precursor cells in the adult dentate gyrus following status epilepticus (Ekdahl, Mohapel et al. 2001). Status epilepticus and hippocampal damage transiently increases dentate gyrus neurogenesis (Hattiangady, Rao et al. 2004). However, the mechanisms underlying the control of precursor cells apoptosis and generation of new neurons are not clear. This may suggest an important role for apoptotic cell death in regulating hippocampal neurogenesis in epilepsy. Therefore, exploring apoptotic cell death of hippocampal progenitor cells and their neural precursors would increase our understating to the control mechanisms of cell death in neurogenesis.

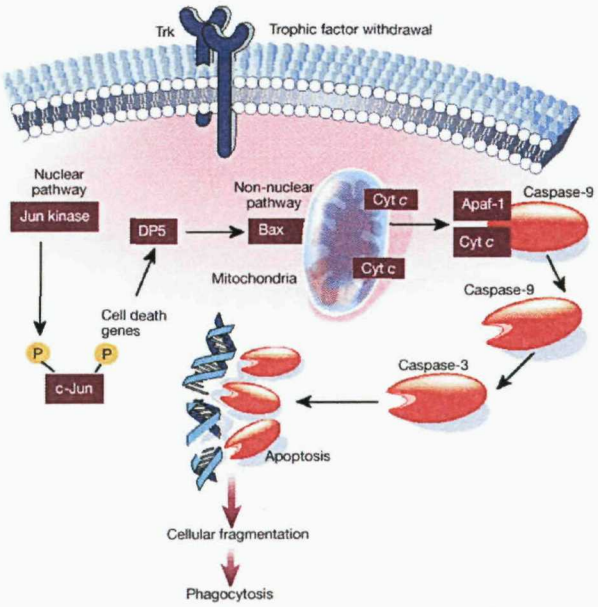


Figure 5.1 Activation of caspase-3 results in apoptosis. The above scheme demonstrates the activation of cascade of factors in cultured sympathetic neurons after tropic factors withdrawal which in turn activates Caspase-3 and results in programmed cell death.(Adopted from (Yuan and Yankner 2000))

As we discussed earlier a growing body of evidence highly implies a role for apoptosis as the main neuronal cell death pathway which have an important role in regulating hippocampal neurogenesis. The activation of caspase-3 has been shown as a sign for this mode of cell death (Yuan and Yankner 2000). Therefore, we will demonstrate, as part of this chapter, the acute effects of Kainate on apoptotic cell death of hippocampal stem / progenitor cells and neuronal precursors using immunocytochemistry for caspase-3.

5.2 Aims of the chapter

The aim of this part of the project is to examine whether Kainate has a role in regulating the survival of hippocampal progenitor cells and their neural precursors or not. We will address the effects of Kainate on the survival of hippocampal cells in cultures. We also aim to investigate the effects of Kainate on caspase-3 immunoreactive BrdU

incorporated cells, nestin-expressing cells, and TuJ1 immuno-positive cells. In addition, we will study the survival role of Kainate on newly-born cells and their fate of choice using time-lapse microscopy methods.

5.3 Methodology and Materials:

5.3.1 Experimental aims

In this section, I will briefly describe the procedures and experimental designs to study the effects of Kainate on cell survival *in vitro*.

5.3.2 Cell cultures

Primary hippocampal cell cultures were prepared from 7-10 day-old Wistar rats as detailed in (**Chapter 2 Section 2.4.2**) with some modifications that suit our experimental design and will be described in relevant sections.

5.3.3 Determining the effects of Kainate on cell survival using MitoTrackers and PI

MitoTracker dyes are fluorescent mitochondrial markers that covalently bind free sulfhydryls (Buckman et al 2001). They were developed commercially by Molecular Probes (Eugene, OR). The MitoTracker dyes (MitoTracker Green FM, MitoTracker Orange CMTMRos, and MitoTracker Red CMXRos) contain chloromethyl moieties that are thought to react with free sulfhydryls within the cells mitochondria. MitoTrackers passively diffuse across the plasma membrane and accumulate in active mitochondria. All three MitoTracker probes are believed to be retained following fixation due to the membrane-impermeant dye complex that is formed when the dyes enter the mitochondria. MitoTracker Green FM is essentially non-fluorescent in aqueous

solutions, only becoming fluorescent once it accumulates in the lipid environment of mitochondria (Molecular Probes, Eugene, OR). Hence, background is negligible, enabling us to clearly visualize mitochondria in live cells immediately following addition of the stain, and without a wash step. Mitochondria in cells stained with MitoTracker Green FM dye (M7514) exhibit bright green, fluorescein-like fluorescence (Keij, Bell-Prince et al. 2000). However, this dye is not well retained after cell permeabilization. We used MitoTrackers (Green and Orange) to stain live cells in cultures and DAPI to label all cells. We imaged cells while they were live and post fixation. The counts of live cells under control and after short terminal pulse of 5μM Kainate were then determined. Furthermore, we developed a protocol where we have applied MitoTracker Green FM, PI, and DAPI to live cells and without washing steps we imaged cells while still alive and examined the effects of terminal 6 h pulse of 5μM Kainate on the counts of live and dead cells (**Figure 5.2**). The above characteristics of MitoTrackers and PI enabled us to measure dead cells as well as dying cells. Cells that are DAPI positive, MitoTracker Green positive and PI positive are considered dying cells whereas cells that are DAPI positive, PI positive but MitoTracker Green negative are considered dead cells. In this regard, live cells were identified as those that are DAPI positive, Mitotracker Green positive and PI negative. In our protocol, we added 5μg/ml PI and 20μg/ml DAPI to the cultures for the last 45 minutes then we applied 50nM Mitotracker Green to cells for the last 15 minutes before imaging as described in **Section 2.4.8**. In another series of experiments, the MitoTracker Orange was used. In this setup, live cells were fist imaged and then fixed before being imaged again post-fixation for DAPI and MitoTracker Orange. This dye (Mitotracker Orange) is well retained after cell fixation and permeabilization, and thus it enables us to measure the number of live cells before and after cell fixation. However, because it exhibits bright red fluorescein-like fluorescent it is not possible to be applied in combination with PI (exhibits red fluorescent). Therefore, we will measure live cells in this series of experiments by counting DAPI positive and MitoTracker Orange positive. All cells that are MitoTracker Orange negative are dead cells. My protocol includes the addition of 20μg/ml DAPI to the cultures for the last 45 minutes. 50nM of MitoTracker Orange was then applied to the same cultures for the last 15 minutes before imaging. Cells were then imaged live

and post fixation for DAPI and MitoTracker Orange as described in **Chapter 2 (Section 2.4.8).** Counts were further analyzed as detailed in **Chapter 2 (Sections 2.4.10)**.

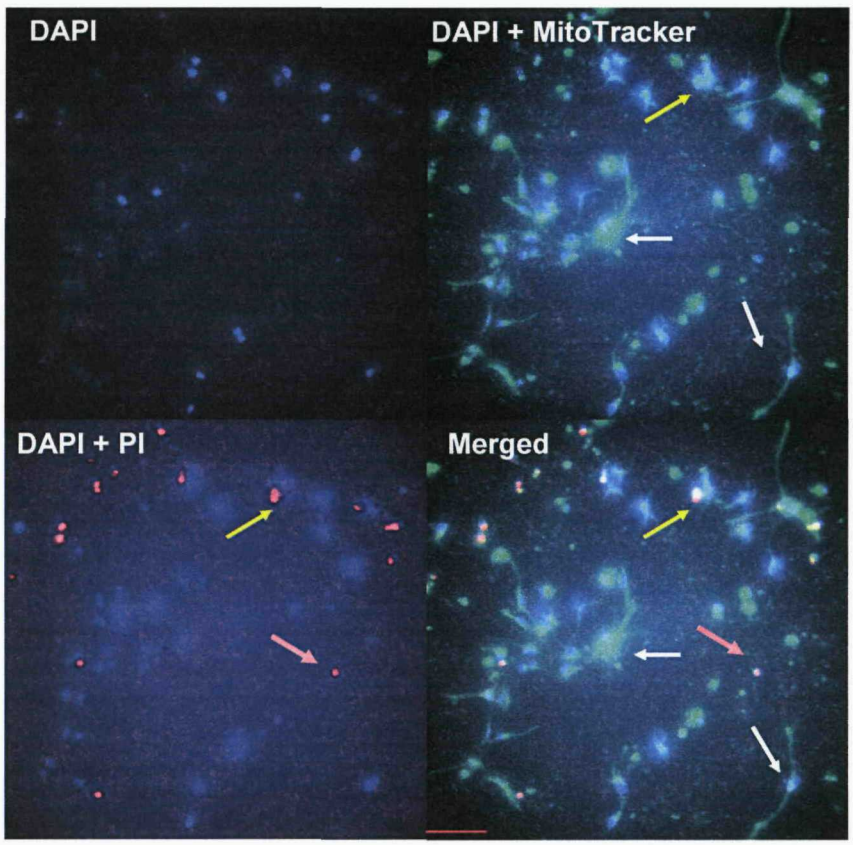


Figure 5.2 Cells survival after Kainate treatments. Cells were cultured under standard control conditions for 3 days. On day three terminal 6 h pulse of 5μM Kainate was applied to a group of cells while another group remained untreated as controls. DAPI and PI were pulsed to cells for the last 45 minutes while 50nM of the MitoTracker Green FM was given to cells 15 minutes before imaging. Cells were then imaged as mentioned in **Chapter Two (Section 2.4.8)**. Data analyzed as detailed in **Chapter Two (Section 2.4.8 and 2.4.10)**. DAPI nuclear staining is displayed in blue. MitoTracker Green FM stains mitochondria of live cells in green. PI enters leaky cells (dead/dying) and appears in red. White arrows show sample of live cells (**DAPI+ MitoTracker + &PI negative**). Yellow arrows display dying cells (**DAPI+ MitoTracker + &PI+**) and pink arrows show dead cells (**DAPI+ MitoTracker negative & PI+**). Imaging was performed on an inverted Leica DM IRB microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). Open Lab image capturing system version 2.1 (Improvision, Lexington, USA) was used to count cells. Scale bar = 50μm.

5.3.4 Examining the effects of Kainate on the survival of both hippocampal cells and newly-born cells under Time-lapse microscopy

Primary hippocampal cells were dissociated and plated into poly-L-lysine coated plastic wells for time-lapse light microscopy. Cells were incubated in a custom-made chamber at 37°C in a humidified atmosphere (5.0% CO_2/ in air) and staged on an Olympus IX 81 inverted microscope system equipped with a digital camera. Differential interference contrast (DIC) images were acquired every 15 minutes with 20x objective for 25 hours. An automatic shutter was used to minimize phototoxicity. The CellˆP software system was used to acquire and process the resulting stacks of images. In each experiment, three randomly selected fields per well were selected and stacked from three different wells under each condition. The resulted time-lapse movies were then carefully studied for newly born cells, cell counts and death. Each newly-born cell was then tracked to determine whether it survived or died. The numbers of newly-born cells that either survived or died and the total counts of live cells were then compared under control conditions with those under terminally Kainate treated conditions. Data were analyzed using Chi square test. $P<0.05$ is considered significant.

5.4 Results

5.4.1 Kainate is trophic for proliferating cells

In **Chapter 3 (Section 3.4.2)**, we have shown that 5μM Kainate is proliferative for hippocampal cells at 3 DIV as indicated by the proportional increase in BrdU incorporated cells. However, at 5 DIV, we found a significant rise in the counts of BrdU with no proportional increase. Therefore, we sought to study the survival effects of Kainate on proliferating hippocampal cells (BrdU incorporated cells) in 5 days cell cultures. Hippocampal cells were again prepared from postnatal Wistar rats 7-10 days old as detailed in **Chapter 2 (Section 2.4.2)**. At day 5, cells were pulsed with a final concentration of 20μM BrdU for the terminal 4 hours before being fixed with 4% PFA. Cells were then immunostained against the proliferative marker BrdU, and the cleaved caspase-3 antigen (**Section 2.4.7**). We measured the proportions of caspase-3 immunoreactive cells with respect to the total cell number (Caspase-3/DAPI) in culture as well as the proportions of cells that were double-labelled for BrdU and caspase-3 (BrdU+ and Caspase-3+/BrdU+).

The significant increase in DAPI cell count after 5 days of Kainate exposure is consistent with what we have described before (**Figure 5.3 A**). While we found that 44.4 ± 1.9 % of overall cells were immunoreactive for caspase-3 under standard growth conditions, this proportion decreased significantly to 32.8 ± 1.0 % after five days of 5μM Kainate treatment (un-paired simple Student's t-test, $p<0.001$) (**Figure 5.3 B**). In (**Figure 5.3 C**), we showed an increase in the counts of BrdU incorporated cells which was significant and consistent with our findings in previous experiments as detailed in **Chapter 3 (Sections 3.4.2, 3.4.3.1, and 3.4.4.1)**. Interestingly, we demonstrated that 53.7 ± 6.1 % of BrdU incorporated cells were expressing caspase-3 under control conditions, however, 5 days of Kainate treatment decreased this proportion to 30.5± 1.0 % **(Figure 5.3 D)**. This decrease was statistically significant (un-paired simple Student's t-test, $p<0.01$).

In this set of experiments, we have shown that 5μM Kainate not only increases the numbers of hippocampal cells that incorporated BrdU but also enhances the survival of

these S-phase labelled (actively dividing) cells in cultures. These results demonstrate significant trophic effects of Kainate on hippocampal cells.

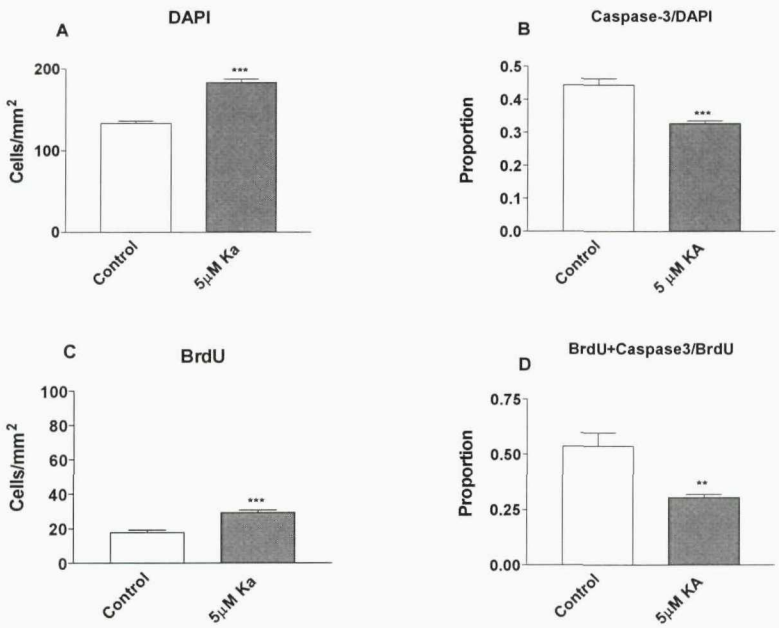


Figure 5.3 Kainate is trophic for proliferating hippocampal cells. Cells were cultured for 5 days in the absence and presence of 5μM Ka. On day three two-thirds of the growth medium with/without treatments were changed. 4 h terminal pulse of a final concentration of 20μM BrdU was given to all cells on day 5 before fixation and immunostaining against caspase-3 and BrdU. Then cells were counterstained for the nuclear stain DAPI. We showed a significant decrease in hippocampal and BrdU incorporated cells that were caspase-3 immuno-positive after 5 days of Kainate treatment. A) The total cell numbers were measured by counting DAPI stained nuclei and showed a significant rise in cell counts under Kainate treatment when compared to control conditions. B) The proportion of the caspase-3 immunoreactive cells in culture decreased significantly under Kainate conditions in comparison with control conditions. C) There was a significant rise in the counts of BrdU incorporated cells after 5 days of Kainate treatment when compared to control conditions. D) The proportion of proliferating immunoreactive caspase-3 cells in primarily hippocampal cultures decreased significantly after treating cells with Kainate for 5 days. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 3 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *un-paired simple Student's t-test*. ** $p<0.01$, *** $p<0.001$

5.4.2 Kainate is trophic for Nestin expressing progenitor cells

To determine whether Kainate has a survival role on nestin expressing sub-population of cells, hippocampal cells dissociated from postnatal rats' hippocampi were cultured for 5 days under control conditions or in the presence of 5μM Kainate as mentioned in **Chapter 2 (Section 2.4.2)**. Two-thirds of the growth medium with/without enrichments was replaced on day three. By day five, cells were fixed and double-labelled for the stem/progenitor cell marker (nestin) and the apoptotic marker caspase-3 as described in **Chapter 2 (Section 2.4.7)**. Finally cells were imaged, counted and data was analyzed as detailed in **Chapter 2 (Sections 2.4.8 &2.4.10)**

In **Figure 5.4,** we showed a significant effect for five days of Kainate treatment on stem/progenitor (nestin expressing) cells as the proportion of caspase-3 immunoreactive nestin expressing cells decreased significantly under Kainate conditions (64.5 ± 2.1% versus 82.0 ± 2.1% under control maintained conditions (un-paired simple Student's t-test, $p<0.001$).

This result implies a significant trophic effect for Kainate on hippocampal progenitor cells.

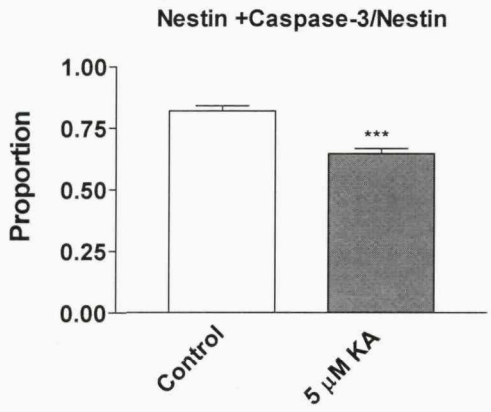


Figure 5.4 Kainate is trophic for Nestin expressing hippocampal cells. Cells were cultured for 5 days in the absence and presence of 5μM Ka. On day 3 two-thirds of the growth medium with/without treatment was changed. Cells were fixed with 4% PFA and followed by immunostaining against both nestin and caspase-3. The figure showed the significant drop in the proportion of nestin immunoreactive caspase-3 cells after 5 days of Kainate exposure and in comparison with control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *un-paired simple Student's t-test*. *** $p<0.001$

5.4.3 Kainate enhanced the survival of neural precursor cells

We reported consistently a significant proportional increase in TuJ1-expressing sub-population of cells in 3 and 5 DIV (**Sections 3.4.3.4 and 3.4.4.4**). We demonstrated a significant proliferative effect of Kainate on TuJ1 cells after 3 DIV as indicated by the increase in raw counts and proportions of TuJ1 positive cells that incorporated BrdU (mitotic index of TuJ1 positive cells) after 3 days of the treatment. However, at 5 DIV, while the numbers of proliferating TuJ1 cells increased under Kainate treatment, there was no proportional increase in this subpopulation of cells. Therefore, we hypothesize that Kainate might have a survival effect on TuJ1 expressing cells. To address this question, 5 days monolayer hippocampal cell cultures were prepared as detailed in **Chapter 2** (**Section 2.4.2**). In this set of experiments, cultures were maintained for 5 days in the presence or absence of 5μM Kainate. Two-thirds of the growth medium

with/without treatments was replenished at day three. Cells were fixed on day 5 with 4% PFA and then double-labelled for TuJ1 and cleaved caspase-3 as detailed in **Chapter 2** (**Section 2.4.7**). Counterstaining of cells with the nuclear stain DAPI was carried out before imaging and analyzing samples as described in **Chapter 2** (**Sections 2.4.8 & 2.4.10**).

Interestingly, our data (**Figure 5.5**) showed that the proportion of TuJ1 expressing cells that were caspase-3 immunoreactive significantly decreased from 92 ± 1.7 % under control conditions to 72.8 ± 5.6 % under 5 μM Kainate treatment (un-paired simple Student's t-test, $p<0.01$). This data suggests a significant survival role for Kainate on TuJ1 sub-population of cells at 5 DIV.

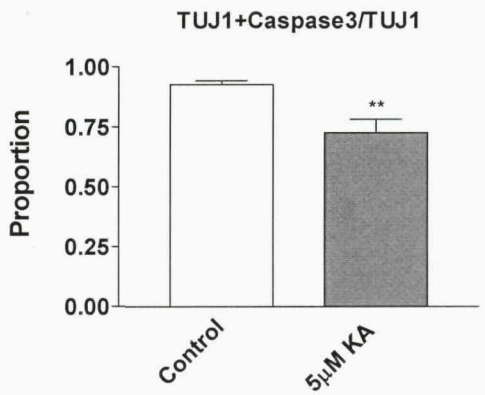


Figure 5.5 Kainate is trophic for TuJ1 expressing hippocampal cells in 5 DIV. Cells were cultured for 5 days in the absence or presence of 5μM Ka. On day 3 two-thirds of the growth medium with/without treatment was changed. Cells were fixed and immunostained against both TuJ1 and caspase-3, then counterstained for the nuclear marker DAPI. The above figure demonstrated a significant decrease in the proportion of TuJ1 immunoreactive caspase-3 cells after 5 days of Kainate exposure when compared to control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *un-paired simple Student's t-test.* ** $p<0.01$

5.4.4 Short pulse of Kainate increased the proportion of MitoTracker positive cells in culture

We have demonstrated in chapter 3 that total cell numbers increase significantly after a short time exposure (6 h) to Kainate at 5, but not at 3 DIV. In addition, our earlier data presented in the early sections of this chapter demonstrated a significant survival role for Kainate on hippocampal cells (**Section 5.4.1**), including both nestin positive progenitor cells and TuJ1 immuno-positive neuronal precursors (**Sections 5.4.2 and 5.4.3**). Therefore, we sought to determine whether this increase in total cell counts after a 6 h pulse of Kainate has resulted from Kainate-enhanced survival effects or not. To address this issue, hippocampal cells were grown under standard control conditions for either 3 or 5 days as detailed in **Chapter 2** (**Section 2.4.2**). On either day 3 or 5, cells in half of the wells were terminally pulsed for 6 h with 5μM Kainate and the others remained untreated as controls. While the cells are alive, DAPI, PI, and MitoTracker Green were applied to measure cell survival in cultures after 6 h Kainate in each group as detailed in **Section 5.3.3**. Using the three markers, we measured the total cell counts, the proportions of live cells and the proportions of dead cells under the different conditions (**Section 5.3.3**).

At day three, there was no significant rise in the total numbers of cells as indicated by counting DAPI positive cells under Kainate treated conditions and in comparison to control conditions (**Figure 5.6 A**). We measured the proportion of cells that were DAPI positive, MitoTracker positive but PI negative (live cells) and we demonstrated a significant increase in the proportion of live cells in cultures in response to 6 h Kainate treatment when compared to control conditions. 37.1 ± 5.2 % of cells were live in control conditions and significantly increased to 63.2 ± 1.8 % after 6 h exposure of Kainate (un-paired simple Student's t-test, $p<0.001$)(**Figure 5.6 B**). Interestingly, the proportion of PI positive (dead/dying) cells decreased significantly under Kainate conditions (33.6 ± 1.5 % versus 43.5 ± 3.6 % under control conditions) (un-paired simple Student's t-test, $p<0.05$) (**Figure 5.6 C**).

At day five, the total cell counts as indicated by DAPI positive cells significantly increased from 119.0 ± 3.5 cells/mm^2 under control conditions to 142.3 ± 2.5 after 6 h of

Kainate exposure (**Figure 5.7 A**) (un-paired simple Student's t-test, $p<0.01$). A parallel significant rise in the counts of live hippocampal cells (DAPI positive and MitoTracker Orange positive) was observed after 6 h of Kainate exposure (61.0 ± 1.3 % versus 48.3 ± 1.3 % under control conditions) (un-paired simple Student's t-test, $p<0.001$) (**Figure 5.7 B**). Moreover, the proportion of dead cells as indicated by measuring DAPI positive but MitoTracker Orange negative cells decreased significantly from 51.2 ± 1.2 % under control conditions to 39.0 ± 1.3 % after 6 h of Kainate treatment (un-paired simple Student's t-test, $p<0.05$) (**Figure 5.7 C**). Sample images are displayed in **Figure 5.8**.

Our results in this part suggest a significant direct survival role for Kainate on cells generated from the postnatal hippocampus after a 6 h Kainate pulse.

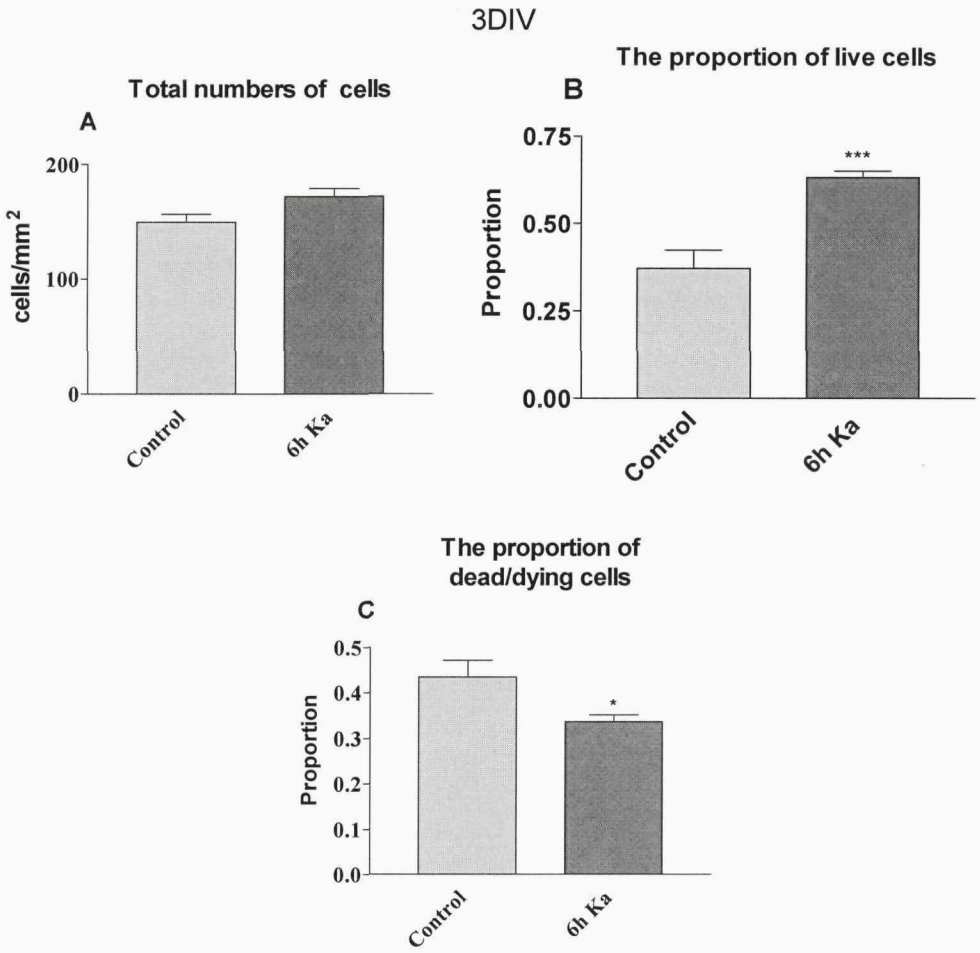


Figure 5.6 Short pulse of Kainate enhanced the survivals of hippocampal cells at day 3 in culture. Cells were grown under standard control conditions for 3 days. On day three terminal 6 h pulse of 5μM Kainate was given to a group of cells while another group remained untreated as controls. DAPI and PI were pulsed to cells for the last 45 minutes while 50nM of the MitoTracker Green FM was given to cells 15 minutes before imaging. Kainate increased the proportion of live cells and decreased the proportion of dead/dying cells at day 3 in culture. A) There was no change in total cell counts in Kainate treated cultures when compared to control conditions. B) We reported a significant proportional increase in live cell counts as measured by counting DAPI positive, MitoTracker positive, and PI negative cells. C) A significant drop in the proportion of dead/dying (PI positive) cells was also found after 6 h of Kainate treatment and in comparison with control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *un-paired simple Student's t-test*. * $p<0.05$, *** $p<0.001$

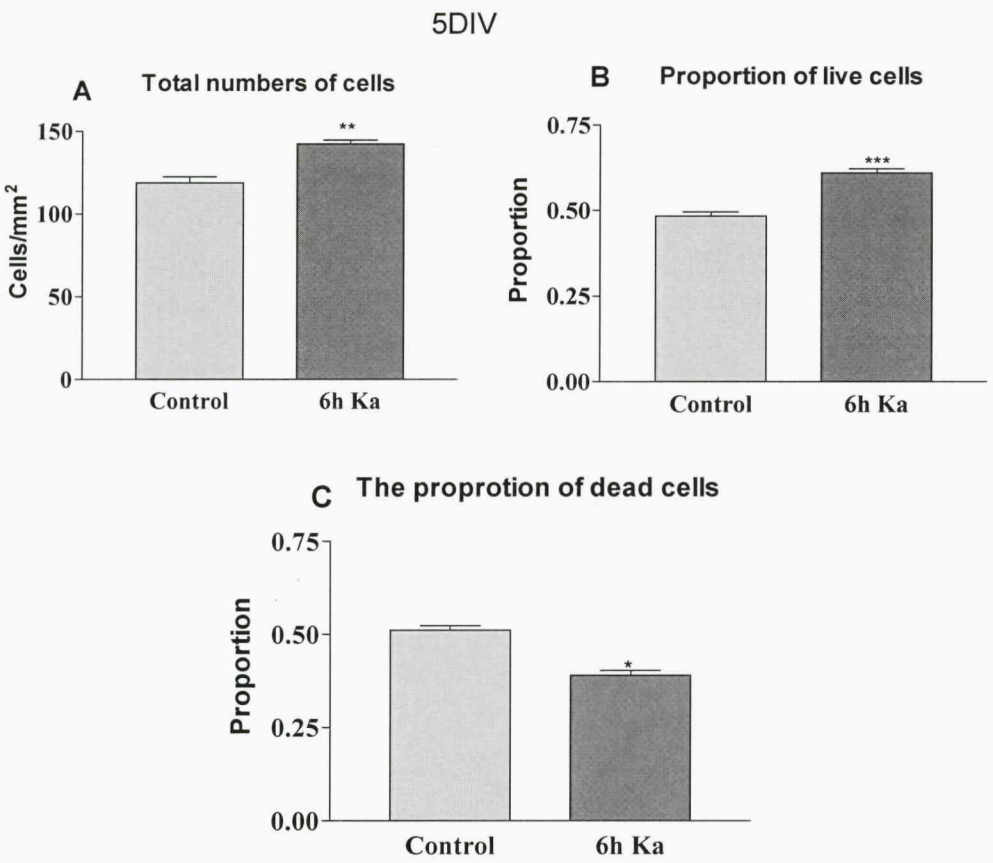


Figure 5.7 Short pulse of Kainate enhanced the survival of hippocampal cells at day 5 in culture. Cells were grown under standard control conditions for 5 days. On day five terminal 6 h pulse of 5μM Kainate was given to a group of cells while another group remained untreated as controls. DAPI and MitoTracker orange were pulsed to cells for the last 45 minutes while 50nM of the MitoTracker orange was given to cells 15 minutes before imaging. Kainate increased the total cell counts and proportion of live cells and decreased the proportion of dead/dying cells at day 5 in culture. A) There was a significant increase in total cell counts in Kainate treated cultures when compared to control conditions. B) We found a significant proportion increase in live cell counts as measured by counting DAPI positive, MitoTracker orange positive cells. C) There was a significant drop in the proportion of dead (DAPI positive but MitoTracker orange negative) cells after 6 h of Kainate exposure and in comparison with control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *un-paired simple Student's t-test.* * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

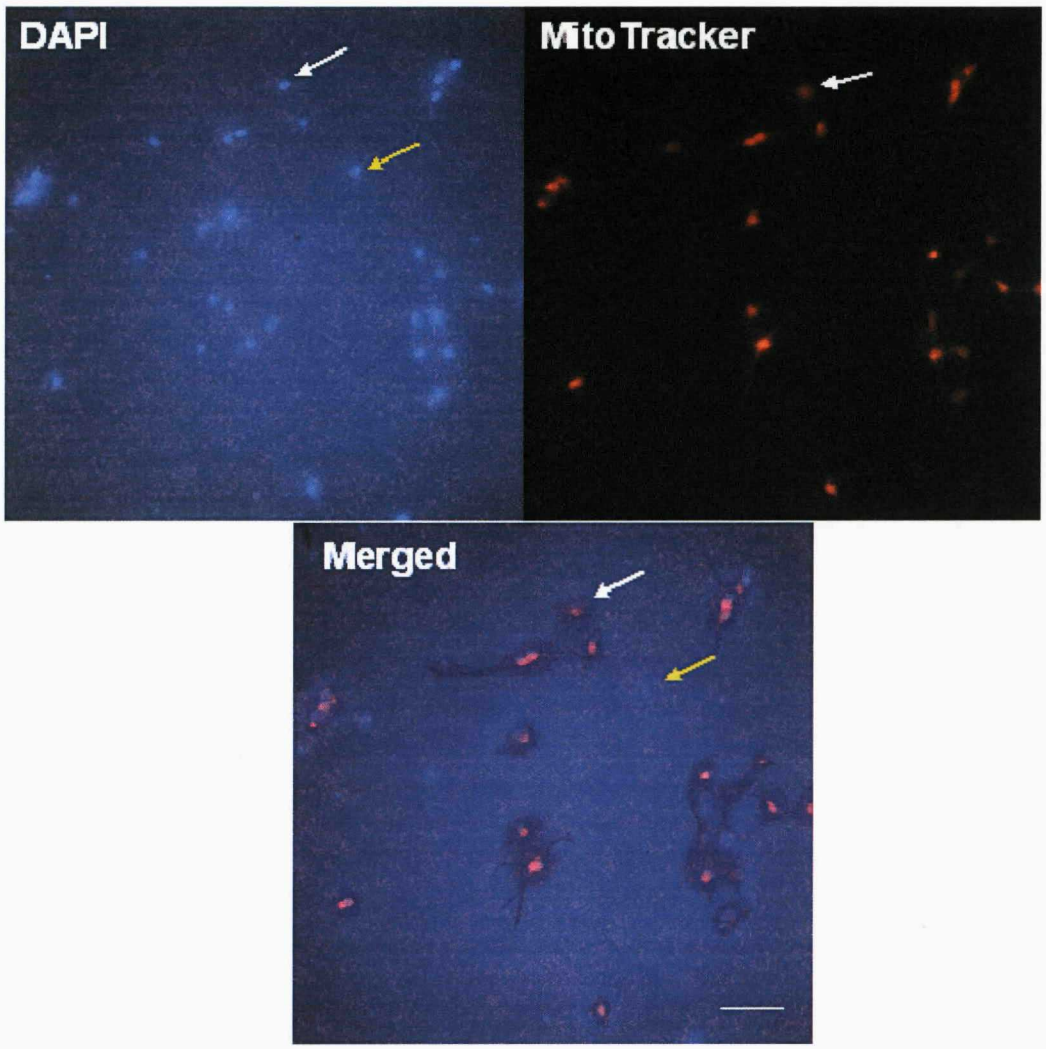


Figure 5.8 Kainate increased the proportion of MitoTracker positive cells at 5 days in culture. Cells were cultured under standard control conditions for 5 days. On day five terminal 6 h pulse of 5μM Kainate was given to a group of cells while another group remained untreated as controls. 20μg/ml DAPI was pulsed to cells for the last 45 minutes while 50nM of the MitoTracker orange was given to cells 15 minutes before imaging. We reported a significant proportional increase in MitoTracker orange positive cells and a proportional decrease in dead cells (DAPI positive and MitoTracker negative). White arrows show sample of live cells (**DAPI (+) and MitoTracker (+)**). Yellow arrows display dead cells (**DAPI (+) but MitoTracker (-)**). Imaging was performed on an inverted Leica DM IRB microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). Open Lab image capturing system version 2.1 (Improvision, Lexington, USA) was used to count cells. Scale bar = 40μm.

5.4.5 Kainate treatment enhanced the survival of hippocampal cells in general and newly-born cells in particular

In **Section 5.4.4,** we have demonstrated a significant survival effect for short pulse of Kainate on hippocampal cells in general and we demonstrated a significant rise in the total cell counts at day 5. We have also shown (**Sections 5.4.1**) that 5 days of Kainate exposure enhanced the survival of proliferating hippocampal cells. Herein, we further investigated terminal 25 h effects of Kainate on non-dividing and newly-born cells under time-lapse microscopy. We have videoed the cells for 25 h in order to explore the mechanism of the increase in the total cell counts and proliferation at day 5 in culture. Cells were prepared as described in **Chapter 2** (**Section 2.4.2**). On day 5, a group of cells was pulsed with 5μM Kainate the other group was maintained untreated as control. Cells were then staged on the time lapse microscope for the next 25 h, where an image was acquired every 15 minutes. Time-lapse movies were then processed and studied. Hippocampal cells particularly the newly-born ones were morphologically followed up to predict its survival state over the 25 h, with cell membrane bursting and disintegration and subsequent cell shrinkage as signs of cell death (**Figure 5.10**). 9 random fields from three different wells for each condition (controls and Kainate) were videoed. We measured the total cell counts under control and Kainate at 0 h and 25 h time points. We then followed up and counted cells that divided (newly-born cells) and cells that survived irrespective of whether they divided or not.

There was no significant difference in the average cell counts per 20x field between Kainate and control conditions at 0 h time point (**Figure 5.9 A**). This indicated a similar cell counts under both conditions at the beginning of imaging. The total number of hippocampal cells that survived under control conditions (196 cells) was significantly different from those who survived under Kainate treatment (279). While 7 cells died under Kainate conditions, 38 died under control conditions (Chi-square = 30.97, p = 0.000) (**Table 5.1**). This suggests a significant survival effect of Kainate on overall hippocampal cells.

We also followed the newly-born cells and found that out of 50 newly-born cells under control conditions 14 died (28%), while 2 newly-born cells died (2.8 %) out of 72 under

Kainate treatment. This difference in the survival and counts of newly-born cells between Kainate and control was statistically significant (Chi-square = 16.47, p = 0.000) (**Table 5.2**).

We further investigated the survival of cells that did not divide in the course of our video imaging (25 h). We found that, under control conditions, while 160 cells survived, 24 died. However, under Kainate conditions, 211 hippocampal cells survived and only 5 cells died. The difference between the two conditions (control and Kainate) was significant (Chi-square = 17.0, p = 0.000) (**Table 5.3**). As a result of the survival effects, we found a significant increase in the average cell counts per 20x field after 25 h of Kainate exposure when compared to control conditions (un-paired simple Student's t-test, $p<0.01$) (**Figure 5.11 B**).

Moreover, we fixed and stained cells for DAPI and found that cells that died either got washed away during the staining process or they have become pyknotic (**Figure 5.12 (13 and 14)**).

Our results indicate that Kainate is trophic for hippocampal cells in general and for newly-born cells in particular.

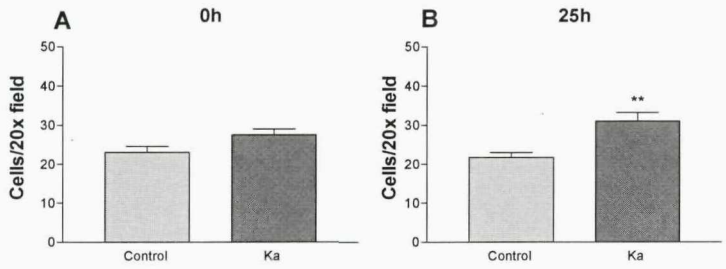


Figure 5.9 Kainate is trophic for dividing and non-dividing hippocampal cells in culture. Cells were grown for 5 days under standard growth conditions. On day five a group of wells was treated with 5μM Kainate while another group was maintained as controls. Cells were staged and imaged on time lapse microscope for the last 25h. We measured the cell counts per 20x field at 0h and 25 h. We found a significant increase in the average cell counte/20x field under Kainate treatment and in comparison with control conditions. A) There was no change in the average cell counts per 20x filed when comparing Kainate conditions to control conditions at 0h time point. B) There was a significant increase in the average cell counts per 20x field when comparing Kainate treated cells to control conditions at 25 h time point. Ka = Kainate. Values represent mean ± SEM based on a sample of 3 wells per condition per experiment. Data points were plotted using the Graph Pad Prism software and means compared using *un-paired simple Student's t-test*. ** $p<0.01$

	Hippocampal cells in culture	
	survived	Died
Control	196	38
Kainate	279	7

Table 5.1 Kainate enhanced the survival of hippocampal cells in culture. Hippocampal cells were grown for 5 days before being pulsed with 5μM Kainate and staged and imaged on time lapse microscope. One image was acquired from each field per 15 minute for 25 h. Three fields per well were selected from three wells per condition. Kainate increased the survival of hippocampal cells in culture. Degrees of freedom: 1, Chi-square = 30.97 and p =0.000.

	Newly-born cells	
	survived	Died
Control	36	14
Kainate	70	2

Table 5.2 Kainate enhanced the survival of newly-born hippocampal cells. Hippocampal cells were grown for 5 days before being pulsed with 5μM Kainate and staged and imaged on time lapse microscope. One image was acquired from each field per 15 minute for 25 h. Three fields per well were selected from three wells per condition. Kainate increased the survival of hippocampal newly-born cells in culture. Degrees of freedom: 1, Chi-square = 16.47 and p =0.000.

	Non dividing Hippocampal cells in culture	
	survived	Died
Control	160	24
Kainate	211	5

Table 5.3 Kainate enhanced the survival of non dividing hippocampal cells. Hippocampal cells were grown for 5 days before being pulsed with 5μM Kainate and staged and imaged on time lapse microscope. One image was acquired from each field per 15 minute for 25 h. Three fields per well were selected from three wells per condition. Kainate increased the survival of non-dividing hippocampal cells in culture. Degrees of freedom: 1, Chi-square = 17.0 and p =0.000.

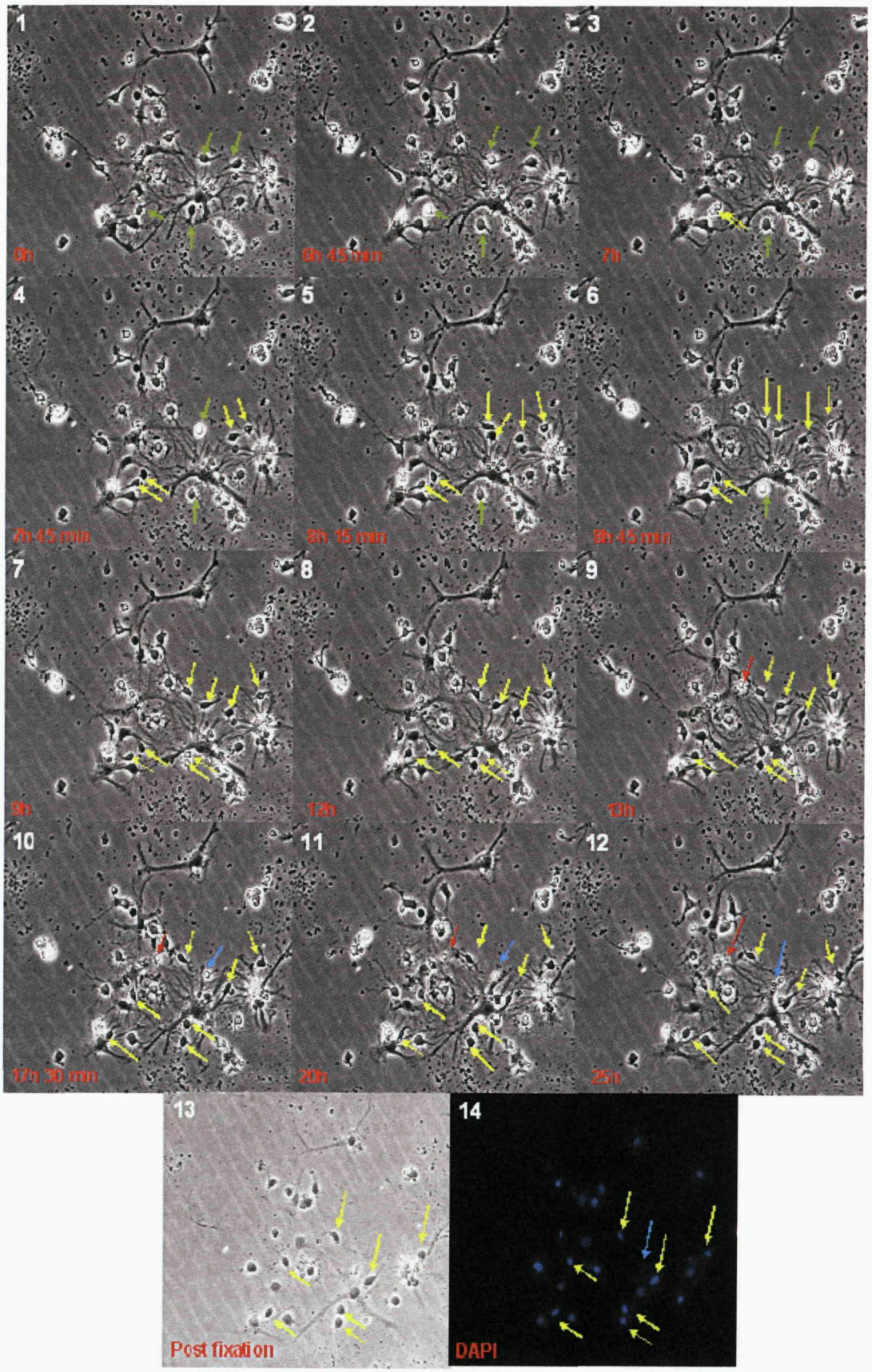

1
0h
2
6h 45 min
3
7h
4
7h 45 min
5
8h 15 min
6
8h 45 min
7
9h
8
12h
9
13h
10
17h 30 min
11
20h
12
25h
13
Post fixation
14
DAPI

Figure 5.10 Kainate enhanced the survival of hippocampal cells and newly-born cells in culture. Hippocampal cells were harvested from postnatal rat pups (P7-10) and grown for 5 days under standard growth conditions. On day 5 a group of cells was treated with 5μM Kainate and another group was untreated and considered as controls. Cells were then staged on an inverted microscope for serial time lapse imaging. An image was acquired every 15 minutes for 25 hours. We counted the hippocampal cells that survived, died and divided (newly-born cells). We found that Kainate enhanced the survival of hippocampal cells in general and both non-dividing and newly-born cells. Green arrows show cells that were observed to divide later within the 25 h time course. Yellow arrows demonstrate newly-born cells. Red arrows show the death course of a non-dividing cell. Blue arrows show the death course of a newly-born cell. Image 13 shows cells post fixation and image 14 demonstrates DAPI staining for hippocampal cells. 1) The reference image at the beginning of the experiment and green arrows show cells that were observed to divide later within the 25 h. 2) This image shows two of arrowed cells (green) become brighter. 3) We observed a division of one of the cells to give two newly-born cells (yellow arrows). 4) Another cell division was observed and another cell became brighter. 5) Third cell divided and the fourth became brighter. 6) Another cell became brighter (green arrow). 7) The fourth cell division was observed. 8) This image displays the 8 newly-born cells in culture (yellow arrows). 9) Red arrows show a non-dividing cell that became shrunk. 10) A newly-born cell (blue arrow) and a non-dividing cell shrank and became pyknotic. 11) The non-dividing cell (red arrow) has burst and fragmented. 12) The newly-born cell (blue arrow) has burst and fragmented. 13) This image shows cells post fixation. 14) This micrograph displays DAPI staining of cells nuclei and shows the fragments that remained from the newly-born cell that died (blue arrow). Images were captured on an Olympus IX 81 inverted microscope system equipped with a digital camera. Differential interference contrast images were acquired every 15 minutes with 20x objective for 25 hours.

5.4.6 Terminal short pulse of Kainate is trophic for progenitor cells and neural precursor cells

In **Chapter 3** (**Sections 3.4.4.3**), we have shown a significant increase in proliferating progenitor cells after a short (6 or 8 h) treatment of Kainate. In addition, we observed a net rise in TuJ1 sub-population of cells after short term of Kainate exposure in the absence of proliferative effects (**Chapter 3, Section 3.4.4.4**). In this **Chapter,** we have consistently demonstrated that short pulse of Kainate enhanced the survival of hippocampal cells and newly-born cells. Therefore, we sought to investigate short term effects of Kainate on the survival of both progenitor cells and neural precursor cells in

an attempt to explore the mechanisms underlying the significant proportional rise in TuJ1 expressing cells at day 5 in cultures.

Hippocampal dissociated cells were grown on glass cover slides for five days under standard growth conditions as detailed in **Chapter 2 (Sections 2.4.2& 2.4.3)**. We replaced two-thirds of the growth medium on day three. At day five, 6 h terminal pulse of 5μM Kainate was given to a group of cells while the other group was maintained under control conditions. After fixation immunocytochemistry double-labelling was carried out for nestin and caspase-3, and for TuJ1 and caspase-3 as described in **Chapter 2 (Section 2.4.7)**. Cell imaging, counting, and analysis were performed as detailed in **Chapter 2 (Sections 2.4.9 &2.4.10)**

We found that terminal 6 h pulse of 5μM Kainate significantly decreased the proportion of cells that were double-labelled for nestin and caspase-3 from 80.3 ± 2.8 % under control conditions to 67.8 ± 1.8 % under Kainate treatment (un-paired simple Student's t-test, $p<0.01$) **(Figure 5.11 A).** Similarly, a proportional decrease in TuJ1 immunoreactive caspase-3 cells was observed after 6 h of Kainate treatment (89.4 ± 1.9 % versus 75.4 ± 1.5 % under control conditions). This difference was statistically significant (un-paired simple Student's t-test, $p<0.001$) **(Figure 5.11 B)**. In **Figure 5.12**, we showed a con-focal micrograph of caspase-3 positive cells (red) and TuJ1 expressing cells (green). We have also shown, in **Figure 5.13**, cells that expressed both nestin (green) and caspase-3 (red).

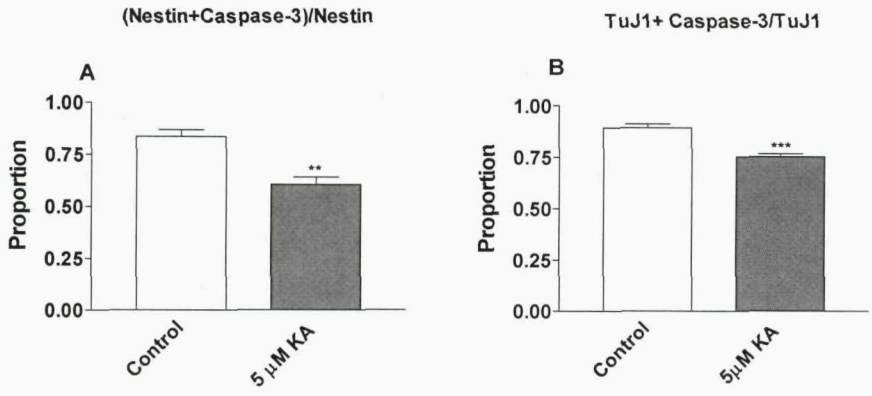


Figure 5.11 Short pulse of Kainate is trophic for both progenitor cells and neural precursors. Cells were cultured for 5 days under standard growth conditions. On day 3 two-thirds of the growth medium was replaced. On day five 6 h terminal pulse 5μM Ka was given to a group of wells and another group was maintained as controls. After cell fixation with 4% PFA, they were immunostaining against either nestin and caspase-3, or TuJ1 and caspase-3 and counterstained for the nuclear marker DAPI. We found a significant drop in the proportion of nestin immuno-positive cells that expressed caspase-3 as well as in TuJ1 positive and caspase-3 immunoreactive cells. A) There was a significant decrease in the proportion of nestin immunoreactive caspase-3 cells after 6 h of Kainate treatments when compared to control conditions. B) The proportion of TuJ1immuno-positive and caspase-3 immunoreactive cells 6 h post Kainate treatments decreased significantly in comparison with control conditions. Ka = Kainate. Values represent mean ± SEM based on a sample of 4-8 wells per condition per experiment from at least 2 separate experiments. Data points were plotted using the Graph Pad Prism software and means compared using *un-paired simple Student's t-test.* ** $p<0.01$, *** $p<0.001$

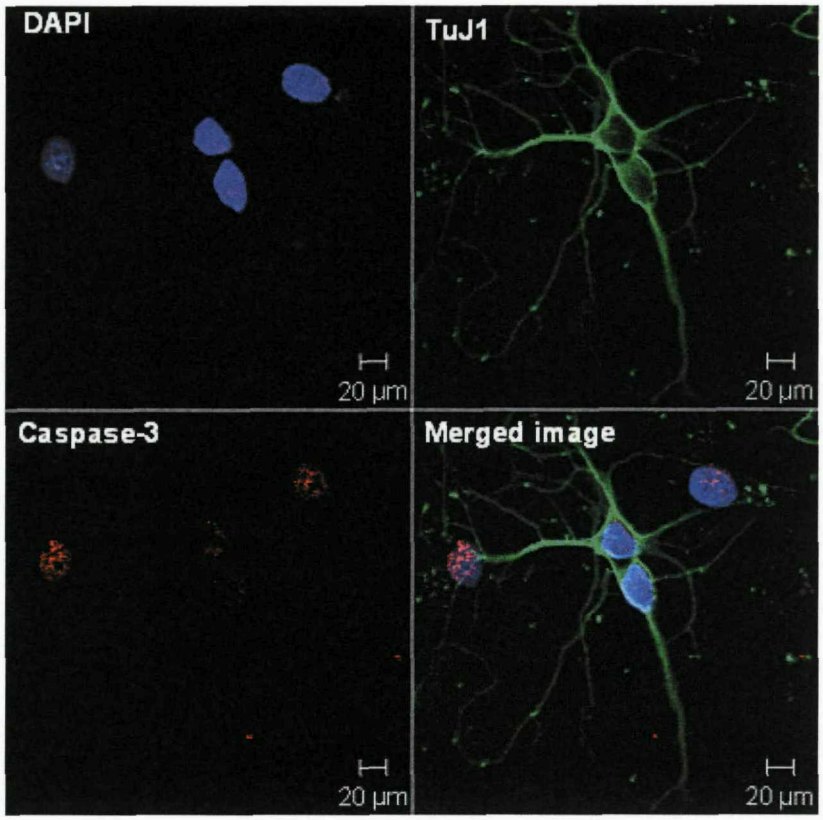


Figure 5.12 Short pulse of Kainate is trophic for TuJ1 expressing sub-population. We cultured primary hippocampal cell on glass slides for 5 days under standard growth conditions. Two-thirds of the growth medium was replaced on day three. 6 h terminal pulse of 5µM Kainate was applied to a group of wells while another group was kept as controls. Fixation for 30 minutes with 4% PFA was done on day 5 before consequent immunocytochemistry. Primary specific anti-sera against the immature neuronal marker (TuJ1) and the cleaved caspase-3 were detected with fluorescently linked secondary antibodies. Counterstaining for the nuclear marker DAPI was carried out at the end of immunocytochemistry. Images were captured with 63x oil immersion objective on a laser scanning confocal microscope and 3D projections reconstructed using the Zeiss LSM software. Images show the same population of cells stained against TuJ1 (green), caspase-3 (red) and counterstained for DAPI (blue). Scale bar = 20µm.

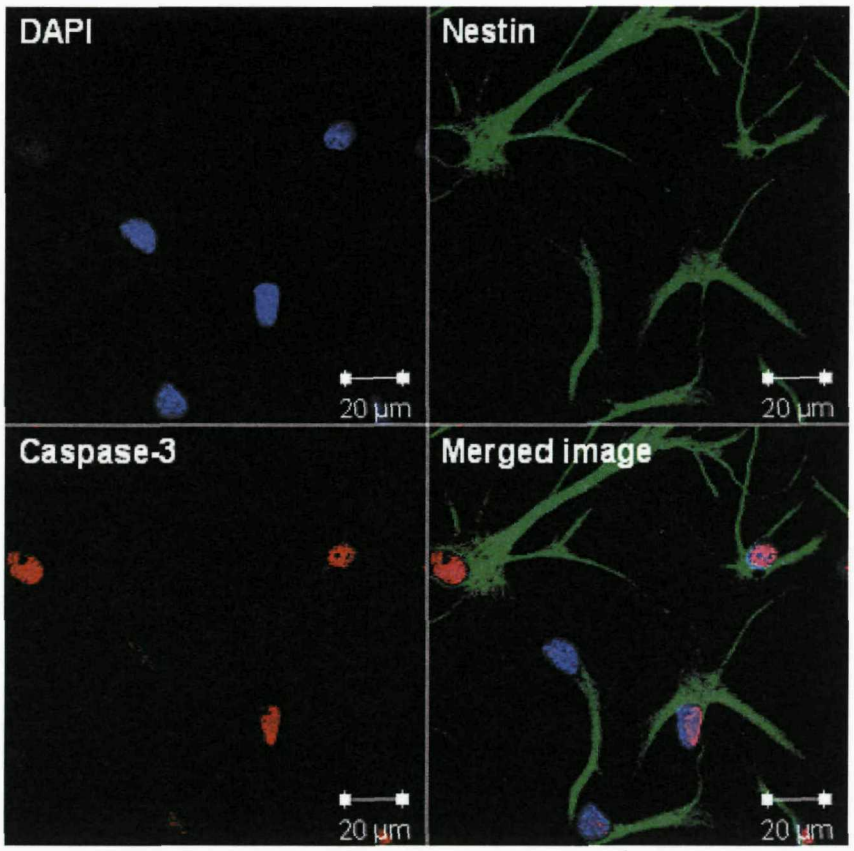


Figure 5.13 Short pulse of Kainate is trophic for Nestin expressing sub-population. Cultures were grown on glass slides for 5 days under standard growth conditions with 6 hours terminal pulse of 5µM Kainate. Two-thirds of the growth medium was replenished on day three. Cell fixation for 30 minutes with 4% PFA was done on day 5 before consequent immunocytochemistry. Primary specific anti-sera against nestin (stem/progenitor marker), and cleaved caspase-3 (death marker) were detected with fluorescently linked secondary antibodies. Counterstaining for the nuclear marker DAPI was carried out at the end of immunocytochemistry. DAPI (blue) stains the nuclei of cells in culture. Nestin (green) stains progenitor cells. Caspase-3 immunoreactive cells are shown in red. Images were captured with 63x oil immersion objective n a laser scanning confocal microscope and 3D projections reconstructed using the Zeiss LSM software. Scale bar = 20µm.

5.4.7 Kainate enhanced the survival of Nestin positive but TuJ1 negative as well as Nestin positive and TuJ1 positive newly-born cells

We have shown that Kainate is trophic for newly-born cells (**Section 5.4.5**) and decreased the proportion of both progenitor nestin immuno-positive and TuJ1-positive cells that expressed caspase-3 (**Section 5.4.6**). In this set of experiments, we investigated the fate of the newly-born cells in terms of their expression of nestin and TuJ1 as markers of putative/ stem cells and neuronal restricted progeny cells, respectively. We generated dissociated cultures from postnatal rats' hippocampi and seeded them into poly-l-lysine coated plastic wells for 5 days as described in **Chapter 2 (Section 2.4.2)**. On day 5, a group of cells was pulsed with 5μM Kainate while the other group was maintained untreated as control. Cells were then staged on the time lapse microscopy for the next 25 h, where an image was acquired every 15 minutes. Time-lapse movies were then processed and studied. Each newly-born cell was morphologically followed up to predict its survival state over the 25 h, with cell membrane rupture and disintegration or subsequent size shrinkage as signs of cell death (**Figure 5.14**). 9 random fields from three different wells for each condition (controls and Kainate) were videoed. We followed up and counted cells that divided (newly-born cells) and whether they survived or died. Cells were then immunostained for both nestin and TuJ1 and counted newly-born cells that were nestin positive and TuJ1 positive, nestin positive and TuJ1 negative, and nestin negative and TuJ1 negative. We could not assess the phenotype of a few newly-born cells that died because they were washed away during immunocytochemistry. None of the newly-born cells that survived washed away during the immunocytochemistry process.

We found that under Kainate conditions 53 newly-born cells that survived were nestin positive but TuJ1 negative and only 1 newly-born cell (1.9%) died. However, 25 newly-born cells survived and 12 (48%) died under control conditions. This difference in the survival of newly-born cells that expressed nestin but not TuJ1 is statistically significant (Chi-square = 16.8, p = 0.000) (**Table 5.4**). Kainate treatment also increased significantly the survival of cells that are double-labelled for both TuJ1 and nestin (10 newly-born cells survived under Kainate conditions compared to 3 newly-born cells

survived under control conditions) (McNemar's test = 5.3 and p = 0.02) (**Table 5.5**). There was no effect of Kainate on newly-born cells that were nestin and TuJ1 negative (**Table 5.6**).

Our results suggest that Kainate enhanced the survival of newly-born progenitor cells that do not express the neuronal marker TuJ1 as well as those newly-born progenitor cells that already committed neuronal lineage by expressing TuJ1. Kainate enhanced the survival of both newly-born precursor and neuronally committed progenitor cells but did not enhance the survival of any other cell type.

	Newly-born cells Fate	
	N+/T- survived	N+/T- Died
Control	25	12
Kainate	53	1

Table 5.4 Kainate enhances the survival of Nestin positive but TuJ1 negative newly-born hippocampal cells. Hippocampal cells were grown for 5 days before being pulsed with 5μM Kainate and staged and imaged on time lapse microscope. One image was acquired from each field per 15 minute for 25 h. Three fields per well were selected from three wells per condition. Degrees of freedom: 1, Chi-square = 16.8 and p =0.000.

	Newly-born cells Fate	
	N+/T+ survived	N+/T+ Died
Control	3	2
Kainate	10	1

Table 5.5 Kainate enhances the survival of Nestin positive and TuJ1 positive newly-born hippocampal cells. Hippocampal cells were grown for 5 days before being pulsed with 5μM Kainate and staged and imaged on time lapse microscope. One image was acquired from each field per 15 minute for 25 h. Three fields per well were selected from three wells per condition. McNemar's test = 5.3 and p = 0.02.

	Newly-born cells Fate	
	N-/T- survived	N-/T- Died
Control	7	0
Kainate	8	0

Table 5.6 Kainate does not affect Nestin negative and TuJ1 negative newly-born hippocampal cells. Hippocampal cells were grown for 5 days before being pulsed with 5μM Kainate and staged and imaged on time lapse microscope. One image was acquired from each field per 15 minute for 25 h. Three fields per well were selected from three wells per condition. Chi-square was not possible.

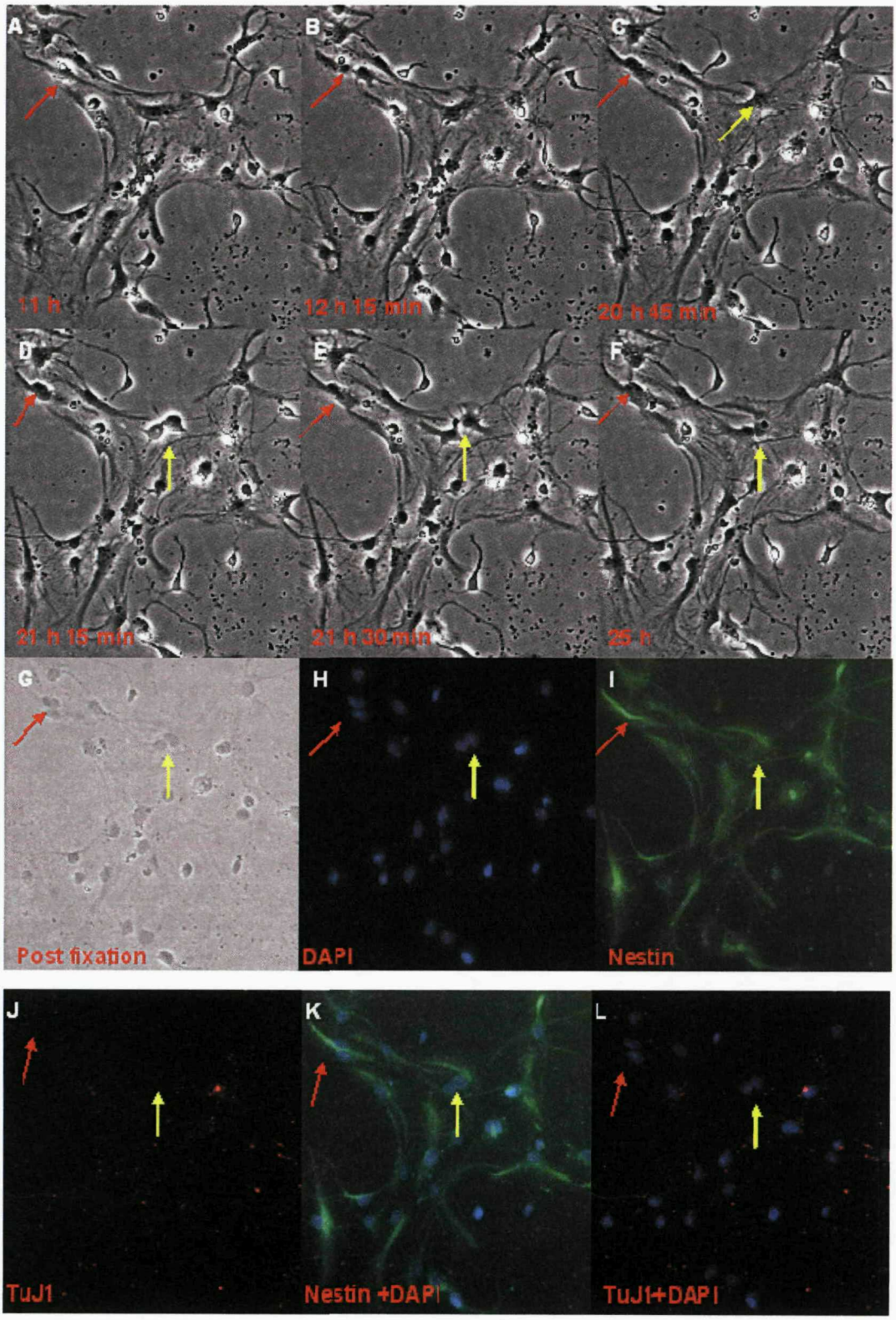

A
11 h
B
12 h 15 min
C
20 h 45 min
D
21 h 15 min
E
21 h 30 min
F
25 h
G
Post fixation
H
DAPI
I
Nestin
J
TuJ1
K
Nestin +DAPI
L
TuJ1+DAPI

Figure 5.14 Kainate enhanced the survival of Nestin (+)/TuJ1 (-) as well as Nestin (+)/TuJ1 (+) newly-born hippocampal cells in culture. Hippocampal cells were harvested from postnatal rat pups (P7-10) and grown for 5 days under standard growth conditions. On day 5 a group of cells was treated with 5μM Kainate and another group was untreated and considered as controls. Cells were then staged on an inverted microscope for serial time lapse imaging. An image was acquired every 15 minutes for 25 hours. We followed up and counted hippocampal cells that divided (newly-born cells) and quantified those newly-born cells that died. Cells were fixed and immunostained against both Nestin and TuJ1 to study the fate of the newly-born cells that survived/died. We found that Kainate enhanced the survival of newly-bonr hippocampal cells that were Nestin (+) and TuJ1 (-) as well as Nestin (+) and TuJ1 (-). Red arrows show the life of one of the newly-born cells that gave at the end 2 newly born cells (Nestin (+)/TuJ1 (-)). Yellow arrows follow up the course of another cell that divided and gave 2 newly bon cells that survived. Images A and B demonstrate the division of hippocampal cell into 2 newly-born cells (red arrows). Images C, D, E and F show the division of another hippocampal cell into 2 newly-born cells (yellow arrows). G) This micrograph shows newly-born cells post fixation (yellow and red arrows). H) DAPI staining demonstrating the nuclei of the newly-born cells. I) This image demonstrates nestin staining and the arrows (red and yellow) point out to the newly-born cells. J) This image displays TuJ1 immunostaining for hippocampal cells. K) This is a merged image for DAPI and nestin staining demonstrating the newly-born cells. L) This is a merged micrograph for DAPI and TuJ1. Images were captured on an Olympus IX 81 inverted microscope system equipped with a digital camera. Differential interference contrast images were acquired every 15 minutes with 20x objective for 25 hours.

5.5 Discussion

Kainate is an excitotoxin and often used to induce neuronal cell loss and hippocampal damage (Ben-Ari and Cossart 2000). Kainate-induced seizure increases dentate gyrus neurogenesis, but the underlying mechanisms are not well-understood (Bengzon, Kokaia et al. 1997; Gray and Sundstrom 1998). It has been suggested that excitotoxic insult causes granule cell death which induces precursor cell proliferation and subsequent increase in neurogenesis (Gould and Tanapat 1997). However, the mechanisms that regulate excitotoxic-induction of hippocampal neurogenesis and the role of cell death are not fully clear. Therefore, we investigated the effects of Kainate on the survival of hippocampal progenitor cells and their neuronal progeny cells *in vitro*. We have demonstrated a significant survival role for Kainate on hippocampal cells in general and on both progenitor and neural precursor cells in particular. We show that 5 days of 5μM Kainate exposure decreases the proportion of hippocampal cells that is caspase-3 immunoreactive as well as caspase-3 immuno-positive cells that incorporated BrdU suggesting a significant survival effect for Kainate on proliferating hippocampal cells. Furthermore, we demonstrate a significant drop in the proportion of both nestin expressing cells and TuJ1 immuno-positive cells that expressed caspase-3 under Kainate conditions after 5 days in culture compared to control conditions. Interestingly, terminal short exposures to Kainate enhance the survival of hippocampal cells as indicated by a significant increase in DAPI+/Mitotracker+ cells as well as an increase in the counts of hippocampal cells that survived when videoed under time-lapses microscopy and in comparison with control conditions. Moreover, short (6 h) treatments of Kainate decrease the proportion of nestin immuno-positive cells that are caspase-3 immuno-positive. The same effect of Kainate treatment was demonstrated on the TuJ1-expressing sub-population of cells. In addition, our time-lapse analysis shows that terminal (25 h) of Kainate treatments increase the survival of newly-born hippocampal cells that are Nestin+/TuJ1- as well as Nestin+/TuJ1+ with no effect on Nestin-/TuJ1- cells.

5.4.1 Kainate has an important survival role when applied to hippocampal cells *in vitro*

Our understating of the mechanisms underlying cell death is hindered by the lack of markers and/or methods to label cell death in live subjects. Classically cell death was investigated either using immunohistochemistry methods to detect TUNEL and/or caspases (or other markers of cell death) or measure the expression of specific proteins that are expressed by dead/dying cells using western blot and/or PCR techniques. However, there is no direct evidence showing how cells die *in vitro*. Therefore, we measured cell death in Kainate treated hippocampal cell cultures using immunocytochemistry against caspase-3 and also applied PI and MitoTrackers on live cell cultures and identified the proportion of live cells as well as dead/dying cells. Furthermore, we videoed cells in culture for 25 h and examined the survival of newly-born cells and their phenotype under both controls and Kainate treatments. Our results (**Section 5.4.4**) suggest that Kainate enhances the survival of hippocampal cells at 3 and 5 DIV. PI enters cells with leaky cell membranes, binding to DNA and fluorescing, thus identifying dead/dying cells in culture. MitoTrackers, on the other hand, enter mitochondria of live cells and emit either green or red fluorescent (according to the MitoTracker) that can be detected using fluorescent microscope (Buckman, Hernandez et al. 2001). These characteristics enabled us to measure the numbers of live cells in culture once MitoTracker is applied in combination with DAPI and PI in the case of MitoTracker Green FM or with DAPI only in the case of MitoTracker Orange. These results show that the significant rise in the total numbers of DAPI cells after 6 h of Kainate treatment is due to enhancing cell survival compared to control conditions where dead/dying cells are either washed away during immunocytochemistry or perhaps cleared up by microglia. These results argue strongly against Kainate-induced death being an indirect trigger for Kainate induced proliferation.

In agreement with our findings that Kainate does not enhance cell death in cultures from postnatal hippocampi, it has been demonstrated that hippocampal cells of the postnatal rats are resistant to Kainate induced-seizures (Sperber, Haas et al. 1991). Indeed, Sperber and colleagues have observed that although Kainate-induced seizures was more

severe in 15 days old rats pups when compared to adult rats, neuronal cell death was less in these postnatal pups. Consistent with this, Haas et al have shown that hippocampal damage due to Kainate-induced seizures in immature rats was significantly less than the damage which was observed in adult rats' hippocampi (Haas, Sperber et al. 2001). Kainate and glutamate have also been found to enhance survival of cells in cultures generated from postnatal Wistar rats' subventricular zone when applied in combination with non-competitive NMDA receptors antagonist KYNA (Brazel, Nunez et al. 2005). Furthermore, this group has demonstrated that up to 1 mM glutamate did not enhance precursor cells death in culture. They have also shown that the mechanism of protection was not accompanied with an elevation of the trophic factors FGF-2, BDNF, and NGF. And thus a direct survival role of glutamate may be implicated. Unlike Brazel et al 2005 who used in situ nick-end labelling to measure cell death in culture; we used different methodological approaches including a combination of DAPI, PI, and MitoTracker, as well as caspase-3 immunocytochemistry, in addition to time-lapse microscopy. We show, in this chapter, significant trophic effect for Kainate on hippocampal cells *in vitro*. Our results further confirm that, in agreement with Sperber et al 1991, Haas et al 2001, and Brazel et al 2005, hippocampal cells are not only resistant to Kainate excitotoxity but also survive more in the presence of 5μM Kainate.

Although we demonstrate that a proportion of the proliferating cells (BrdU incorporated cells) are immunoreactive to caspase-3, we show a significant decrease in this proportion after 5 days of Kainate treatment (**Section 5.4.1**). It has been suggested that cells undergoing apoptosis in other systems can express markers of proliferating cells (Herrup and Busser 1995). In this regard, Herrup and co-workers have demonstrated that a fraction of cerebellar granule cells from postnatal mice that incorporated BrdU are dying naturally by apoptotic cell death. In agreement with this, we have shown that around half of BrdU incorporated cells are expressing the apoptotic cell marker caspase-3. Interestingly, Kainate treatment rescued slightly over 20% of proliferating (BrdU incorporated) hippocampal cells from death (**Section 5.4.1**). In addition, we further confirm under time-lapse that newly-born cells have better survival rates under Kainate conditions compared to control conditions (**Section 5.4.4**). These results support the hypothesis that Kainate does not enhance cell death; on the contrary it enhances the

survival of proliferating cells. It is interesting to speculate whether the default condition for newly generated cells is to die by apoptosis and that the environment controls neurogenesis by rescuing a greater or lesser proportion of these cells from death. In agreement with this idea, Yan et al have found that a population of BrdU incorporated cells express caspase-3 in the SVZ of postnatal Wistar rats (Yan, Najbauer et al. 2001). We confirm that approximately 50% of BrdU incorporated cells in our cultures are expressing caspase-3. However, Kainate treatment decreased this proportion by half. And as such, this may suggest that a substantial proportion of proliferating precursor cells perhaps is instructed to commit apoptosis unless they are instructed otherwise. An alternative explanation is that our culture conditions are adverse for the survival of these cells in the absence of Kainate or indeed glutamate activation of AMPA receptors (**see chapter 4**). Indeed, it has been elegantly shown that AMPA receptors protect cerebellar neurons generated from postnatal pups through a mechanism involving BDNF release, synthesis, and activation of TrkB receptors (Wu, Zhu et al. 2004). In chapter 4 we have shown that the effects of Kainate were abolished by blocking AMPA receptor. Therefore, we conclude that AMPA receptor subtypes indeed modulate the survival of proliferating hippocampal cells.

5.4.2 Kainate enhances the survival of hippocampal progenitor cells and their neuronal precursors in culture

Caspase inhibitors have been reported to increase the survival of progenitor cells and their progeny cells in the adult dentate gyrus after status epilepticus (Ekdahl, Mohapel et al. 2001). In this study, a reduction in apoptosis was suggested to be associated with increased neurogenesis. In addition, it has been demonstrated that neuronal cell death after seizure-induced status epilepticus is through caspase-3 dependent pathway (Ekdahl, Zhu et al. 2003). Furthermore, caspase-3 mediated cell death in the subgranular zone of the dentate gyrus has also been shown to be associated with enhanced neurogenesis (Bingham, Liu et al. 2005). These studies suggest a key role for caspase-3 in both cell death and its association with neurogenesis after excitotoxic insult such as Kainate. Therefore, we double-labelled either nestin or TuJ1 positive cells with caspase-3 and

measured the proportion of nestin and TuJ1-apoptotic cell death under both Kainate and control conditions. We find a significant reduction in the proportion of caspase-3 immunoreactive cells that express the progenitor marker nestin (**Section 5.4.2**). In this part, Kainate decreases the proportion of nestin expressing cells that are caspase-3 immunoreactive by approximately 20%. This suggests a significant survival effect of Kainate on hippocampal progenitor cells that is caspase-3 pathway dependent. We also show a significant decrease in the proportion of TuJ1 cells that express caspase-3 after 5 days of Kainate treatment (**Section 5.4.3**) which implies that Kainate is trophic for neuronal progeny in culture as well. Interestingly, we also demonstrate (**Section 5.4.6**) that short exposure to Kainate at day 5 enhances the survival of hippocampal progenitor cells and TuJ1 immuno-positive cells as indicated by the decrease in the proportions of both nestin immuno-positive cells and TuJ1 expressing cells that are double-labelled with caspase-3. In line with these results Brazel and co-workers have shown a reduction in apoptosis after terminal 24 h treatment of 3μM Kainate and 500nM KYNA and in comparison with control conditions in cultures from SVZ of postnatal Wistar rats. In addition, SVZ precursor cells survival under Kainate conditions was not associated with an increase in trophic factors (Brazel, Nunez et al. 2005). These results may suggest direct trophic effect of Kainate on precursor cells. In our model of hippocampal cell culture we demonstrate that both long (5 days) and short (6 h or 25 h) exposure to Kainate is enhancing the survival of nestin expressing hippocampal progenitor cells as well as TuJ1 immuno-positive sub-population of cells. In this context, Kainate perhaps has similar mechanisms of action like caspase inhibitors and may also inactivate cell death genes and thus reduce apoptotic cell death. From our work we can confirm that the survival effects of Kainate are modulated via a pathway involving caspase-3, however, the involvement of other caspases and cell death genes like DP5 or C-Jun would be a good avenue for future work. As far as our effects are modulated via AMPA receptors (**Chapter 4**), we further suggest that AMPA receptors may regulate caspses-3 mediated apoptotic cell death. However, what is confirmed so far is that cell death after seizures is at least in part regulated via caspase-3 dependent pathway (Ekdahl, Mohapel et al. 2001; Ekdahl, Zhu et al. 2003; Mohapel, Ekdahl et al. 2004).

Our time-lapse set of experiments (**Section 5.4.7**) show that 72.6% of newly-born cells that are Nestin+/TuJ1- survive under terminal 25 h Kainate conditions versus 47.1% of Nestin+/TuJ1- newly-born cells survive under control conditions. This provides direct evidence that Kainate is trophic for Nestin+/TuJ1- newly-born cells. Furthermore, we demonstrate that 13.7% of newly-born Nestin+/TuJ1+ cells survive under Kainate treatments versus 5.9% survive under control conditions. There is no significant survival effect for Kainate on newly-born cells that are Nestin-/TuJ1-. None of the newly-born cells that survived was washed away during immunocytochemistry. However, a few of the newly-born cells that died were washed away and we were unable to determine their phenotype. These results imply an important trophic role for Kainate on progenitor cells and their neuronal lineage.

5.5 Conclusions

We have shown that Kainate enhanced the survival of hippocampal cells in cultures. We demonstrated that 5 days of Kainate exposure decreased the proportion of hippocampal cells, BrdU-incorporated cells, nestin immuno-positive cells, and TuJ1 expressing cells that were dual labelled for the apoptotic marker caspase-3. Moreover, terminal 6 h of Kainate exposure at day 5 enhanced the overall survival of hippocampal cells as measured by DAPI+/MitoTracker Orange+ and both progenitor cells and TuJ1 sub-population of cells in particular. We also showed, using time-lapse video microscopy, that 25 h of Kainate treatment at day 5 enhanced the survival of hippocampal cells and newly-born cells. Interestingly, we demonstrated that Kainate at day 5 and under time-lapse video increased the survival of newly-born cells that were nestin+/TuJ1- and nestin+/TuJ1+. We further showed that cells that survived were not washed away during immunocytochemistry while dead/dying cells or part of them were washed away. We conclude that Kainate has an important survival role which regulates the survival of hippocampal progenitor cells and their neuronal progeny cells.

Chapter Six

Clonal Proliferation and cell death in an *in vivo* Kainate model of temporal lobe epilepsy

6.1 Introduction

The work described in chapters 2-5 focuses on the development of a novel *in vitro* model for investigating the acute effects of Kainate on hippocampal progenitor cells and neurogenesis from immature animals. However, this is not the only possible approach to the investigation of increased neurogenesis following Kainate-induced seizures. There are already several models of seizures described *in vivo*, in which neurogenesis can be studied. The principle model of seizures-induced neurogenesis that our lab uses relies on the chemo-convulsant Kainate (Mitchell, Sundstrom et al. 1993; Sundstrom, Mitchell et al. 1993; Gray and Sundstrom 1998; Gray, May et al. 2002; Huttmann, Sadgrove et al. 2003). In primary hippocampal cell cultures a known concentration of Kainate is easily added to the growth medium and for a specified period of time, however, methods of applications are more complicated *in vivo*. Some studies use unilateral intracerbroventricular (ICV) or intrahippocampal Kainate infusion to generate seizure activity (Nadler and Cuthbertson 1980). Other studies use intraperitoneal injection to induce seizures; effects with this technique produce bilateral changes (Covolan, Ribeiro et al. 2000; Nakagawa, Aimi et al. 2000; Tokuhara, Sakuma et al. 2007). However, in the previous studies, researchers mainly focused at investigating the effects of Kainate and/or seizures on hippocampal neuronal cell damage and induction of neurogenesis thereafter. We, in this project, will study the acute effects of Kainate/seizures on the proliferation of a pre-labelled and un-labelled 'clones' of dentate gyrus progenitor cells. We will also examine the effects of Kainate/seizures on granule cell survival and neuroblasts formation. Comparing Kainate/seizures effects *in vivo* with *in vitro* Kainate results would help us unravel whether Kainate *in vitro* is having similar effects to Kainate/seizures *in vivo* or not.

6.1.1 Models of temporal lobe epilepsy

Status Epilepticus (SE) models with spontaneous seizures and neurodegeneration are the most commonly used animal models of TLE (Leite, Garcia-Cairasco et al. 2002). In these models spontaneous seizures are evoked by systemic or intracerebral injection of

excitotoxins such as Kainate or pilocarpine or by tetanic electrical stimulation of temporal lobe structures (Leite, Garcia-Cairasco et al. 2002). The neuronal loss induced by SE in such preparations might be considered an equivalent of the initial precipitating injury event, usually a prolonged febrile convulsion, which is commonly found in patients with mesial temporal lobe epilepsy (Mathern, Babb et al. 1996).

Kindling is another model of TLE. In this model temporal lobe structures are repeatedly stimulated by short electrical pulse trains (McIntyre, Poulter et al. 2002). The animals respond to the stimulation by displaying an electrical discharge on the electroencephalogram (EEG) and abnormal behavior. Rats display more complex after-discharges or more severe seizures with increasing number of stimulations. Once rats consistently display tonic-clonic seizures they are termed fully kindled. Although large amount of stimulations are required to induce spontaneous seizures, gross morphological damage, seen in the kindling model, is much less severe than in other models of SE (McIntyre, Poulter et al. 2002). Therefore, because of the controlled induction of epileptogenesis, the kindling model is a valuable tool in studying the role of events associated with epileptogenesis (McIntyre, Poulter et al. 2002). However, there is no convincing evidence for kindling in human temporal lobe epilepsy.

In many animal models of TLE, status epilepticus has been demonstrated to increase neural stem cells proliferation and neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (DG) (Bengzon, Kokaia et al. 1997; Parent, Yu et al. 1997; Gray and Sundstrom 1998; Madsen, Treschow et al. 2000; Nakagawa, Aimi et al. 2000; Scott, Wojtowicz et al. 2000; Ekdahl, Mohapel et al. 2001) but the contributions of neural stem cells and their control mechanisms are not clear. Hippocampal injury induces an initial transient proliferative surge in the SGZ with the number of neurons increasing several folds during the first few weeks after injury (Parent, Yu et al. 1997; Nakagawa, Aimi et al. 2000; Hattiangady, Rao et al. 2004). Although it has been shown that several neurotrophic factors and Neuropeptide Y are up-regulated in the hippocampus after Kainate-induced seizures (Lowenstein, Seren et al. 1993; Shetty, Zaman et al. 2003; Howell, Silva et al. 2007), our knowledge of the acute mechanisms underlying progenitor cells control and contributions to the epileptic brain is far from complete.

6.1.2 The significance of seizures induced neurogenesis

Kainate-induced seizures enhances DG neurogenesis *in vivo* (Gray and Sundstrom 1998) and to lesser extent in hippocampal slice cultures *in vitro* (Routbort, Bausch et al. 1999; Sadgrove, Chad et al. 2005). However, there is still a considerable debate about the role of neurogenesis and its biological mechanisms in the adult DG. Most TLE patients have learning and memory impairments (Devinsky 2004). In animal models, spatial memory impairment has been reported after 5 months of epilepsy (Detour, Schroeder et al. 2005). While the vast majority of newly-born neurons in the SGZ migrate into the GCL in normal conditions, a large number of newly-born neurons migrate away from the GCL into the dentate hilus in SE (Parent, Yu et al. 1997; Scharfman, Goodman et al. 2000; Hattiangady, Rao et al. 2004). Likewise ectopic granule and progenitor cells were also found in the hilus and molecular layer of epileptic human DG (Parent, Elliott et al. 2006). Although neurogenesis persists in chronic TLE, a significant reduction in neurogenesis and GABA-ergic interneurons has been reported (Hattiangady, Rao et al. 2004). On this basis, a hypothesis emerges that newly-born granule cells migrate into the hilus and/or molecular layer after SE and contribute to the development of chronic epilepsy (Scharfman, Goodman et al. 2000). Moreover, the reduction in neurogenesis and the inhibitory GABA-ergic interneurons may contribute to cognitive and memory impairments in chronic TLE. In an interesting study, it has been demonstrated that intracerebroventricular infusion of the antimitotic agent cystosine-b-D-arabinofuranoside (Ara-C) significantly reduced spontaneous recurrent motor seizures in pilocarpine-induced SE when compared to control animals (Jung, Chu et al. 2004). This group has also reported a reduction in ectopic granule cells in SE animals after Ara-C treatment. These results support the notion that SE enhances the ectopic migration of newly-born granule cells which contributes to the development of chronic epilepsy. However, the complete effects of SE on SGZ progenitor cells have yet to be established. Although SE induces SGZ cell proliferation, it is not well understood which progenitor subtype(s) is/are affected, at which stage progenitor cells migrate into ectopic areas and whether SE has a proliferative effects on hilar and molecular layer hippocampal cell or not. Thus, there is a need to investigate the mechanisms underlying the acute changes in SGZ progenitor cells after status epilepticus. Furthermore, in an interesting study,

Jessberger et al 2007 have demonstrated that, after labelling dividing progenitor cells with retrovirus, Kainate-induced seizures enhanced the production of new neurons with altered polarity, migration, and integration (Jessberger, Zhao et al. 2007). Indeed, they have reported that Kainate-induced seizures altered synaptic integration of newly-born neurons and also dentate gyrus connectivity. These abnormal changes started shortly after seizures and were long lasting. In addition, Jakubs and colleagues have demonstrated that newly-born neurons showed increased inhibitory and reduced excitatory input onto granule cells that were born after electrically induced SE compared to granule cells generated in the running animals (physiologically produced) (Jakubs, Nanobashvili et al. 2006). This research clearly demonstrates that seizures abnormally affect newly-born neurons. In contrast to other researches, Jakubs work suggests an inhibitory compensatory effect after the generation of new neurons after seizures. Thus, rigorous studies at early points after acute SE in animal models of TLE are needed to elucidate further the links between acute seizures and hippocampal neurogenesis.

6.1.3 Possible mechanisms of increased cell proliferation after seizures

There are two possible theories to explain the increase in cell proliferation after seizures; increased rates of division of existing active progenitor cells through decreased cell cycle times, and activation of quiescent or slowly dividing population of progenitor cells. In many experimental models, a point/pulse labelling method was used to identify cell proliferation after seizure induction (Parent, Yu et al. 1997; Nakagawa, Aimi et al. 2000). Seizures are induced, and then a 'pulse' of a proliferation marker such as BrdU is administered. The animal is sacrificed shortly after the proliferation marker has been incorporated; the number of labelled cells detected represents a proportion of the total proliferating population at the time of sacrifice. This method of identifying cell proliferation has demonstrated that cell proliferation increases between day 1 and day 3, remains elevated until around day 7 and then falls to normal or slightly less than normal levels (Parent, Yu et al. 1997; Nakagawa, Aimi et al. 2000). However, point labelling cannot identify the populations of cells responsible for these increases, and therefore the

underlying changes in the dynamics of precursor cell proliferation after status epilepticus remain unclear.

An emerging body of evidence supports the hypothesis that neurogenesis in the SGZ is dependent on two sub-populations of dividing cells, a stem cell sub-population probably derived from astrocytes, which generate a second restricted progenitor sub-population of cells (Alvarez-Buylla, Seri et al. 2002). In line with this, it has been shown that astrocyte-like progenitor cells significantly increased 72 hours after Kainate treatment in the subgranular zone (SGZ) of adult mice (Huttmann, Sadgrove et al. 2003). Interestingly, it has been demonstrated that in the SGZ quiescent neural progenitor cells give rise to an amplifying neural progenitor cells that generates neurons (Encinas, Vaahtokari et al. 2006). In an elegant study; It has been demonstrated that immature (1 month) and adult (3 months) rats produce the same number of BrdU labelled cells in response to intracerebroventricular (ICV) Kainate induced seizures despite a 30% decrease in cell proliferation in the ICV saline injected control animals between the two age groups (Gray, May et al. 2002). This study implies both mechanisms of increased cell proliferation are involved. For the 1 month group, the significant rise of cell proliferation is unlikely to be due to the significant reduction in the cell cycle time as at this age the cell cycle is close to the minimum cycle time of postnatal rats (P1-12) (Lewis 1978). These results suggest a significant recruitment for quiescent cells at this age. The cell cycle is longer by 3 months (Cameron and McKay 2001) and hence the basal proliferation is lower, although, seizure-induced cell proliferation is the same. Indeed it appears, from the previous studies, that the two theories of increased cell proliferation are implicated in SGZ neurogenesis. Furthermore, it has been shown that Kainate-induced seizures enhanced the proliferation of so called type-3 precursor cells that expressed the neuronal marker Doublecortin (Dcx) at nine days after seizures (Jessberger, Romer et al. 2005). However, the acute effects of seizures on Dcx and the other sub-populations of cells are only partially understood.

Cell death might have a role in regulating hippocampal neurogenesis. It has been detected in several *in vivo* models, including excitotoxic and mechanical lesions of the granule cell layer (GCL) (Gould and Tanapat 1997). Although granule cells are resistant to seizures-induced death, it has been found that granule cells die after seizures in adult

rats (Bengzon, Mohapel et al. 2002). However, the severity of seizures appears to play an important role, with more severe seizures decreasing the survival of new neurons (Mohapel, Ekdahl et al. 2004). On the other hand, Ekdahl and co-workers have proposed that seizures modify the survival of new neurons and a transient reduction of apoptotic cell death might be associated with increased neurogenesis (Ekdahl, Mohapel et al. 2001). Furthermore, spontaneous seizures that follow SE do not appear to affect SGZ cell survival (Scharfman, Goodman et al. 2000; Ekdahl, Zhu et al. 2003; McCloskey, Hintz et al. 2006). It therefore appears that the role of cell death in DG neurogenesis is a matter of controversy with no concrete evidence explaining the contribution of cell death to hippocampal neurogenesis.

6.1.4 *In vivo* markers of cell proliferation

BrdU is currently the preferred marker for birth dating and monitoring cell proliferation (Taupin 2007). Identification of S-phase (BrdU incorporated) cells in the brain was made following the development of immunohistochemistry (Miller and Nowakowski 1988), and this method allows the estimation of labelled cells, even in thick sections, by stereological counting methods (Scharfman, Goodman et al. 2005). BrdU is also used to measure the survival of specific cell phenotypes because of its persistence in postmitotic cells, allowing multiple labelling with phenotype markers at longer survival times after administration (Kempermann, Gast et al. 2003). Although a combined use of BrdU with neuronal markers has been used to study neurogenesis (Eriksson, Perfilieva et al. 1998; Kempermann, Gast et al. 2003), other proliferation markers exist. The expression of markers of the cell cycle, like proliferating nuclear antigen (PCNA) and Ki-67 has been used to study cell proliferation (Taupin 2007). Although these markers are poorly identifying cell phenotype in survival experiments, they are useful markers as point proliferation markers. The cell cycle marker PCNA is expressed in cells undergoing DNA repair, and in some non-proliferating neurons (Ino and Chiba 2000). Ki-67, an endogenous cell cycle protein, is expressed in all phases of the cell cycle except the resting phase and for short period at the beginning of G1 after initial recruitment into the cell cycle (Zacchetti, van Garderen et al. 2003). Ki-67 is not detectable during DNA

repair process, has a very short half life, and is absent in quiescent cells (Scholzen and Gerdes 2000; Zacchetti, van Garderen et al. 2003). Furthermore, the quantification of Ki-67 immuno-positive cells has been demonstrated to reflect DG cell proliferation in a manner consistent with BrdU labelling in the adult rats (Eadie, Redila et al. 2005). These characteristics support the use of Ki-67 as marker of cell proliferation for studying neurogenesis.

Intraperitoneal (IP) injections of BrdU can be used to follow the fate of a group of dividing cells under control conditions, however introducing a seizure or treatment into the model might alter the permeability of the blood brain barrier (Bolton and Perry 1998). Therefore, disruption of the blood brain barrier might induce increases in the numbers of BrdU labelled cells. If BrdU is available for incorporation at the time of seizures, changes in the blood brain barrier could alter the bioavailability of BrdU and therefore its incorporation into dividing cells. This might lead to a difference in BrdU incorporated cells between control and treated conditions. However, if a BrdU pulse is administered at least 1 cell cycle before seizure induction then many of the cells dividing at the time of seizures will be BrdU labelled, and unincorporated BrdU will have been cleared from the body eliminating the possibility of confounding effects from the blood brain barrier disruption (Taupin 2007). Therefore, IP administration of BrdU 24 hours before seizures induction will overcome the potential confounding effects of the blood brain barrier disruption on BrdU incorporation. A 'pulse' of BrdU might be diluted after a number of cell divisions. Once a 'pulse' of BrdU is incorporated into dividing cells, only a limited quantity is present in each cell. Every cell division therefore results in a dilution in the BrdU density within the dividing cells, after a number of cell divisions the density of BrdU labelling will have decreased below the threshold required for detection and thus underestimate the counts of BrdU labelled cells. However, it has been demonstrated that BrdU dilutions probably occurs after about 4 cell cycles (Hayes and Nowakowski 2002; Dayer, Ford et al. 2003). In addition, the length of the cell cycle in adult rats was found to be 25 h (Cameron and McKay 2001). Therefore, IP application of BrdU 24 hours before seizures induction will enable us to follow BrdU incorporated cells up to 3 cell cycles after seizures before BrdU being diluted and undetected.

6.1.5 Labelling a 'clone' of proliferating cells before seizure induction

Ki-67 has been used as a point proliferation marker and BrdU as a 'clonal' marker to study cell death and the dilution of BrdU in a proliferating 'clone' of DG progenitors (Dayer, Ford et al. 2003). In this study, double labelling of BrdU and Ki-67 at different time points has shown that BrdU is detectable up to 4 days in dividing cells, indicating that any decrease in the number of BrdU incorporated cells after 4 days is likely to reflect cell death rather than BrdU dilution. We will use this approach to explore the underlying mechanisms of proliferating SGZ cells under control conditions and after seizures. BrdU labelling alone could identify the total number of 'clonal' cells produced between administration and sacrifice, and Ki-67 labelling alone could identify the total number of cells in the cell cycle at the time of sacrifice. However, the combined use of both BrdU and Ki-67 will identify 3 different sub-populations of cells, those positive for BrdU only, those positive for BrdU and Ki-67, and those positive for Ki-67 only. BrdU only cells are postmitotic cells derived from a cell that was dividing when BrdU was applied and which have exited the cell cycle between BrdU additions and sacrifice (**Figure 6.1**).

BrdU positive and Ki-67 negative cells = Postmitotic sub-population of cells

Ki-67 and BrdU positive cells are actively dividing 'clonal' cells, derived from cells that were dividing at the time of BrdU administration and which are dividing at sacrifice (**Figure 6.1**).

BrdU positive and Ki-67 positive cells = Proliferating pre-labelled 'clone' of cells

Cells that only express Ki-67 are cells that are dividing at the time of sacrifice but not derived from a cell in the S-phase when BrdU was applied (**Figure 6.1**).

BrdU negative and Ki-67 positive cells = Un-labelled proliferating cells

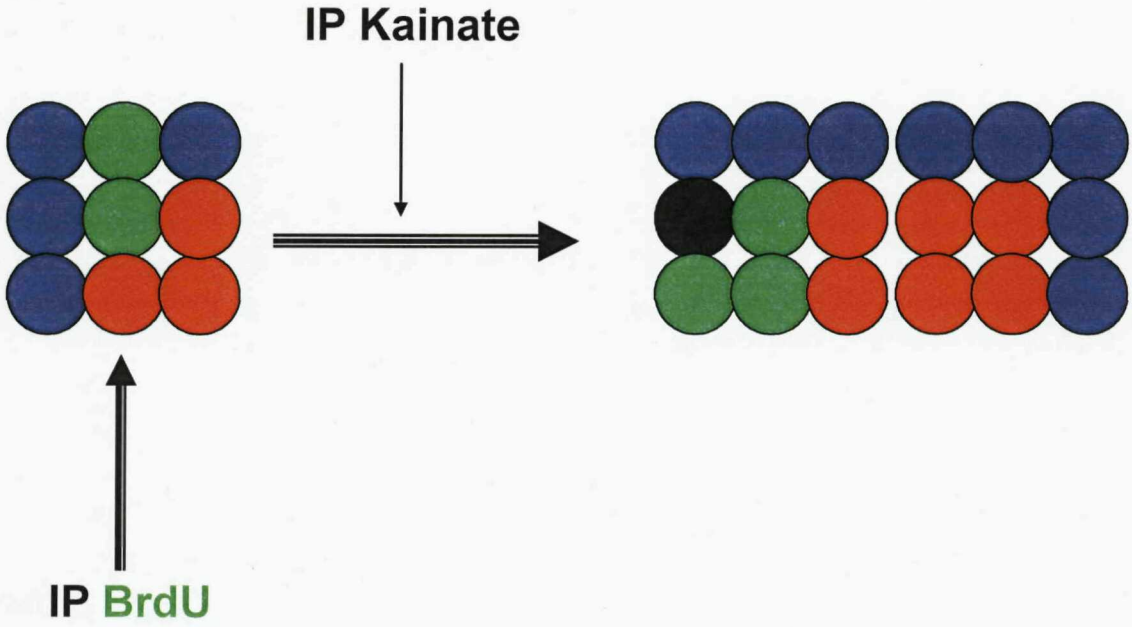


Figure 6.1 Schematic representations for the distribution of proliferation markers. Cells that are not diving do not incorporate BrdU or express Ki-67 ; these cells form the majority of granule cell layer cells and are not detected by this experimental model. All cells that enter the cell cycle and start to divide express Ki-67 while they are in the cell cycle, but cease to express it shortly after leaving the cell cycle . In addition to expressing Ki-67, cells in the S-phase of division at the time of BrdU pulse incorporated BrdU . If these BrdU labelled cells cease to divide (exit the cell cycle) then they cease to express Ki-67, but BrdU labelling persists and detected up to 4 cell cycles .

= Granule cell (non-dividing cells in our experimental paradigm). = cells in the cell cycle (Ki-67+ cells). = cells in S-phase of the cell cycle (BrdU+ and Ki-67+). = cells that incorporated BrdU and exited the cell cycle (BrdU+ and Ki-67-) (postmitotic cells).

6.2 Chapter aims

The main aim of these experiments is to examine the general profile of cell proliferation through time after status epilepticus, while simultaneously following the survival and proliferation of a cohort of cells dividing at the time of the precipitating status. IP Kainate injection will be used to generate seizures/SE. The labelling of a cohort of cells will be achieved with a single 'pulse' of BrdU 24 hours before Kainate treatment, the size of the cohort can be then compared at different time points after Kainate and saline injections. Point proliferation will be quantified by Ki-67 immunostaining, double labelling of BrdU and Ki-67 will identify the 'clonal' proliferation (changes in BrdU+ and Ki-67+ cohort of cells and their progenies), postmitotic cells (BrdU+ and Ki-67- cells), and an un-labelled 'clone' of proliferating cells (BrdU- and Ki-67+). Caspase-3 immunostaining will be used to quantify cell death and Doublecortin immunostaining will identify immature neurons. These observations should permit comparisons with existing control and Kainate *in vitro* experiments.

6.3 Methods and Materials

We outlined below the generals methods for the chapter. The details of the exact tissue sampling are outlined immediately before each accompanying results section

6.3.1 Animals

Fifty four adult male Wistar rats (250-300g) were used. All animal procedures were licensed and performed in strict accordance with the Animal Scientific Procedures' Act 1986 and with consent of the University of Southampton Bioethics Committee. Rats were housed six per cage throughout the experiment and were maintained on a 12:12 light: dark cycle and provided with unlimited access to food and water for the duration of the experiment.

6.3.2 Kainate and BrdU Injections

A single intraperitoneal (IP) injection of BrdU (50 mg/kg, 10mg/ml in 0.007M NAOH/0.9% sterile saline; Sigma) was given to all rats after weighing. 24 hours after the BrdU injections 6 animals were sacrificed (see below) forming an un-operated control group. In the remaining 48 animals IP injections of Kainate (10 mg/kg dissolved in sterile 0.9% saline) were given to half of them (24 animals), a further 24 rats were given IP injections of sterile saline. Kainate treatment resulted in development of wet dog shakes, head nodding, facial clonus, continued rearing and falling, and generalized tonic-clonic seizures in all rats at 30-60 minutes after Kainate injections. Groups of 6 rats per condition were then sacrificed 6 hours, 24 hours, 48 hours, and 72 hours later.

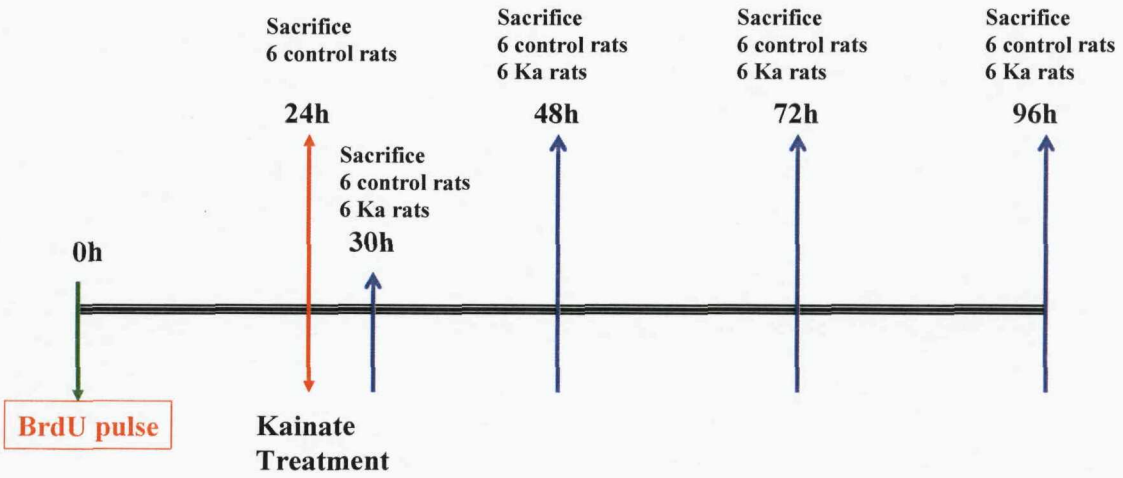


Figure 6.2 ***In vivo*** **experimental paradigm.** Adult rats received single intraperitoneal injection of BrdU (50 mg/kg) at 0h. 24 hours later 6 animals were sacrificed while 24 animals were injected with Kainate (10 mg/kg IP). At 30 h (6 h after Kainate treatment) 6 control animals and 6 Kainate treated animals were sacrificed. The same was carried out at 48 h, 72 h, and 96 h post BrdU pulse.

6.3.3 Sacrifice and tissue preparation

For all rats, sacrifice was by administration of a terminal dose of phenobarbitone. This was immediately followed by transcardiac perfusion initially with 50 mls 0.9% Saline, followed by 50 mls 4% paraformaldehyde (PFA) PH 7.4, whole brains were then swiftly removed and post fixed in 4% PFA and stored at 4°C until sectioned. Sections containing hippocampal formation were produced for immunohistochemistry on a Leica VT100M vibrotome cutting in the coronal plane at a thickness of 40 μm; approximately 100 sections, comprising the entire hippocampus from each brain, were collected and stored in a sequential manner - such that the position within the hippocampus of any individual section can be identified.

6.3.4 Immunohistochemistry

Single immunohistochemistry for caspase-3 was performed on sections dried onto gelatinized slides. Immunohistochemistry for Doublecortin (Dcx) was performed on free-floating sections. Double labelling of BrdU and Ki-67 was carried out on sections mounted and dried onto gelatinized slides. All immunohistochemistry was performed on systematically sampled tissue, with the initial section selected randomly and subsequent sections being taken at constant intervals thereafter, ensuring the entire dentate gyrus is sampled. BrdU and Ki-67 double labelling were performed on 12 sections per animal. Caspase-3 single labelling was performed on 3 sections per animal. Likewise, Dcx single labelling was also carried out on 3 sections per animal.

6.3.4.1 BrdU and Ki-67 double labelling

For double stain immunohistochemistry of BrdU and Ki-67, sections were mounted onto slides, which were placed in citrate buffer at room temperature (0.01M, PH 6.0; 300 ml) and heated in a microwave oven at full power for 3 minutes and 30 seconds and then simmered for further 6 minutes and 30 seconds at low power. Slides were removed from the microwave oven and left to stand on the bench in the hot citrate buffer for 25 minutes while it cooled. Slides were then transferred to 2M HCl and heated in a water

bath (37C°; 30 minutes), followed by repeated Tris-Buffered Saline (0.01M TBS; PH 7.6) washes, and incubation with 3% H_2O_2 and 10% methanol in distilled water for 30 minutes at room temperature to eliminate non-specific peroxidase activity. Further TBS washes were followed by 1 hour incubations in 2% horse serum and 3% goat serum in 0.25% Triton X-100 in TBS (TBS.T) (blocking solution) at room temperature. This was followed by overnight incubation with a cocktail of primary antibodies to BrdU (rat monoclonal 1:1000; Oxford Biotech) and Ki-67 (mouse monoclonal 1:50; Novocastra) in blocking solution at 4C°. Following multiple washes in blocking solution, tissue was incubated in TBS.T with biotinylated secondary antibody, raised against mouse (horse 1:200 Vector) for 1 hour at room temperature. TBS washes were repeated and HRP-ABC (1:200 Vector) in TBS.T was applied for 1 hour, further washing in TBS was followed by visualization with DAB (Vector). More TBS washes were followed by incubation with biotinylated secondary antibody, raised against rat (goat 1:200 Vector) for 1 hour at room temperature. TBS washes were repeated and Alkaline Phosphatase conjugated Streptavidin-biotin complex (AP-ABC, 1:200; Vector) in TBS.T was appied for 1 hour, further washing in TBS followed by visualization with Fast Red (Vector). Sections were then wet mounted in moviol.

6.3.4.2 Caspase-3 immunohistochemistry

All tissue processed for caspase-3 immunohistochemistry received triple washes in Phosphate-Buffered Saline (PBS; 0.1M, PH 7.4), followed by incubation with 3% H_2O_2 and 20% methanol in distilled water for 30 minutes at room temperature to eliminate non-specific peroxidase activity. Further PBS washes were followed by 1 hour incubation in 5% goat serum in 0.3% Triton X-100 (PBS.T) at room temperature. This was followed by 24 hours incubation with rabbit polyclonal cleaved caspase-3 primary antibody (1:200 Cell signaling) at 4C° in 5% goat serum in PBS.T. Following multiple washes in PBS, tissue was incubated in PBS.T with biotinylated secondary antibody raised against rabbit (goat 1:200 Vector) for 1 hour at room temperature. PBS washes were repeated and a Horseradish peroxidase conjugated Streptavidin-biotin complex (HRP-ABC, 1:200 Vectors) in PBS.T was applied for 1 hour, further washing in PBS

was followed by visualization with 3'3-Diaminobenzidine (DAB, brown; Vector) and a final set of washes. All sections were dehydrated through 1 minute washes in alcohol (70%, 90%, and 2x 100%) and then xylene before cover-slipping with DPX (Sigma).

6.3.4.3 Doublecortin immunostaining

For Dcx immunohistochemistry, free floating tissue received triple washes in Phosphate-Buffered Saline (PBS; 0.1M, PH 7.4), followed by incubation with 3% H_2O_2 and 20% methanol in distilled water for 30 minutes at room temperature to eliminate non-specific peroxidase activity. Further PBS washes were followed by 1 hour incubation in 10% horse serum in 0.3% Triton X-100 in PBS (PBS.T) at room temperature. This was followed by 24 hours incubation with Dcx primary antibody (1/200 Sc;8066 Santa Crus Biotechnology , Santa Crus, CA, USA) at 4C° in 10% horse serum in PBS.T. Following multiple washes in PBS, tissue was incubated in PBS.T with biotinylated secondary antibody raised against goat (horse 1:200 Vector) for 1 hour at room temperature. PBS washes were repeated and a Horseradish peroxidase conjugated Streptavidin-biotin complex (HRP-ABC, 1:200 Vectors) in PBS.T was applied for 1 hour, further washing in PBS was followed by visualization with DAB, brown (Vector) and a final set of washes. All sections were dehydrated through 1 minute washes in alcohol (70%, 90%, and 2x 100%) and then xylen before cover-slipping with DPX (Sigma).

6.3.5 Cell Quantification and Statistical analysis

A blind counting methodology was employed for all quantification, with slides coded such that the examiner was blind to the treatment of the tissue; the code was not broken until all cell counting was complete.

6.3.5.1 BrdU and Ki-67 cell counting

In all groups (Saline and Kainate-treated rats), BrdU and Ki-67 immuno-positive cells were exhaustively counted throughout the subgranular zone (SGZ), granule cell layer

(GCL), hilus, and Molecular layer (Ml). The SGZ was defined as a two-nucleus-wide band below the apparent border between the granule cell layer (GCL) proper and the hilus (Kempermann, Gast et al. 2002). Masks of the SGZ, GCL, hilus, and ML were generated with the contour tool in StereoInvestigator™ (Ver. 5.0; Microbrightfield) software package on each dentate gyrus (DG). Using 20x objective magnification on a Stereology Microscope (Dialux 22, Leitz) BrdU and Ki-67 Immuno-positive cells were then counted in each area. While Ki-67 staining was visualized under bright-phase microscopy, the presence of fluorescent filters on this microscope enabled us to visualize BrdU staining in red on the same area of interest. Counts from all sections (every 8th section per brain) in each animal were added, this total was then multiplied by the intersectional interval (8) to calculate a count per dentate, from which a mean and standard error of the mean were obtained. Overestimation might arise because all cells in a 40μm section are counted. Cells that are near to the cut surface of the section can be cut in two by the sectioning process, these cells would then be counted twice if all sections were counted, when only one cell existed. This overestimation would also apply if the cell counts expressed per 40μm section. In order to minimize over estimating cell counts, we used the optical dissector methods in order to count objects in three dimensions; a depth of focus is defined within the counting area (typically 20 microns), any object that comes into focus in those 20 microns is counted. The use of the optical dissector produces a more accurate estimate because in one of the two sections cells that have been cut in half will start in focus and so will not be counted, in the other section the cell will not start in focus but appear in the section and so will be counted, ensuring all cells are now only counted once.

6.3.5.2 Caspase-3 immuno-positive cell counting

In saline and Kainate-treated rats, Caspase-3 immuno-positive cells were exhaustively counted throughout the SGZ, GCL, hilus, and Ml as described in the previous section (**6.3.5.1**). Cell counts were performed on every 36th section per brain which resulted in systemic sampling of 3 sections per animal. The counts from these sections were added, this total was then multiplied by the intersectional interval (36) to calculate a count per

dentate, from which a mean and standard error of the mean were obtained. Sections were taken from both Kainate and saline injected rats at 0 hours, 6 hours and 48 hours time points. We used bright-field Stereology Microscope (Dialux 22, Leitz) to count caspase-3 immunostained cells. An objective of 20x magnification was used to count caspase-3 positive cells.

6.3.5.3 Doublecortin immunostained cell counting

In saline and Kainate conditions, Doublecortin (Dcx) cell counts were exhaustively carried out throughout the SGZ. Quantification was performed on every 36^{th} section per brain, resulting in systematic sampling of three 40μm sections per animal. The sum of these cell counts was then multiplied by the intersectional interval (36) to calculate a count per dentate, from which a mean and standard error of the mean were obtained. Sections were taken from both Kainate and saline injected rats at 72 hours time point. Bright-field Stereology Microscope (Dialux 22, Leitz) equipped with 40x oil objective was used to count Dcx immuno-positive cells.

6.3.5.4 Statistical Analysis

Two variables are considered in all analysis, treatment type (Kainate or saline injections) (considered as column factor), and time after treatment (row factor). Comparisons are made between brains (cell counts in both dentates) in saline injected groups and Kainate injected groups.

Multiple two-way ANOVA's were used to investigate responses to the factors mentioned above, with Bonferroni post hoc comparison testing for specific differences between means of the column factors at each time point (row factor) using Prism (GraphPad, San Diego, CA). All data are expressed as means ± standard error of the mean. These values are expressed per brain (two dentates), and all statistical differences reported are Bonferroni post hoc comparisons unless otherwise stated.

6.4 Results

Generalized tonic-clonic seizures occurred in all rats treated with 10 mg/kg Kainate, and the severity of the status epilepticus was the same among all animals. Two out of 24 rats died during the course of the experiment (72 hours). One rat died 6 h after Kainate treatment. Another one followed the same fate after 48 h of Kainate injection. Postmortem was carried out where perforated bowels and/or peritonitis were excluded as the cause of death. These animals were therefore excluded from subsequent analysis. The remaining animals were distributed across the time points as follows: 0 hours (6 controls), 6 hours (6 control and 6 Kainate), 24 hours (6 control and 5 Kainate), 48 hours (6 control and 6 Kainate), and 72 hours (6 control and 5 Kainate). We studied the effects of Kainate/seizures on cell proliferation (by measuring Ki-67 immuno-positive cells) and a pre-labelled 'clone' of proliferating cells in the subgranular zone (SGZ), granule cell layer (GCL), hilus, and molecular layer (Ml). We also measured caspase-3 immuno-positive cells at 0 hours, 6 hours, and 48 hours time point in both Kainate and saline conditions in the different dentate gyrus (DG) sub-areas (SGZ, GCL, hilus, and Ml). We further examined the effect of 72 hours of Kainate/seizures on Doublecortin (Dcx) immunostained cells. Kainate/seizures induced cell proliferation in the SGZ after 72 hours; therefore, it is highly unlikely that there will be an increase in neurogenesis before this time point. As such we examined the effects of Kainate/seizures on Dcx expressing cells after 72 hours.

6.4.1 The effects of Kainate/seizures in the subgranular zone (SGZ)

6.4.1.1 Kainate/seizures enhanced cell proliferation in the SGZ

Cell proliferation induced by intraperitoneal injection of Kainate was monitored by immunohistochemistry staining for Ki-67. This marker labels cells that are dividing/ cycling at the time of sacrifice.

The total number of Ki-67 immuno-positive cells overall was increased in animals receiving Kainate injections (two-way ANOVA, $p < 0.05$), with significant increase

(Kainate, 23070 ± 1361 cells per SGZ per brain, vs. saline, 15620 ± 453 cells per SGZ per brain) (two-way ANOVA with Bonferroni post hoc test, $p < 0.01$) after 72 hours of Kainate treatment (**Figure 6.3**). Sample image is displayed in **Figure 6.4**.

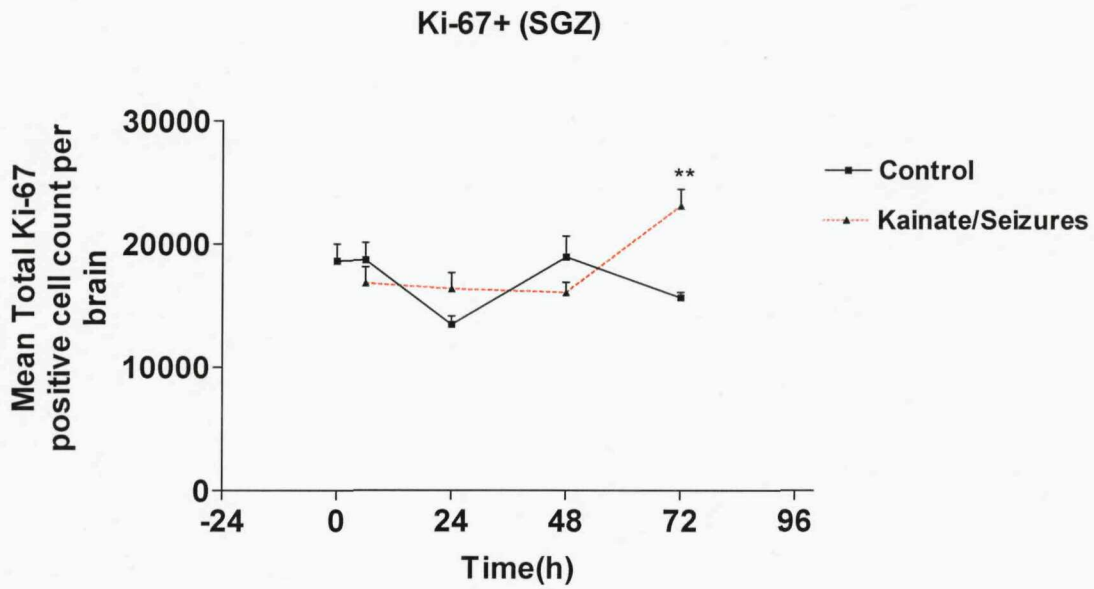


Figure 6.3 Kainate increased SGZ cell proliferation 72 hours after the treatment. Cell proliferation at the time of sacrifice was investigated using Ki-67 which labels cells that are in the cell cycle. There was a significant increase in Ki-67 immunostained cells in the SGZ after 72 hours of Kainate (dotted lines) treatment and in comparison with saline (solid lines) treated animals. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8th brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc test. **$p<0.01$

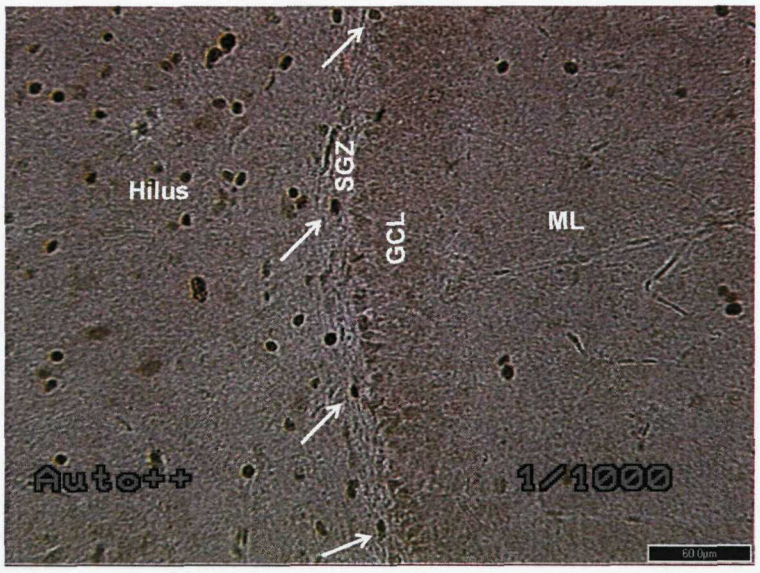


Figure 6.4 Cell proliferation in the SGZ increased after 72 hours of Kainate/seizures. Ki-67 immunohistochemistry was used to measure point cell proliferation between 0 h and 72 h. We demonstrated a significant increase in the number of Ki-67 immuno-positive cells in the SGZ at 72 hours time point in Kainate injected animals when compared to saline injected ones. Every 8th section was sampled from each brain and exhaustive cell counting for Ki-67 expressing cells was carried out. Counts from all sections in each animal were added, this total was then multiplied by the intersectional interval (8) to calculate a count per dentate, from which a mean and standard error of the mean were obtained. We used bright-field Stereology Microscope (Dialux 22, Leitz) equipped with 20x objective to image and count Ki-67 positive cells. SGZ = subgranular zone; GCL = granule cell layer; ML = molecular layer. White arrows show Ki-67 immuno-positive cells in the SGZ. Scale bar = 60µm.

6.4.1.2 Kainate/seizures did not affect the survival or proportional proliferation of the pre-labelled 'clone' of precursor cells in the SGZ

The number of pre-labelled proliferating progenitor cells in SGZ was measured by counting BrdU immuno-positive cells in both Kainate treated animals and saline injected animals.

In the SGZ, there was no change in the number of BrdU incorporated cells at any of the time points examined when comparing Kainate treated animals with saline ones (**Figure**

6.5 A) suggesting that Kainate induced status epilepticus/ seizure did not differentially affect the survival of these cells compared to controls. Interestingly, we found that Kainate/seizures increased the number of cells that incorporated BrdU and remained in the cell cycle (BrdU+ Ki-67+) (Kainate/seizures 2131 ± 578.5 cells per SGZ per brain, vs. saline, 1323 ± 183.3 cells per SGZ per brain) (**Figure 6.5 B**). Furthermore, the proportion of cells that was tagged with BrdU with respect to the cells that were in the cell cycle at the time of sacrifice (BrdU+ and Ki-67+/Ki-67+) in Kainate injected animals was similar to the proportion in saline injected rats (**Figure 6.5 C**). In this region of interest, we found approximately 10% of cycling cells (Ki-67+) were pre-labelled with BrdU under both Kainate and saline conditions. There was a tendency towards an increase in the proportion of pre-labelled 'clone' of cells with respect to the total numbers of the pre-labelled 'clone' when comparing the Kainate group with the saline group (**Figure 6.5 D**). However, this increase was statistically insignificant (two-way ANOVA with Bonferroni post hoc test, $p > 0.05$).

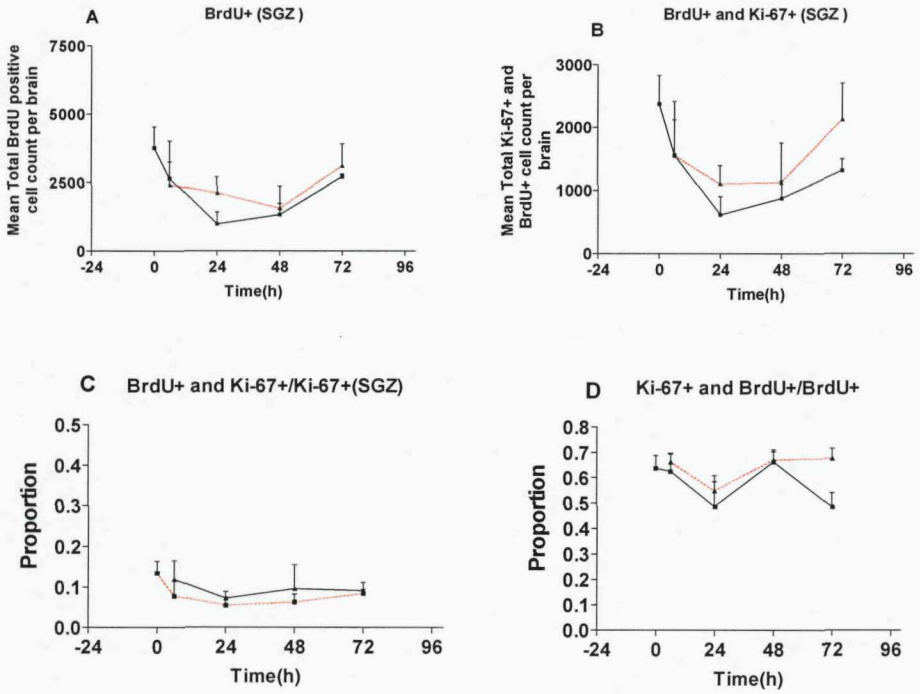


Figure 6.5 Kainate did not affect the survival or proportional proliferation of SGZ pre-labelled 'clone' of cells. A 'clone' of proliferating progenitor cells was tagged with BrdU 24 hours before treating the animals with Kainate. Immunohistochemistry was used to detect BrdU incorporated cells at each time point. The proportion of cells that incorporated BrdU and continued to divide with respect to the total cells that were dividing at the time of sacrifice was also measured (BrdU+ and Ki-67+/Ki-67+). There was no significant effect for Kainate (dotted lines) on this 'clone' of proliferating cells when compared to saline (control) (solid lines) treated animals over time and at any of the time points. A) There was no significant increase in the numbers of BrdU pre-labelled cells in the Kainate (dotted lines) group and in comparison with the saline (solid lines) group. B) There was a tendency towards an increase in the number of cells that were BrdU+ and Ki-67+ at 72 h time point in the Kainate (dotted lines) group when compared to the saline (solid lines) group. C) The proportion of pre-labelled 'clone' of proliferating cells with respect to the total cells that were dividing at the time of sacrifice did not change in Kainate (dotted lines) treated animals when compared to saline (solid lines) injected rats. D) There was a trend towards an increase in the proprotion of pre-labelled 'clone' of cells that continued to divide with respect to the total number of pre-labelled cells when comparing the Kainate (dotted lines) group with the saline (solid lines) group. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8th brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc test.

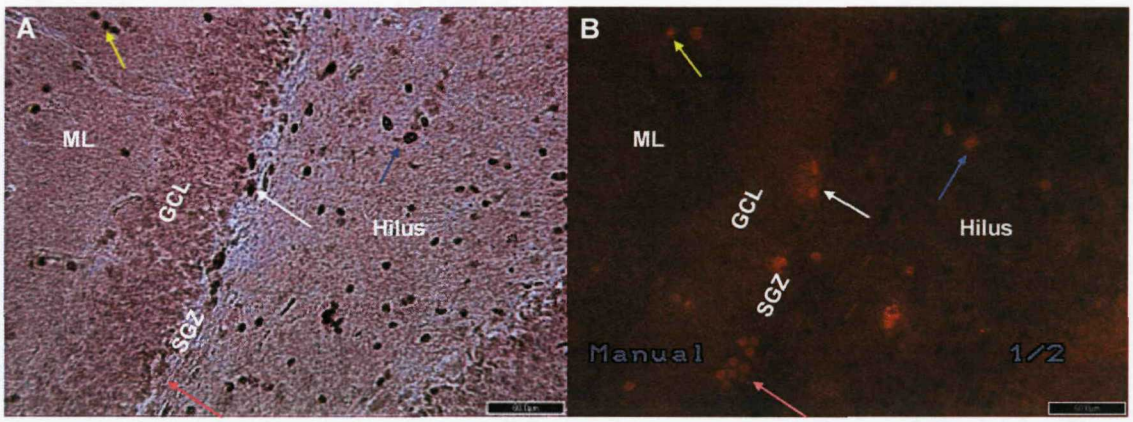


Figure 6.6 Kainate/seizures had no effect on the proportion of proliferating pre-labelled clone of cells in the SGZ. BrdU was injected to all animals 24 hours before inducing seizures by Kainate in half of them. Immunohistochemistry was used to identify BrdU incorporated cells. Double-labelling of cells for Ki-67 and BrdU was carried out to determine cells that continued to divide (BrdU+ and Ki-67+), cells that exited the cell cycle and became postmitotic (BrdU+ and Ki-67-), and cells that were recruited to the cell cycle (BrdU- and Ki-67+). There was no effect for Kainate/seizures on the pre-labelled 'clone' of proliferating cells in the SGZ. Every 8th section was sampled from each brain and exhaustive cell counting for Ki-67 expressing cells (A) as well as BrdU positive cells (B) was carried out. Counts from all sections in each animal were added, this total was then multiplied by the intersectional interval (8) to calculate a count per dentate, from which a mean and standard error of the mean were obtained. Using 20x objective magnification on a Stereology Microscope (Dialux 22, Leitz) BrdU and Ki-67 Immuno-positive cells were counted in each area. While Ki-67 staining was visualized under bright-phase microscopy, the presence of fluorescent filters on this microscope enabled us to visualize BrdU staining in red on the same area of interest. SGZ = subgranular zone; GCL = granule cell layer; ML = molecular layer. White arrows show BrdU+ and Ki-67+ in the SGZ. Blue arrows demonstrate BrdU+ and Ki-67+ in the hilus. Yellow arrows show BrdU+ and Ki-67+ in the molecular layer. Pink arrows demonstrate BrdU+ and Ki-67- (postmitotic) cells in the SGZ and inner third of GCL. Scale bar = 60μm.

6.4.1.3 BrdU Un-labelled proliferating cell numbers increased in the SGZ after Kainate/seizures

Cells that expressed the proliferative marker Ki-67 at the time of sacrifice but did not express BrdU were termed as un-labelled proliferating progenitor cells. We measured the un-labelled proliferating progenitor cells in the dentate gyrus SGZ by identifying cells that were Ki-67 immuno-positive but BrdU immuno-negative at the following time points (0h, 6h, 24h, 48h, and 72h).

The numbers of un-labelled proliferating SGZ (Ki67+ and BrdU-) progenitor cells increased significantly after 72 hours of Kainate treatment (20940 ± 1118 cells/SGZ/brain versus 14300 ± 333 cells/SGZ/brain in saline treated animals) (two-way ANOVA with Bonferroni post hoc test, $p < 0.01$) (**Figure 6.7 A**). However, there was no proportional increase in Ki-67 only positive cells with respect to all cells in the cell cycle at any of the time points examined (**Figure 6.7 B**).

These results suggest that Kainate/seizures increased proliferation of the SGZ un-labelled precursor cells. In the SGZ, 20940± 1118 cells/brain out of 23070 ± 1361 cells/brain were Ki-67 immunopositive after 72 hours of Kainate insult when compared to the basal proliferation (Ki-67 immunopositive) of cells under control conditions (14300 ± 333 cells/SGZ/brain out of 15620 ± 453 cells/SGZ/brain). However, these results along with the previous section's results imply that both pre-labelled and un-labelled 'clones' of proliferating cells are contributing equally to cell division after Kainate/seizures and, therefore, there is no proportional increase in either the pre-labelled or the un-labelled 'clones' of proliferating precursor cells at any of the time points examined.

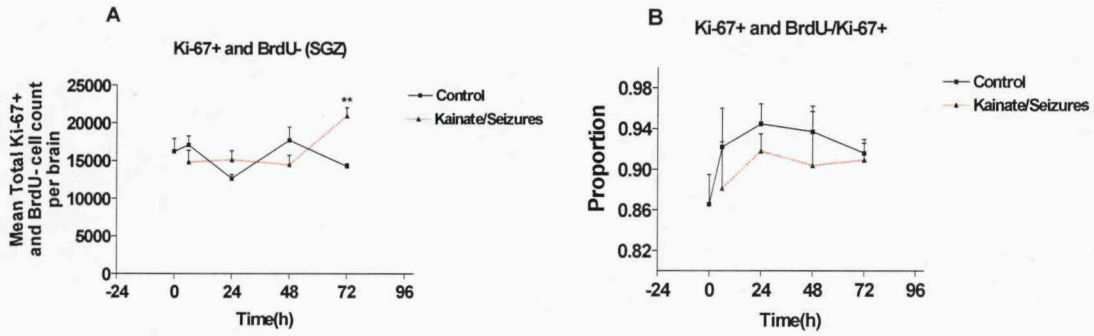


Figure 6.7 Kainate increased un-labelled proliferating cells in the SGZ. A clone of proliferating progenitor cells was tagged with BrdU 24 hours before treating the animals with Kainate. Immunohistochemistry double-labelling for BrdU and Ki-67 was used to detect Ki-67+ and BrdU- cells at each time point. Cells that were Ki-67+ but BrdU- were considered as un-labelled proliferating cells. A) There was a significant increase in the un-labelled proliferating cells in the SGZ in Kainate (dotted lines) treated animals when compared to saline (solid lines) injected rats. B) There was no proportional increase in Ki-67 only positive cells with respect to total Ki-67 positive cells after Kainate treatment at any of the time points. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8th brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc test. **$p<0.01$

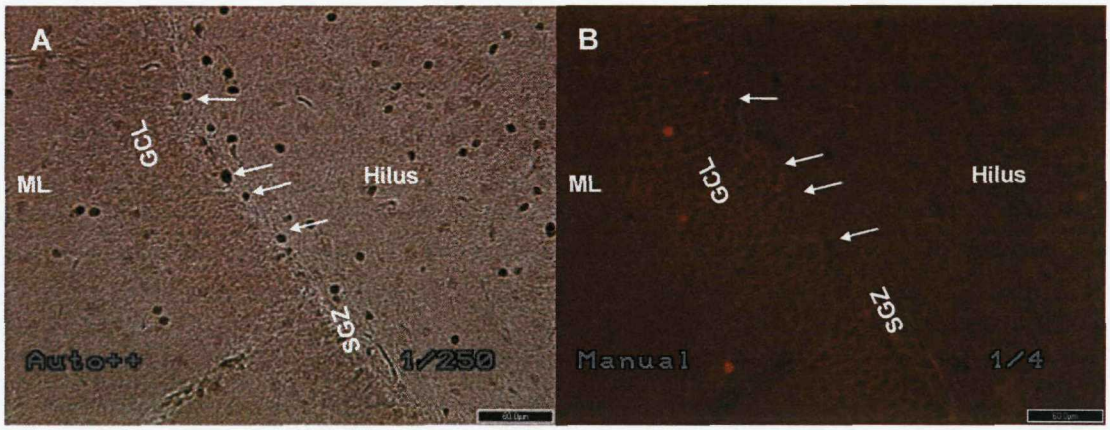


Figure 6.8 Kainate/seizures increased both pre-labelled and un-labelled precursor cell proliferation in the SGZ. BrdU was injected to all animals 24 hours before inducing seizures by Kainate in half of them. Immunohistochemistry was used to identify BrdU incorporated cells. Double-labelling of cells for Ki-67 and BrdU was carried out to determine cells that were recruited to the cell cycle by counting BrdU- and Ki-67+. 72 hours of Kainate/seizures recruited quiescent precursor cells in SGZ to become actively dividing. Every 8th section was sampled from each brain and exhaustive cell counting of Ki-67 expressing cells (A) that do not express BrdU (B) was carried out. Counts from all sections in each animal were added, this total was then multiplied by the intersectional interval (8) to calculate a count per dentate, from which a mean and standard error of the mean were obtained. Using 20x objective magnification on a Stereology Microscope (Dialux 22, Leitz) BrdU and Ki-67 Immuno-positive cells were counted in each area. While Ki-67 staining was visualized under bright-field microscopy, the presence of fluorescent filters on this microscope enabled us to visualize BrdU staining in red on the same area of interest. SGZ = subgranular zone; GCL = granule cell layer; ML = molecular layer. White arrows show Ki-67+ and BrdU- in the SGZ. Scale bar = 60μm.

6.4.1.4 Kainate/seizures tended to reduce the postmitotic sub-population of BrdU pre-labelled cells in SGZ

Pre-labelling a 'clone' of proliferating progenitor cells and the immunohistochemical detection of both BrdU and Ki-67 allowed us to investigate the effects of Kainate/seizures on cells that have become post-mitotic (exited the cell cycle). We measured cells that were BrdU+ (incorporated BrdU at some stage) but Ki-67- (exited the cell cycle) at the time of sacrifice in both Kainate injected rats and saline injected group. Cells that were BrdU+ and Ki-67- were considered as postmitotic.

In the SGZ, there was no significant effect for Kainate/seizures on number of cells that became postmitotic (**Figure 6.9 A**). Approximately, one-third of BrdU incorporated cells became postmitotic 72 hours after Kainate/seizures when compared to around half of BrdU incorporated cells that became post mitotic under control conditions (Kainate 35.6 ± 5.6%, vs. saline conditions 51.5 ± 6.0 %) (**Figure 6.9 B**). Although this difference was not statistically significant there is a trend towards a reduction in the Kainate group.

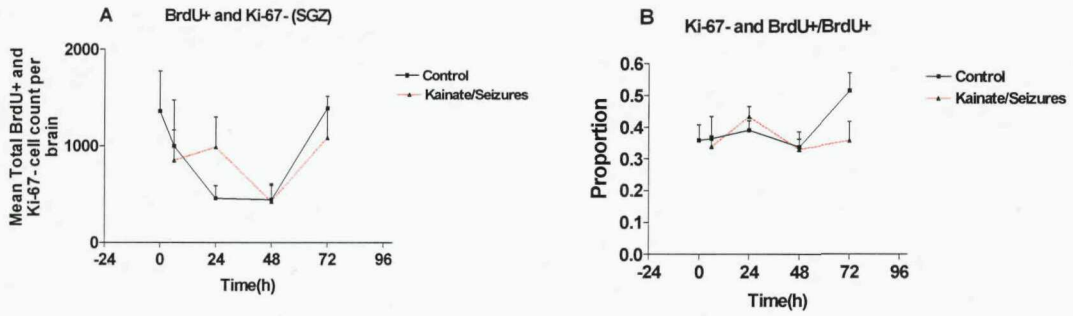


Figure 6.9 Kainate/seizures tended to reduce the SGZ postmitotic sub-population of cells. A 'clone' of proliferating progenitor cells was tagged with BrdU 24 hours before treating the animals with Kainate. Immunohistochemistry double-labelling for BrdU and Ki-67 was used to detect BrdU+ and Ki-67- cells at each time point. Cells that were Ki-67- but BrdU+ were considered as pre-labelled proliferating cells that became postmitotic. A) There was no significant change in the numbers of pre-labelled proliferating cells in the SGZ that became postmitotic in Kainate (dotted lines) treated animals when compared to saline (solid lines) injected rats. B) There was no significant proportional change in the pre-labelled 'clone' of cells that became postmitotic, although there was a trend towards a decrease in this sup-population of cells in Kainate group. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8^{th} brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc test.

6.4.1.5 Kainate/seizures did not enhance cell death in the SGZ

Cell death is a well documented consequence of Kainate-induced seizures within the hippocampus. It is generally reported as being localized in the CA3 pyramidal neurons and to lesser extent in CA1 area. However, some studies reported cell death in the dentate, and in chapter 5 we have identified cell death of hippocampal progenitor cells and their neuronal progeny cells by immunohistochemistry against the apoptotic cell death marker caspase-3. Furthermore, as research has suggested that cell proliferation may be driven by cell death, possibly of progenitor/precursor cells, the 'clonal' proliferation paradigm is ideally suited to further study a potential correlation between proliferation and cell death.

We did not observe significant proliferative effects for Kainate/seizures on the SGZ cells before 72 hours. Therefore, we decided to investigate whether cell death counterbalanced cell proliferation and/or an increase in cell death preceded the enhanced cell proliferation seen at 72 hours. In order to examine this hypothesis, we have investigated the effects Kainate/seizures on caspase-3 immuno-positive cells at the following time points (0 hours, 6 hours, and 48 hours).

There was no significant change in the number of SGZ cells that expressed caspase-3 at any time up to and including 48 hours post seizures when comparing Kainate injected animals to saline injected animals (**Figure 6.10**). However, there is a trend towards a reduction in caspase-3 immunoreactive cells in Kainate group. Sample images of caspase-3 immunoreactive cells are shown in **Figure 6.11**.

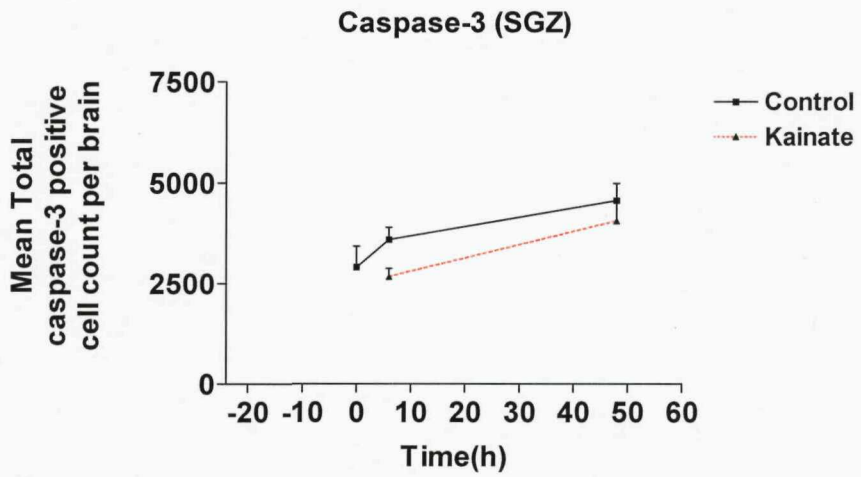


Figure 6.10 Kainate/seizures did not enhance SGZ cell death. We have investigated cell death using immunohistochemistry for activated caspase-3. Every 36th section per brain from 0 hours, 6 hours and 48 hours time points was sampled and immunostained for caspase-3 from both saline (control) (solid lines) injected and Kainate (dotted lines) injected animals. Activated caspase-3 cells within the SGZ were then measured. There was no change in the number of caspase-3 activated SGZ cells when comparing Kainate (dotted lines) injected rats to saline (solid lines) injected ones. Values are expressed as mean ± SEM, means represent the total cell count in the SGZ per brain (the two dentates) and every 36th brain section was sampled from each animal at the above mentioned time points. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc test.

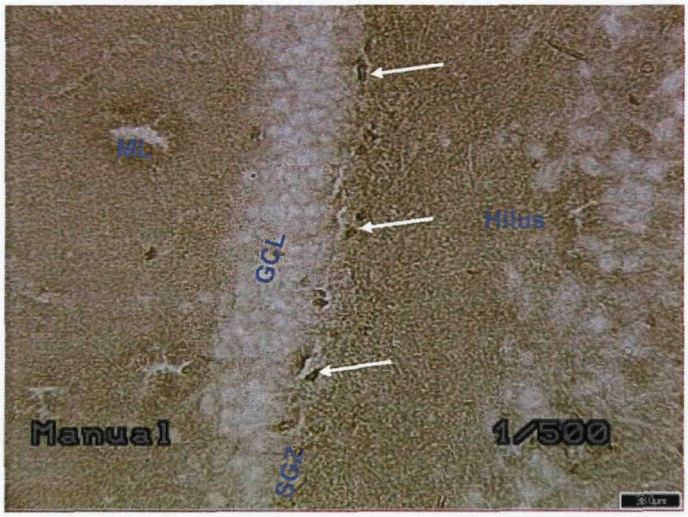


Figure 6.11 Kainate/seizures did not increase caspase-3 immunopositive cells in the SGZ. Immunohistochemistry was used to identify Caspase-3 activated cells and thus measure cell death in Kainate and saline. Every 36th section was sampled from each brain and exhaustive cell counting for caspase-3 immuno-positive cells was carried out. Counts from all sections in each animal were added, this total was then multiplied by the intersectional interval (36) to calculate a count per dentate, from which a mean and standard error of the mean were obtained. Using 20x objective magnification on a Stereology Microscope (Dialux 22, Leitz) caspase-3 Immuno-positive cells were counted in each area. Caspase-3 staining was visualized under bright-field microscopy. SGZ = subgranular zone; GCL = granule cell layer; ML = molecular layer. White arrows show activated caspase-3 cells in the SGZ. Scale bar = 38μm.

6.4.2 Kainate/seizures effects in the granule cell layer (GCL)

6.4.2.1 Kainate/Seizures increased the GCL cell proliferation

Kainate/seizures-induced cell proliferation in the dentate gyrus GCL was measured by immunohistochemistry staining for Ki-67. At 48 hours, there was a significant increase in Ki-67 expressing cells in the GCL in Kainate injected animals when compared to saline injected group (Kainate 20770 ± 618 cells/GCL/brain, vs. saline14690 ± 1580 cells/GCL/brain) (two-way ANOVA with Bonferroni post hoc test, $p< 0.01$) (**Figure 6.12**). A similar significant increase was also found at 72 hours time point in Kainate injected animals when compared to saline treated animals (13150 ± 1793 cells per GCL

per brain in saline conditions, vs. 23060 ± 2226 cells per GCL per brain in Kainate treated conditions) (two-way ANOVA with Bonferroni post hoc test, $p < 0.001$) (**Figure 6.12**).

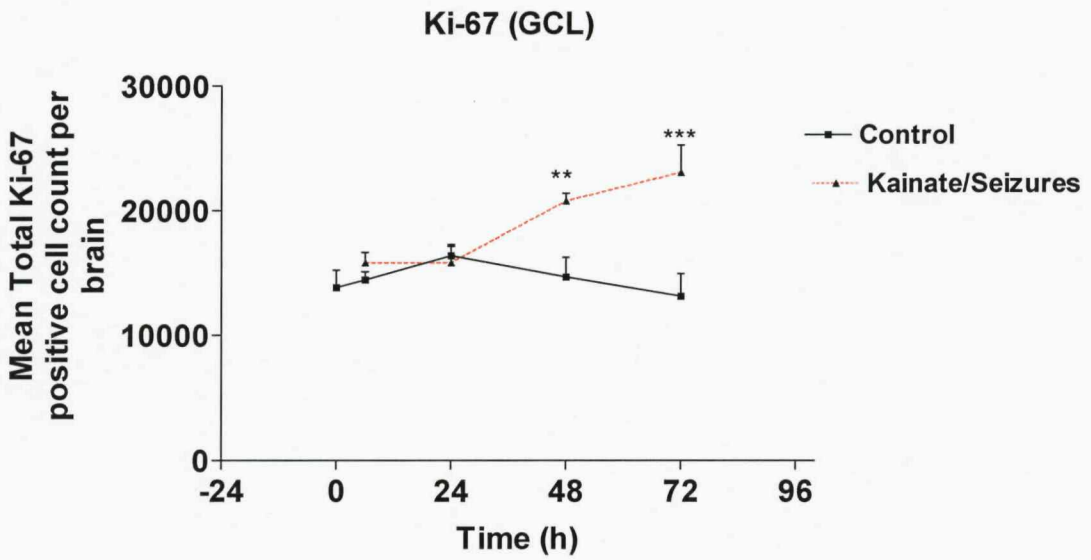


Figure 6.12 Kainate/seizures increased GCL cell proliferation. Cell proliferation at the time of sacrifice was investigated using Ki-67 which labels cells that are in the cell cycle. There was a significant increase in Ki-67 immunostained cells in the GCL after 48 and 72 hours of Kainate (dotted lines) treatment and in comparison with saline (solid lines) treated animals. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8th brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc test. **$p<0.01$, ***$p<0.001$

6.4.2.2 Kainate/seizures enhanced the proliferation of a pre-labelled 'clone' of GCL proliferating progenitor cells

We used BrdU to label a 'clone' of proliferating progenitor cells 24 hours before treating animals with Kainate. BrdU incorporated cells that were in the S-phase of the cell cycle before Kainate insult were then detected by immunohistochemistry techniques before

and after Kainate treatment. This paradigm enabled us to investigate the effects of Kainate/seizures on the proliferation of this 'clone' of progenitor cells.

Although there was a slight increase in BrdU immuno-positive cells at 24 hours time point in Kainate injected animals when compared to saline injected ones, this increase was statistically insignificant (**Figure 6.13 A**). However, Kainate treatment significantly increased the number of BrdU labelled cells (13370 ± 4167 cells/GCL/brain versus 2624 ± 1376 cells/GCL/brain in saline treated animals) after 72 hours of Kainate injections (two-way ANOVA with Bonferroni post hoc test, $p < 0.001$) (**Figure 6.13 A**).

Interestingly, in the GCL, while 5.5 ± 2.6 % of proliferating (Ki-67+) cells were pre-labelled with BrdU in saline treated rats at 24 hours time point, 16.1 ± 2.8 % of this 'clone' of cells continued to divide in Kainate treated rats at the same time point (**Figure 6.13 B**). This increase in the pre-labelled 'clone' of proliferating cells was statistically significant (two-way ANOVA with Bonferroni post hoc test, $p < 0.05$). Furthermore, a significant increase in the proportion of pre-labelled 'clone' of proliferating progenitor cells was also found 72 hours after Kainate injection (Kainate, 31.9 ± 7.9 %, vs. saline, 5.8 ± 1.1 %) (two-way ANOVA with Bonferroni post hoc test, $p < 0.001$) (**Figure 6.13 B**). Interestingly, there was no difference between control and Kainate animals at 48 h post injection, suggesting that this pre-labelled clone had exited the cell cycle by this time point, followed by either a reentry by 72 hours or recruitment of a different sub 'clone' of the pre-labelled population. Intriguingly, the proportion of the pre-labelled clone that was in the cell cycle (Ki-67+BrdU+/BrdU+) decreased at 24 h returned to control levels at 48 h and increased at 72 h suggesting a progressive recruitment of the pre-labelled clone by 72 h (**Figure 6.13 C**).

These results show a significant effect of Kainate/seizures on the pre-labelled 'clone' of proliferating cells that enhanced their proliferation 24 h after the treatment. However, there was no difference in the proliferation of this 'clone' of cells at the 48 h time point which may suggest either dilution of the BrdU below detection or a cell cycle time of less than 24 h. The former is unlikely given the larger increase in BrdU labelled cells seen at 72 hours. Importantly, the pre-labelled 'clone' of cells proliferated more under Kainate conditions when compared to saline conditions at 72 hours time point,

suggesting that this clone of cells re-entered the cell cycle at least one more time and possibly were cycling faster.

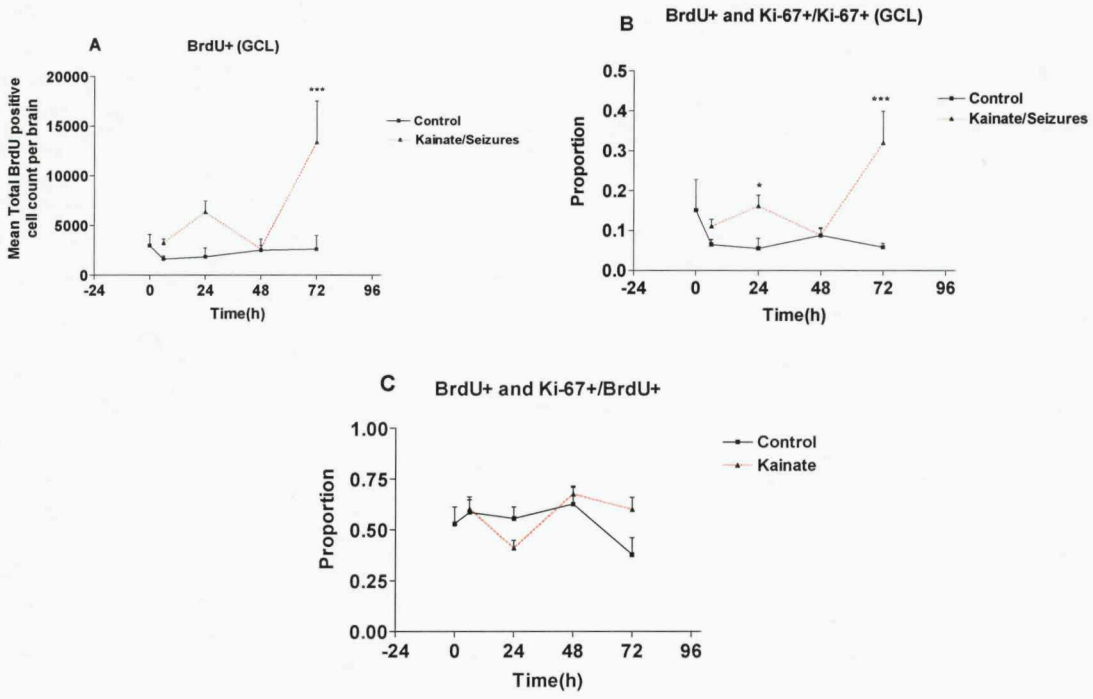


Figure 6.13 Kainate increased the GCL pre-labelled 'clone' of proliferating cells. A 'clone' of proliferating progenitor cells was tagged with BrdU 24 hours before treating the animals with Kainate. Immunohistochemistry was used to detect BrdU incorporated cells at each time point. The proportion of cells that incorporated BrdU and continued to divide with respect to the total cells that were diving at the time of sacrifice was also measured (BrdU+ and Ki-67+/Ki-67+). There was a significant effect for Kainate (dotted lines) on this 'clone' of proliferating cells when compared to saline (control) (solid lines) treated animals at 72 hours time point. A) There was a significant increase in the number of BrdU incorporated cells (pre-labelled 'clone') when comparing Kainate (dotted lines) treated animals with saline (solid lines) treated animals at 72 h time point. B) The proportion of pre-labelled 'clone' of proliferating cells with respect to the total cells that were dividing at the time of sacrifice significantly increased in Kainate (dotted lines) treated animals when compared to saline (solid lines) injected rats. C) There was no significant change in the proportion of cells that pre-labelled with BrdU and continued to divide with respect to the total number of cells that was pre-labelled with BrdU. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8th brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc test. * $p<0.05$, *** $p<0.001$

6.4.2.3 There were more GCL post-mitotic cells after Kainate/seizures but there was a trend towards a lower proportion of BrdU cells becoming post-mitotic after Kainate/seizures

In our *in vivo* paradigm, a pre-labelled 'clone' of GCL proliferating progenitor cells that exited the cell cycle (became postmitotic) was immunohistochemically detected by measuring BrdU immuno-positive and Ki-67 immuno-negative cells at the time of sacrifice in Kainate and saline injected animals. Cells that were BrdU+ and Ki-67- were considered as postmitotic.

Kainate/seizures increased the GCL numbers of cells that were BrdU+ and Ki-67- 72 hours after Kainate treatment when compared to saline treated animals (**Figure 6.14 A**). This increase was statistically significant (Kainate, 5616 ± 2185 cells/GCL/brain, vs. saline, 1856 ± 1184 cells/GCL/brain) (two-way ANOVA with Bonferroni post hoc test, $p< 0.05$). Interestingly, there was a tendency towards a decrease in the proportion of BrdU only cells (postmitotic) with respect to the total number of cells that was pre-labelled with BrdU (**Figure 6.14 B**). However, this decrease just failed to reach statistical significance (two-way ANOVA with Bonferroni post hoc test, $p= 0.065$).

These results demonstrated that although Kainate/seizures increased the number of postmitotic cells at 72 h time point, it tended to decrease the proportion of postmitotic sub-population of cells in the GCL. This may imply an enhancement of cell cycle re-entry of the pre-labelled 'clone' of precursors or a fraction of them.

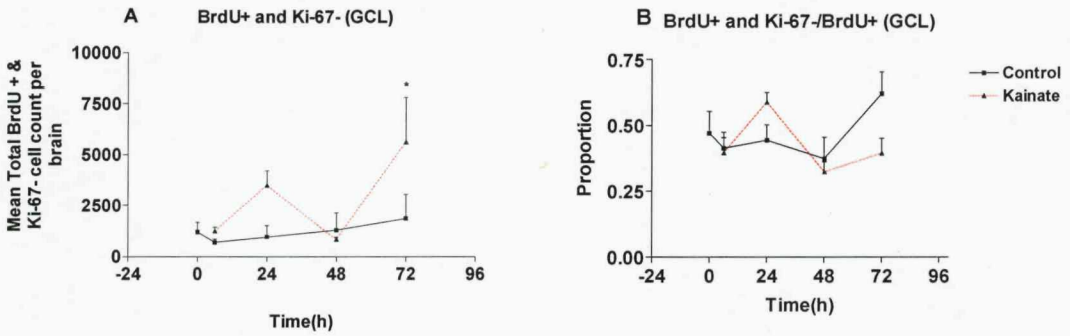


Figure 6.14 Kainate/seizures tended to reduce the proportion of GCL postmitotic cell population. A 'clone' of proliferating progenitor cells was tagged with BrdU 24 hours before treating the animals with Kainate. Immunohistochemistry double-labelling for BrdU and Ki-67 was used to detect BrdU+ and Ki-67- cells at each time point. Cells that were Ki-67- but BrdU+ were considered as pre-labelled proliferating cells that became postmitotic. A) Kainate (dotted lines) treatment increased the number of pre-labelled sub-population of proliferating cells that exited the cell cycle at 72 hours time point when compared to saline (solid lines) conditions. B) Kainate/seizures tended to reduce the proportion of cells that became postmitotic at 72 h time point. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8th brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc. * $p<0.05$

6.4.2.4 Although BrdU un-labelled proliferating cells increased transiently in the GCL after Kainate/seizures, their proportional contribution to total cell proliferation was decreased

Cells that were Ki-67 immuno-positive at the time of sacrifice and did not express BrdU were termed as un-labelled proliferating progenitor cells. We measured the un-labelled proliferating progenitor cells in the GCL of the DG by identifying cells that were Ki-67 immuno-positive but BrdU immuno-negative at the following time points (0h, 6h, 24h, 48h, and 72h).

Although 48 hours of Kainate treatment significantly increased the GCL number of Ki-67+ and BrdU- immuno-positive cells to (18950 ± 787 cells/GCL/brain versus 13460 ±

1665 cells/GCL/brain in saline conditions) (two-way ANOVA with Bonferroni post hoc test, $p< 0.05$) (**Figure 6.15 A**), there was no significant effect for Kainate treatment on the un-labelled proliferating cells at any of the other time points. However, Kainate/ seizures reduced the proportion of un-labelled 'clone' of cells with respect to the total cell numbers in the cell cycle at 24 and 72 h time points (**Figure 6.15 B**). For example, at 24 h 83 ± 2.7 % of cells were un-labelled in Kainate group, while 94.5 ± 2.5 % of cells were un-labelled in the control group. This difference was statistically significant (two-way ANOVA with Bonferroni post hoc test, $p< 0.05$). Likewise, 72 h of Kainate/seizures dramatically decreased the proportion of un-labelled sub-population of cells (Kainate 68.1 ± 1.0 %, vs. saline, 94.1 ± 1.1 %) (two-way ANOVA with Bonferroni post hoc test, $p< 0.001$).

These results are consistent with **section 6.4.2.2** and imply perhaps a significant recruitment of the pre-labelled 'clone' of cells to enter the cell cycle again and divide.

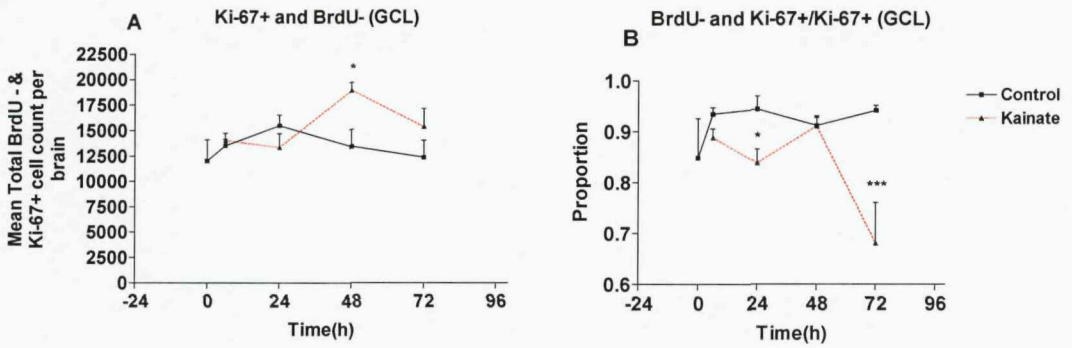


Figure 6.15 Kainate/seizures transiently increased the un-labelled proliferating cells in the GCL; however, it decreased their proportion. A 'clone' of proliferating progenitor cells was tagged with BrdU 24 hours before treating the animals with Kainate. Immunohistochemistry double-labelling for BrdU and Ki-67 was used to detect Ki-67+ and BrdU- cells at each time point. Cells that were Ki-67+ but BrdU- were considered as un-labelled proliferating cells. A) Although Kainate (dotted lines) treatment increased the proliferation of the un-labelled sub-population of cells at 48 hours time point, there was no significant effect for Kainate (dotted lines) treatment on this sub-population of cells at any other time point and in comparison with saline (solid lines) conditions. B) There was a significant reduction in the proportion of pre-labelled 'clone' of cells at 24 and 72 h time points in the Kainate (dotted lines) group. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8^{th} brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc. * $p<0.05$, *** $p<0.001$

6.4.2.5 Caspase-3 immunostained cells increased after Kainate/seizures over time in the GCL

We have demonstrated that Kainate-induced seizures are proliferative in the dentate gyrus GCL as indicated by the increase in Ki-67 immuno-positive cells after 48 and 72 hours of the treatment. Moreover, the proportion of BrdU incorporated cells that continued to divide with respect to the total cells that were dividing at the time of sacrifice (BrdU+ and Ki-67+/Ki-67+) increased 24 hours after Kainate/seizures when compared to saline conditions. However, this proportion decreased in Kainate treated animals at 48 hours time point and increased again at 72 hours. Therefore, we

investigated if this drop in the proportion of pre-labelled 'clone' of cells at 48 hours is associated with enhanced general cell death in the same area. And thus, in this section, we will address the effects of Kainate/seizures on activated caspase-3 cells in the GCL at 0 hours, 6 hours, and 48 hours time points.

We found that Kainate/seizures increased the number of caspase-3 immuno-positive cells over time (two-way ANOVA with Bonferroni post hoc test, $p< 0.001$) with no significant difference between Kainate and saline injected animals (**Figure 6.16**). However, 2616 ± 281 cells/GCL/brain were caspase-3 immunoreactive at 6 hours time point in Kainate conditions and increased significantly to 4104 ± 308 cells/GCL/brain at 48 hours in Kainate conditions (one-way ANOVA with Bonferroni post hoc test, $p< 0.01$).

Our results demonstrated that although there was no difference between Kainate and saline conditions, caspase-3 activated cells in the GCL increased in Kainate treated animals after 48 hours of the treatment.

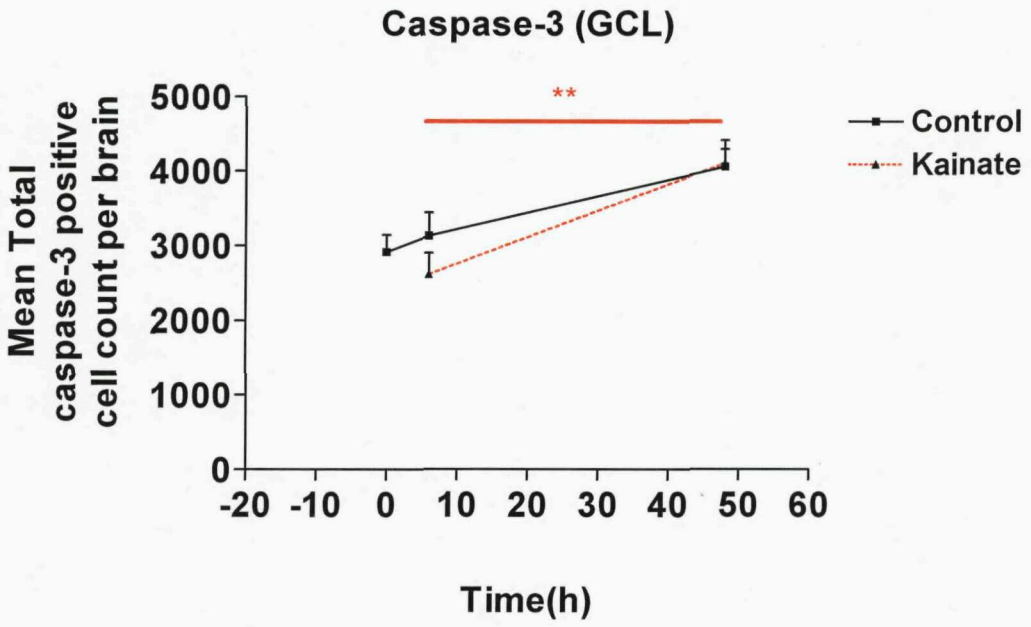


Figure 6.16 Kainate/seizures increased caspase-3 immunoreactive cells in the GCL over time. We have investigated cell death using immunohistochemistry for activated caspase-3. Every 36th section per brain from 0 hours, 6 hours and 48 hours time points was sampled and immunostained for caspase-3 from both saline (control) (solid lines) injected animals and Kainate (dotted lines) injected animals. Activated caspase-3 cells within the GCL were then measured. There was no significant difference in the number of activated caspase-3 in the GCL when comparing Kainate (dotted lines) injected animals with saline (solid lines) injected ones; however, 48 hours of Kainate (dotted lines) treatment increased caspase-3 activated cells when compared to 6 hours Kainate (dotted lines) conditions. Values are expressed as mean ± SEM, means represent the total cell count in the GCL per brain (the two dentates) and every 36th brain section was sampled from each animal at the above mentioned time points. Data points were plotted using the Graph Pad Prism software and means compared using One-way ANOVA with Bonferroni post hoc or Two-way ANOVA with Bonferroni post hoc test was used. Red asterisks denote significant difference between Kainate conditions at different time points, $**p<0.01$ (One-way ANOVA with Bonferroni post hoc test).

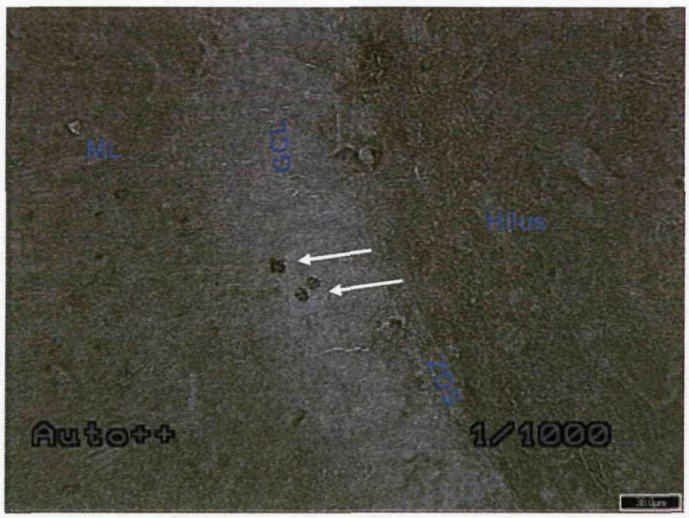


Figure 6.17 Caspase-3 activated cells in the GCL increased over time in Kainate conditions with no difference from controls. Immunohistochemistry was used to identify Caspase-3 activated cells in the GCL and thus measure cell death in Kainate and saline. Every 36th section was sampled from each brain and exhaustive cell counting for caspase-3 immuno-positive cells was carried out. Counts from all sections in each animal were added, this total was then multiplied by the intersectional interval (36) to calculate a count per dentate, from which a mean and standard error of the mean were obtained. Using 20x objective magnification on a Stereology Microscope (Dialux 22, Leitz) caspase-3 Immuno-positive cells were counted in each area. Caspase-3 staining was visualized under bright-filed microscopy. SGZ = subgranular zone; GCL = granule cell layer; ML = molecular layer. White arrows show activated caspase-3 cells in the GCL. Scale bar = 38μm.

6.4.3 The effects of Kainate/seizures on cells in the hilus

6.4.3.1 Kainate/seizures enhanced the proliferation of a pre-labelled 'clone' of cells in the hilus

In the previous sections, we have demonstrated the effects of Kainate/seizures in the SGZ and GCL. Herein, we will show Kainate/seizures effects on cell proliferation (Ki-67 immuno-positive cells) and pre-labelled 'clone' of proliferating progenitor cells (BrdU+, BrdU+ and Ki-67-, BrdU+ and Ki-67+/Ki-67+) as well as Ki-67 only expressed cells in the hilus.

There was an overall increase in the number of Ki-67 immuno-positive cells in the hilus over time when comparing Kainate conditions with saline condition (two-way ANOVA with Bonferroni post hoc test, $p< 0.01$) (**Figure 6.18 A**). However, there was no significant difference in the number of Ki-67 expressing cells between Kainate injected animals and saline injected ones at any of the time points examined. Interestingly, a significant rise in the pre-labelled 'clone' of proliferating cells as indicated by BrdU+ cells was found after 72 hours of Kainate injection and in comparison with saline injected rats (Kainate, 22320 ± 9544 cells/hilus/brain, vs. saline, 2176 ± 1513 cells/hilus/brain) (two-way ANOVA with Bonferroni post hoc test, $p< 0.001$) (**Figure 6.18 A**). Moreover, 72 hours of Kainate treatment recruited a pre-labelled 'clone' of proliferating progenitor cells to divide as indicated by the increase in the proportion of cells that was expressing both BrdU and Ki-67 with respect to the total number of cells in the cell cycle at 72 hours time point (Kainate, 18.7 ± 6.0 %, vs. saline, 1.2 ± 0.5 %) (two-way ANOVA with Bonferroni post hoc test, $p< 0.001$) (**Figure 6.18 C**). In addition, we found that 72 hours of Kainate treatment significantly enhanced the transition of a sub-population of pre-labelled proliferating cells to exit the cell cycle and become postmitotic (Kainate, 11950 ± 5722 cells/hilus/brain, vs. saline, 1632 ± 1253 cells/hilus/brain) (two-way ANOVA with Bonferroni post hoc test, $p< 0.001$) (**Figure 6.18 D**). However, there was no proportional change in the postmitotic sub-population of cells at any time point when comparing Kainate group with saline one (**Figure 6.18 E**). Interestingly, Kainate/seizures did not recruit un-labelled proliferating cells to divide as there was no change in the number of cells that were Ki-67+ and BrdU- (**Figure 6.18 F**).

These findings may suggest that Kainate/seizures targeted the pre-labelled 'clone' of cells in the dentate hilus and enhanced their proliferation most probably by inducing cell cycle re-entry and perhaps also shortened the cell cycle.

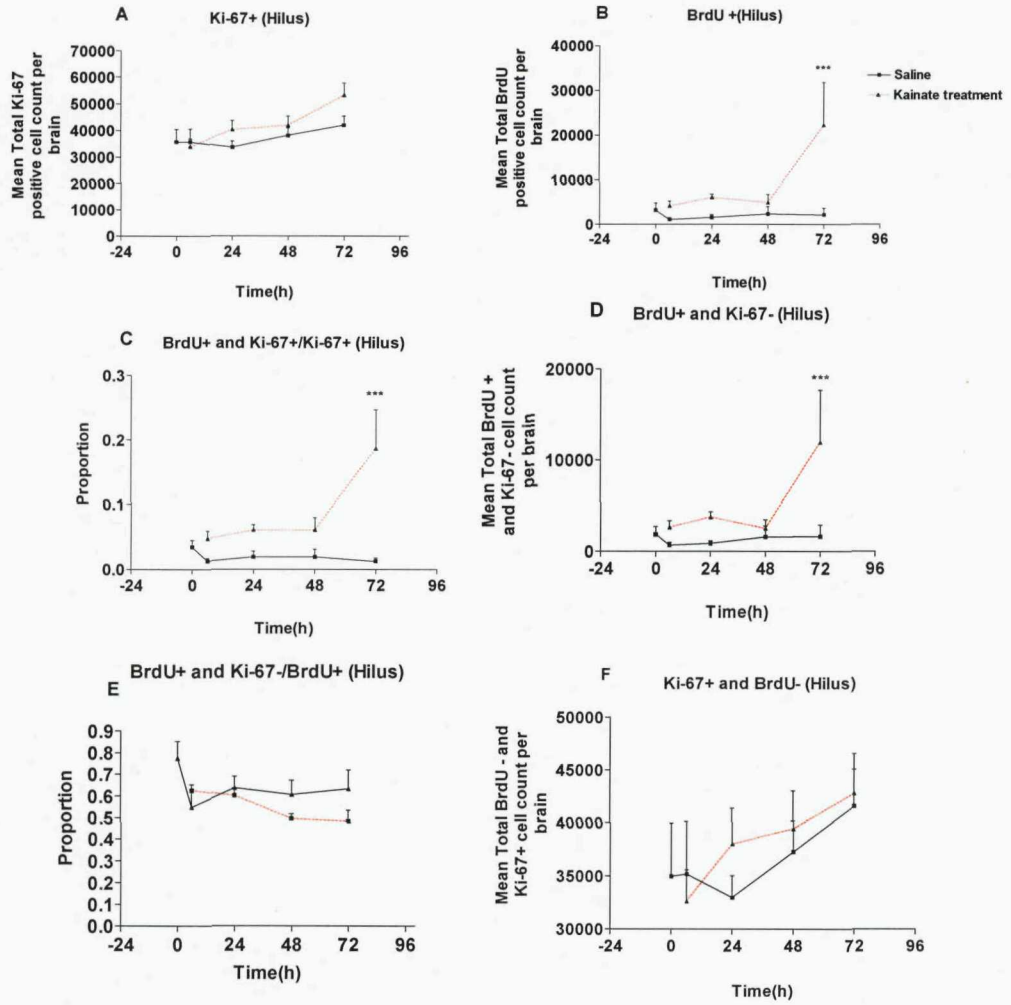


Figure 6.18 Kainate enhanced the proliferation of a pre-labelled clone of cells in the hilus. A 'clone' of proliferating progenitor cells was tagged with BrdU 24 hours before treating the animals with Kainate. Immunohistochemistry double-labelling for BrdU and Ki-67 was used to detect BrdU+ and Ki-67+ cells as well as BrdU+ and Ki-67- cells at each time point. The proportion of cells that were BrdU+ and Ki-67+ with respect to the total dividing/cycling cells at the time of sacrifice was measured. Cells that were Ki-67- but BrdU+ were considered as pre-labelled proliferating cells that became postmitotic. We demonstrated an over all significant proliferative effect for Kainate (dotted lines) on hilus cells as indicated by the positive shift in the curve of Ki-67 immuno-positive cells in Kainate (dotted lines) treatment. We also showed a recruitment for the pre-labelled sub-population of cells to divide as measured by (BrdU+ and Ki-67+/Ki-67+). Kainate (dotted lines) also drove a substantial fraction of the pre-labelled cells to become postmitotic after 72 hours of the treatment. A) There was an overall significant increase in Ki-67 immuno-positive cells in Kainate (dotted lines) conditions when compared to saline (solid lines) conditions. B) 72 hours of Kainate (dotted lines)

treatment increased the number of pre-labelled sub-population of proliferating progenitor cells (BrdU+) when compared to saline (solid lines) conditions. C) Kainate (dotted lines) recruited a pre-labelled 'clone' of proliferating cells to divide at 72 hours time point. D) There was a significant increase in the hilus pre-labelled proliferating cells that exited the cell cycle in Kainate (dotted lines) injected animals when compared to saline (solid lines) treated animals at 72 hours time point. E) There was no significant effect of Kainate/seizures (dotted lines) on the proportion BrdU only cells (postmitotic) with respect to the total number of cells that incorporated BrdU. F) There was no significant effect for Kainate (dotted lines) on un-labelled proliferating cells when compared to saline (solid lines) conditions. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8th brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc test. ***$p<0.001$

6.4.3.2 Caspase-3 activated cells transiently decreased in the hilus after Kainate/seizures

In the previous section, we have investigated the effects of Kainate-induced seizures on cell proliferation in the hilus. We found a significant effect for Kainate/seizures on a pre-labelled 'clone' of cells. Here, we will address if cell proliferation is associated with cell death. Therefore, the effects of Kainate/seizures on activated caspase-3 cells in the hilus was measured at 0 hours, 6 hours, and 48 hours time points in both Kainate and saline injected animals.

Interestingly, in the hilus, there was a significant drop in caspase-3 immuno-positive cells 6 hours after Kainate injections when compared to saline injected animals (Kainate, 2136 ± 124 cells/Hilus/brain, vs. saline, 2988 ± 266 cells/Hilus/brain) (two-way ANOVA with Bonferroni post hoc test, $p< 0.05$) (**Figure 6.19**). However, Kainate/seizures increased the number of caspase-3 immuno-positive cells to 3612 ± 251 cells/Hilus/brain at 48 hours time point. This increase was statistically significant when compared to 6 hours Kainate treatment (one-way ANOVA with Bonferroni post hoc test, $p< 0.05$).

Our results demonstrated a transient survival effects for Kainate/seizures on cells in the hilus. However, Kainate/seizures enhanced apoptotic cells death 48 hours later when

compared to 6 hours Kainate conditions with no significant difference between Kainate and saline conditions.

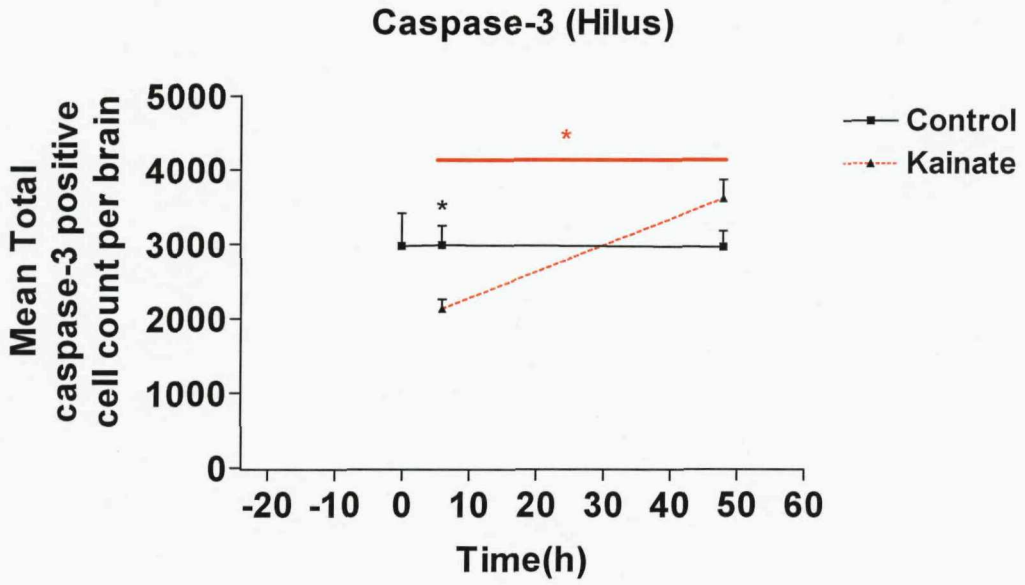


Figure 6.19 Kainate/seizures transiently decreased cell death in the hilus. We have investigated cell death using immunohistochemistry for activated caspase-3. Every 36th section per brain from 0 hours, 6 hours and 48 hours time points was sampled and immunostained for caspase-3 from both saline (control) (solid lines) injected animals and Kainate (dotted lines) injected animals. Activated caspase-3 cells within the hilus were then measured. There was a significant decrease in the number of activated caspase-3 in the hilus when comparing Kainate (dotted lines) injected animals with saline (solid lines) injected ones at 6 hours time point; however, 48 hours of Kainate (dotted lines) treatment increased caspase-3 activated cells when compared to 6 hours Kainate (dotted lines) conditions. Values are expressed as mean ± SEM, means represent the total cell count in the SGZ per brain (the two dentates) and every 36th brain section was sampled from each animal at the above mentioned time points. Data points were plotted using the Graph Pad Prism software and means compared using One-way ANOVA with Bonferroni post hoc or Two-way ANOVA with Bonferroni post hoc test. Black asterisks denote significant effect between Kainate and saline conditions, * $p<0.05$ (Two-way ANOVA with Bonferroni post hoc test), red asterisks denote significant difference between Kainate conditions at different time points, * $p<0.05$ (One-way ANOVA with Bonferroni post hoc test).

6.4.4 The effects of Kainate/seizures in the molecular layer (Ml)

6.4.4.1 Kainate/seizures enhanced cell proliferation in the molecular layer of the dentate gyrus

In this part, we will investigate the effects of Kainate/seizures on cell proliferation (Ki-67 immuno-positive cells) and pre-labelled 'clone' of proliferating progenitor cells (BrdU+, BrdU+ and Ki-67-, BrdU+ and Ki-67+/Ki-67+) as well as Ki-67 only expressed cells in the molecular layer of the dentate gyrus.

There was an overall increase in the number of Ki-67 immuno-positive cells in the molecular layer over time (two-way ANOVA with Bonferroni post hoc test, $p< 0.05$) and when comparing Kainate conditions with saline condition (two-way ANOVA with Bonferroni post hoc test, $p< 0.001$) (**Figure 6.20 A**). Moreover, there was a significant increase in Ki-67 immuno-positive cells in Kainate injected animals when compared to saline injected ones at 24 hours time point (Kainate, 53580 ± 3364 cells/Ml/brain, vs. saline, 40440 ± 1900 cells/Ml/brain) (two-way ANOVA with Bonferroni post hoc test, $p< 0.05$) (**Figure 6.20 A**). Likewise, 72 hours of Kainate/seizures increased the number of Ki-67 immunostained cells to 62160 ± 6514 cells/Ml/brain versus 31420 ± 2245 cells/Ml/brain (two-way ANOVA with Bonferroni post hoc test, $p< 0.001$) (**Figure 6.20 A**). Interestingly, significant rise in the pre-labelled 'clone' of proliferating cells as indicated by BrdU+ cells was found after 72 hours of Kainate injection and in comparison with saline injected rats (Kainate, 39050 ± 20980 cells/Ml/brain, vs. saline, 4608 ± 2526 cells/Ml/brain) (two-way ANOVA with Bonferroni post hoc test, $p< 0.05$) (**Figure 6.20 B**). Moreover, 72 hours of Kainate treatment recruited a pre-labelled 'clone' of proliferating progenitor cells to divide as indicated by the increase in the proportion of cells that was expressing both BrdU and Ki-67 with respect to the total number of cells in the cell cycle at 72 hours time point (Kainate, 34.6 ± 24.6 %, vs. saline, 3.9 ± 1.4 %) (two-way ANOVA with Bonferroni post hoc test, $p< 0.05$) (**Figure 6.20 C**). In addition, we found that 72 hours of Kainate treatment significantly enhanced the transition of a sub-population of pre-labelled proliferating cells to exit the cell cycle

and become postmitotic (Kainate, 22900 ± 13260 cells/Ml/brain, vs. saline, 3328 ± 1996 cells/Ml/brain) (two-way ANOVA with Bonferroni post hoc test, $p < 0.05$) (**Figure 6.20 D**). Although Kainate/seizures had an overall significant effect on Ki-67 only positive cells when comparing Kainate condition with saline conditions (two-way ANOVA with Bonferroni post hoc test, $p < 0.01$), there was no significant difference between the two conditions at any of the time points (**Figure 6.20 E**).

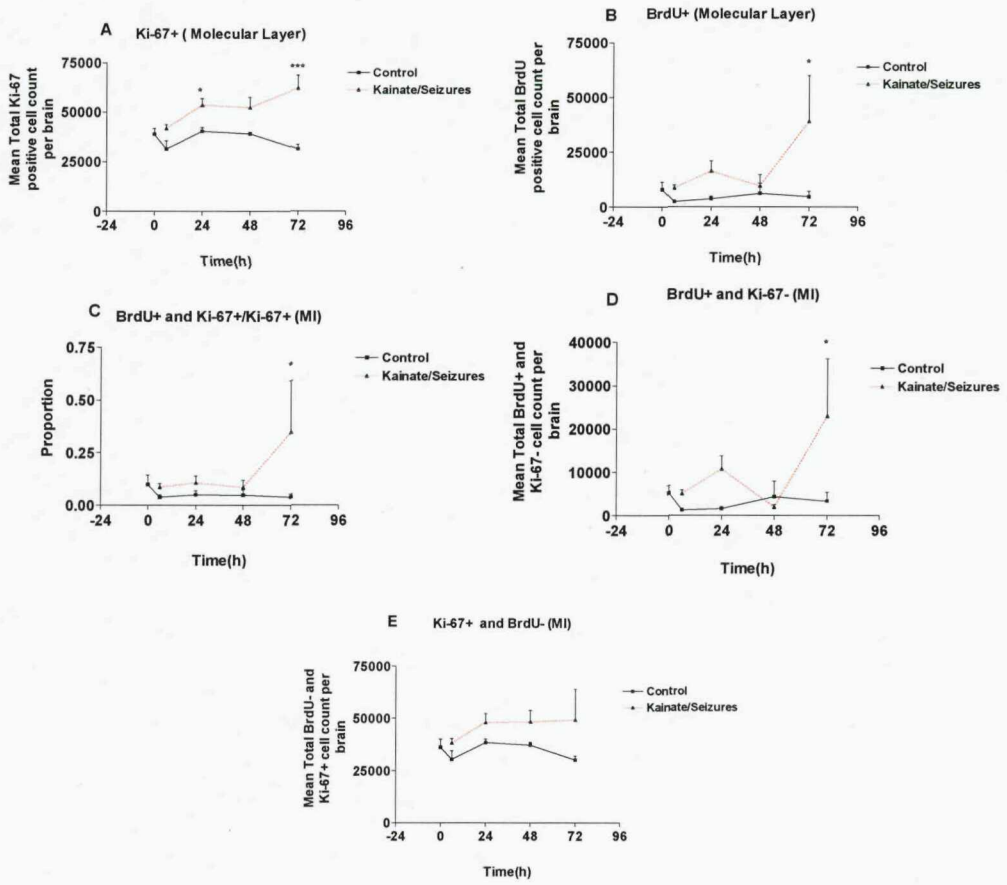


Figure 6.20 Kainate enhanced cell proliferation in the molecular layer of the DG. A 'clone' of proliferating progenitor cells was tagged with BrdU 24 hours before treating the animals with Kainate. Immunohistochemistry double-labelling for BrdU and Ki-67 was used to detect BrdU+ and Ki-67+ cells as well as BrdU+ and Ki-67- cells at each time point examined. The proportion of cells that were BrdU+ and Ki-67+ with respect to the total dividing/cycling cells at the time of sacrifice was measured. Cells that were Ki-67- but BrdU+ were considered as pre-labelled proliferating cells that became postmitotic. We demonstrated an over all significant proliferative effect for Kainate (dotted lines) on MI cells as indicated by the positive shift in the curve of Ki-67 immuno-positive cells in Kainate (dotted lines) treatment. We also showed a recruitment for the pre-labelled sub-population of cells to divide as measured by (BrdU+ and Ki-67+/Ki-67+). Kainate (dotted lines) also drove a sub-population of the pre-labelled cells to become postmitotic after 72 hours of the treatment. A) There was an overall significant increase in Ki-67 immuno-positive cells in Kainate (dotted lines) conditions when compared to saline (solid lines) conditions with significant increases in Kainate treated animals at 24 hours and 72 hours time points. B) 72 hours of Kainate (dotted lines) treatment increased the number of pre-labelled sub-population of proliferating progenitor cells (BrdU+) when compared to saline (solid lines) conditions. C) Kainate (dotted lines) recruited a pre-labelled 'clone'

of proliferating cells to divide at 72 hours time point. D) There was a significant increase in the Ml pre-labelled proliferating cells that exited the cell cycle in Kainate (dotted lines) injected animals when compared to saline (solid lines) treated animals at 72 hours time point. E) There was an overall significant effect for Kainate (dotted lines) on the un-labelled proliferating cells when compared to saline (solid lines) conditions, however, no significant change was found, at any of the time points examined, when comparing Kainate injected animals with saline injected ones. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8th brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc test. * $p<0.05$, ***$p<0.001$

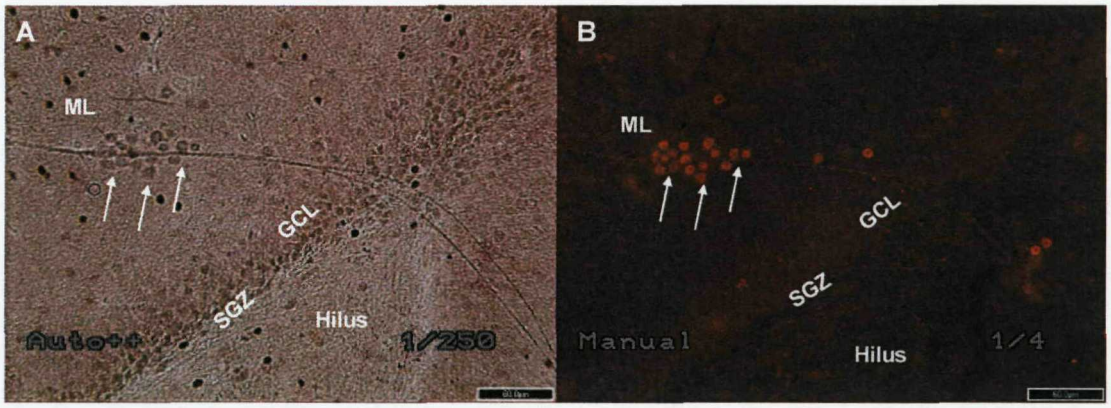


Figure 6.21 Kainate/seizures drove a substantial fraction of pre-labelled 'clone' of proliferating precursors to become postmitotic in the Ml. BrdU was injected to all animals 24 hours before inducing seizures by Kainate in half of them. Immunohistochemistry was used to identify BrdU incorporated cells. Double-labelling of cells for Ki-67 and BrdU was carried out to determine cells that became postmitotic by counting BrdU+ and Ki-67-. 72 hours of Kainate/seizures drove a substantial sub-population of pre-labelled 'clone' of cells to exit the cell cycle in the Ml of the DG. Every 8th section was sampled from each brain and exhaustive cell counting for BrdU immuno-positive cells (B) that do not co-localize with Ki-67 expressing cells (A) was carried out. Counts from all sections in each animal were added, this total was then multiplied by the intersectional interval (8) to calculate a count per dentate, from which a mean and standard error of the mean were obtained. Using 20x objective magnification on a Stereology Microscope (Dialux 22, Leitz) BrdU and Ki-67 Immuno-positive cells were counted in each area. While Ki-67 staining was visualized under bright-filed microscopy, the presence of fluorescent filters on this microscope enabled us to visualize BrdU staining in red on the same area of interest. SGZ = subgranular zone; GCL = granule cell layer; ML = molecular layer. White arrows show and BrdU+ and Ki-67- in the molecular layer of the DG. Scale bar = 60μm.

6.4.4.2 Kainate/seizures transiently decreased cell death in the molecular layer of the dentate gyrus

In the previous section, we have investigated the effects of Kainate-induced seizures on cell proliferation in the molecular layer (Ml). We found a significant effect for Kainate/seizures on cell proliferation (Ki-67 expressed cells) and on a pre-labelled 'clone' of cells. Although there was an increase in Ki-67 immuno-positive cells after 48 hours of Kainate injection, this increase was not statistically significant. As such, there is a possibility of an ongoing cell death. Here, we will address if cell proliferation is associated with cell death. Therefore, the number of activated caspase-3 cells in the Ml was measured at 0 hours, 6 hours, and 48 hours time points in both Kainate and saline injected animals.

Surprisingly, in the Ml, there was a significant decrease in caspase-3 immuno-positive cells 6 hours after Kainate injections when compared to saline injected animals (Kainate, 2928 ± 297 cells/Ml/brain, vs. saline, 4314 ± 390 cells/Ml/brain) (two-way ANOVA with Bonferroni post hoc test, $p < 0.05$) (**Figure 6.22**). Although Kainate/seizures increased the number of caspase-3 immuno-positive cells to 4680 ± 227 cells/Ml/brain at 48 hours time point, this increase was statistically insignificant when compared to 6 hours Kainate treatment (one-way ANOVA with Bonferroni post hoc test, $p>0.05$).

Our results demonstrated a transient survival effect of Kainate/seizures on cells in the Ml. However, Kainate/seizures slightly enhanced apoptotic cells death 48 hours later when compared to 6 hours Kainate conditions with no significant difference between Kainate and saline conditions.

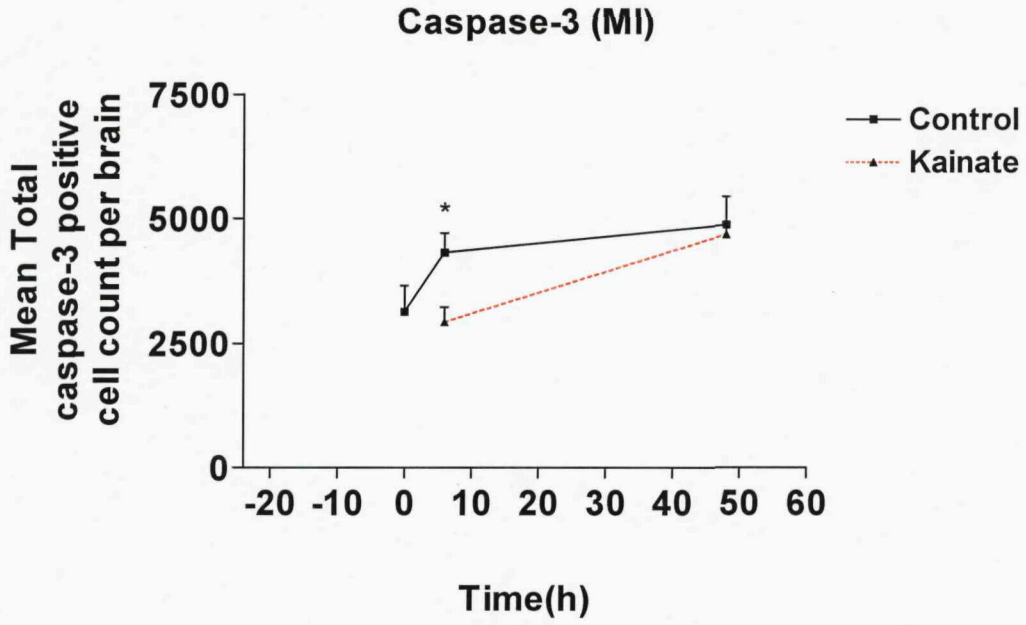


Figure 6.22 Kainate/seizures transiently decreased cell death in the MI. We have investigated cell death using immunohistochemistry for activated caspase-3. Every 36th section per brain from 0 hours, 6 hours and 48 hours time points was sampled and immunostained for caspase-3 from both saline (control) (solid lines) injected animals and Kainate (dotted lines) injected animals. Activated caspase-3 cells within the molecular layer were then measured. There was a significant decrease in the number of activated caspase-3 in the MI when comparing Kainate (dotted lines) injected animals with saline (solid lines) injected ones at 6 hours time point; however, 48 hours of Kainate (dotted lines) treatment slightly (insignificant) increased caspase-3 activated cells when compared to 6 hours Kainate (dotted lines) conditions. Values are expressed as mean ± SEM, means represent the total cell count in the SGZ per brain (the two dentates) and every 36th brain section was sampled from each animal at the above mentioned time points. Data points were plotted using the Graph Pad Prism software and means compared using One-way ANOVA with Bonferroni post hoc or Two-way ANOVA with Bonferroni post hoc test. Black asterisks denote significant effect between Kainate and saline conditions, * $p<0.05$, (Two-way ANOVA with Bonferroni post hoc test)

6.4.5 The effects of Kainate/seizures in the combined SGZ and GCL

6.4.5.1 Kainate/Seizures enhanced progenitor cell proliferation in the combined SGZ and GCL

In the previous sections, we have demonstrated the effects of Kainate/seizures in the dentate gyrus sub-areas (SGZ, GCL, Hilus, and Ml). In order to get further insight on the mechanisms by which Kainate/seizures induced cell proliferation, we will show the effects of Kainate/seizures on cell proliferation of the different 'clones' of cells in the combined SGZ and GCL area. To achieve this we measured the effects of Kainate/seizures on cell proliferation in general (Ki-67 immuno-positive cells), pre-labelled 'clone' of proliferating progenitor cells (BrdU+) and the proportions of pre-labelled 'clone' of cells that continued to divide with respect to the total number of cells in the cell cycle as well as the total number of BrdU incorporated cells (BrdU+ & Ki-67+/Ki-67+ and BrdU+ & Ki-67+/BrdU+, respectively) in the combined SGZ and GCL.

The number of Ki-67 immuno-positive cells increased significantly from 28770 ± 1991 cells/SGZ+GCL /brain in saline treated animals to 46140 ± 2458 cells/ SGZ+GCL/brain in Kainate treated rats at 72 hours time point (Two-way ANOVA with Bonferroni's post hoc test, $p< 0.05$) (**Figure 6.23 A**). Moreover, the number of the pre-labelled proliferating progenitor cells significantly increased from 5341 ± 1325 cells/SGZ+GCL/brain in saline treated animals to 16480 ± 4053 cells/SGZ+GCL/brain in Kainate injected animals after 72 hours of the treatment (two-way ANOVA with Bonferroni's post hoc test, $p< 0.01$) (**Figure 6.23 B**). Moreover, there was a significant increase in the numbers of pre-labelled 'clone' of cells that continued to divide at 72 h time point when comparing Kainate group with the saline ones (Kainate, 9787 ± 2279 cells/SGZ+GCL/brain, vs. saline 2091 ± 59.1 cells/SGZ+GCL/brain) (Two-way ANOVA with Bonferroni's post hoc test, $p< 0.001$) (**Figure 6.23 C**). Importantly, 72 hours of Kainate treatment recruited a pre-labelled 'clone' of proliferating progenitor cells to divide as indicated by the increase in the proportion of cells that was expressing both BrdU and Ki-67 with respect to the total number of cells in the cell cycle at 72

hours time point (Kainate, 21.0 ± 4.8 %, vs. saline, 7.3 ± 3.2%) (Two-way ANOVA with Bonferroni's post hoc test, $p<0.001$) (**Figure 6.23 D**). In addition, we found that 72 hours of Kainate treatment increased the proportion of pre-labelled 'clone' of cells with respect to the total number of the pre-labelled 'clone', however, this increase was statistically insignificant(Kainate, 60.3 ± 5.0 %, vs. saline, 43.2 ± 8.3 %) (Two-way ANOVA with Bonferroni's post hoc test, $p = 0.1$) (**Figure 6.23 E).**

These results suggest an important effect of Kainate/seizures on overall cell proliferation in general and on the pre-labelled 'clone' of precursors in particular in the combined SGZ and GCL areas.

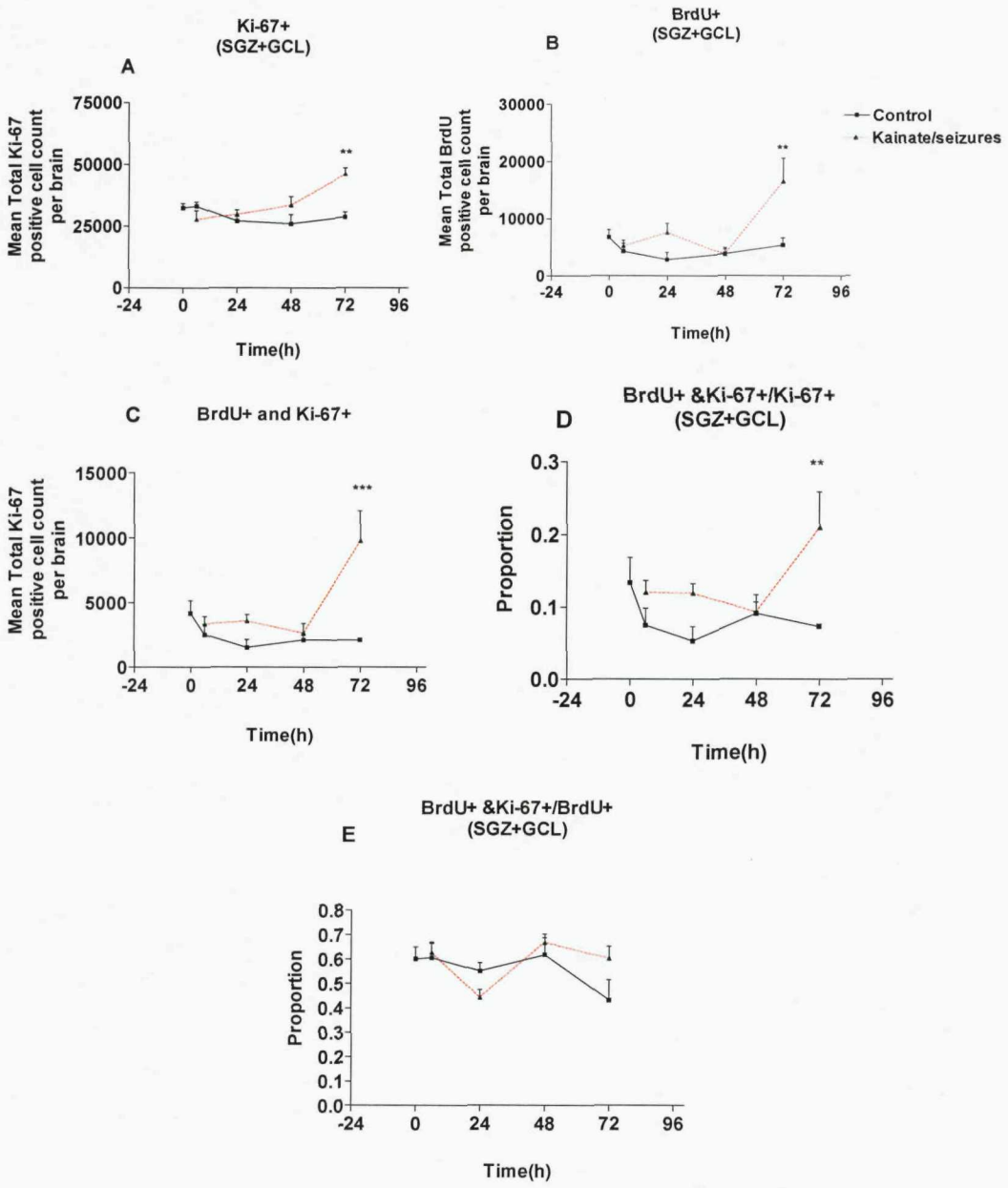


Figure 6.23 Kainate enhanced cell proliferation in the combined SGZ and GCL. A clone of proliferating progenitor cells was tagged with BrdU 24 hours before treating the animals with Kainate. Immunohistochemistry double-labelling for BrdU and Ki-67 was used to detect BrdU+ and Ki-67+ cells as well as BrdU+ and Ki-67- cells at each time point. The proportion of cells that were BrdU+ and Ki-67+ with respect to the total dividing/cycling cells at the time of sacrifice was measured. Cells that were Ki-67- but BrdU+ were considered as pre-labelled proliferating cells that became postmitotic. We demonstrated a significant proliferative effect for Kainate (dotted lines) on

the combined SGZ and GCL cells as indicated by the rise in Ki-67 immuno-positive 72 hours after Kainate (dotted lines) treatment. We also showed a recruitment for the pre-labelled sub-population of cells to divide as measured by (BrdU+ and Ki-67+/Ki-67+). A) There was a significant increase in Ki-67 immuno-positive cells after 48 hours and 72 hours of Kainate (dotted lines) treatment when compared to saline (solid lines) conditions. B) 72 hours of Kainate (dotted lines) treatment increased the number of pre-labelled sub-population of proliferating progenitor cells (BrdU+) when compared to saline (solid lines) conditions. C) There was a significant increase in the numbers of pre-labelled 'clone' of cells that continued to divide at 72 h time point in the Kainate (dotted lines) group. D) Kainate (dotted lines) recruited a pre-labelled 'clone' of proliferating cells to divide at 72 hours time point. E) There was a significant increase in the dentate gyrus pre-labelled proliferating cells that exited the cell cycle in Kainate (dotted lines) injected animals when compared to saline (solid lines) treated animals at 72 hours time point. Values are expressed as mean ± SEM, means represent the total cell count in the region of interest per brain (the two dentates) and every 8^{th} brain section was sampled from each animal at each time point. Data points were plotted using the Graph Pad Prism software and means compared using Two-way ANOVA with Bonferroni post hoc test. $^{**}p<0.01$, $^{***}p<0.001$

6.4.6 Kainate/Seizures increased neurogenesis as determined by Doublecortin immuno-positive cells

Increases in both cell proliferation and neurogenesis have been reported as consequences of Kainate-induced seizures. Although, BrdU pre-labelling protocol did not detect the same magnitude of cell proliferation that is observed using post-labelling protocols, we have demonstrated a significant proliferative effect for 72 hours of Kainate/seizures in the SGZ and GCL (as measured by Ki-67 immuno-positive cells). However, it is important to establish that the increase in proliferation results in neuronal formation. Doublecortin (Dcx) immunostaining was used to assess the number of immature neurons, and hence the level of neurogenesis, in the dentate gyrus at 72 hours after either an IP Kainate or saline injections.

Quantification of the number of Dcx immuno-positive cells within the SGZ/inner one-third of GCL was performed on every 36^{th} section, resulting in sampling three 40μm sections per brain, the sum of Dcx immuno-positive cell counts was then multiplied by

the intersectional interval. Sections were sampled from both Kainate and saline injected animals at 72 hours time point.

There was a significant increase in the number of Dcx immuno-positive cells when comparing Kainate injected animals to saline injected rats (Kainate, 8474 ± 162 cells/SGZ (and inner one-third of GCL)/brain, vs. saline, 6111 ± 424 cells/SGZ (and inner one-third of GCL)/brain) (unpaired simple students' t-test , $p<0.001$) (**Figure 6.24**). **Figure 6.25** showed a sample of Dcx images that were analyzed.

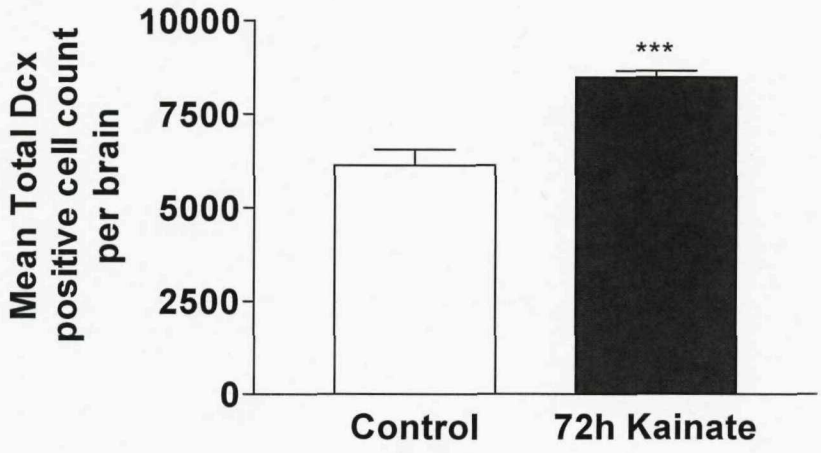


Figure 6.24 Kainate/seizures increased Dcx immuno-positive cells. We have investigated the effects of Kainate/seizures on immature neurons by immunostaining every 36th brain section sampled from either Kainate or saline injected animals at 72 hours time point. Immuno-positive Dcx cells within SGZ and inner one-third of the GCL were then counted. There was a significant increase in the number of Dcx immunostained cells in Kainate injected rats when compared to saline injected animals. Values are expressed as mean ± SEM, means represent the Dcx cell count in the SGZ and inner one-third per brain (the two dentates) and every 36th brain section was sampled from each animal at the above mentioned time point. Data points were plotted using the Graph Pad Prism software and means compared using unpaired simple Student's t-test ***$p<0.001$

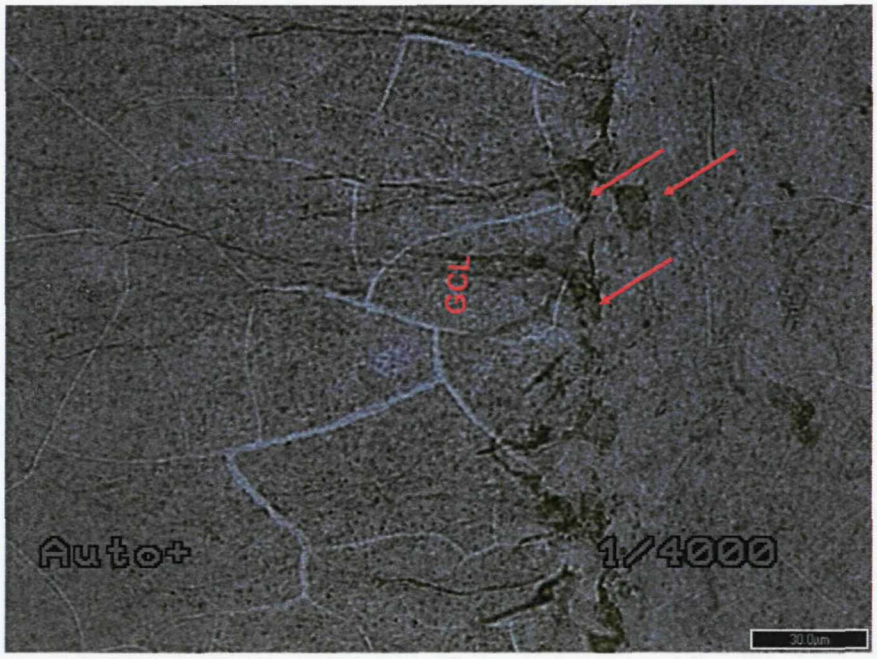


Figure 6.25 Kainate/seizures enhanced neurogenesis 72 hours after the treatment. Doublecortin (Dcx) immunohistochemistry was used to measure neuroblasts number at 72 hours in both Kainate and saline treated animals. We demonstrated a significant increase in the number of Dcx immuno-positive cells in the SGZ and inner one-third of the GCL at 72 hours time point in Kainate injected animals when compared to saline injected ones. Every 36th section was sampled from each brain and exhaustive cell counting for Dcx immuno-positive cells was carried out. Counts from all sections in each animal were added, this total was then multiplied by the intersectional interval (36) to calculate a count per dentate, from which a mean and standard error of the mean were obtained. We used bright-field Stereology Microscope (Dialux 22, Leitz) equipped with 40x oil objective to image and count Dcx positive cells. GCL = granule cell layer. White arrows show Dcx immuno-positive cells. Scale bar = 30µm.

6.5 Discussion

To investigate possible mechanisms underlying the neurogenic response to status epilepticus, we applied IP Kainate to adult Wistar rats after labelling a 'clone' of proliferating cells with BrdU 24 hours before Kainate injection. As we applied BrdU 24 hours before seizures-induction we could not use BrdU to study point proliferation. PCNA was reported to be expressed in cells undergoing DNA repair, and some non-proliferating neurons (Hall, McKee et al. 1993; Ino and Chiba 2000). We therefore used Ki-67, as a marker of cell proliferation. Ki-67 is detected in all cells in the cell cycle, has a very short half life, is not detectable during DNA repair, and is strongly down-regulated/absent in quiescent cells (Taupin 2007). As such, Ki-67 is a reliable marker of cell proliferation. Since Ki-67 is expressed in all cells in the cell cycle, we will detect more cells than BrdU (which only labels S-phase cells) when used as a proliferation marker at the time of sacrifice.

This paradigm enabled us to elucidate the effects of Kainate/seizures on a 'clone' of pre-labelled proliferating cells (BrdU+ and Ki-67+) and un-labelled proliferating cells (Ki-67+ only) in the DG and its sub-areas (SGZ, GCL, hilus, and Ml). Interestingly, measuring the proportion of pre-labelled cells that continued to proliferate with respect to the total cell number in the cell cycle (BrdU+ and Ki-67+/Ki-67+) enabled us to investigate the contribution of the pre-labelled 'clone' to the post-Kainate proliferation, at different time points. The proportion of pre-labelled cells that continued to proliferate (Ki67+ and BrdU+/ Ki67+) was used to determine whether a sub-population of pre-labelled cells was recruited to divide again. Furthermore, by measuring BrdU+ and Ki-67- cells we determined the number of cells that became postmitotic after Kainate/seizure at different time points. In addition, we explored the possibility that Kainate/seizures-induced cell death influences neurogenesis by measuring caspase-3 activated cells at 0 hours, 6 hours, and 48 hours in both Kainate and saline treated animals. Moreover, net neurogenesis was determined using Doublecortin (Dcx) immuno-positive cells identified in the SGZ and the inner one-third of the GCL under both Kainate/seizures and saline conditions at 72 hours time point.

Our results identified major differences in the dentates of animals receiving IP Kainate injections when compared to saline injected controls. These include, increased precursor cell proliferation as measured by Ki-67+ cells in the SGZ, GCL, and Ml. There is a proportional increase in the contribution of the pre-labelled 'clone' of cells to all cells in the cell cycle (BrdU+ and Ki-67+/Ki-67+), in the GCL, hilus, and Ml. However, in the SGZ, Kainate/seizures enhanced equally the proliferation of both the pre-labelled and un-labelled 'clone' of cells. Furthermore, Kainate/seizures increased the numbers of Ki-67 only positive cells (un-labelled proliferating cells) in the SGZ alone. Interestingly, there is a trend towards a decrease in the proportion of BrdU pre-labelled 'clone' of cells that became postmitotic in the Kainate group when compared to the saline group at the 72 h time point in the SGZ and GCL. Importantly, Kainate/seizures enhanced neurogenesis as measured by Doublecortin (Dcx) immunostaining. Kainate/seizures had no effect on cell death as identified by caspase-3 activated cell counts in the SGZ and resulted in a transient decrease of cell death (in the Hilus and Ml) at 6 h post Kainate induced status.

6.5.1 Kainate-induced seizures enhance cell proliferation

Cell proliferation was determined by measuring Ki-67 immuno-positive cells at the time of sacrifice in the DG sub-areas. In **section 6.4.1.1**, we show a significant increase in the SGZ number of cells that are Ki-67 immuno-positive after 72 hours of Kainate/seizures when compared to saline injected animals. This proliferative SGZ response is consistent with previous reports. Huttmann et al, using transgenic mice, have demonstrated an increase in the SGZ progenitor cell proliferation 72 hours after Kainate/seizures in adult mice (Huttmann, Sadgrove et al. 2003). Furthermore, Nakagawa et al. studied cell proliferation after Kainate-induced seizures and reported an elevated cell proliferation on days 3, 5 and 7 after Kainate/seizures (Nakagawa, Aimi et al. 2000). Although BrdU was used to label proliferating cells in the previous study, 3 hours single pulse of BrdU was administered to examine cell proliferation and thus would identify proliferating cells thereafter. Likewise, and in agreement with our results, Parent et al have described an increased SGZ progenitor cell proliferation 3 days after pilocarpine-induced status

epilepticus in adult rats (Parent, Yu et al. 1997). In the previous study, they used a series of 4 IP BrdU injections at 2 hours intervals, given 1, 3, 6, 13 or 27 days after pilocarpine injection had induced seizures, animals were sacrificed 24 hours after BrdU administration. Moreover, it has been shown that Kainate-induced seizures enhanced cell proliferation as early as 3 days after the treatment, as indicated by an increase in PCNA immuno-positive cells, in the SGZ of adult mice (Ledergerber, Fritschy et al. 2006).

Interestingly, Ki-67 immuno-positive cells increased earlier at 48 h following Kainate insult in the GCL when compared to saline injected rats (**Section 6.4.2.1**). Similarly, the number of Ki-67 expressing cells in the GCL increased again after 72 hours of Kainate treatment when compared to saline conditions. These results suggest that cells proliferate earlier in the GCL after Kainate and thus contribute to the increase in net neurogenesis seen at the 72 h time point. In line with this, it has been shown that over 20% of Dcx immunopositive cells are proliferating and these cells are type-3 precursor cells (see also **section 6.5.5**) (Jessberger, Romer et al. 2005; Plumpe, Ehninger et al. 2006). Dcx positive cells are more likely to lie outside of the SGZ and within the GCL and this cell type has been previously reported to respond to seizures (Jessberger, Romer et al. 2005). Therefore, an increase in the proliferation of Dcx expressing cells in the Kainate group between 48 h and 72 h time points would likely contribute to the net rise in neurogenesis observed at 72 h time point.

Several lines of evidence have indicated that newly-generated granule cells display ectopic migration patterns after seizures-induced cell proliferation (Parent, Yu et al. 1997; Scharfman, Goodman et al. 2000; Pierce, Melton et al. 2005; McCloskey, Hintz et al. 2006). Although it was proposed that proliferating progenitor cells migrate from the SGZ and GCL to other ectopic DG sub-areas, it remains unsettled, however whether precursor cell proliferation was induced shortly after status epilepticus in other areas of the DG like the hilus and molecular layer. Interestingly, we have found that the numbers of Ki-67 positive cells increased significantly in the combined SGZ and GCL (**Section 6.4.5.1**). These data indicate that cell proliferation is an ongoing process in this combined area which raises the possibility of perhaps dispersion of the stem cell niche. Furthermore, we have investigated cell proliferation in the hilus and molecular layer

shortly after Kainate/seizures. Although there is no significant difference in Ki-67 cell numbers up to 72 hours between Kainate and saline conditions in the hilus, there is an overall increase in Ki-67 expressing cells with time in Kainate treatment (**Section 6.4.3.1**). We demonstrate that approximately 53000 cells are Ki-67 positive at 72 hours under Kainate conditions while around 41000 are expressing Ki-67 in saline injected animals. In addition, we show that 34000 are Ki-67 immuno-positive after 6 hours of Kainate injections which was similar to saline injected animals. We conclude that Kainate/seizures are enhancing cell proliferation in the hilus, although their phenotype is unknown.

The molecular layer (Ml) is another part of the dentate gyrus that is vulnerable to Kainate induced neuronal damage (Nadler 1981). It has been demonstrated that ectopic granule cells were found in the molecular layer of the DG after pilocarpine induced seizures and in specimens of humans with temporal lobe epilepsy (Parent, Elliott et al. 2006). However, it is still unclear whether precursor cells in this region proliferate and/or have a role after Kainate/seizures. Therefore, we examined the effects of Kainate/seizures on Ml cell proliferation. We describe that a substantial number of Ml cells are expressing Ki-67 after Kainate/seizures (**Section 6.4.4.1**). Surprisingly, over 53000 cells were Ki-67 positive in the Ml 24 hours after Kainate injection which was significantly higher than saline injected animals (slightly over 40000 cells/Ml). Although the number of Ki-67 positive cells was not statistical significant at 48 hours time point when comparing Kainate/seizures conditions to saline conditions, Ki-67 positive cell numbers were doubled after 72 hours of Kainate/seizures and in comparison with saline conditions. However, the phenotype of these proliferating cells yet to be studied. An interesting question arises as to the role of this level of cell proliferation in the molecular layer under normal physiological conditions and how it is perturbed after Kainate induced status epilepticus, and whether this cell proliferation modifies the neurogenic process. However, more experiments need to be conducted in order to explore the contribution of this cell proliferation to both SGZ and ectopic neurogenesis and functional integration.

6.5.2 Kainate-induced 'clonal' cell proliferation increased in the different DG sub-areas

Examining cell proliferation at the time of sacrifice clearly demonstrated that cell proliferation in the SGZ and the rest of the DG sub-areas is altered by seizures, and the temporal profile for these changes is now well documented. However, the mechanisms through which these changes occur have yet to be elucidated. In order to follow the fates of a limited number of dividing cells and their progeny as they progressed through the consequences of seizures induction we used a 'clonal' BrdU labelling protocol. This methodology has been described in adult normal mice (Hayes and Nowakowski 2002). Using this protocol, we have investigated the effects of Kainate/seizures on a 'clone' of proliferating cells that were pre-labelled with BrdU 24 hours before Kainate injections.

Surprisingly, the number and proportion of BrdU incorporated cells did not change after Kainate treatment in the SGZ when compared to saline injected animals (**Section 6.4.1.2**). Our results show that approximately 10% of Ki-67 expressing cells are BrdU immuno-positive throughout the whole time period examined (0h-72h). We are expecting that BrdU incorporated a fraction of proliferating cells in the SGZ because we have injected the animals with a single pulse of 50mg/kg BrdU 24 hours before Kainate treatment. In agreement with our interpretation, it has been shown that a single pulse of 300mg/kg BrdU 24 hours before sacrificing the animals labelled 50% of proliferating Ki-67 expressing cells in adult Wistar rats' dentate gyrus (Kee, Sivalingam et al. 2002). The fact that there was no difference in the number of the pre-labelled 'clone' of cells between Kainate/seizures and control conditions indicate that these cells survived the insult. As such, we conclude that Kainate/seizures did not kill pre-labelled SGZ precursor cells within the first 72 hours after seizure induction.

There was no evidence of proliferative dilution of the BrdU labelled 'clone' of cells over the first 72 h in either controls or post Kainate since the proportion of BrdU+ Ki-67+/ Ki-67+ cells remained remarkably constant over time and across both conditions. In agreement with this, it has been reported that BrdU can be traced and detected up to 4 cell cycle after being administered (Hayes and Nowakowski 2002; Dayer, Ford et al. 2003) and the length of the cell cycle is 25 h in adult rats (Cameron and McKay 2001),

therefore, BrdU incorporated cells will be still detected up to 100 h after being administered. As such we would not expect a dilution in BrdU unless there was a very significant shortening of the cell cycle with re-entry under Kainate conditions. There was a tendency to a decrease in the number of proliferating (Ki-67+) BrdU+ cells over the first 24 hours with a subsequent recovery between 48 and 72 h in both groups consistent with either an initial stress related decrease in general proliferation or a successive proliferative cycles with an intervening G_0 resting phase. The former is unlikely as the level of total cell proliferation in the SGZ was remarkably constant up to the 48 h time point.

Importantly, these results also show that Kainate/seizures did enhance the proliferation of the pre-labelled 'clone' in the SGZ as there was a significant increase in Ki67+ cells at 72 h with an unchanged ratio of BrdU+ & Ki-67+/Ki-67+ cells. Consistent with this was a clear increase in the numbers of Ki-67+ & BrdU+ cells at 72 h which just failed to reach statistical significance on its own. This is because the pre-labelled 'clone' of cells identified a small fraction of proliferating cells and thus the effects of Kainate/seizures on the SGZ pre-labelled 'clone' of cells were masked by the substantial increase in the un-labelled 'clone' of proliferating cells. Therefore, we conclude that, in the SGZ, Kainate/seizures increased the proliferation of both pre-labelled and un-labelled 'clones' of precursor cells with no preferential effect on the pre-labelled 'clone'. These results are entirely consistent with the lack of an overall change in the number of SGZ BrdU labelled cells, as some BrdU cells will be post mitotic, many of the dividing ones at 72 h may not have yet undergone mitosis and many may have migrated out of the SGZ into the GCL (*vide infra*).

Interestingly, however, Kainate/seizures significantly increased the number and proportion of proliferating pre-labelled cells at the 72 hours time point in the GCL as measured by the proportion of cells that are BrdU+ and Ki-67+ with respect to overall Ki-67+ cell numbers (BrdU+ and Ki-67+/Ki-67+) (**Section 6.4.2.2**). Here we demonstrate a five-fold increase in the proportion of pre-labelled 'clone' of proliferating cells after 72 hours of Kainate treatment when compared to saline conditions. This may suggest that Kainate/seizures cause dispersion of SGZ BrdU+ cells and/or enhance the proliferation of GCL pre-labelled 'clone', or both. In agreement with this, our results in

Section 6.4.5 demonstrate a significant increase in the numbers and proportion of the pre-labelled 'clone' of cells in the combined SGZ and GCL. Consistent with our results, Nakagawa et al have shown that Kainate-induced seizures enhanced progenitor cell proliferating in the adult rats as early as 3 days after the insult and BrdU+ cells were distributed in the GCL and not SGZ (Nakagawa, Aimi et al. 2000). In this previous study, a single pulse of BrdU was used to label proliferating cells before seizure induction and thus it was unclear if BrdU incorporated cells continued to divide or exited the cell cycle and became postmitotic. However, in our paradigm, the combined use of Ki-67 and BrdU enabled us to measure cells that continued to proliferate (BrdU+ and Ki-67+) as well as those that exited the cell cycle as indicated by counting BrdU+ and Ki-67- cells (see the next section). On this basis, a robust assessment of proliferation of the pre-labelled 'clone' can be obtained after Kainate/seizures. In another study, although Parent et al have demonstrated a proportional increase in cell proliferation after pilocarpine-induced seizures in the SGZ, there was a substantial migration of proliferating cells into the GCL (Parent, Yu et al. 1997). In addition, Parent and co-workers administered 4 IP injections of BrdU after seizure-induction and thus we expect to observe higher proportion of BrdU cells after seizures than would be after one single injection (as in our model). Moreover, in Parents study, it is not possible to identify cells that have become postmitotic. In addition, seizures might change the permeability of the blood brain barrier and thus BrdU counts may be inaccurately estimated. In our study, the BrdU was administered 24 hours before Kainate over a short bioavailability of 2 hours after IP injection and so increases in BrdU counts are not attributable to altered bioavailability and must reflect altered cell cycle kinetics.

Interestingly, in the hilus and molecular layer, Kainate/seizures increased the proportion of pre-labelled 'clone' of cells more than ten-folds (**Sections 6.4.3.1 and 6.4.4.1**). These results strongly suggest substantial recruitment of the pre-labelled 'clone' of precursor cells after Kainate induced status epilepticus. This raises the possibility that the proliferation of this 'clone' of cells may be the major contributor of altered precursor proliferation to the pathology of Kainate-induced status epilepticus.

6.5.3 Kainate-induced seizures tend to reduce postmitotic cells in the SGZ and GCL while it preferentially enhances the proliferation of pre-labelled 'clones' of precursors in the GCL

The labelling of a 'clone' of proliferating cells that we have termed 'precursor' cells made it possible to investigate the effects of Kainate/seizures on a sub-population of cells that was pre-labelled as well as un-labelled sub-population of precursors. We measured these sub-populations of cells by counting Ki-67+ and BrdU- (un-labelled), BrdU+ and Ki-67+ (pre-labelled that continued to divide), BrdU+ and Ki-67- (postmitotic). We further measured the proportion of these sub-populations of cells. A proportional increase suggests a preferential effect on that specific sub-population of cells.

Significantly, we find that approximately 21000 cells /SGZ are expressing Ki-67 only at 72 hours time point in Kainate injected animals and was significantly higher than Ki-67 expressing cells (nearly 14300 cells/SGZ) in saline injected animals (**Section 6.4.1.3**). This substantial increase in the number of Ki-67 only expressing cells was observed in the SGZ and not in the GCL or the other DG sub-areas.

However, the proportion of proliferating un-labelled cells (Ki-67 positive BrdU negative) cells with respect to the total numbers of cells in the cell cycle (Ki-67+&BrdU-/Ki-67+) is the same in both Kainate and control groups suggesting that both un-labelled and labelled populations contributed equally to the increased cell proliferation after Kainate. On the other hand, Kainate/seizures reduced the proportion of cells that became postmitotic by 20%. Although this decrease is not statistically significant, it suggests that Kainate/seizures are increasing the number of pre-labelled cells that continued to be in the cell cycle. In other words, it enhances cell cycle re-entry. It may also enhance self-renewal of at least a fraction of the pre-labelled 'clone' of cells. The fact that there is no increase in the counts or the proportion of BrdU pre-labelled 'clone' of cells in the SGZ again suggests equal effects of Kainate/seizures on the proliferation of both pre-labelled and un-labelled precursors. In line with this, Huttmann and colleagues have shown that Kainate/seizures induced progenitor cell proliferation in the adult mice SGZ by 72 hours after the insult (Huttmann, Sadgrove et al. 2003).

However, it was not clear, in the previous study, whether Kainate/seizures affected/recruited quiescent precursor cells or had an effect on postmitotic cells. In addition, in the SGZ, it has been demonstrated that new neurons or a fraction of them are derived from radial glia which in turn divide into so-called D cells that ultimately become dentate gyrus granule cells (Seri, Garcia-Verdugo et al. 2004). We conclude that precursor cells that give rise to new neurons reside in the SGZ and thus our results demonstrate an enhancement of proliferation of precursor cells which gives birth to new neurons. Moreover, these distinguishing findings are a hallmark of the SGZ only and these results are in line with describing the SGZ as a stem/progenitor cell niche.

However, in the GCL, there is a transient recruitment of un-labelled precursor cells after 48 hours of Kainate/seizures which was not evident thereafter (**Section 6.4.2.4**). These results suggest that the increase in cell proliferation after Kainate/seizures at 48 h time point is due to shift in the induction of proliferation towards an un-labelled sub-population of cells. This population is likely to be composed of a "clone" of Dcx positive cells that were older than the pre-labelled 'clone' and which will have undergone mitosis to result in an increase in total Dcx cells by 72 h. Contrary to this, at 72 h Kainate/seizures' effects are shifted towards enhancing more pre-labelled cells to continue dividing. These results also suggest that Kainate/seizures might enhance cell cycle re-entry and perhaps reduce the length of the cell cycle.

An intriguing feature of our *in vivo* paradigm is that it enabled us to examine the effects of Kainate/seizures on pre-labelled cells that became postmitotic at the time of sacrifice.

We show that Kainate-induced seizures have a strong tendency to reduce the proportion of postmitotic sub-population of cells in the SGZ (**Section 6.4.1.4**). Indeed, the number of cells that are BrdU+ and Ki-67- is lower in the Kainate/seizures group than the saline group at 72 h time point. Interestingly, Kainate/seizures reduced the proportion of postmitotic cells from 68% under control conditions to 48% under Kainate/seizures conditions. Therefore, Kainate/seizures may enhance cell cycle re-entry and may also shorten the cell cycle length. This also implies that Kainate/seizures do not enhance cell proliferation of the pre-labelled 'clone' of cells in a steady state pattern that was described by Hayes et al 2002 (Hayes and Nowakowski 2002). If BrdU pre-labelled

cells are proliferating in a steady state then we are expecting an increase in the number of BrdU cells as well as those that become postmitotic. Consistent with our hypothesis, Kainate/seizures are reducing the postmitotic sub-population of cells, or tending to do so, and therefore, the pre-labelled 'clone' of cells is not proliferating in a steady state described by Hayes. Furthermore, Hayes and colleagues described cell proliferation in the whole DG and not in each specific sub-area and since the SGZ is the stem cell niche we are not expecting all proliferating cells to behave similarly. To circumvent this, we analyzed the different sub-areas of the DG separately

Another possibility is that Kainate/seizures enhanced the proliferation of the pre-labelled 'clone' of cells in the SGZ but this effect was associated with dispersion of these BrdU+ cells into the GCL. Since more pre-labelled cells are contributing to the proliferating cells in the GCL at 72 h time point which is not evident before that although there is a significant increase in Ki-67 positive cells at 48 h, this suggests that perhaps a proportion of SGZ BrdU pre-labelled cells are dispersed in the GCL by 72 h. In line with this, our results from the combined SGZ and GCL anaylsis indicate that the pre-labelled 'clone' of cells increased signficnatly in this combined area suggesting an encncemnet of cell cycle re-entry as well as dispersion of the pre-labelled 'clone' into the GCL.

Likewise, Kainate/seizures have a tendency to decrease the proportion of the pre-labelled 'clone' of cells that exited the cell cycle at 72 h in the GCL (**Section 6.4.2.3**). Although the numbers of postmitotic (BrdU+ and Ki-67-) cells increased in GCL in the Kainate group at 72 h time point, there is a reduction in the proportion of postmitotic sub-population of cells in the GCL in the Kainate group (**Section 6.4.2.3**). However, this 20% decrease in the postmitotic cells fails to reach statistical significance. This may suggest that Kainate/seizures enhanced the proliferation of pre-labelled GCL precursor cells perhaps by enhancing cell cycle re-entry. Another possibility to explain the increase in pre-labelled 'clone' of cells and reduction in the postmitotic sub-populations is shortening of the cell cycle of precursors after Kainate/seizures. Although exact changes in the cell cycle length cannot be measured directly in our system, it is possible to predict differences from changes in the different BrdU+ populations. Since BrdU only cell counts and BrdU+ and Ki-67+ cell number are significantly increased after Kainate/seizures in the GCL, it is likely that Kainate/seizures shortened the length of the

cell cycle and/or enhanced cell cycle re-entry. The overall increase in the total BrdU positive population could probably be achieved if Kainate caused a switch to exponential cell division, with all daughter cells remaining in the cell cycle. However, there will be no decrease in BrdU only sub-population (postmitotic) which was not the case in our experiments. A decrease in the cell cycle time from the reported 25 hours in 10 weeks old rats (Cameron and McKay 2001) to the shortest reported time which is 16 hours in the postnatal rats (Lewis 1978) is sufficient to permit a completion of at least an additional cell cycle between 6 hours and 72 hours points examined. This would allow the generation of BrdU only cells. However, it appears that the pre-labelled 'clone' of cells is dividing with slight shift from the steady state because we report an increase in the proportion of BrdU+ and Ki-67+ sub-population as well. The increase in the BrdU+ and Ki-67+ double-labelled cells can only arise as a consequence of the dividing 'clone' cells switching to regenerate themselves more frequently after Kainate/seizures.

On the other hand, in the hilus and molecular layer of the DG, we show that approximately half of BrdU incorporated cells are postmitotic at 72 hours time point in Kainate conditions which is significantly higher than saline conditions (**Sections 6.4.3.1 and 6.4.4.1**). However, no difference in the proportion of the cells that became postmitotic in the Kainate group was observed when compared to the saline group. These results suggest that Kainate/seizures have complex effects on precursor cells in both the hilus and the molecular layer. This is perhaps due to the nature of these sub-areas, as we are not expecting proliferating cells in either area to behave like the stem cell niche. However, shortening the cell cycle of pre-labelled 'clone' of precursor cells in these areas might contribute to the increase in cell proliferation. In addition, an increase in the proportion of BrdU and Ki-67 double-labelled cells indicates an ongoing regeneration of this 'clone' of cells. Another possibility is that a fraction of the pre-labelled SGZ 'clone' migrated to these areas. However, we can conclude the pre-labelled 'clone' of cells is preferentially affected by Kainate/seizures in the hilus and Ml of the DG. Determining the phenotype of the proliferating cells in these areas would help unveil the effects of Kainate/seizures on these cells.

While following the proliferation of BrdU pre-labelled 'clone' of cells we should rule out the possible dilution of BrdU. It has been demonstrated that BrdU dilution below

detection levels occurs after 3-4 cell cycles in adult mice (Hayes and Nowakowski 2002). Furthermore, in another study, Dayer et al demonstrated that BrdU can be detected up to 4 days after application (Dayer, Ford et al. 2003). To circumvent this dilution issue, we followed the fate of BrdU incorporated cells up to 96 hours (4 days). Consistent with this, there is no drop in BrdU positive cell number in any of the DG sub-areas throughout the course of the experiment under control conditions. However, we propose that part of the increase in BrdU+ cells perhaps due to the shortening of the cell cycle and thus BrdU might be diluted. In contrast to this, there is a significant increase in the proportion of pre-labelled 'clone' of cells (BrdU incorporated cells) at 72 hours after Kainate/seizures in the GCL. This suggests that, in our system, we are still able to detect BrdU+ cells up to 72 hours post Kainate treatment. Another possibility is that a fraction of BrdU incorporated cells that re-entered the cell cycle proliferated 3-4 times before exiting the cell cycle and become postmitotic and therefore, BrdU is detected in postmitotic cells. Cells that exit the cell cycle retain BrdU and can be detected anytime thereafter.

In conclusion, Kainate/seizure enhances equally the proliferation of the pre-labelled 'clone' of cells as well as the un-labelled sub-population of cells in the SGZ. It also tends to reduce postmitotic cells by enhancing cell cycle re-entry and it may also shorten the cell cycle length. However, in the GCL, there is a preferential effect of Kainate/seizures on the pre-labelled 'clone' of cells at 72 h as well as a tendency to reduce postmitotic cells. Furthermore, Kainate/seizures affect the proliferation of the pre-labelled 'clone' of cells in the hilus and Ml with no effect on postmitotic cells.

6.5.4 Kainate/seizures transiently enhance the survival of cells in the hilus and molecular layer without affecting cell death in the SGZ and GCL

It is now well accepted that newly-born granule cells mature into functional neurons in the adult mammalian brain (van Praag, Schinder et al. 2002). Furthermore, granule cells born during adulthood that become integrated into circuits and survive to maturity are

very stable and may replace granule cells born during development (Dayer, Ford et al. 2003). However, it has been shown that 0.004% of granule cells are a contribution of newly-born neurons in the adult monkeys (Kornack and Rakic 1999). Furthermore, in an elegant study, it has been demonstrated, using transgenic mice, that nestin expressing cells contribute to no more than 1% of DG granule cell layer (Lagace, Whitman et al. 2007). In spite of the fact that stem/precursor cells contribution to adult neurogenesis is very small, interestingly, it has emerged that adult-born granule cells that survive and mature into functional neurons contribute to hippocampal dependent memory formation (Kee, Teixeira et al. 2007). Therefore, we decided to investigate the effects of Kainate/seizures on cell death in our *in vivo* paradigm and thus unravel the association of cell death with proliferation. This may help understating of mechanisms underlying the increase in proliferating precursor cells and thus their contribution in learning and memory.

We measured cell death by identifying caspase-3 activated cells in the SGZ, GCL, hilus, and ML. Determining activated caspase-3 cells in status epilepticus is a well known method to study cell death (Ekdahl, Zhu et al. 2003; Dhanushkodi and Shetty 2008). Interestingly; there is no effect for Kainate/seizures on caspase-3 activated cells in the SGZ (**Section 6.4.1.5**). Consistent with this, the number of BrdU incorporated cells (pre-labelled 'clone') in the SGZ is the same in Kainate injected animals and saline ones. This suggests that Kainate/seizures did not enhance cell death in the SGZ pre-labelled 'clone' of cells. In agreement with our study, it has been demonstrated that Kainate-induced status epilepticus hardly damages dentate gyrus granule cells when compared to pilocarpine-induced status epilepticus (Covolan, Ribeiro et al. 2000). In line with this, there is no significant increase in caspase-3 activated cells in the GCL when comparing Kainate/seizures conditions to saline conditions (**Section 6.4.2.5**). However, we observe an overall increase in caspase-3 activated cells at 48 hours time point under Kainate/seizures conditions when compared to 6 hours Kainate/seizures conditions, with no significant difference in caspase-3 immuno-positive cells between saline and Kainate conditions at the two time points examined. This might be perhaps as a result of the slight decrease in activated caspase-3 cells at 6 hours time point under Kainate/seizures

conditions when compared to controls which increased under both conditions at the same rate thereafter.

Surprisingly, in **Sections 6.4.3.2 and 6.4.4.2**, we show a significant decrease in the number of activated caspase-3 cells after 6 hours of Kainate treatment when compared to controls in the hilus and molecular layer of the dentate gyrus. Although cell death increased after 48 hours of Kainate treatment when compared to 6 hours Kainate conditions, there is no difference between Kainate conditions and controls at this time point. The increase in apoptotic cell death as indicated by caspase-3 activated cells in both Kainate and saline groups at 48 hours time point might be due to the effect of acute stress as a result of handling and injecting the animals. Consistent with this, it has been shown that acute stress decreases neurogenesis (Tanapat, Galea et al. 1998). Therefore, the increase in cell death (caspase-3 positive cells) in both groups (Kainate and saline) at 48 h time point is perhaps due to acute stress that both groups of animals experienced while being injected and handled. Since there is no difference between Kainate injected animals and saline ones, it is highly unlikely that Kainate is inducing cell death.

It is still unclear why Kainate-induced status epilepticus decreased caspase-3 activated cells at 6 hours time point. However, in an elegant study, Zucchini et al 2005 have demonstrated an increase in FGF-2 after Kainate-induced status epilepticus (Zucchini, Barbieri et al. 2005). Another study has demonstrated a marked increase in mRNA of FGF-2 after Kainate-induced seizures (Bugra, Pollard et al. 1994). Furthermore, it is well known that FGF-2 enhances not only the proliferation but also the survival of progenitor cells *in vivo* and *in vitro* (Lowenstein and Arsenault 1996; Kuhn, Winkler et al. 1997; Liu, Stafstrom et al. 1997). On this basis, there might be perhaps an initial up regulation of FGF-2 that protects against Kainate induced cell death. In agreement with this idea, Yoshimura and co-workers described an increase in FGF-2 level in adult mice hippocampi after Kainate-induced status epilepticus which peaked at 1 day post treatment (Yoshimura, Takagi et al. 2001). In the previous study, importantly, they found that 10mg/kg of IP Kainate induced seizures which also increased DG cell proliferation without affecting DG cell death. By and large, our results are consistent with Yoshimura et al as we used 10 mg/kg of Kainate to induce status epilepticus. There

might be an initial up regulation of trophic factors in the hippocampus that prevents cell death thereafter.

Moreover, Kainate-induced seizures increased the levels of Neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP) in the adult rats' hippocampus (Marksteiner, Sperk et al. 1989). In addition, it has been shown in our lab that NPY increased precursor cell proliferation (Howell, Scharfman et al. 2003) and VIP is trophic for hippocampal precursor cells (unpublished data). Although VIP is trophic for DG precursor cells, its role after seizures is yet to be determined. Although Howell et al have demonstrated that cell proliferation is lower in NPY knockout mice than age-matched controls; he showed that the relative increase in cell proliferation after Kainate was the same in the wild-type and NPY knockout mice (Howell, Silva et al. 2007). On this basis, he suggested that NPY is not entirely responsible for modulating cell proliferation after seizures. However, in the previous study, cell proliferation was examined after 5 days of seizures-induction and therefore, it is unclear if NPY has an earlier effect on precursor cells. On the other hand, it has been shown that Kainate-induced seizures increased the expression of NPY in adult Wistar rats and is associated with less severe epileptic activity (El Bahh, Auvergne et al. 2001). In line with this, brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) were found to be up regulated with subsequent increase in NPY (Croll, Wiegand et al. 1994). Consistent with this, it has been demonstrated that BDNF, NGF, FGF-2 and Epidermal growth factor (EGF) increased after 4 hours in pilocarpine-induced seizures model (Hagihara, Hara et al. 2005). Likewise, it has been reported that Kainate-induced seizures increased neurotrophic factors (Lowenstein, Seren et al. 1993; Shetty, Zaman et al. 2003; Shetty, Rao et al. 2004). In addition, Scharfman et al have described an increase in the GCL and hilus cell proliferation after BDNF infusion (Scharfman, Goodman et al. 2005). The previous studies may suggest a role for neurotrophic factors after perhaps Kainate-induced seizures which may explain the transient increase in cell survival shortly after Kainate treatment.

Kainate-induced seizures are usually associated with enhanced cell death in CA1-3 depending on the severity of the insult and the mode of Kainate administration with preservation of the dentate granule cells (Covolan, Ribeiro et al. 2000; Tokuhara, Sakuma et al. 2007). Although we did not quantify caspase-3 activated cell numbers in

CA1-3, we observed that caspase-3 is substantially expressed in CA1-3 regions in Kainate treated animals but not in controls (**Figure 6.26**).

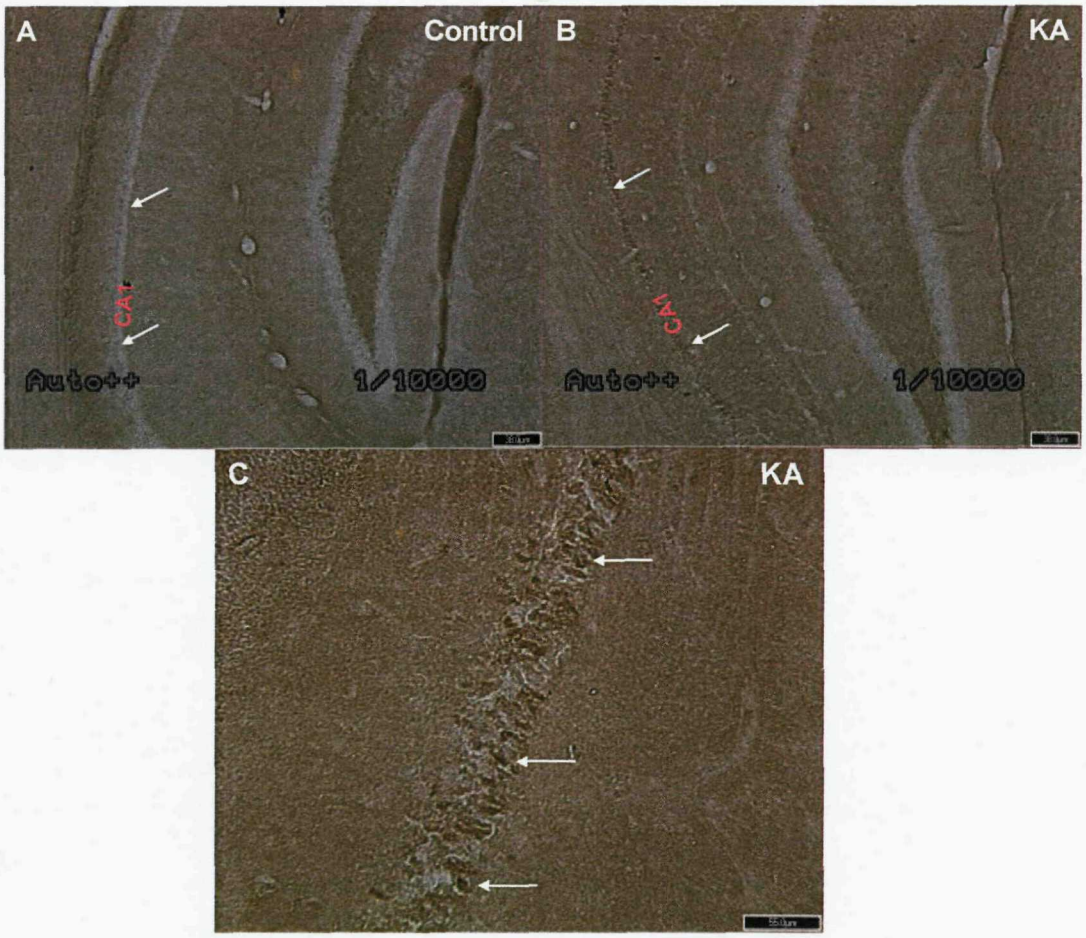


Figure 6.26 Kainate/seizures enhanced the expression of Caspase-3 in CA1-3 of the hippocampus. While doing immunohistochemistry to identify caspase-3 activated cells in the DG we observed an increased expression of caspase-3 in the areas CA1-3 of the hippocampus in Kainate conditions (B) to saline conditions (A). C) It shows higher magnification of caspase-3 expression in CA3 of the hippocampus after 48 h of Kainate treatment. Using 20x objective magnification on a Stereology Microscope (Dialux 22, Leitz) caspase-3 Immuno-positive cells were observed in CA1-3 areas. Caspase-3 staining was visualized under bright-field microscopy. White arrows show activated caspase-3 cells in the CA1 in control animals and Kainate injected animals. Scale bar = 38μm (A and B). Scale bar = 55μm (C).

6.5.5 Increased neurogenesis is induced by Kainate/seizures

In addition to the increase in cell proliferation, we observe an increase in neurogenesis. The increase in neurogenesis determined by Dcx immunostaining was found after 72 hours of Kainate treatment (**Section 6.4.6**). Our results show about 25% increase in neuroblasts at 72 hours time point when comparing Kainate injected animals with controls. It has been demonstrated that Kainate-induced seizures increased a sub-population of type-3 neural progenitor cells in the adult DG (Jessberger, Romer et al. 2005), which are mitotic cells that express an immature neuronal phenotype (Seri, Garcia-Verdugo et al. 2004). Furthermore, Gray et al have elegantly shown a bilateral increase in the adult DG neurogenesis after unilateral ICV Kainate infusion (Gray and Sundstrom 1998). In the previous study, most of the proliferating DG cells expressed the neuroblasts marker, class III ß-tubulin, and rarely expressed the astrocytic marker, GFAP; however, the mechanisms underlying the acute effects of Kainate/seizures on DG proliferation and neurogenesis remained to be elucidated. Importantly, Shapiro and colleagues have recently reported a significant neuroplastic changes in DG Dcx sub-population of cells after pilocarpine-induced seizures examined between days 1 and 5 (Shapiro, Figueroa-Aragon et al. 2007). Indeed, Shapiro and co-workers demonstrated a significant increase in Dcx number after 3 days of pilocarpine/seizures. This is strongly agrees with what we have shown in our study. Moreover, the increase in a fraction of the proliferating Dcx positive cells after Kainate-induced seizures reported by Jessberger et al 2005 supports our findings. Consistent with this, it has been demonstrated that approximately 20% of Dcx immuno-positive cells are in the cell cycle as indicated by their expression of Ki-67 marker (Plumpe, Ehninger et al. 2006). Indeed, Plumpe et al 2006 have reported maturation of Dcx expression in 3 days. In our model, we show approximately 35 % and 45% increases in precursor cell proliferation in the SGZ and GCL, respectively, at 72 hours time point. In addition, 25% increase in Dcx positive cells under Kainate conditions. This may suggest that a substantial fraction of these Dcx positive cells were formed after Kainate treatment. Interestingly; although it has been shown that Kainate/seizures increased astrocyte-like precursor cells in the SGZ (Huttmann, Sadgrove et al. 2003), there was no increase in TUC-4 neuronal marker. Probably TUC-4 did not detect early proliferating neuroblasts or newly-born neurons

which resulted in underestimating neuroblasts counts after Kainate/seizures. Another possibility is that Kainate-induced seizures enhanced the proliferation of a fraction of Dcx expressing cells. In this regard, we reported that an increase in cell proliferation started at 48 h time point in the GCL of the Kainate group. We also know from previous studies that slightly over 20% of Dcx cells (type-3 precursors) are proliferating as measured by their expression to Ki-67 (Jessberger, Romer et al. 2005; Plumpe, Ehninger et al. 2006). Therefore, it is most likely that Kainate-induced seizures enhanced the proliferation of type-3 precursor cells (proliferating Dcx cells) and thus we observe an increase in neurogenesis at this early stage (72 h).

Here, we demonstrate a significant effect of Kainate/seizures on neurogenesis identified by Dcx; however, the effects of Kainate/seizures on astrocytes remain to be examined.

6.6 Conclusions

We conclude that Kainate/seizures enhanced neurogenesis in the DG of adult rats as indicated by the increase in doublecortin positive cells after 72 hours of the treatment. Interestingly, there was no induction of cell death in the SGZ, GCL, hilus and ML after Kainate/seizures as measured by activated caspase-3. This was further confirmed by the survival of the pre-labelled 'clone' of cells which was the same under Kainate/seizures and controls. Our *in vivo* paradigm enabled us to elucidate the effects of Kainate/seizures on a pre-labelled 'clone' of precursor cells (BrdU+), un-labelled precursors (Ki-67+ and BrdU-), and postmitotic cells (BrdU+ and Ki-67-). We found that Kainate/seizures equally enhanced the proliferation of pre-labelled and un-labelled precursors in the SGZ and also had a strong tendency to reduce the proportion of cells that exit the cell cycle and become postmitotic most likely by enhancing cell cycle re-entry and possibly shortening the length of the cell cycle. However, in the GCL, Kainate/seizures preferentially enhanced the proliferation of pre-labelled 'clone' of cells with a tendency to reduce postmitotic sub-population of cells. In addition, our results from the combined SGZ and GCL indicate that the increase in the proliferation of the GCL pre-labelled 'clone' of cells might be a result of dispersion of the SGZ stem cell niche after Kainate/seizures. In the hilus and molecular layer, the pre-labelled 'clone' of cells contributed even more to cell proliferation after Kainate/seizures with no effect on the proportion of postmitotic cells. Overall Kainate/seizures most probably enhanced the proliferation of pre-labelled 'clone' of precursor cells by shortening their cell cycle and re-entering the cell cycle. These results demonstrate a complex interplay on different precursor cell populations in the SGZ and GCL after Kainate- induced seizures.

Chapter Seven

General Discussion

7.1 Overview and summary of the major findings

An accumulating body of evidence over the past five decades has led to the widespread recognition that neurogenesis, the generation of new neurons, persists in the hippocampal dentate gyrus of the postnatal and adult mammalian brain (Altman and Das 1965; Gould, Reeves et al. 1999; Gage 2000; Howell, Scharfman et al. 2003; Howell, Doyle et al. 2005; Howell, Silva et al. 2007). There is much interest in elucidating the role of adult hippocampal neurogenesis in the normal brain, where it is associated with hippocampal-dependent learning and memory (Shors, Miesegaes et al. 2001; van Praag, Schinder et al. 2002) as well as the control of mood (Santarelli, Saxe et al. 2003).

Approximately 50 million people in the USA have epilepsy and around 40% of these patients suffer from temporal lobe epilepsy (TLE) (Shetty and Hattiangady 2007). Over a third of patients with TLE have chronic seizures that are resistant to antiepileptic drugs (Engel 2001; McKeown and McNamara 2001). The majority of TLE patients suffer from learning and memory dysfunctions (Devinsky 2004; Helmstaedter, Brosch et al. 2004) as well as depression (Mazza, Orsucci et al. 2004). Hippocampal lesions inflicted by acute status epilepticus primarily lead to epileptogenic structural changes which in turn progress into hippocampal dysfunction typified by chronic epilepsy (Shetty and Hattiangady 2007). Learning and memory impairments are associated with chronic epilepsy which concurs with a reduction in hippocampal neurogenesis (Hattiangady, Rao et al. 2004; Helmstaedter and Kockelmann 2006).

Although neurogenesis is reduced in chronic TLE, Kainate-induced seizures and status epilepticus (SE) initially transiently enhance DG neurogenesis (Bengzon, Kokaia et al. 1997; Parent, Yu et al. 1997; Gray and Sundstrom 1998; Covolan, Ribeiro et al. 2000; Nakagawa, Aimi et al. 2000; Gray, May et al. 2002; Hattiangady, Rao et al. 2004; Jessberger, Zhao et al. 2007) before later falling below normal levels in chronic epilepsy. However, neurogenesis is chronically altered immediately after status epilepticus as evidenced by abnormal connectivity of the newly generated neurons, which are only later diminished in number. Chronic epilepsy usually occurs after a latent period following the initial precipitating injury (Mathern, Pretorius et al. 1995). Therefore, a promising intervention strategy to curb epileptogenesis should be applied

shortly after the injury. The mechanisms underlying seizure-altered neurogenesis are incompletely understood. However, altered neurogenesis clearly involves changes in stem/precursor cells proliferation, differentiation as well as survival and integration and/or death. Therefore, understating the control mechanism of neurogenesis processes would increase the possibilities of successfully targeting stem/precursor cells after status epilepticus to prevent or ameliorate cognitive dysfunction, abnormal mood control or even perhaps mesial temporal epileptogenesis itself. Although there is currently little evidence for the pervasive spontaneous replacement of dead neurons in the hippocampus after status epilepticus, there is enormous interest for stimulating endogenous neural stem/precursor cells in the neurogenic niches to generate new neurons to participate in brain repair after injury. As such, TLE is also a possible clinical target for stem/precursor cell based therapies.

Summary of the major in vitro findings:

The primary hippocampal cell culture we used is a relatively well controlled method which enables us to quantify the variable effects of Kainate on different cell-types' proliferation, survival and differentiation over specific time periods (Brewer 1997; Brewer 1999; Howell, Scharfman et al. 2003; Howell, Doyle et al. 2005; Howell, Silva et al. 2007). Using this *in vitro* paradigm we have demonstrated that Kainate increased overall cell numbers and the number of proliferating hippocampal cells in culture. We have found that 5μM Kainate enhanced cell proliferation as indicated by the increase in the mitotic index (BrdU/DAPI) and the speed of the cell cycle (labelling index) at 3 DIV. While we described that 3 days of Kainate treatment and 6h/8h of Kainate exposure at day three were proliferative for hippocampal nestin expressing cells as indicated by the increase in both the mitotic index and the labelling index of nestin immuno-positive cells, only 3 days of Kainate treatment enhanced the proliferation of neuronal precursor cells (TuJ1 positive). Moreover, our results have demonstrated an increase in the total cell count as well as a proportional rise in the TuJ1 sub-population of cells after five days in culture. We have further shown that either 5 days of Kainate treatment or 6h/8h of Kainate exposure at day five were proliferative for nestin immuno-

positive cells as indicated by the increase in the proportion of nestin positive cells (BrdU+ nestin+ /nestin+). We also demonstrated that an 8 hour pulse of Kainate significantly increased the speed of the cell cycle of nestin positive cells as measured by the labelling index (BrdU+ nestin+ Ki-67+/nestin+ Ki-67+). We examined cell phenotype in culture and reported an increase in nestin, TuJ1, and GFAP immunopositive cells in response to 5 days of Kainate treatment with a proportional rise seen only in the TuJ1 sub-population of cells. We showed that 5 days of Kainate treatment has a combination of proliferation and differentiation effects on progenitor (nestin positive) cells towards a neuronal lineage, which is not detected after 3 DIV. Moreover, 5 days of Kainate treatment caused a significant increase in the number of cells that express nestin and GFAP as well as cells that express nestin only. In order to study whether the effects of Kainate *in vitro* are modulated via AMPA and/or Kainate receptors, we applied NBQX (AMPA and Kainate receptors antagonist) and GYKI52466 (AMPA receptor antagonist) onto cells in culture. We demonstrated that the addition of either NBQX or GYKI52466 in conjunction with Kainate abolished the proliferative effects of Kainate on cultured hippocampal cells as well as the increases in both nestin and TuJ1 immuno-positive cells. Moreover, we also found that, like Kainate, (S)-AMPA, the AMPA receptor selective agonist, increased the total numbers of hippocampal cells in culture.

We have also looked at the effects of Kainate on the survival of hippocampal precursor cells and their neuronal progeny cells in cultures. We demonstrated that 5 days of Kainate exposure enhanced the survival of nestin positive cells, TuJ1 expressing cells, as well as proliferating cells (BrdU incorporated cells) as indicated by the decrease in the proportion of their expression of caspase-3 antigen. Moreover, terminal 6 h exposure of Kainate at day 5 enhanced overall the survival of hippocampal cells in general as measured by DAPI+/MitoTracker Orange+ and in particular on progenitor cells including the TuJ1 sub-population of cells. We time-lapse videoed cells in culture and found that 25 h of Kainate treatment at day 5 enhanced the survival of hippocampal cells and newly-born cells that were nestin+/TuJ1- and nestin+/TuJ1+.

Summary of the major in vivo findings:

In vivo, we pre-labelled a 'clone' of proliferating precursors with BrdU 24 hours before seizures were induced by Kainate in an animal model of SE. We found that Kainate/seizures enhanced neurogenesis in the DG of adult rats as indicated by the increase in doublecortin positive cells after 72 hours of the treatment. Interestingly, there was no induction of cell death in the SGZ, GCL, hilus and ML after Kainate/seizures as measured by activated caspase-3. This was further confirmed by the survival of the pre-labelled 'clone' of cells which was the same under Kainate/seizures and controls. Our *in vivo* paradigm enabled us to elucidate the effects of Kainate/seizures on a pre-labelled 'clone' of precursor cells (BrdU+), un-labelled precursors (Ki-67+ and BrdU-), and postmitotic cells (BrdU+ and Ki-67-). We found that Kainate/seizures equally enhanced the proliferation of pre-labelled and un-labelled precursors in the SGZ, (although there was a trend towards a greater proliferation of the unlabelled clone), and also had a tendency to reduce the proportion of cells that became postmitotic. It also dispersed the SGZ stem cell niche into the GCL as indicated by the dramatic increase in the pre-labelled 'clone' of precursors in the combined SGZ and GCL area. On the other hand, in the GCL, Kainate/seizures preferentially enhanced the proliferation of pre-labelled 'clone' of cells, at a significantly earlier time point (48h), with a tendency to reduce the postmitotic sub-population of cells. Combining the cell counts from the SGZ and GCL showed a significant increase (3 fold) in the numbers of 'clonal' BrdU labelled cells and proliferating BrdU labelled cells in the Kainate group at 72 h post status, consistent with both increased cell cycle re-entry and a shortened cell cycle time, in the 'clonally' labelled cells under Kainate conditions. There was also some evidence to support increased recruitment of the pre-labelled 'clone', but this alone could not have explained the dramatic increase in cell number noted.

Kainate/seizures mainly enhanced the proliferation of the pre-labelled 'clone' of cells in the hilus and molecular layer with no effect on the proportion of postmitotic cells. In general, Kainate/seizures most likely enhanced the proliferation of pre-labelled 'clone' of precursor cells by shortening their cell cycle and enhancing cell cycle re-entry.

7.2 Kainate is proliferative for hippocampal precursor cells

The generation of cells that eventually will become neurons is the first step in neurogenesis. It is now well established that neurogenesis in both the DG and olfactory bulb is evidently tied to the production of new daughter cells from a stem/precursor population via mitosis (Christie and Cameron 2006). BrdU immunohistochemistry has been instrumental to study birth dating and monitor cell proliferation in the mammalian brain, including in human (Taupin 2007). BrdU is a thymidine analog that incorporates into the DNA of dividing cells during the S-phase of the cell cycle (Nowakowski, Lewin et al. 1989). Therefore, short terminal pulses of BrdU will only label cells that entered the S-phase of the cell cycle over that time period. Measuring the proportion of cells that incorporated BrdU with respect to the overall cell population in culture (DAPI positive cells) will determine the average mitotic index (MI) (Joo, Kim et al. 2007). An increase in the overall mitotic index indicates an increase in the proliferation of an averaged cell population. Likewise, identifying the proportion of a specific cell phenotype (nestin or TuJ1 expressing cells) that incorporated BrdU with respect to total number of the same cell phenotype will determine the mitotic index of the specific cell phenotype. However, an increase in cell proliferation could be either due to an increase in the average cell cycle speed or as a result of recruitment of quiescent cells into the cell cycle or a combination of both. Measuring the speed of the cell cycle necessitates the use of another proliferative marker along with BrdU. Therefore, we used the cell cycle phase specific protein Ki-67, which is expressed in all phases of the cell cycle except for the resting phase (G_O) (Zacchetti, van Garderen et al. 2003), to determine the size of the proliferating population and the speed of the cell cycle (Contestabile, Fila et al. 2007; Mandyam, Harburg et al. 2007; Olariu, Cleaver et al. 2007) (by measuring the proportion of S-phase entering cells of the total proliferating cell population). Furthermore, identifying the proportion of Ki-67 positive cells with respect to the total cell number (DAPI) will determine the growth fraction (GF) (**Figure 7.1 and 7.2**). An increase in the growth fraction indicates recruitment of quiescent cells. In our in vitro paradigm, a short pulse of BrdU measures the fraction of cycling precursor cells (Ki-67 positive) in the S-phase of the cell cycle and identifies the labelling index (LI) (**Figures 7.1 and 7.2**) (Dehay, Savatier et al. 2001; Olariu, Cleaver et al. 2007). As such, an

increased labelling index would indicate an increase in proliferation rate (**Figure 7.2**) (Dehay, Savatier et al. 2001). Similarly, measuring the LI of precursor cells (nestin positive) would determine the effect of a factor on the precursor cell proliferation rate (speed of the cell cycle).

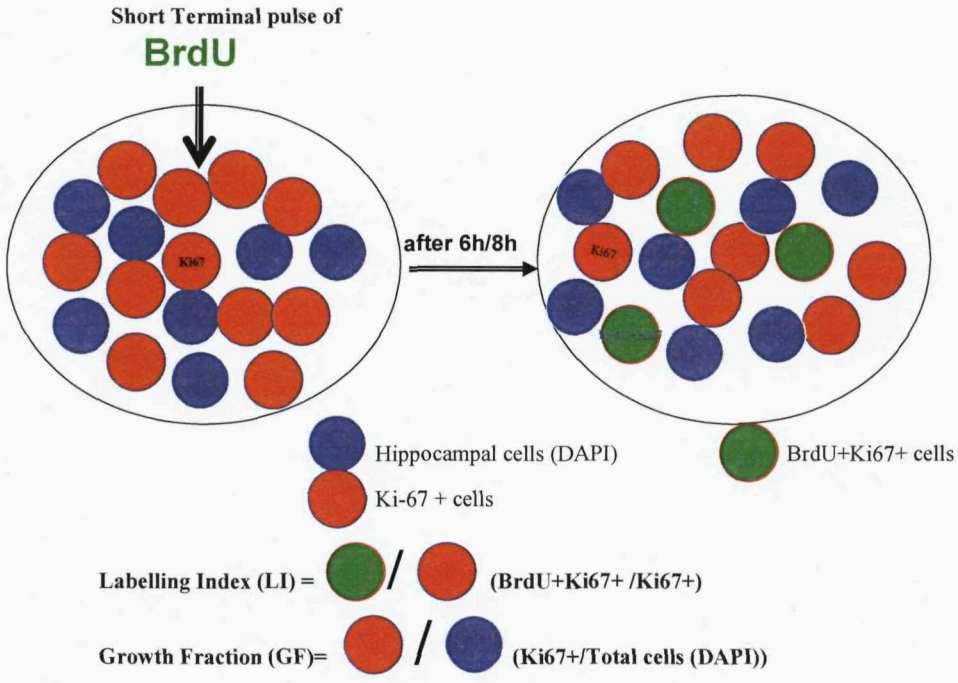


Figure 7.1 Cell proliferation markers. The combined use of both Ki-67, which is an endogenous protein that is expressed in all phases of the cell cycle except G_0, and BrdU, which labels cells that are in the S-phase of the cell cycle, enabled us to determine the labelling index (BrdU+&Ki-67+/Ki-67+) and the growth fraction (Ki-67+/DAPI+). An increased labelling index indicates an enhanced speed of the cell cycle while an increased growth fraction implies recruitment of quiescent cells. Note that all cells (BrdU+ and/or Ki-67+) are DAPI positive.

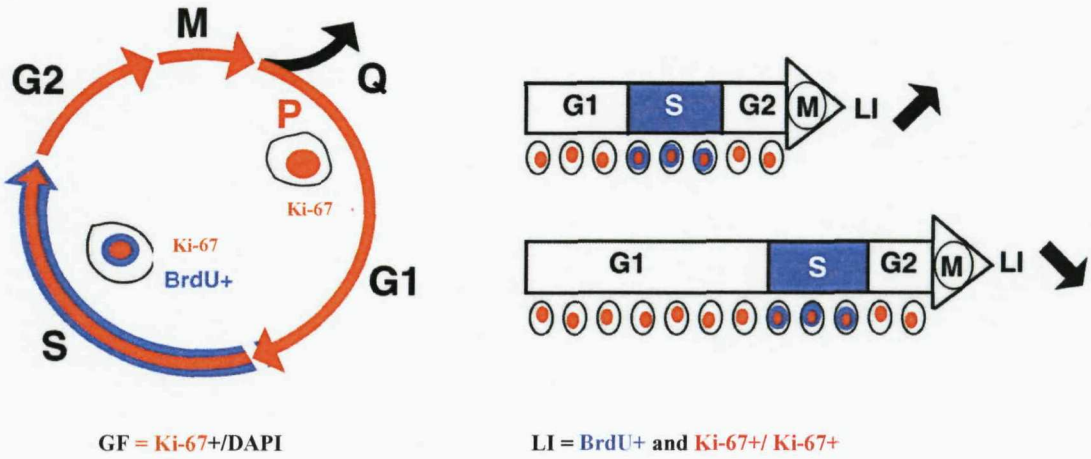


Figure 7.2 Measurements of the cell cycle parameters. Asymmetric divisions give rise to a precursor cell and a neuroblast. Symmetric divisions give rise to two precursor cells. BrdU incorporation identifies the fraction of precursors (Ki-67+) in S-phase of the cell cycle and determines the labelling index (LI). Since the length of S-phase is invariant, variations in the LI reflect changes in the total length of the cell cycle (Schmahl 1983; Dehay, Savatier et al. 2001).

It has long been reported that, in the animal models of human temporal lobe epilepsy, Kainate-induced seizures alter hippocampal neurogenesis (Gray and Sundstrom 1998; Covolan, Ribeiro et al. 2000; Nakagawa, Aimi et al. 2000; Hattiangady, Rao et al. 2004; Jessberger, Romer et al. 2005; Sadgrove, Chad et al. 2005; Jessberger, Zhao et al. 2007). While it has been shown that Kainate-induced seizures enhanced DG neurogenesis acutely, this effect declines in chronic epilepsy conditions (Hattiangady, Rao et al. 2004). Furthermore, it has been elegantly demonstrated that Kainate-induced seizures enhanced DG neurogenesis in adult rats (3 months) up to the same level as in immature rats (1 month), although the baseline neurogenesis in the adult was far less than in immature animals (Gray, May et al. 2002). In agreement with previous studies, Kainate-induced seizures were found to enhance hippocampal neurogenesis in the adult rats, although this effect declines dramatically in middle age and aged animals (Hattiangady, Rao et al. 2008). However, to date, the mechanisms underlying precursor cell

proliferation have not been fully addressed. We therefore looked at the proliferative effects of Kainate on overall hippocampal cells and particularly neural precursor cells.

5μM Kainate increased the mitotic index (MI) of overall hippocampal cells and precursor cells (nestin positive) after 3 DIV and after short pulse of Kainate at day 3. However, while 3 days of Kainate treatment elicited an increase in the MI of neuroblasts (TuJ1 cells that incorporated BrdU), short exposure of Kainate at day 3 had no effect. The possible mechanisms of a net increase in the proliferating cells are: Firstly, increased speed of the cell cycle; secondly, recruitment of quiescent cells to enter the cell cycle; thirdly, enhanced survival of proliferating cells; fourthly, any combination of the previously mentioned mechanisms. We, therefore, looked at the labelling index and found that Kainate increased the labelling index of overall hippocampal cells and nestin positive cells at day 3. The proportion of a proliferating population in S-phase of the cell cycle corresponds to the ratio between the length of the S-phase to the total cycle length. Since S-phase of the cell cycle is relatively invariant (Schmahl 1983), the increased ratio between BrdU incorporated cells (S-phase marker) and Ki-67 labelled cells (marker of all cell cycle phases) can be inferred that Kainate reduced the total length of the cell cycle (**Figure 7.2**). These results also indicate that Kainate facilitates the transitions of cells from G_1 phase of the cell cycle to the S-phase and/or shortens G_1 phase of the cell cycle. As such, Kainate increased the average speed of the cell cycle of hippocampal cells and precursor cells. We did not study the labelling index of TuJ1 cells because short exposure of Kainate had no effect on the proliferating neuroblasts (MI) and therefore it will not affect the labelling index. The labelling index is a well recognized approach to differentiate between proliferative and trophic effects of a factor (Lu, Black et al. 1996). In this context, an increase in the LI with no increase in total number of cells indicates pure proliferative effects. In our system, we demonstrated an overall increase in the LI with no increase in the total cell numbers after short exposure of Kainate at day 3 and thus implying a pure proliferative effect on hippocampal cells. However, there was an increase in the total number of nestin positive cells along with an increase in their labelling index at days 3 and 5 in culture. This suggests a survival role in addition to a proliferative effect of Kainate. This is because an increase in the labelling index on its own indicates a pure proliferative effect, but a combined increase

in the total numbers as well as the LI of a sub-population of cells (nestin in our case) indicates an additional trophic effect on newly-born cells (Lu, Black et al. 1996). On the other hand, because Kainate treatments could not elicit an increase in the total number of cells at 3 DIV and had no direct effects on TuJ1 expressing cells, we sought to investigate the effects of Kainate after either 5 DIV or after short exposures of Kainate at day 5. We found that 5 days of Kainate treatment increased the total number of cells (DAPI), precursor cells (nestin positive), and TuJ1 positive cells with a proportional increase in TuJ1 positive cells. Surprisingly, 6 and/or 8 h of Kainate exposure at day 5 increased the total number of cells (DAPI) as well. Therefore, again we examined the MI and LI of hippocampal cells and precursor cells. A short pulse of Kainate at day 5 increased the number of BrdU positive cells but had no effect on either the MI or the LI. Similarly, this short exposure of Kainate had no effect on the MI of TuJ1 positive cells that incorporated BrdU. In contrast, the MI and LI of nestin positive cells increased significantly after a short (6 or 8 h) pulse of Kainate exposure at day 5. These results imply that Kainate in addition to its proliferative effect on nestin cells most likely had survival effects on overall hippocampal cells as well as TuJ1 expressing cells after 5 DIV (**see Section 7.6**).

The finding that there was no increase in the overall growth fraction (GF) or the GF of nestin positive cells after either 3 or 5 DIV or short exposures at days 3 or 5 indicates no effect of Kainate on recruitment of quiescent cells in culture. In fact, 100% of nestin positive cells were Ki-67 positive at either 3 or 5 DIV which suggests that all precursor cells are cycling / proliferating. Interestingly, these results imply that all nestin positive cells are Ki-67 positive and thus are in the cell cycle. This suggests that postnatal hippocampal precursor cells are actively dividing and not quiescent. In agreement with this, Namba et al 2005 have demonstrated that the majority of precursor cells in the dentate of postnatal Wistar rats were Ki-67 immunopositive. Although he did not double label nestin cells with Ki-67 since this was not the aim of his study, he demonstrated that radial-glia stem like cells generate the GCL neurons postnatally in Wistar rats. We further demonstrated that the effects of Kainate are selectively acting on precursor cells. Indeed, our data showed increased numbers of nestin positive and GFAP (astrocytic marker) positive cells as well as nestin positive and GFAP negative cells with no effect

on the pure GFAP sub-population (GFAP positive but nestin negative) (**Figure 7.3**). On contrary, there was a tendency towards a reduction in this sub-population of cells; consistent with a Kainate induced phenotypic shift towards the nestin population. In line with Namba and others (Seri, Garcia-Verdugo et al. 2004; Namba, Mochizuki et al. 2005), we show that a substantial population of precursor cells are astrocyte-like precursor cells (GFAP+ and nestin+) and we further demonstrate that these cells were targeted by Kainate. In agreement with this, it has been recently demonstrated, in a study using transgenic mice, that GFP+ precursor cells under Sox2 promoter have two morphologically distinct cell types in the SGZ of adult DG (Suh, Consiglio et al. 2007). One sub-population of GFP+ cells showed radial-glial like morphology with long processes and was called radial. In contrast, the second sub-population of Sox2-GFP cells lacked radial processes, however, some of these cells showed short processes and were termed non-radial. Importantly, under normal conditions Suh and colleagues (Suh, Consiglio et al. 2007) have reported an enhanced cell proliferation of the non-radial type. However, under physiological stimulation (wheel running) the other sub-population of precursors (radial) was found to be proliferating, albeit at lower level. The morphology of these precursors described by Suh et al 2007 is relevant to what we have observed in our culture system. In **Figure 7.3**, which represents the morphology of cells in our cultures, we have shown two morphological cell types. The first one includes cells that co-express nestin and GFAP, have long processes which match with the radial glial-like cells of Suh et al 2007. The second one consisted of cells that express nestin only and had no or very short processes. The later sub-population of cells was termed as highly-amplifying progenitors. We further show that Kainate increased the numbers of both sub-populations of cells in culture. The fact that there was no proportional increase in either of the two sub-populations of precursor after Kainate treatment may suggest that Kainate perhaps enhances both the symmetric and asymmetric cell divisions. This is because if a population is proliferating asymmetrically there would be no change in the number of this population while there would be an increase in the asymmetric daughter population (e.g. a neuronal one) (Suh, Consiglio et al. 2007). However, in our study, we observe an increase in the numbers of GFAP+ and nestin+ and nestin+ and GFAP- as well as a proportional increase in TuJ1 positive cells. These results indicate that the

nestin+ and GFAP- population of cells divides asymmetrically (by which these cells regenerated themselves and gave neurons) and also divides symmetrically by which they gave more nestin+ and GFAP- cells and thus we observe an increase in the numbers of this population of cells. The similar scenario is likely occurring for nestin+ and GFAP+ population as well. We have also to consider an interplay between these two subpopulations of precursor cells where there is an additional possibility that nestin+ and GFAP+ population is also giving rise to nestin+ and GFAP- population. However, we do not have a direct proof of this hypothesis.

Therefore, the likely interpretation is that Kainate may have enhanced the self-renewal of these precursor cells as well as have given rise to new neurons, which resulted in an increase in neurogenesis as well as these precursors (as we found). **Figure 7.4** confirms the morphology of proliferating nestin precursor cells, although it was not possible to do quadruple immunostaining to rule out the existence of GFAP staining. However, our data, in chapter 3, showed no expression of GFAP in non-radial nestin positive cells. Interestingly, the speed of the cell cycle of precursor cells decreased over time in control cultures. This is indicated by a reduction in the labelling index of nestin positive cells by approximately 50% between days 3 and 5 in cultures generated from the same batch. Although Kainate increased the LI and thus the speed of the cell cycle at day 5 in these cultures, it failed to reach the same level as day 3. We therefore conclude that the age of precursor cells in culture is critical for determining an effect of a factor. In line with this, Kuhn and colleagues have shown that FGF-2 and EGF have differential proliferative effects on precursor cells depending on the age of the cultures (Kuhn, Winkler et al. 1997).

In addition, our initial analysis of time-lapse microscopy experiments shows that approximately 70% of newly born cells are nestin only positive cells and around 15% of newly-born cells are expressing both nestin and TuJ1 markers under both control and Kainate treatments with no difference between the two conditions. However, these newly-born cells (nestin only positive cells as well as nestin positive and TuJ1 positive cells) increased significantly after Kainate treatment with no preferential shift towards nestin only positive cells or nestin positive and TuJ1 positive sub-population of cells. As

such, we conclude that Kainate also enhances the survival of symmetrically and asymmetrically divided cells with no preferential effects towards any of them.

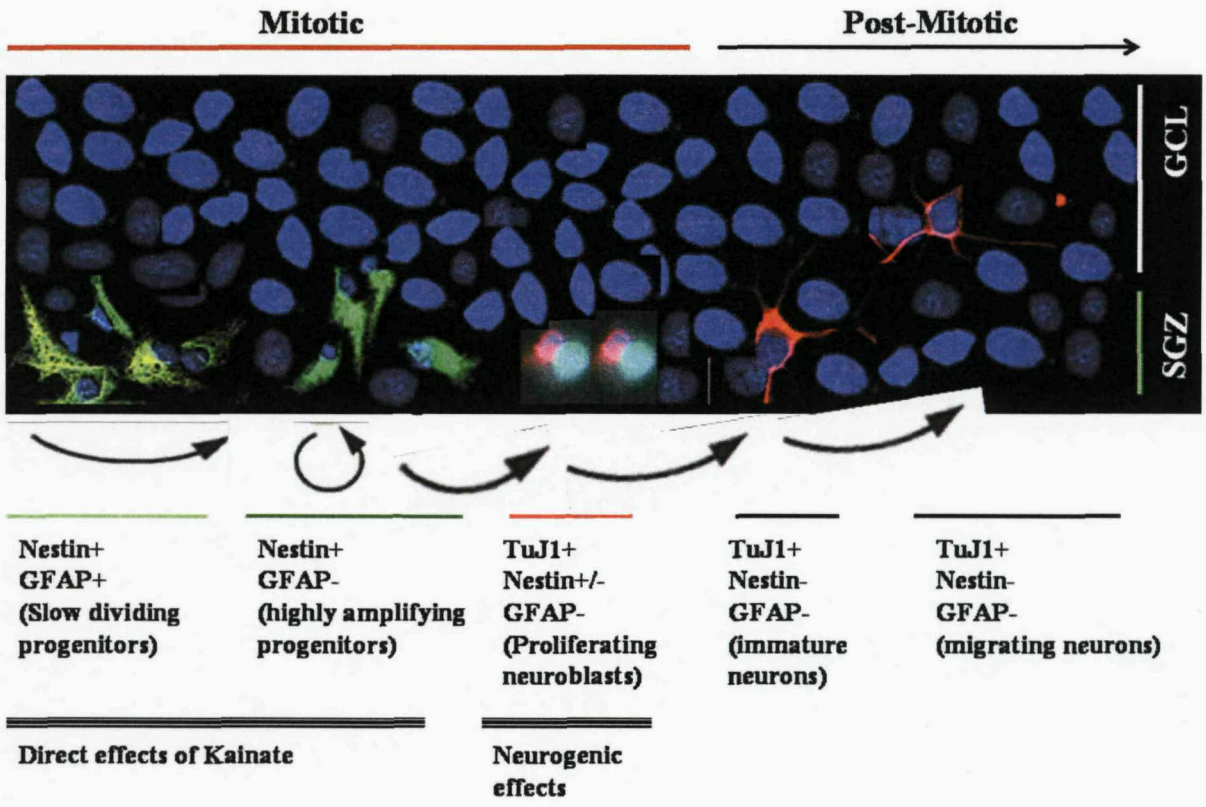


Figure 7.3 Schematic representation of cell phenotypes in culture and Kainate effects on them. Kainate has direct proliferative and survival effects on slow dividing as well as highly amplifying precursor cells in culture. It also enhances the proliferation of neuroblasts after 3 DIV. In addition it has a direct survival effects on neuroblasts.

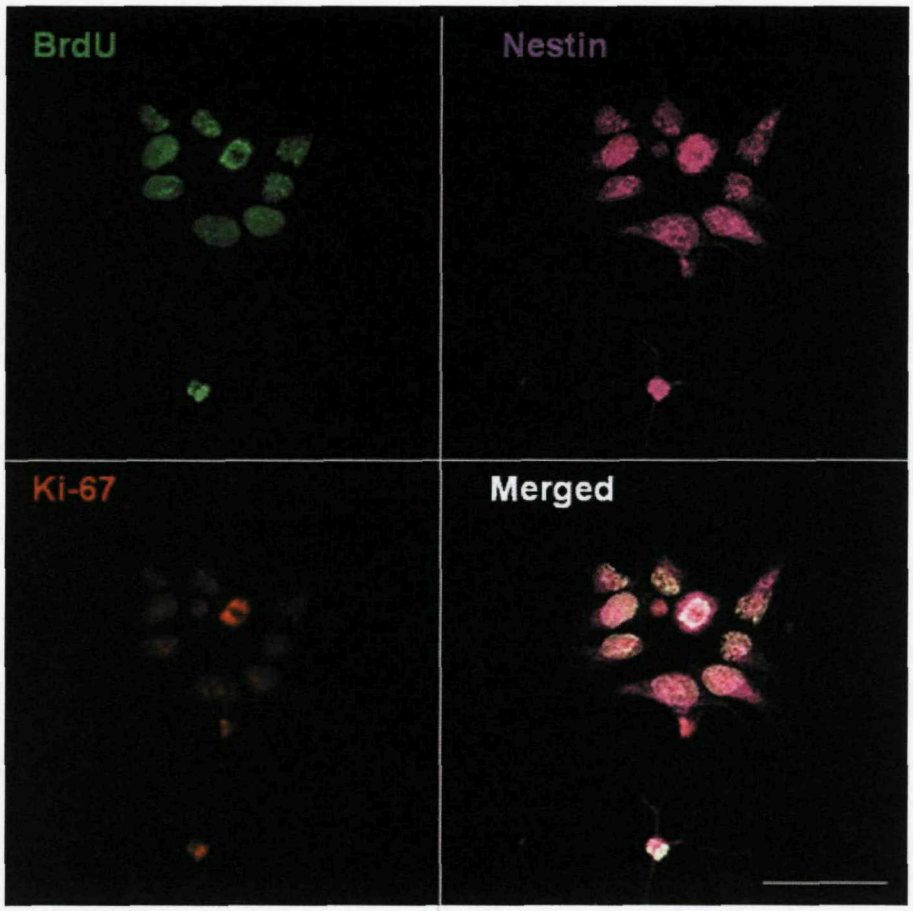


Figure 7.3 The morphology of proliferating nestin positive cells in culture. Cells were grown for 5 days under standard growth conditions. On day 5 they were terminally pulsed with BrdU and 5μ Kainate. After fixation, triple immunohistochemistry was done where cells were probed for BrdU (green), Ki-67 (red), and nestin (purple). We observed that nestin positive cells with no or short processes were the main cont4rinutor to the dividing population of precursor cells in vitro. Images were captured with 40x oil objective on a laser scanning con-focal microscope and 3D projections reconstructed using Leica Microsystems LAS AF lite software. Control experiments with the primary antibody omitted were devoid of staining. Scale bar = 57μm.

7.3 The effects of Kainate are modulated via AMPA receptors

The chemoconvulsant Kainate is an agonist of AMPA/Kainate receptors (Lerma 1997; Huettner 2003; Lerma 2006). Kainate and AMPA receptor subtypes are ubiquitously distributed in the CNS, especially in the hippocampus (Huettner 2003; Catarzi, Colotta et al. 2007). Therefore, Kainate effects on hippocampal cells might involve, at least in

part, actions that are modulated via these receptors. To elucidate this hypothesis, we blocked AMPA and Kainate receptors by adding 30μM NBQX to both control and Kainate conditions (Wilding and Huettner 1996). Similarly, we selectively antagonized AMPA receptors by applying 10μM of the noncompetitive AMPA receptors antagonist GYKI52466 to control and Kainate conditions (Paternain, Morales et al. 1995).

We have shown that neither NBQX nor GYKI52466 have effects on overall hippocampal cells and their neural precursors under control conditions. However, the combined application of either NBQX or GYKI52466 with Kainate abolished the proliferative effects of Kainate on cultured hippocampal cells. Indeed, the increase in the LI after 6/8 h of Kainate exposure was abolished in the presence of either NBQX or GYKI52466. These results imply that blocking AMPA and Kainate receptors by NBQX abolished the effects of Kainate. Similarly, antagonizing AMPA receptors only by GYKI52466 abolished the increase in the LI after Kainate exposure. Therefore, AMPA receptors directly modulate the increase in the speed of the cell cycle after Kainate treatment. Interestingly, we have shown that Kainate, in addition to its 'proliferative' characteristics, has a survival role when applied to cells in culture. Although we did not test the survival role of AMPA receptors directly, our results indirectly suggest that AMPA receptors modulate the survival effects of Kainate on overall hippocampal cells, nestin positive cells, and TuJ1 expressing cells. This is because the addition of either GYKI52466 or NBQX in conjunction with Kainate on hippocampal cell cultures completely abolished the Kainate-induced increases in both nestin and TuJ1 immuno-positive cells. The net increase in TuJ1 immunopositive cells after 5 days in culture resulted from the additional survival and differentiation effects of Kainate on these newly-born precursor cells. Thus, we conclude that Kainate enhances the survival of newly-born precursor cells and their neuronal progeny cells via a mechanism involving, at least in part, AMPA receptors. We also suggest that AMPA receptors may play a role in precursor cells differentiation to a neuronal lineage. This is an indirect and tentative conclusion, because blockade of AMPA receptors abolished all effects of Kainate including a differentiation effect on precursor cells towards a neuronal lineage.

The next question that we addressed, if indeed AMPA receptors modulate hippocampal neurogenesis what is the effect of AMPA receptor agonist on hippocampal cells in

culture? To answer this question, I examined the effects of (S)-AMPA, selective agonist of AMPA receptor, on hippocampal cells in culture. Consistent with our hypothesis, we have shown that (S)-AMPA, like Kainate, increased the total number of hippocampal cells in culture.

The preliminary finding that MK-801 increased overall cell proliferation and nestin expressing cells in cultures suggests that glutamate is involved in regulating hippocampal neurogenesis in our *in vitro* system. In this regard, it has been demonstrated that blockade of NMDA receptors by MK-801 enhanced the trophic effects of Kainate on cerebellar granule cells generated from postnatal rats (Balazs, Hack et al. 1990). In addition, the combined application of Kainate and the NMDA receptor antagonist kynurenic acid (KYNA) enhanced the survival of precursor cells cultured from SVZ of postnatal Wistar rats (Brazel, Nunez et al. 2005). Interestingly, Brazel and co-workers showed that glutamate has the same trophic effect on cells as Kainate when applied in combination with KYNA. Taken together, these data along with ours may suggest that glutamate plays an important proliferative role in postnatal neurogenesis mainly via non-NMDA receptors including AMPA receptors.

In primary cultures generated from embryonic (E18) rats' hippocampi, it has been demonstrated the AMPA receptors are expressed in nestin positive cells as well as in TuJ1 positive cells (Hagimura, Tsuzuki et al. 2004). Therefore, most likely AMPA receptors are expressed in precursor cells in culture and Kainate activates and/or enhances the expression of these receptors. Interestingly, it has also been demonstrated that LY451646 (an AMPA receptor potentiator) enhanced DG proliferation as indicated by the increase in BrdU clusters (Bai, Bergeron et al. 2003). However, the previous study, Bai and colleagues failed to show a significant increase in the numbers of BrdU cells. This may suggests an indirect role for AMPA receptors in modulating DG cell proliferation in vivo. In another study, it has been demonstrated that ICV administration of (S)-AMPA increased Ki-67 positive cells as well as doublecortin expressing cells in the SVZ of postnatal rats (Xu, Ong et al. 2005). Furthermore, it has been shown that AMPA receptor subtypes (GluR2/3) were up-regulated in the dentate gyrus of adult rats after Kainate-induced seizures (Friedman, Pellegrini-Giampietro et al. 1994). Similarly, the expression of GluR2/3 was increased on cells in the GCL and hilus of humans with

TLE (de Lanerolle, Eid et al. 1998). These studies support the hypothesis that Kainate via AMPA receptors regulates the proliferation and survival of precursor cells in our system and may suggest a role of AMPA receptors in vivo. Moreover, it has been shown that AMPA enhanced the survival of cerebellar neurons in cultures from postnatal rats (Wu, Zhu et al. 2004). Indeed, this group has demonstrated that AMPA treatment increased the release of BDNF and its mRNA expression which was blocked by NBQX. In this regard, it has been elegantly shown that BDNF plays an important role in baseline neurogenesis where it appears to regulate the proliferation and survival of newly-born neurons in the DG of adult mice (Lee, Duan et al. 2002; Sairanen, Lucas et al. 2005). A role of AMPA receptors has been implicated in anti-depressants effects and neurogenesis (Alt, Nisenbaum et al. 2006; Encinas, Vaahtokari et al. 2006). This may suggest that AMPA receptors could be a target for control of mood disorders.

In summary, our results indicate both proliferative and trophic roles for AMPA receptors in modulating hippocampal neurogenesis in the normal brain and after Kainate treatments.

7.4 Kainate/seizure has differential effects on proliferation kinetics in the subgranular zone and granule cell layer

A significant increase in the total Ki-67 cell counts was observed in the SGZ of animals injected with Kainate after 72 h of the treatment. Similarly, Kainate/seizures increased the numbers of Ki-67 positive cells at 48 h and 72 h time points when compared to age-matched controls. In order to elucidate the mechanisms by which Kainate/seizures enhanced precursor cell proliferation in the SGZ/GCL, we employed BrdU to label a 'clone' of proliferating precursors in all animals 24 h before seizures were induced by Kainate in half of them. We sought to explore the possible effects of Kainate/seizures on a sub-population of cells that are in S-phase of the cell cycle. The relevant features of our experimental design are: BrdU will incorporate into cells before seizure induction and thus eliminate any confounding effect that might result from disruption of the blood brain barrier after seizures (Bolton and Perry 1998), un-incorporated BrdU will be cleared from the circulation before seizures which excludes any bioavailability effects

after seizures which may alter the blood brain barrier permeability of BrdU and thus inaccurately estimates the number of proliferating cells in epileptic animals. BrdU would also be transferred into daughter cells and thus can be used to follow the fate of these newly-born cells for up to 4 cell cycles (Hayes and Nowakowski 2002; Dayer, Ford et al. 2003). Since the length of the cell cycle in adult rats is estimated to be 25 h (Cameron and McKay 2001), we are not expecting any dilution effect during the course of our experiment (96 h) at least under control conditions. In addition, the combined use of BrdU and Ki-67 enabled us to identify cells that were dividing before seizures and remained in the cell cycle (BrdU+ and Ki-67+ cells) (pre-labelled 'clone'), cells that were recruited to divide after seizures induction (un-labelled 'clone') (Ki-67+ and BrdU- cells), and cells that became postmitotic (BrdU+ and Ki-67-).

The mechanisms by which Kainate/seizures enhances cell proliferation might include increasing the rate of cell divisions and/or recruiting quiescent cells to become actively dividing. Dissecting the effects of Kainate/seizures on both the pre-labelled 'clone' of cells as well as the un-labelled sub-population of cells revealed that in the SGZ we observed an equal contribution of these two sub-populations of cells to seizure-induced cell proliferation. The fact that there was no statistically significant increase in the proportion of pre-labelled 'clone' of cells was because there was a very substantial increase in the numbers of Ki-67 only positive cells (un-labelled) which masked the small increase in the pre-labelled 'clone' of precursors. The numbers of cells that are BrdU + and Ki-67+ doubled after 72 h of Kainate/seizures, however, this rise it is not statistically significant. Because the proportion of cells that are BrdU+ and Ki-67+ with respect to the total number of proliferating cells (Ki-67+) was only around 10% throughout the course of the experiment, we did not observe a significant increase in this sub-population of cells. In agreement with our results, it has been shown that only a small proportion of pre-labelled precursor cells is dividing in the SGZ after Kainate/seizures (Huttmann, Sadgrove et al. 2003). In addition, type-3 precursors (Dcx positive cells) were reported to be increased after Kainate-induced seizures (Jessberger, Romer et al. 2005). Moreover, the proportion of proliferating Dcx positive cells has been found to be around 20 % of the total Dcx positive population (Plumpe, Ehninger et al. 2006).

There are two possible interpretations of the increase in the cell proliferation after Kainate/seizures. Firstly, the increased proliferation of the pre-labelled 'clone' of cells may have led to the expansion of the size of this sub-population of cells. This model is reminiscent of the increase in stem cell population during brain development, in which precursor cells undergo symmetric cell division prior to neurogenesis. If this is the case, the numbers of the proliferating 'clone' of precursors should increase which agreed with our results. The second interpretation is that the increased proliferation of the pre-labelled 'clone' of cells is associated with asymmetric contribution of daughter cells. In this regard, the phenotype of proliferating pre-labelled 'clone' have to be determined in order to examine whether a pre-labelled precursor cell gave rise to another precursor and a Dcx positive cells or not.

Analysing the possible mechanisms by which Kainate/seizures enhanced cell proliferation in the SGZ suggests that Kainate/seizures enhanced self-renewal of the pre-labelled 'clone' of cells by enhancing cell cycle re-entry (as indicated by the enhanced proliferation of the pre-labelled 'clone' of cells in the combined SGZ and GCL see **section 7.5**). In support of this, we also find a reduction in the postmitotic sub-population which suggests that more cells have remained in the cell cycle. Another possibility is shortening of the cell cycle; however, this mechanism on its own will explain the increase in cell proliferation but not the reduction in postmitotic cells which we observe in our study. Therefore, we conclude that Kainate/seizure enhances cell cycle re-entry of the pre-labelled 'clone' of cells with a possible reduction in the length of the cell cycle. It is important to keep in mind that a single IP injection of 50 mg/kg BrdU will label a small fraction of proliferating cells and therefore, we speculate that inducing cell cycle re-entry is the main mechanism of increased cell proliferation (of both pre-labelled 'clone' and un-labelled population) after Kainate/seizures. Since we have administered BrdU 24 h before seizures induction, it was not possible to study the effects of Kainate/seizures on the length of the cell cycle. In order to circumvent this and to get a quantitative sense of the effects of Kainate/seizures on the length of the cell cycle, we would suggest a model where a 'clone' of proliferating cells can be labelled with BrdU just 2 hours before seizures induction by Kainate. Then animals have to be sacrificed at 0 h, 6 h, 12 h, 18 h and 24 h after Kainate treatment. Double-labelling cells

for BrdU and Ki-67 at each time point would determine the length of the S-phase of the cell cycle as well as the total cell cycle length of dividing cells and show possible effects of Kainate/seizures.

The other possibility of increased cell proliferation is recruitment of quiescent cells into the cell cycle. In our paradigm, although Kainate/seizures significantly increased the numbers of Ki-67 only positive cells (un-labelled population), there was no proportional increase in this sub-population of cells. We found that the proportion of un-labelled 'clone' of cells is approximately 90% in the SGZ in both Kainate and saline groups throughout the course of my experiment. It is therefore still unclear whether recruitment of quiescent cells (Ki67-) contributes to Ki-67 only positive cells or not. Therefore triple labelling of cells for BrdU, Ki-67 and Sox2, a precursor cell marker (Hattiangady and Shetty 2008), would clearly demonstrate Kainate/seizures effects on quiescent cells.

Analysing the effects of Kainate/seizures on cell proliferation in GCL would also help at elucidating the mechanisms underlying cell proliferation not only in the GCL but also in the SGZ. Accrued body of evidence suggests that chemically-induced seizures, like Kainate and pilocarpine, enhanced SGZ cell proliferation with dispersion of these proliferating cells into the GCL (Parent, Yu et al. 1997; Nakagawa, Aimi et al. 2000; Jessberger, Romer et al. 2005). Although the previous studies did not elucidate the origin or the mechanisms of increased cell proliferation after Kainate/pilocarpine–induced seizures, they pointed out to the dispersion of the proliferating cells after the insult. We therefore conclude that, in our model, Kainate/seizures dispersed the pre-labelled 'clone' of cells into the GCL which might also explain why there was no proportional increase in this 'clone' of cells in the SGZ.

Interestingly, in the GCL, Ki-67 cell numbers increased significantly in the Kainate group at 48 and 72 h time points. These results also suggest an equal contribution of the pre-labelled and un-labelled 'clones' of cells to cell proliferation at 48 h time point with shift towards a major contribution of the pre-labelled 'clone' of cells at 72 h time point. This conclusion is based on the observations that at 48 h there was an increase in Ki-67 positive cells and Ki-67 only positive cells (Ki-67+ and BrdU-) with no effect on the proportion of pre-labelled 'clone' of cells with respect to the total number of cells in the

cell cycle (BrdU+ and Ki-67+ /Ki-67+) or the proportion of Ki-67 only positive cells with regard to the total Ki-67 expressing cells (Ki-67+ and BrdU-/Ki-67+). However, at 72 h, Kainate/seizures increased BrdU+, BrdU+ and Ki-67+/Ki-67+ which was associated with a decrease in Ki-67+ and BrdU-/Ki-67+. These results imply that the pre-labelled 'clone' of cells is the main contributor of cell proliferation in the GCL at 72 h. The fact that the pre-labelled 'clone' of cell increased in the Kainate group after 72 h and not earlier as Ki-67 + cells, indicate a dispersion of the SGZ pre-labelled 'clone' of cells into the GCL. The precise analysis of 72 h of Kainate/seizures effects on the pre-labelled and un-labelled 'clones' of cells revealed that there is a reduction in BrdU+ and Ki-67-/BrdU+ by 20% although this was not significant. This suggests again that there is a tendency towards a decrease in the proportion of postmitotic sub-population of cells. Although the numbers of BrdU+ and Ki-67- increased in the Kainate group at 72 h, the proportion of this sub-population of cells with respect to the total number of pre-labelled 'clone' of cells is more accurate indicator of the whole postmitotic population of cells. Taken together, like the SGZ, Kainate/seizures enhancing the regeneration of the pre-labelled 'clone' of cells by increasing cell cycle re-entry (see **section 7.5**).

Our results also show that Kainate/seizures enhanced cell proliferation at 48 h after Kainate/seizures in the GCL (as indicated by the increase in Ki-67 positive cells). However, a similar increase was observed in the SGZ after 72 h and not earlier. These findings may suggest that Kainate/seizures recruited a quiescent population of cells in this region. Answering this question is currently underway. Altogether, our data suggest that Kainate/seizures have differential effects on the kinetics of precursors in the stem cell niche and also disperse(s) the niche shortly after the insult.

We further employed our model to study the effects of Kainate/seizures on cell proliferation and its mechanisms in the hilus and molecular layer (Ml) of the DG. We showed that the pre-labelled 'clone' of cells is the main cell population that has undergone cell division in these regions. While Dissecting Kainate/seizures effects in the hilus and Ml, we found that there was no reduction in the postmitotic sub-population of cells. In fact these results suggest an increase in the postmitotic population in relative to other regions (i.e. SGZ, GCL). The most likely explanation is that Kainate/seizures enhanced the proliferation and migration of the pre-labelled 'clone' of cells from the

SGZ/GCL to the hilus and Ml. Consistent with this, several lines of evidence have demonstrated that newly-granule cells display ectopic migration patterns after seizures-induced cell proliferation (Parent, Yu et al. 1997; Scharfman, Goodman et al. 2000; Pierce, Melton et al. 2005; McCloskey, Hintz et al. 2006). The previous studies investigated the effects of pilocarpine-induced seizures on ectopic migration of cells in the hilus. We further demonstrated that these cells are most likely derived from the pre-labelled 'clone' of cells. In addition, we show that similar effect is evident in the Ml. A likely explanation is that these cells in the hilus and Ml are microglia or astrocytes. Thus, determining the phenotype of these cells would help in elucidating the effects of Kainate/seizures on cell proliferation and gliogenesis in the hilus and Ml.

7.5 Kainate/seizures enhance(s) cell cycle re-entry and shorten(s) the cell cycle length of precursor cells in the combined SGZ and GCL area

To get further insight on the mechanism by which Kainate/seizures dispersed the stem cell niche and enhanced pre-labelled precursor cell proliferation, we additionally analysed the effects of Kainate/seizures on the proliferation of the pre-labelled 'clone' of cells in the combined SGZ and GCL. Importantly, we found that Kainate/seizures increase(s) the overall numbers of pre-labelled 'clone' of precursors (as indicated by a three-fold increase in BrdU positive cells at 72 h time point). Moreover, we demonstrated a significant increase in the pre-labelled precursors that continued to divide after 72 of Kainate/seizures (as measured by counting cells that are positive for both BrdU and Ki-67). The increase in BrdU positive cells indicates that the pre-labelled 'clone' of cells has self-renewed itself giving rise to new BrdU positive cells. Since the increase in both the overall BrdU positive cells and BrdU and Ki-67 positive cells occurred between 48 h and 72 h in the Kainate group, this indicates that Kainate is enhancing cell cycle re-entry and shortening of the cell cycle length. In fact, this fraction of the pre-labelled 'clone' of cells, which might be highly amplifying progenitors, re-entered the cell cycle and divided more than once within 24 h time scale (between 48 and 72 h time points examined) which indicate an enhancing cell cycle re-entry and also considerably shortening the length of the cell cycle of this specific sub-population of

precursors. The fact that BrdU positive cells increased in the Kainate group, but did not change in the control group, implies that BrdU dilution did not happen at these time points. In order to dissect what is happening in vivo, we have demonstrated that Kainate targeted highly amplifying precursor cells (nestin + and GFAP-) as well as quiescent precursor cells (nestin+ and GFAP+). Therefore, Kainate/seizures *in vivo* may also had the same effect of enhancing the proliferation of quiescent precursor cells and giving rise to highly amplifying precursor which indeed divided quickly and gave rise to new BrdU+ cells. In agreement with this, our in vitro data shows that Kainate decreased the length of the cell cycle of nestin positive cells as indicate by the rise in their labelling index. Furthermore, unpublished time-lapse microscopy data in our lab show that the cell cycle length of highly amplifying precursors is around 12 h. In line with this, Suh et al have found that non-radial Sox2 positive precursors divide more often after physiological enhancement of cell division (Suh, Consiglio et al. 2007). These non-radial precursors are equivalent to our nestin positive and GFAP negative sub-population of precursors. Moreover, Encinas and colleagues have reported an enhanced cell division of highly amplifying precursors (nestin+ and GFAP-) after treating transgenic mice with selective serotonin re-uptake inhibitors (Encinas, Vaahtokari et al. 2006). In addition, it has been demonstrated that adrenalectomy (which enhances neurogenesis) dramatically increased the proliferation of a population of cells that expresses nestin but does not express GFAP (Battista, Ferrari et al. 2006). These studies along with ours support hypothesis that Kainate/seizures enhance(s) the proliferation of highly amplifying precursors which in turn resulted in an increase in the pre-labelled 'clone' of cells.

We also show that the proportion of cells that incorporated BrdU, and continued to divide with respect to the total proliferating cells (expressing Ki-67), was significantly increased at 72 h in the Kainate group (Kainate/seizures approximately 20%, vs. saline, around 10%)(two-way ANOVA with Bonferroni's post hoc test, $p< 0.01$). Moreover, while 43.3 ± 8 % of BrdU + cells were in the cell cycle in the control group at 72, this proportion increased to 60.4 ± 5 % in the Kainate group. However, this increase was not statistically significant (two-way ANOVA with Bonferroni's post hoc test, $p = 0.1$). Dissecting the effects of Kainate/seizures on the pre-labelled 'clone' in an area that

injury survived for at least 3 weeks. It has been demonstrated that ICV Kainate induced pyramidal neuronal cell loss in postnatal rats' hippocampi that enhanced DG and CA3 neurogenesis (Dong, Csernansky et al. 2003). However, there was no evidence of induction of cell death in the GCL. Likewise, in other studies, GCL cell death was minimal or absent in Kainate models of epilepsy (Covolan, Ribeiro et al. 2000; Sloviter, Zappone et al. 2003). To date, the relationship between precursor cell death and neurogenesis has not been fully addressed.

Understanding the precise role of cell death in Kainate-induced seizures and neurogenesis models has been hampered by the lack of proper markers of cell death. Therefore, we adopted a multi-approach policy to study hippocampal cell death in culture to unravel the mechanisms underlying precursor cell death after Kainate treatment. Cell survival investigations were mainly focused on cultures maintained for 5 DIV; where Kainate was applied onto cells either for 5 days or for 6-25 h at day 5. This is because 3 DIV of Kainate and/or 6-8 h of Kainate exposure at day 3 are mainly proliferative for overall hippocampal cells and their precursors. However, the proliferative characteristics of Kainate failed to explain the increase in the total cell numbers (DAPI) and neuronal precursor cells after 5 DIV of Kainate treatment and/or short pulse of Kainate at day 5. One approach in studying cell death was measuring the overall proportion of caspase-3 activated cells in culture, the proportion of both nestin and TuJ1 cells that are reactive to caspase-3 after 5 DIV of Kainate treatments and also after short exposure (6 h) to Kainate at day 5. The overall proportion of caspase-3 immunopositive cells decreased significantly under both 5 DIV of Kainate and short treatments of Kainate at day 5. Interestingly, we showed that 50% of DAPI cells were caspase-3 immunoreactive under control conditions. However Kainate treatments rescued 20% of cells in culture. Likewise, double-labelling of caspase-3 with either BrdU, nestin, or TuJ1 demonstrated that Kainate treatments (6-8 h or 5 days) reduced the proportion of BrdU-incorporated cells, nestin positive cells as well as TuJ1 expressing cells that were caspase-3 immunopositive. The findings that half cells in culture are instructed to commit suicide is not surprising since an accumulating body of evidence suggested that around half of newly-born DG cells die shortly after their birth (Yuan and Yankner 2000; Dayer, Ford et al. 2003) and apoptosis is the main pathway of

includes both the SGZ and GCL suggests that the pre-labelled 'clone' of cells is the major contributor to cell proliferation.

Altogether, our in vivo work shows that Kainate/seizures increased the pre-labelled 'clone' of cells in the combined SGZ and GCL between 48 and 72 h time points by enhancing cell cycle re-entry and also shortening the length of the cell cycle of a fraction of this 'clone' of cells. The finding that BrdU numbers and proportions did not increase in the SGZ and increased in the combined SGZ and GCL supports the hypothesis that Kainate/seizures indeed dispersed the SGZ stem cell niche into the GCL.

7.6 Hippocampal precursors and their neural progeny cells survive more under Kainate treatments *in vitro*

An accumulating body of evidence suggests that the survival of newly-born hippocampal precursor cells plays an important role in cognitive functions such as learning and memory (Shors, Miesegaes et al. 2001; Shors, Townsend et al. 2002; Bruel-Jungerman, Laroche et al. 2005; Winocur, Wojtowicz et al. 2006). Although substantial numbers of new granule cells, the principal neuronal type in the DG, are generated from dividing cells confined in the SGZ, many of these newly-born neurons die before they mature (Dayer, Ford et al. 2003; Tashiro, Makino et al. 2007). The surviving neurons are functionally integrated into existing neuronal circuits within one month (van Praag, Schinder et al. 2002; Jessberger and Kempermann 2003). However, the magnitude of the contribution of these newborn neurons into the adult DG was unclear until it has recently revealed that approximately 4% of newborn neurons form new circuits in the adult mice DG and thus contribute to learning and memory (Tashiro, Makino et al. 2007).

It has long been held that the chemoconvulsant Kainate kills neurons within the hippocampus (Nadler 1981; Ben-Ari 1985) and therefore it has been used to model human temporal lobe epilepsy in animals. It has been suggested that excitotoxins and/or mechanical damage enhanced DG neuronal cell death that in turn triggers DG precursor cell proliferation in the adult rats (Gould and Tanapat 1997). However, it was not clear in the previous study whether DG precursor cells or their neuronal progeny cells were affected by the damage. In contrast, large numbers of the newly-born neurons after the

neuronal cell death (Yuan and Yankner 2000). Surprisingly, we found that 75% of nestin positive cells and 90% of TuJ1 expressing cells were caspase-3 immunoreactive under control conditions. These results suggest that the majority of precursor cells and their neuronal progeny cells are instructed to die unless this cell death signal is changed. Another possibility is that our cultures conditions are biased towards stem cells and not supporting the growth of neurons or astrocytes. In our study, Kainate treatments consistently reduced the proportion of nestin positive cells as well as TuJ1 positive cells that were caspase-3 positive by about 20%. Importantly, further support for a trophic effect was the consistent increase in the total number of cells (DAPI) after short (6/8 h) of Kainate exposure at day 5. Since there was no effect for 6/8 h of Kainate exposure on the MI and LI index and there was an increase in the total number of cells, this suggests a survival effect on proliferating cells (Lu, Black et al. 1996). As such, we have developed a protocol where we applied propidium iodide (PI), MitoTracker (Green) and DAPI onto live cells in culture. PI enters cells with compromised cell membranes and fluoresces in red (Pringle, Benham et al. 1996; Pringle, Sundstrom et al. 1996; Pringle, Iannotti et al. 1997; Masuda, Monahan et al. 2005; Xu and Zheng 2007). MitoTracker (Green) is a dye which specifically gets incorporated by the mitochondria of respiring cells and emits green fluorescent (de la Monte, Neely et al. 2001; Waters and Smith 2003). DAPI stains the nuclei of all cells (live and dead) in culture (Howell, Scharfman et al. 2003; Howell, Doyle et al. 2005; Howell, Silva et al. 2007). The relevant use of a combination of these three markers (PI, MitoTrackers, and DAPI) enabled us to determine the number of live cells (DAPI positive, MitoTracker positive, and PI negative), dead cells (DAPI positive, MitoTracker negative, and PI positive) as well as dying cells (DAPI positive, MitoTracker positive, and PI positive) in primary hippocampal cultures. Although studying the morphology of DAPI positive cells (the nuclei being shrunken and condensed (pyknotic) or fragmented (blebbing)) could help in studying cell death, the use of the new protocol (triple staining of live cells) avoided the confounding effect that may result from washing away dead/dying cells while doing immunostaining procedures and thus underestimating cell death. Our results confirm that short treatments of Kainate at day 5 increased the survival of hippocampal cells as indicated by the increase in the proportion of live cells in culture. However, it was not

possible to look at the phenotype of cell survival using this protocol. Therefore, and in order to elucidate the mechanism underlying cell survival of nestin and TuJ1 cells *in vitro,* we videoed cell cultures for 25 h under time-lapse microscopy. We found that Kainate treatment enhanced the survival of the overall hippocampal cell numbers, particularly the newly-born cells. The survival effect of Kainate was on nestin positive and TuJ1 negative cells as well as on nestin positive and TuJ1 positive newly-born cells. We further confirmed that cell death is higher under control conditions compared to Kainate conditions and these cells de-fragment and become pyknotic. Because of this, dead/dying cells showed either small pyknotic DAPI staining or were washed away during immunocytochemistry and thus were undetected. Interestingly, none of the newly-born cells that survived was washed away. The latter is a significant methodological confounding variable that has not been addressed in published studies to date.

The increase in the mitotic index might be due to an overestimation of a survival effect of a factor on the newly-born cells. This is because most of dead/dying cell are washed away during immunohistochemistry. However, the increase in the labelling index is an accurate parameter to differentiate between cell trophism and cell proliferation (Lu, Black et al. 1996). This approach will test if the effects of a factor involve a survival effect (an increase in the total number of cells) or just have proliferative effect (an increase in the LI). In our system, we show an increase in the overall LI with no increase in the total cell numbers indicating an enhancement of the cell cycle speed (in 3 DIV). In contrast, in 5 DIV, there is an increase in the total numbers after short exposure of Kainate with no effect on the LI. This indicates a survival role. On the other hand, the LI and the total numbers of nestin precursor cells were increased shortly after Kainate and, thus, clearly demonstrate a combined survival and proliferative effects of Kainate on precursor cells.

In line with our results, Kainate and glutamate decrease apoptotic cell death and increase thymidine incorporation in neurospheres cultured from the SVZ of postnatal rats (Brazel, Nunez et al. 2005). In addition, it has been shown that treatment of hippocampal neuronal cultures with sub-toxic doses of NMDA evoked the release of the trophic factor BDNF immediately (after 2 min) and increased its mRNA 3 h after the treatment (Jiang,

Tian et al. 2005). In this regard, Kainate may induce the release of neurotrophic factors and enhance the survival of hippocampal precursor cells. Furthermore, in agreement with our results, it has been demonstrated that Kainate (25-50 μM Kainate) enhanced the survival of cerebellar granule cells *in vitro* (Balazs, Hack et al. 1990). However, higher concentration of Kainate induced cell death, although the trophic mechanism was not clear in the previous study.

7.7 Acute SE does not enhance DG cell death *in vivo*

Systemic administration of Kainate induces a period of generalized seizures, followed by a defined pattern of neuronal death within the hippocampus (Nadler and Cuthbertson 1980; Sperk, Lassmann et al. 1983). Kainate/seizures induced neuronal death exhibits characteristics of apoptosis (Filipkowski, Hetman et al. 1994; Simonian, Getz et al. 1996). Apoptosis pathways that have been identified in mammals involve members of the Bcl-2 and caspase gene families (Basu and Haldar 1998; Thornberry 1998). Caspase-3 is a cysteine protease that cleaves a specific aspartate residue in proteins in a diversity of structures which leads to cell death (Liu, Zou et al. 1997; Thornberry 1998). Under normal conditions, caspase-3 is synthesized as a latent pro-enzyme, which is proteolytically processed to the active form in response to apoptotic stimuli (Cohen 1997). Moreover, it is thought that caspase-3 protease activity contributes to Kainate-induced cell death in cultures generated from the fetal cerebellum (Nath, Probert et al. 1998). In an interesting study, it has been demonstrated that Kainate-induced seizures increased caspase-3 immunoreactive cells in the hippocampus (CA1-CA3/4) of sensitive (FVB/N) strain of mice, but not in the resistant strain (129/SvEMS) (Faherty, Xanthoudakis et al. 1999). Importantly, Faherty and co-workers showed that caspase-3 positive cells were expressed 2 days after Kainate insult in the sensitive strain. Subsequent studies have suggested an important role of apoptosis involving caspase-3 in regulating hippocampal neurogenesis in models of SE (Ekdahl, Mohapel et al. 2001; Ekdahl, Zhu et al. 2003; Dhanushkodi and Shetty 2008). Consistently, our *in vitro* results demonstrated a reduction in the proportion of caspase-3 immunoreactive neuroblasts after Kainate treatment indicating a survival effect. We therefore sought to

determine the numbers of caspase-3 immunoreactive cells after Kainate-induced SE in the DG of adult rats. We looked at cells expressing caspase-3 in the SGZ, GCL, hilus, and ML between 0 h and 48 h time points in both saline and Kainate injected animals. We aimed at elucidating if cell death in the stem cell niche precedes cell proliferation which was elicited at 48 in the GCL and 72 in the SGZ in Kainate treated animals.

Interestingly, we showed that Kainate/seizures had no effect on the number of capsase-3 immunopositive cells in the SGZ. Consistently, there was no difference in caspase-3 immunopositive cells in GCL when comparing saline to Kainate injected animals at 6 h and 48 h. In line with these results, there was no drop in the number of BrdU incorporated cells (pre-labelled 'clone') under Kainate conditions throughout the course of our experiment (0-72 h) indicating that the pre-labelled 'clone' indeed survived after Kainate/seizures. Consistent with this, we reported that the number of pre-labelled 'clone' of cells increased after Kainate treatment in the GCL supporting the hypothesis Kainate/seizures did not enhance cell death in the GCL of the DG. It has been shown that the GCL of adult DG is relatively resistant to Kainate-induced seizures when compared with other chemoconvulsants like pilocarpine (Covolan, Ribeiro et al. 2000). Surprisingly, there was a reduction in the number of caspase-3 positive cells in the SGZ and GCL after 6 h of Kainate/seizures; however, this decrease was not significant. In addition, 6 h of Kainate/seizures significantly reduced the counts of caspase-3 immunoreactive cells in the hilus and Ml of the DG. Although we did not phenotype caspase-3 immunoreactive cells, we suggest that Kainate/seizures enhance(s) the survival of DG cells transiently after the insult. The up-regulation of FGF-2 and its mRNA after Kainate-induced SE has been described in many independent studies which may suggest an initial survival effects perhaps related to FGF-2 and other neurotrophic factors (Bugra, Pollard et al. 1994; Yoshimura, Takagi et al. 2001; Zucchini, Barbieri et al. 2005). Consistent with this, it has been shown that pilocarpine-induced seizures increased the release of trophic factors such as, BDNF, NGF, FGF-2, and EGF after 4 h of the treatment/seizures. Similarly, Shetty et al have observed increased levels of neurotrophic factors after Kainate-induced seizures in rodents (Shetty, Zaman et al. 2003; Shetty, Rao et al. 2004).

In conclusion, our data confirm that the pre-labelled 'clone' of cells survived after Kainate/seizures and also show that no enhancement of cell death within the SGZ/GCL. On the contrary, there is a tendency towards an enhanced cell survival in SGZ/GCL which is evident transiently in the hilus and Ml.

7.8 Kainate-induced SE enhances hippocampal neurogenesis

We have demonstrated that Kainate treatments *in vitro* enhanced neurogenesis as implied by the proportional increase in TuJ1 positive cells. *In vivo,* we induced status epilepticus (SE) by IP Kainate injections 24 hours after labelling a 'clone' of proliferating precursor cells with BrdU. At 72 hours time point, I have found that Kainate/seizures significantly increased the numbers of doublecortin (Dcx) positive cells and consequently neurogenesis. Dcx, a protein associated with cell migration, expressed in newly-born neurons, has been widely used to study the acute effects of a factor on neurogenesis (Hattiangady, Shuai et al. 2007; Olariu, Cleaver et al. 2007; Rai, Hattiangady et al. 2007; Hattiangady, Rao et al. 2008; Hattiangady and Shetty 2008; Noonan, Choi et al. 2008). The mechanism underlying the increase in Dcx positive cells would include asymmetric proliferation of precursors, differentiation effects on precursor cells towards a neuronal lineage, and/or symmetric proliferation of type-3 precursor cells in the SGZ/GCL. Jessberger et al 2005 have demonstrated that Kainate-induced seizures had no effect on nestin-expressing early precursors in the SGZ/GCL of adult mice (Jessberger, Romer et al. 2005). Instead, he reported an increased proliferation of type-3 precursors (Dcx). In the light of this, we examined the effects of Kainate/seizures on Dcx positive cells at 72 h as there was no enhancement of cell proliferation in the SGZ at any of the time points examined before 72 h. We know that newly-born neurons are confined to the SGZ and inner one-third of the GCL (Jessberger, Romer et al. 2005; Plumpe, Ehninger et al. 2006). In view of these facts, it seems unlikely that Dcx positive cells would be increased before 72 h. In addition, in our study, Kainate/seizures elicited an increase in Ki-67 positive cells starting at 48 hours in the GCL and thus if a fraction of proliferating cells is induced by Kainate/seizures, it seems likely that we could detect an effect at 72 h time point. In support of this hypothesis, it

has been shown that slightly over 20% of Dcx cells in the SGZ/GCL are expressing the proliferative marker Ki-67 (Plumpe, Ehninger et al. 2006). Proliferating neuroblasts or so-called type-3 precursor cells are considered putative progenitor cells with limited self renewal ability (Kempermann, Jessberger et al. 2004; Seri, Garcia-Verdugo et al. 2004). Type-3 precursors, or at least a fraction of them, express the neuronal marker Dcx. Consistent with this, it has recently emerged that indeed a fraction of so-called neuroblasts-1 expresses Dcx and proliferate in the SGZ/GCL of the adult rodents (Encinas and Enikolopov 2008). In our model, cell proliferation in the GCL was mainly a contribution of the pre-labelled 'clone' of cells (BrdU+ and Ki-67+) and the most likely mechanism of enhancement of the proliferation of this 'clone' is by inducing cell cycle re-entry. As such, the increase in Dcx positive cells is mainly due to increased cell cycle re-entry of the pre-labelled 'clone' of cells. Jessberger et al have reported dispersion of Dcx positive cells in the GCL after Kainate/seizures, but his study did not point out to the origin of these precursor cells (Jessberger, Romer et al. 2005). In addition, an increase in GFP+ cells under GFAP promoter was reported after 72 h of Kainate/seizures in the SGZ of adult mice (Huttmann, Sadgrove et al. 2003). These studies have indicated an early enhancement of precursor cell proliferation in the stem cell niche of adult rodents. Another interpretation of our results is an enhanced asymmetric cell division of a precursor cell giving rise to Dcx precursor cells. This will lead to a self renewal of these precursor cells and an increase in neurogenesis. This model of cell division has been described recently as the main mechanism that causes an increase in neurogenesis in the SGZ of adult mice after running exercise (Suh, Consiglio et al. 2007). This may indicate that Kainate/seizures enhanced asymmetric cell division of the pre-labelled 'clone' and also enhanced symmetric cell division of the un-labelled 'clone'.

In summary, we demonstrate that Kainate/seizures elicited an increase in Dcx positive cells as early as 72 after the insult. We further suggest that this increase is either due to the proliferation of type-3 precursor cells and/or asymmetric cell division of pre-labelled 'clone' of precursor cells. If Kainate/seizures increased the proliferation of type-3 precursors (proliferating Dcx cells), this effect is most likely due to an enhanced cell

cycle re-entry of these proliferating type-3 precursors in the SGZ and dispersing them into the GCL.

7.9 The role of precursor cells in the postnatal and adult hippocampus in normal conditions and after Kainate/seizures

The century-old presumption of "no new neurons after birth" and the concepts of restricted regenerative capacity of the mammalian brain (Cajal 1928) are widely accepted as requiring alteration. The hippocampal dentate gyrus (DG) is one of two neurogenic niches, along with the olfactory interneurons generated in the subventricular zone, where new neurons are added to the existing neural circuitry throughout life (Altman and Das 1965; Kempermann, Kuhn et al. 1997; Cameron and McKay 1998; Gage 2000; Christie and Cameron 2006; Ehninger and Kempermann 2008).

The GCL is formed by precursor cells that were originally proliferating within the periventricular zone of the medial part of the cerebral cortex, and then migrated to the prospective dentate region during the perinatal period (Altman and Bayer 1990; Nakahira and Yuasa 2005). These migrating cells form the outer shell of the GCL and reside within the hilus by P5 in rats (Altman and Bayer 1990). Then, the inner shell of the GCL is formed by newly-born cells that are generated in the hilus and SGZ (Altman and Bayer 1990). Thus, more than half of the GCL cells are born postnatally (Bayer 1980; Namba, Mochizuki et al. 2005) and precursor cells inhabit the SGZ throughout life (Seri, Garcia-Verdugo et al. 2001; Garcia, Doan et al. 2004; Namba, Mochizuki et al. 2005). Therefore, studying postnatal hippocampal neurogenesis is an important issue because it would provide key information about how precursor cells are controlled in this special region and thus contribute to our understanding of adult neurogenesis. Many studies aimed at investigating the effects of Kainate on the severity of seizures and its relation with neuronal loss as well as cognitive impairment between postnatal and adult rodents (Sarkisian, Tandon et al. 1997; Haas, Sperber et al. 2001). For example, Sarkisian and co-workers have compared the effects of Kainate-induced seizures on spatial memory in immature (P 20-26) and adult rats (P 60-66). They have described that immature rats were spared the cognitive and pathological sequelae of Kainate-induced

seizures when compared to adult rats. They have also found minimal or no neuronal cell loss after seizures in pups in comparison with adult rats. Another study has demonstrated significant neuronal cell loss in CA1-4 in adult rats after Kainate-induced seizures, but postnatal rats were resistant to neuronal cell loss although they suffered sever seizures (Haas, Sperber et al. 2001). However, the previous study did not show an effect on neurogenesis or cell loss in the SGZ/GCL, but they clearly have shown a distinct pattern of resistance to the effects of Kainate-induced seizures suggesting that the postnatal brain perhaps have the capability of self repair. However, the role of precursor cells and neurogenesis was not clear. In our study, we have shown that hippocampal precursor cells are not only resistant to Kainate-induced cell death, but also proliferate and survive more in the presence of Kainate. This may explain the spared cognition after 1 month of multiple injections of Kainate in postnatal rats (Sarkisian, Tandon et al. 1997).

Seizure activity in humans leads to a diversity of structural changes in the brain, especially within hippocampal formation (Theodore and Gaillard 2002). The mechanisms that regulate the propagation of single seizures into epilepsy are not fully understood, but clearly are associated with cognitive impairments (Elger, Helmstaedter et al. 2004)). Accrued data that precursor cell proliferation and subsequent net neurogenesis are considerably enhanced in a variety of rodent models of TLE opened up new stage of complexity in understanding the pathogenesis and consequences of epilepsy (Gray and Sundstrom 1998; Gray, May et al. 2002; Parent 2002; Jessberger, Romer et al. 2005; Parent, Elliott et al. 2006; Jessberger, Zhao et al. 2007). Many reports have shown that seizure-induced neurogenesis is associated with abnormal morphological features that are not observed under normal conditions, including basal dendrites extending into the hilus (Dashtipour, Wong et al. 2003; Shapiro and Ribak 2006). Moreover, Jessberger and Zhao et al 2007 have revealed that hilar basal dendrites showed a substantial number of spines that had matured into large mushroom spines after 4 weeks of the retrovirus injection in the Kainate group only. Strikingly, it has been demonstrated that the size of the spine head positively correlates with the amounts of AMPA-receptors and also influences Ca^{2+} dynamics (Matsuzaki, Ellis-Davies et al. 2001). This may suggest a strong synapse and a role in synaptic plasticity (Hayashi and Majewska 2005). Furthermore, the enlargement of the spine head has also been

associated with an increase in AMPA-receptor-mediated currents at the stimulated synapse as well as long term potentiation (LTP) (Matsuzaki, Honkura et al. 2004). However, how the change in spine geometry after seizures translates into function has yet to be elucidated. Taken together, these studies suggest an important role for AMPA receptors after seizures which may also affect learning and memory. On the other hand, an ectopic location of the newly-generated neurons after seizures has been described in rodents (Scharfman, Goodman et al. 2000; Parent, Elliott et al. 2006) and humans (Parent, Elliott et al. 2006) which may persist for several months after the insult. In an attempt to elucidate the relation between the time of epileptic insult and the appearance of abnormal morphology of new neurons, Jessberger and colleagues (Jessberger, Zhao et al. 2007) have labelled dividing progenitor cells and their neuronal progeny cells in the adult DG with a retrovirus expressing green fluorescent protein (GFP) one week before and one week after Kainate-induced seizures. Then he followed these newly-born neurons for 4 weeks, 3 months and 1 year. Interestingly, he has found that new neurons born at any stage after seizures induction were abnormal. This abnormality was long-lasting as well. However, cells that were born one week before seizures-induction were normal even after Kainate insult. Another interesting study has shown that neuroplastic changes in Dcx expressing cells occur 1 day after pilocarpine-induced seizures and were long lasting (Shapiro, Figueroa-Aragon et al. 2007). Other studies have demonstrated that alterations in adult neurogenesis after Kainate-induced seizures occurred at the progenitor cell level (Huttmann, Sadgrove et al. 2003; Jessberger, Romer et al. 2005). These progenitor cells have been described by other investigators in Alvarez-Buylla lab as astrocyte-like stem cells (Seri, Garcia-Verdugo et al. 2001; Seri, Garcia-Verdugo et al. 2004; Ihrie and Alvarez-Buylla 2008). We conclude from the previous studies that abnormal alterations in neurogenesis start immediately after seizures induction. Although Jessberger et al 2007 have demonstrated abnormal long lasting changes in newly-born neurons; his study has failed to show the immediate contribution of precursor cells to these abnormalities. This may be due the fact that he labelled the dividing cells one week before seizures. Thus, rigorous stereological studies at early points after acute SE in animal models of TLE are needed to elucidate further the links among acute seizures and hippocampal neurogenesis. Therefore, an important approach

would include labelling the dividing cells (or cells in S-phase of the cell cycle) shortly before seizure-induction (as in our model) in order to elucidate the contribution of these dividing cells to the abnormal changes observed after SE. We have shown that Kainate/seizures enhanced the proliferation of the pre-labelled 'clone' of cells in the SGZ/GCL region and this most likely contributes to the increase in Dcx positive cells either by enhancing proliferation of type-3 precursors or increasing asymmetric cell division of a pre-labelled precursor cell. However, the current challenge is to develop a system in order differentiate between the effects of Kainate and seizures on precursor cells *in vivo*. This would be an interesting avenue for future research. The next section will discuss what our project added to the current knowledge about hippocampal precursor cells role in epeilpesy and their possible control mechanisms.

7.10 The possible contributions of hippocampal precursor cells to epilepsy

Epilepsy and cognitive impairments are most likely associated with a variety of structural and molecular changes in the course of the disease. I discussed, in the previous section, the current knowledge about the possible role of neurogenesis in TLE. However, the mechanism underling altered neurogenesis immediately after SE is unclear. Likewise, the role of precursor cells after seizures is incompletely understood. Clearly, there is a critical period during which neural precursor cells are vulnerable to external stimuli. However, the molecular mechanisms underlying the distinct vulnerability of precursor cells to these stimuli are unknown. My in vitro results demonstrated direct proliferative and survival effects of Kainate on precursor cells that caused a net increase in neurogenesis. Surprisingly, while Kainate did not kill these precursors, in contrast it enhanced their survival. However, it has been shown that glutamate increases cell proliferation in the embryonic rat ventricular zone (VZ) (Haydar, Wang et al. 2000). It has also been shown that the majority of hippocampal precursor cells are formed postnatally (Namba, Mochizuki et al. 2005) and are hypothesised to be direct descendents of the embryonic VZ (Pleasure, Collins et al. 2000). In addition, it has been elegantly shown that within 2-3 weeks of the birth of neurons, irrespective of animals

age, they are depolarized by GABA because of their high [Cl^-] (Ge, Goh et al. 2006). Importantly, Ge and co-workers have also reported that these immature neurons exhibit a depolarizing tonic response to GABA. These results suggest that the ambient GABA is released by local interneurons as indicated by enhanced tonic activation by the stimulation of interneurons (Ge, Goh et al. 2006). Interestingly, two independent studies have shown that SGZ neural precursors that express GFP under the control of nestin promoter in the adult transgenic mice exhibited both spontaneous and evoked GABAergic synaptic transmission (Tozuka, Fukuda et al. 2005; Wang, Kempermann et al. 2005). For example, Tozuka et al have demonstrated significant differentiation effects of precursor cells towards a neuronal lineage. Previous studies showed that GABA decreased cell proliferation in neocortical implants as indicated by the decrease in BrdU incorporated cells (LoTurco, Owens et al. 1995). Therefore, the precise mechanisms underlying the role of GABA in neurogenesis are not clear. As such, rigorous investigations need to be carried out in order to determine the mechanisms of the temporal role of GABA in neurogenesis.

The novel demonstration that the effects of Kainate were modulated via AMPA receptors deepened the insight towards the role of these receptors in regulating neurogenesis and cognitive and mood disorders in epilepsy. In support with our data, it has been shown that AMPA enhanced the survival of cerebellar granule cells *in vitro* via mechanisms involving BDNF and its receptors (Wu, Zhu et al. 2004). Furthermore, AMPA enhanced the proliferation of SVZ precursor cells cultured from postnatal rats (Xu, Ong et al. 2005). Interestingly, AMPA receptors were reported to be predominantly expressed in neuronal progenitor cells of the rat hippocampus (Hagimura, Tsuzuki et al. 2004). Moreover, it has been shown that AMPA receptor potentiator (LY451646) has an effect on precursor cell proliferation of the adult rats DG (Bai, Bergeron et al. 2003). In this regard, Bai and colleagues have shown that acute and chronic administration of LY451646 enhanced the proliferation of BrdU-incorporated cells that were arranged in clusters in the SGZ/GCL. However, their study failed to show significant effects of LY451646 treatment on the numbers of BrdU-incorporated cells although there was a trend towards an increase. We here show that AMPA receptors have an important role in regulating hippocampal neurogenesis in Kainate model. Altogether, this may suggest

that AMPA receptors activity in animals contributes, at least in part, to the regulation of cell proliferation in the postnatal hippocampus.

We have identified a critical time period where proliferating precursor cells respond to Kainate in vitro and to Kainate induced seizures in vivo. Indeed, our *in vivo* paradigm enabled us to examine the effects of Kainate/seizures on a pre-labelled and un-labelled 'clones' of DG precursor cells. Our data revealed that proliferating cells at the time of seizures induction play a major role in neurogenesis. We found that the proliferation of the pre-labelled 'clone' of cells is increased by Kainate induced-seizures through enhancing cell cycle re-entry and that precursors / progenitors are dispersed into the GCL suggesting disruption of the niche. The finding that cell proliferation was elicited either 48 h or 72 h after the Kainate/seizures suggests that the effects are indirect and may be through the release of mitogenic factors such as FGF-2 and BDNF (Shetty, Zaman et al. 2003; Shetty, Rao et al. 2004; Zucchini, Barbieri et al. 2005). However, we demonstrated a direct effect on cultures over a 6-8 h time period in vitro and we further showed that cell death was not enhanced after Kainate/seizures. In contrast, they may enhance the survival of these cells transiently. The published data have suggested that chemoconvulsant-induced seizures elicited the birth of abnormal neurons and these abnormalities were long lasting (Jessberger, Zhao et al. 2007; Shapiro, Figueroa-Aragon et al. 2007). In addition, Kainate-induced seizures were associated with dispersion of Dcx immunopositive cells within the SGZ and inner one third of the GCL (Jessberger, Romer et al. 2005). However, the mechanism by which precursor cells may contribute to this abnormality was unclear. Our study revealed that the early increase in neurogenesis (as indicated by an increase in Dcx positive cells) which was observed in the SGZ and inner one-third of the GCL was due to a mainly contribution from a mixed clone of precursors that were dividing 24h before the onset of SE. Our data also demonstrates a dispersion of the SGZ stem cell niche into the GCL where we observed a major contribution of the pre-labelled 'clone' of cells to cell proliferation after Kainate-induced seizures

7.11 Conclusions

Our project revealed the mechanism of Kainate effects on precursor cells and their neuronal progeny cells in cultures generated from the postnatal rat hippocampus. Briefly, Kainate enhanced symmetric and asymmetric precursor cell proliferation by increasing the speed of the cell cycle. It also instructs newly-born precursor cells and immature neurons to survive more. In addition, it has differentiation effects on nestin expressing cells towards a neuronal lineage. Our work identifies AMPA receptors as a key regulator of precursor cells proliferation, differentiation, and survival in Kainate model. *In vivo*, we have elucidated the acute effects of Kainate-induced SE on precursor cells. Here, we show that proliferating precursor cells are uniquely influenced by the insult and they further contributed to the development of new neurons that were found by others to be abnormal. Our *in vivo* results imply that while Kainate-induced SE does not kill precursor cells, it enhances a net increase in neurogenesis after 72 hours of seizures induction. We also propose a critical time at which proliferating precursor cells and their niche might be targeted to prevent the production of abnormal neurons which may contribute to epileptogenesis. Therefore, targeting precursor cells immediately after the insult might be of importance in the prevention of hippocampal neurogenesis dependant cognitive dysfunction in epilepsy. Likewise, these data suggest that AMPA receptors should be considered for their potential role to target alterations in stem / precursor cell proliferation and survival as well as in associated cognitive and mood disorders.

8. References:

Abrous, D. N., M. Koehl, et al. (2005). "Adult neurogenesis: from precursors to network and physiology." Physiol Rev **85**(2): 523-69.

Aimone, J. B., J. Wiles, et al. (2006). "Potential role for adult neurogenesis in the encoding of time in new memories." Nat Neurosci **9**(6): 723-7.

Alessio, A., B. P. Damasceno, et al. (2004). "Differences in memory performance and other clinical characteristics in patients with mesial temporal lobe epilepsy with and without hippocampal atrophy." Epilepsy Behav **5**(1): 22-7.

Alt, A., E. S. Nisenbaum, et al. (2006). "A role for AMPA receptors in mood disorders." Biochem Pharmacol **71**(9): 1273-88.

Altman, J. and S. A. Bayer (1990). "Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods." J Comp Neurol **301**(3): 365-81.

Altman, J. and S. A. Bayer (1990). "Mosaic organization of the hippocampal neuroepithelium and the multiple germinal sources of dentate granule cells." J Comp Neurol **301**(3): 325-42.

Altman, J. and G. D. Das (1965). "Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats." J Comp Neurol **124**(3): 319-35.

Altman, J. and G. D. Das (1966). "Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions." J Comp Neurol **126**(3): 337-89.

Alvarez-Buylla, A. and D. A. Lim (2004). "For the long run: maintaining germinal niches in the adult brain." Neuron **41**(5): 683-6.

Alvarez-Buylla, A., B. Seri, et al. (2002). "Identification of neural stem cells in the adult vertebrate brain." Brain Res Bull **57**(6): 751-8.

Ambrogini, P., R. Cuppini, et al. (2000). "Spatial learning affects immature granule cell survival in adult rat dentate gyrus." Neurosci Lett **286**(1): 21-4.

Anderson, M. F., M. A. Aberg, et al. (2002). "Insulin-like growth factor-I and neurogenesis in the adult mammalian brain." Brain Res Dev Brain Res **134**(1-2): 115-22.

Andrade, L., J. J. Caraveo-Anduaga, et al. (2003). "The epidemiology of major depressive episodes: results from the International Consortium of Psychiatric Epidemiology (ICPE) Surveys." Int J Methods Psychiatr Res **12**(1): 3-21.

Antonopoulos, J., I. S. Pappas, et al. (1997). "Activation of the GABAA receptor inhibits the proliferative effects of bFGF in cortical progenitor cells." Eur J Neurosci **9**(2): 291-8.

Arvidsson, A., Z. Kokaia, et al. (2001). "N-methyl-D-aspartate receptor-mediated increase of neurogenesis in adult rat dentate gyrus following stroke." Eur J Neurosci **14**(1): 10-8.

Babb, T. L., W. J. Brown, et al. (1984). "Temporal lobe volumetric cell densities in temporal lobe epilepsy." Epilepsia **25**(6): 729-40.

Babb, T. L., J. P. Lieb, et al. (1984). "Distribution of pyramidal cell density and hyperexcitability in the epileptic human hippocampal formation." Epilepsia **25**(6): 721-8.

Bai, F., M. Bergeron, et al. (2003). "Chronic AMPA receptor potentiator (LY451646) treatment increases cell proliferation in adult rat hippocampus." Neuropharmacology **44**(8): 1013-21.

Balazs, R., N. Hack, et al. (1990). "Selective stimulation of excitatory amino acid receptor subtypes and the survival of cerebellar granule cells in culture: effect of kainic acid." Neuroscience **37**(1): 251-8.

Balazs, R., O. S. Jorgensen, et al. (1988). "N-methyl-D-aspartate promotes the survival of cerebellar granule cells in culture." Neuroscience **27**(2): 437-51.

Banasr, M., M. Hery, et al. (2004). "Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone." Neuropsychopharmacology **29**(3): 450-60.

Bangasser, D. A., D. E. Waxler, et al. (2006). "Trace conditioning and the hippocampus: the importance of contiguity." J Neurosci **26**(34): 8702-6.

Barnea, A. and F. Nottebohm (1994). "Seasonal recruitment of hippocampal neurons in adult free-ranging black-capped chickadees." Proc Natl Acad Sci U S A **91**(23): 11217-21.

Barres, B. A., W. J. Koroshetz, et al. (1990). "Ion channel expression by white matter glia: the type-1 astrocyte." Neuron **5**(4): 527-44.

Basu, A. and S. Haldar (1998). "The relationship between Bcl2, Bax and p53: consequences for cell cycle progression and cell death." Mol Hum Reprod **4**(12): 1099-109.

Battista, D., C. C. Ferrari, et al. (2006). "Neurogenic niche modulation by activated microglia: transforming growth factor beta increases neurogenesis in the adult dentate gyrus." Eur J Neurosci **23**(1): 83-93.

Bauer, S. and P. H. Patterson (2005). "The cell cycle-apoptosis connection revisited in the adult brain." J Cell Biol **171**(4): 641-50.

Bayer, S. A. (1980). "Development of the hippocampal region in the rat. I. Neurogenesis examined with 3H-thymidine autoradiography." J Comp Neurol **190**(1): 87-114.

Bedard, A., M. Cossette, et al. (2002). "Proliferating cells can differentiate into neurons in the striatum of normal adult monkey." Neurosci Lett **328**(3): 213-6.

Beisker, W. and W. N. Hittelman (1988). "Measurement of the kinetics of DNA repair synthesis after uv irradiation using immunochemical staining of incorporated 5-bromo-2'-deoxyuridine and flow cytometry." Exp Cell Res **174**(1): 156-67.

Ben-Ari, Y. (1985). "Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy." Neuroscience **14**(2): 375-403.

Ben-Ari, Y. (2001). "Cell death and synaptic reorganizations produced by seizures." Epilepsia **42 Suppl 3**: 5-7.

Ben-Ari, Y. (2002). "Excitatory actions of gaba during development: the nature of the nurture." Nat Rev Neurosci **3**(9): 728-39.

Ben-Ari, Y. and R. Cossart (2000). "Kainate, a double agent that generates seizures: two decades of progress." Trends Neurosci **23**(11): 580-7.

Ben-Ari, Y., E. Tremblay, et al. (1981). "Electrographic, clinical and pathological alterations following systemic administration of kainic acid, bicuculline or pentetrazole: metabolic mapping using the deoxyglucose method with special reference to the pathology of epilepsy." Neuroscience **6**(7): 1361-91.

Ben-Yaakov, G. and H. Golan (2003). "Cell proliferation in response to GABA in postnatal hippocampal slice culture." Int J Dev Neurosci **21**(3): 153-7.

Bendel, O., I. A. Langmoen, et al. (2004). "Crush injury induces NMDA-receptor-dependent delayed nerve cell death in rat entorhinal-hippocampal slice cultures." Brain Res **1025**(1-2): 35-42.

Bengzon, J., Z. Kokaia, et al. (1997). "Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures." Proc Natl Acad Sci U S A **94**(19): 10432-7.

Bengzon, J., P. Mohapel, et al. (2002). "Neuronal apoptosis after brief and prolonged seizures." Prog Brain Res **135**: 111-9.

Benraiss, A., E. Chmielnicki, et al. (2001). "Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain." J Neurosci **21**(17): 6718-31.

Bernabeu, R. and F. R. Sharp (2000). "NMDA and AMPA/kainate glutamate receptors modulate dentate neurogenesis and CA3 synapsin-I in normal and ischemic hippocampus." J Cereb Blood Flow Metab **20**(12): 1669-80.

Bernier, P. J., A. Bedard, et al. (2002). "Newly generated neurons in the amygdala and adjoining cortex of adult primates." Proc Natl Acad Sci U S A **99**(17): 11464-9.

Bertrand, N., D. S. Castro, et al. (2002). "Proneural genes and the specification of neural cell types." Nat Rev Neurosci **3**(7): 517-30.

Best, N., L. E. Sundstrom, et al. (1996). "Pre-exposure to subtoxic levels prevents kainic acid lesions in organotypic hippocampal slice cultures: effects of kainic acid on parvalbumin-immunoreactive neurons and expression of heat shock protein 72 following the induction of tolerance." Eur J Neurosci **8**(6): 1209-19.

Bettler, B., J. Boulter, et al. (1990). "Cloning of a novel glutamate receptor subunit, GluR5: expression in the nervous system during development." Neuron **5**(5): 583-95.

Bettler, B., J. Egebjerg, et al. (1992). "Cloning of a putative glutamate receptor: a low affinity kainate-binding subunit." Neuron **8**(2): 257-65.

Bingham, B., D. Liu, et al. (2005). "Ischemia-stimulated neurogenesis is regulated by proliferation, migration, differentiation and caspase activation of hippocampal precursor cells." Brain Res **1058**(1-2): 167-77.

Bjornebekk, A., A. A. Mathe, et al. (2005). "The antidepressant effect of running is associated with increased hippocampal cell proliferation." Int J Neuropsychopharmacol **8**(3): 357-68.

Blackstone, C. D., A. I. Levey, et al. (1992). "Immunological detection of glutamate receptor subtypes in human central nervous system." Ann Neurol **31**(6): 680-3.

Blackstone, C. D., S. J. Moss, et al. (1992). "Biochemical characterization and localization of a non-N-methyl-D-aspartate glutamate receptor in rat brain." J Neurochem **58**(3): 1118-26.

Bleakman, D., B. A. Ballyk, et al. (1996). "Activity of 2,3-benzodiazepines at native rat and recombinant human glutamate receptors in vitro: stereospecificity and selectivity profiles." Neuropharmacology **35**(12): 1689-702.

Bleakman, D. and D. Lodge (1998). "Neuropharmacology of AMPA and kainate receptors." Neuropharmacology **37**(10-11): 1187-204.

Bliss, T. V. and G. L. Collingridge (1993). "A synaptic model of memory: long-term potentiation in the hippocampus." Nature **361**(6407): 31-9.

Blumcke, I., J. C. Schewe, et al. (2001). "Increase of nestin-immunoreactive neural precursor cells in the dentate gyrus of pediatric patients with early-onset temporal lobe epilepsy." Hippocampus **11**(3): 311-21.

Bolton, S. J. and V. H. Perry (1998). "Differential blood-brain barrier breakdown and leucocyte recruitment following excitotoxic lesions in juvenile and adult rats." Exp Neurol **154**(1): 231-40.

Boris-Moller, F. and T. Wieloch (1998). "Changes in the extracellular levels of glutamate and aspartate during ischemia and hypoglycemia. Effects of hypothermia." Exp Brain Res **121**(3): 277-84.

Boulter, J., M. Hollmann, et al. (1990). "Molecular cloning and functional expression of glutamate receptor subunit genes." Science **249**(4972): 1033-7.

Brazel, C. Y., J. L. Nunez, et al. (2005). "Glutamate enhances survival and proliferation of neural progenitors derived from the subventricular zone." Neuroscience **131**(1): 55-65.

Brecht, S., M. Gelderblom, et al. (2001). "Caspase-3 activation and DNA fragmentation in primary hippocampal neurons following glutamate excitotoxicity." Brain Res Mol Brain Res **94**(1-2): 25-34.

Brewer, G. J. (1997). "Isolation and culture of adult rat hippocampal neurons." J Neurosci Methods **71**(2): 143-55.

Brewer, G. J. (1999). "Regeneration and proliferation of embryonic and adult rat hippocampal neurons in culture." Exp Neurol **159**(1): 237-47.

Brewer, G. J., J. Espinosa, et al. (2001). "Culture and regeneration of human neurons after brain surgery." J Neurosci Methods **107**(1-2): 15-23.

Brewer, G. J. and J. R. Torricelli (2007). "Isolation and culture of adult neurons and neurospheres." Nat Protoc **2**(6): 1490-8.

Brezun, J. M. and A. Daszuta (1999). "Depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats." Neuroscience **89**(4): 999-1002.

Brezun, J. M. and A. Daszuta (2000). "Serotonin may stimulate granule cell proliferation in the adult hippocampus, as observed in rats grafted with foetal raphe neurons." Eur J Neurosci **12**(1): 391-6.

Brown, J., C. M. Cooper-Kuhn, et al. (2003). "Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis." Eur J Neurosci **17**(10): 2042-6.

Brown, J. P., S. Couillard-Despres, et al. (2003). "Transient expression of doublecortin during adult neurogenesis." J Comp Neurol **467**(1): 1-10.

Bruel-Jungerman, E., S. Laroche, et al. (2005). "New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment." Eur J Neurosci **21**(2): 513-21.

Buckman, J. F., H. Hernandez, et al. (2001). "MitoTracker labeling in primary neuronal and astrocytic cultures: influence of mitochondrial membrane potential and oxidants." J Neurosci Methods **104**(2): 165-76.

Buckmaster, P. S., G. F. Zhang, et al. (2002). "Axon sprouting in a model of temporal lobe epilepsy creates a predominantly excitatory feedback circuit." J Neurosci **22**(15): 6650-8.

Bugra, K., H. Pollard, et al. (1994). "aFGF, bFGF and flg mRNAs show distinct patterns of induction in the hippocampus following kainate-induced seizures." Eur J Neurosci **6**(1): 58-66.

Cajal, R. S. (1928). "Degeneration and Regeneration of the Nervous System.

." New York: Hafner.

Cameron, H. A. and E. Gould (1994). "Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus." Neuroscience **61**(2): 203-9.

Cameron, H. A., T. G. Hazel, et al. (1998). "Regulation of neurogenesis by growth factors and neurotransmitters." J Neurobiol **36**(2): 287-306.

Cameron, H. A., B. S. McEwen, et al. (1995). "Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus." J Neurosci **15**(6): 4687-92.

Cameron, H. A. and R. McKay (1998). "Stem cells and neurogenesis in the adult brain." Curr Opin Neurobiol **8**(5): 677-80.

Cameron, H. A. and R. D. McKay (1999). "Restoring production of hippocampal neurons in old age." Nat Neurosci **2**(10): 894-7.

Cameron, H. A. and R. D. McKay (2001). "Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus." J Comp Neurol **435**(4): 406-17.

Cameron, H. A., P. Tanapat, et al. (1998). "Adrenal steroids and N-methyl-D-aspartate receptor activation regulate neurogenesis in the dentate gyrus of adult rats through a common pathway." Neuroscience **82**(2): 349-54.

Cameron, H. A., C. S. Woolley, et al. (1993). "Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat." Neuroscience **56**(2): 337-44.

Cao, L., X. Jiao, et al. (2004). "VEGF links hippocampal activity with neurogenesis, learning and memory." Nat Genet **36**(8): 827-35.

Caragine, L. P., H. K. Park, et al. (1998). "Real-time measurement of ischemia-evoked glutamate release in the cerebral cortex of four and eleven vessel rat occlusion models." Brain Res **793**(1-2): 255-64.

Catarzi, D., V. Colotta, et al. (2007). "Competitive AMPA receptor antagonists." Med Res Rev **27**(2): 239-78.

Cater, H. L., C. D. Benham, et al. (2001). "Neuroprotective role of monocarboxylate transport during glucose deprivation in slice cultures of rat hippocampus." J Physiol **531**(Pt 2): 459-66.

Cavazos, J. E., I. Das, et al. (1994). "Neuronal loss induced in limbic pathways by kindling: evidence for induction of hippocampal sclerosis by repeated brief seizures." J Neurosci **14**(5 Pt 2): 3106-21.

Cendes, F. (2004). "Febrile seizures and mesial temporal sclerosis." Curr Opin Neurol **17**(2): 161-4.

Chatterton, J. E., M. Awobuluyi, et al. (2002). "Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits." Nature **415**(6873): 793-8.

Chittajallu, R., M. Vignes, et al. (1996). "Regulation of glutamate release by presynaptic kainate receptors in the hippocampus." Nature **379**(6560): 78-81.

Christie, B. R. and H. A. Cameron (2006). "Neurogenesis in the adult hippocampus." Hippocampus **16**(3): 199-207.

Cohen, G. M. (1997). "Caspases: the executioners of apoptosis." Biochem J **326 (Pt 1)**: 1-16.

Contestabile, A., T. Fila, et al. (2007). "Cell cycle alteration and decreased cell proliferation in the hippocampal dentate gyrus and in the neocortical germinal matrix of fetuses with Down syndrome and in Ts65Dn mice." Hippocampus **17**(8): 665-78.

Cooper-Kuhn, C. M., M. Vroemen, et al. (2002). "Impaired adult neurogenesis in mice lacking the transcription factor E2F1." Mol Cell Neurosci **21**(2): 312-23.

Coskun, V., D. L. Falls, et al. (2007). "Subventricular zone neuronal progenitors undergo multiple divisions and retract their processes prior to each cytokinesis." Eur J Neurosci **26**(3): 593-604.

Covolan, L., L. T. Ribeiro, et al. (2000). "Cell damage and neurogenesis in the dentate granule cell layer of adult rats after pilocarpine- or kainate-induced status epilepticus." Hippocampus **10**(2): 169-80.

Coyle, J. T. (1987). "Kainic acid: insights into excitatory mechanisms causing selective neuronal degeneration." Ciba Found Symp **126**: 186-203.

Craig, C. G., V. Tropepe, et al. (1996). "In vivo growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain." J Neurosci **16**(8): 2649-58.

Creuzet, C., J. Loeb, et al. (1995). "Fibroblast growth factors stimulate protein tyrosine phosphorylation and mitogen-activated protein kinase activity in primary cultures of hippocampal neurons." J Neurochem **64**(4): 1541-7.

Croll, S. D., S. J. Wiegand, et al. (1994). "Regulation of neuropeptides in adult rat forebrain by the neurotrophins BDNF and NGF." Eur J Neurosci **6**(8): 1343-53.

Curtin, J. F. and T. G. Cotter (2003). "Live and let die: regulatory mechanisms in Fas-mediated apoptosis." Cell Signal **15**(11): 983-92.

Dahlstrand, J., L. B. Zimmerman, et al. (1992). "Characterization of the human nestin gene reveals a close evolutionary relationship to neurofilaments." J Cell Sci **103 (Pt 2)**: 589-97.

Darzynkiewicz, Z., S. Bruno, et al. (1992). "Features of apoptotic cells measured by flow cytometry." Cytometry **13**(8): 795-808.

Dash, P. K., S. A. Mach, et al. (2001). "Enhanced neurogenesis in the rodent hippocampus following traumatic brain injury." J Neurosci Res **63**(4): 313-9.

Dashtipour, K., P. H. Tran, et al. (2001). "Ultrastructural features and synaptic connections of hilar ectopic granule cells in the rat dentate gyrus are different from those of granule cells in the granule cell layer." Brain Res **890**(2): 261-71.

Dashtipour, K., A. M. Wong, et al. (2003). "Temporal profile of hilar basal dendrite formation on dentate granule cells after status epilepticus." Epilepsy Res **54**(2-3): 141-51.

Dayer, A. G., A. A. Ford, et al. (2003). "Short-term and long-term survival of new neurons in the rat dentate gyrus." J Comp Neurol **460**(4): 563-72.

de la Monte, S. M., T. R. Neely, et al. (2001). "Ethanol impairs insulin-stimulated mitochondrial function in cerebellar granule neurons." Cell Mol Life Sci **58**(12-13): 1950-60.

de Lanerolle, N. C., T. Eid, et al. (1998). "Glutamate receptor subunits GluR1 and GluR2/3 distribution shows reorganization in the human epileptogenic hippocampus." Eur J Neurosci **10**(5): 1687-703.

Degterev, A., M. Boyce, et al. (2003). "A decade of caspases." Oncogene **22**(53): 8543-67.

Dehay, C., P. Savatier, et al. (2001). "Cell-cycle kinetics of neocortical precursors are influenced by embryonic thalamic axons." J Neurosci **21**(1): 201-14.

DeLorenzo, R. J., D. A. Sun, et al. (2007). "An in vitro model of stroke-induced epilepsy: elucidation of the roles of glutamate and calcium in the induction and maintenance of stroke-induced epileptogenesis." Int Rev Neurobiol **81**: 59-84.

Detour, J., H. Schroeder, et al. (2005). "A 5-month period of epilepsy impairs spatial memory, decreases anxiety, but spares object recognition in the lithium-pilocarpine model in adult rats." Epilepsia **46**(4): 499-508.

Devinsky, O. (2004). "Therapy for neurobehavioral disorders in epilepsy." Epilepsia **45 Suppl 2**: 34-40.

DeVries, S. H. and E. A. Schwartz (1999). "Kainate receptors mediate synaptic transmission between cones and 'Off' bipolar cells in a mammalian retina." Nature **397**(6715): 157-60.

Dhanushkodi, A. and A. K. Shetty (2008). "Is exposure to enriched environment beneficial for functional post-lesional recovery in temporal lobe epilepsy?" Neurosci Biobehav Rev **32**(4): 657-74.

Dingledine, R., K. Borges, et al. (1999). "The glutamate receptor ion channels." Pharmacol Rev **51**(1): 7-61.

Doetsch, F. and A. Alvarez-Buylla (1996). "Network of tangential pathways for neuronal migration in adult mammalian brain." Proc Natl Acad Sci U S A **93**(25): 14895-900.

Doetsch, F., I. Caille, et al. (1999). "Subventricular zone astrocytes are neural stem cells in the adult mammalian brain." Cell **97**(6): 703-16.

Doetsch, F., J. M. Garcia-Verdugo, et al. (1997). "Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain." J Neurosci **17**(13): 5046-61.

Dong, H., C. A. Csernansky, et al. (2003). "Hippocampal neurogenesis follows kainic acid-induced apoptosis in neonatal rats." J Neurosci **23**(5): 1742-9.

Dong, H., B. Goico, et al. (2004). "Modulation of hippocampal cell proliferation, memory, and amyloid plaque deposition in APPsw (Tg2576) mutant mice by isolation stress." Neuroscience **127**(3): 601-9.

Dranovsky, A. and R. Hen (2006). "Hippocampal neurogenesis: regulation by stress and antidepressants." Biol Psychiatry **59**(12): 1136-43.

Drapeau, E., W. Mayo, et al. (2003). "Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis." Proc Natl Acad Sci U S A **100**(24): 14385-90.

Duncan, J. S., J. W. Sander, et al. (2006). "Adult epilepsy." Lancet **367**(9516): 1087-100.

Eadie, B. D., V. A. Redila, et al. (2005). "Voluntary exercise alters the cytoarchitecture of the adult dentate gyrus by increasing cellular proliferation, dendritic complexity, and spine density." J Comp Neurol **486**(1): 39-47.

Earnshaw, W. C., L. M. Martins, et al. (1999). "Mammalian caspases: structure, activation, substrates, and functions during apoptosis." Annu Rev Biochem **68**: 383-424.

Easter, S. S., Jr., L. S. Ross, et al. (1993). "Initial tract formation in the mouse brain." J Neurosci **13**(1): 285-99.

Ehninger, D. and G. Kempermann (2008). "Neurogenesis in the adult hippocampus." Cell Tissue Res **331**(1): 243-50.

Ekdahl, C. T., P. Mohapel, et al. (2001). "Caspase inhibitors increase short-term survival of progenitor-cell progeny in the adult rat dentate gyrus following status epilepticus." Eur J Neurosci **14**(6): 937-45.

Ekdahl, C. T., C. Zhu, et al. (2003). "Death mechanisms in status epilepticus-generated neurons and effects of additional seizures on their survival." Neurobiol Dis **14**(3): 513-23.

El Bahh, B., R. Auvergne, et al. (2001). "Decreased epileptic susceptibility correlates with neuropeptide Y overexpression in a model of tolerance to excitotoxicity." Brain Res **894**(2): 209-17.

Elger, C. E., C. Helmstaedter, et al. (2004). "Chronic epilepsy and cognition." Lancet Neurol **3**(11): 663-72.

Emsley, J. G., B. D. Mitchell, et al. (2005). "Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells." Prog Neurobiol **75**(5): 321-41.

Encinas, J. M. and G. Enikolopov (2008). "Identifying and quantitating neural stem and progenitor cells in the adult brain." Methods Cell Biol **85**: 243-72.

Encinas, J. M., A. Vaahtokari, et al. (2006). "Fluoxetine targets early progenitor cells in the adult brain." Proc Natl Acad Sci U S A **103**(21): 8233-8.

Endl, E. and J. Gerdes (2000). "The Ki-67 protein: fascinating forms and an unknown function." Exp Cell Res **257**(2): 231-7.

Engel, J., Jr. (2001). "A proposed diagnostic scheme for people with epileptic seizures and with epilepsy: report of the ILAE Task Force on Classification and Terminology." Epilepsia **42**(6): 796-803.

Engel, J., Jr., C. Wilson, et al. (2003). "Advances in understanding the process of epileptogenesis based on patient material: what can the patient tell us?" Epilepsia **44 Suppl 12**: 60-71.

Eriksson, P. S., E. Perfilieva, et al. (1998). "Neurogenesis in the adult human hippocampus." Nat Med **4**(11): 1313-7.

Ernst, C., A. K. Olson, et al. (2006). "Antidepressant effects of exercise: evidence for an adult-neurogenesis hypothesis?" J Psychiatry Neurosci **31**(2): 84-92.

Esposito, M. S., V. C. Piatti, et al. (2005). "Neuronal differentiation in the adult hippocampus recapitulates embryonic development." J Neurosci **25**(44): 10074-86.

Fabel, K., K. Fabel, et al. (2003). "VEGF is necessary for exercise-induced adult hippocampal neurogenesis." Eur J Neurosci **18**(10): 2803-12.

Faherty, C. J., S. Xanthoudakis, et al. (1999). "Caspase-3-dependent neuronal death in the hippocampus following kainic acid treatment." Brain Res Mol Brain Res **70**(1): 159-63.

Fahrner, A., G. Kann, et al. (2007). "Granule cell dispersion is not accompanied by enhanced neurogenesis in temporal lobe epilepsy patients." Exp Neurol **203**(2): 320-32.

Fanarraga, M. L., J. Avila, et al. (1999). "Expression of unphosphorylated class III beta-tubulin isotype in neuroepithelial cells demonstrates neuroblast commitment and differentiation." Eur J Neurosci **11**(2): 517-27.

Filipkowski, R. K., M. Hetman, et al. (1994). "DNA fragmentation in rat brain after intraperitoneal administration of kainate." Neuroreport **5**(12): 1538-40.

Fisher, P. D., E. F. Sperber, et al. (1998). "Hippocampal sclerosis revisited." Brain Dev **20**(8): 563-73.

Fiszman, M. L., L. N. Borodinsky, et al. (1999). "GABA induces proliferation of immature cerebellar granule cells grown in vitro." Brain Res Dev Brain Res **115**(1): 1-8.

Forsgren, L., E. Beghi, et al. (2005). "The epidemiology of epilepsy in Europe - a systematic review." Eur J Neurol **12**(4): 245-53.

Francis, F., A. Koulakoff, et al. (1999). "Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons." Neuron **23**(2): 247-56.

Frederiksen, K. and R. D. McKay (1988). "Proliferation and differentiation of rat neuroepithelial precursor cells in vivo." J Neurosci **8**(4): 1144-51.

French, J. A., P. D. Williamson, et al. (1993). "Characteristics of medial temporal lobe epilepsy: I. Results of history and physical examination." Ann Neurol **34**(6): 774-80.

Friedman, L. K., D. E. Pellegrini-Giampietro, et al. (1994). "Kainate-induced status epilepticus alters glutamate and GABAA receptor gene expression in adult rat hippocampus: an in situ hybridization study." J Neurosci **14**(5 Pt 1): 2697-707.

Fukuda, S., F. Kato, et al. (2003). "Two distinct subpopulations of nestin-positive cells in adult mouse dentate gyrus." J Neurosci **23**(28): 9357-66.

Gage, F. H. (2000). "Mammalian neural stem cells." Science **287**(5457): 1433-8.

Gage, F. H., P. W. Coates, et al. (1995). "Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain." Proc Natl Acad Sci U S A **92**(25): 11879-83.

Gage, F. H., G. Kempermann, et al. (1998). "Multipotent progenitor cells in the adult dentate gyrus." J Neurobiol **36**(2): 249-66.

Gallo, V., M. Pende, et al. (1995). "Expression and regulation of kainate and AMPA receptors in uncommitted and committed neural progenitors." Neurochem Res **20**(5): 549-60.

Garcia, A. D., N. B. Doan, et al. (2004). "GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain." Nat Neurosci 7(11): 1233-41.

Ge, S., E. L. Goh, et al. (2006). "GABA regulates synaptic integration of newly generated neurons in the adult brain." Nature **439**(7076): 589-93.

Ge, S., D. A. Pradhan, et al. (2007). "GABA sets the tempo for activity-dependent adult neurogenesis." Trends Neurosci **30**(1): 1-8.

Geiger, J. R., T. Melcher, et al. (1995). "Relative abundance of subunit mRNAs determines gating and Ca2+ permeability of AMPA receptors in principal neurons and interneurons in rat CNS." Neuron **15**(1): 193-204.

Gleeson, J. G., P. T. Lin, et al. (1999). "Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons." Neuron **23**(2): 257-71.

Gould, E., A. Beylin, et al. (1999). "Learning enhances adult neurogenesis in the hippocampal formation." Nat Neurosci **2**(3): 260-5.

Gould, E., H. A. Cameron, et al. (1992). "Adrenal hormones suppress cell division in the adult rat dentate gyrus." J Neurosci **12**(9): 3642-50.

Gould, E., H. A. Cameron, et al. (1994). "Blockade of NMDA receptors increases cell death and birth in the developing rat dentate gyrus." J Comp Neurol **340**(4): 551-65.

Gould, E., B. S. McEwen, et al. (1997). "Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation." J Neurosci **17**(7): 2492-8.

Gould, E., A. J. Reeves, et al. (1999). "Hippocampal neurogenesis in adult Old World primates." Proc Natl Acad Sci U S A **96**(9): 5263-7.

Gould, E. and P. Tanapat (1997). "Lesion-induced proliferation of neuronal progenitors in the dentate gyrus of the adult rat." Neuroscience **80**(2): 427-36.

Gould, E., P. Tanapat, et al. (1997). "Adrenal steroids suppress granule cell death in the developing dentate gyrus through an NMDA receptor-dependent mechanism." Brain Res Dev Brain Res **103**(1): 91-3.

Gould, E., P. Tanapat, et al. (1999). "Neurogenesis in adulthood: a possible role in learning." Trends Cogn Sci **3**(5): 186-192.

Gould, E., P. Tanapat, et al. (1998). "Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress." Proc Natl Acad Sci U S A **95**(6): 3168-71.

Gray, W. P., K. May, et al. (2002). "Seizure induced dentate neurogenesis does not diminish with age in rats." Neurosci Lett **330**(3): 235-8.

Gray, W. P. and L. E. Sundstrom (1998). "Kainic acid increases the proliferation of granule cell progenitors in the dentate gyrus of the adult rat." Brain Res **790**(1-2): 52-9.

Gutierrez, R. (2003). "The GABAergic phenotype of the "glutamatergic" granule cells of the dentate gyrus." Prog Neurobiol **71**(5): 337-58.

Haas, K. Z., E. F. Sperber, et al. (2001). "Resistance of immature hippocampus to morphologic and physiologic alterations following status epilepticus or kindling." Hippocampus **11**(6): 615-25.

Hagg, T. (2005). "Molecular regulation of adult CNS neurogenesis: an integrated view." Trends Neurosci **28**(11): 589-95.

Hagihara, H., M. Hara, et al. (2005). "Tonic-clonic seizures induce division of neuronal progenitor cells with concomitant changes in expression of neurotrophic factors in the brain of pilocarpine-treated mice." Brain Res Mol Brain Res **139**(2): 258-66.

Hagimura, N., K. Tsuzuki, et al. (2004). "Predominant expression of GluR2 among the AMPA receptor subunits in neuronal progenitor cells of the rat hippocampus." Brain Res Dev Brain Res **152**(2): 213-23.

Hall, P. A., P. H. McKee, et al. (1993). "High levels of p53 protein in UV-irradiated normal human skin." Oncogene **8**(1): 203-7.

Hansel, D. E., B. A. Eipper, et al. (2001). "Neuropeptide Y functions as a neuroproliferative factor." Nature **410**(6831): 940-4.

Harvey, A. S., S. F. Berkovic, et al. (1997). "Temporal lobe epilepsy in childhood: clinical, EEG, and neuroimaging findings and syndrome classification in a cohort with new-onset seizures." Neurology **49**(4): 960-8.

Hastings, N. B. and E. Gould (1999). "Rapid extension of axons into the CA3 region by adult-generated granule cells." J Comp Neurol **413**(1): 146-54.

Hattiangady, B., M. S. Rao, et al. (2004). "Chronic temporal lobe epilepsy is associated with severely declined dentate neurogenesis in the adult hippocampus." Neurobiol Dis **17**(3): 473-90.

Hattiangady, B., M. S. Rao, et al. (2008). "Plasticity of hippocampal stem/progenitor cells to enhance neurogenesis in response to kainate-induced injury is lost by middle age." Aging Cell.

Hattiangady, B., M. S. Rao, et al. (2005). "Brain-derived neurotrophic factor, phosphorylated cyclic AMP response element binding protein and neuropeptide Y decline as early as middle age in the dentate gyrus and CA1 and CA3 subfields of the hippocampus." Exp Neurol **195**(2): 353-71.

Hattiangady, B. and A. K. Shetty (2008). "Aging does not alter the number or phenotype of putative stem/progenitor cells in the neurogenic region of the hippocampus." Neurobiol Aging **29**(1): 129-47.

Hattiangady, B., B. Shuai, et al. (2007). "Increased dentate neurogenesis after grafting of glial restricted progenitors or neural stem cells in the aging hippocampus." Stem Cells **25**(8): 2104-17.

Hayashi, Y. and A. K. Majewska (2005). "Dendritic spine geometry: functional implication and regulation." Neuron **46**(4): 529-32.

Haydar, T. F., F. Wang, et al. (2000). "Differential modulation of proliferation in the neocortical ventricular and subventricular zones." J Neurosci **20**(15): 5764-74.

Hayes, N. L. and R. S. Nowakowski (2002). "Dynamics of cell proliferation in the adult dentate gyrus of two inbred strains of mice." Brain Res Dev Brain Res **134**(1-2): 77-85.

Heinrich, C., N. Nitta, et al. (2006). "Reelin deficiency and displacement of mature neurons, but not neurogenesis, underlie the formation of granule cell dispersion in the epileptic hippocampus." J Neurosci **26**(17): 4701-13.

Helmstaedter, C. (2002). "Effects of chronic epilepsy on declarative memory systems." Prog Brain Res **135**: 439-53.

Helmstaedter, C., T. Brosch, et al. (2004). "The impact of sex and language dominance on material-specific memory before and after left temporal lobe surgery." Brain **127**(Pt 7): 1518-25.

Helmstaedter, C. and E. Kockelmann (2006). "Cognitive outcomes in patients with chronic temporal lobe epilepsy." Epilepsia **47 Suppl 2**: 96-8.

Helmstaedter, C., M. Kurthen, et al. (2003). "Chronic epilepsy and cognition: a longitudinal study in temporal lobe epilepsy." Ann Neurol **54**(4): 425-32.

Herr, I. and K. M. Debatin (2001). "Cellular stress response and apoptosis in cancer therapy." Blood **98**(9): 2603-14.

Herrup, K. and J. C. Busser (1995). "The induction of multiple cell cycle events precedes target-related neuronal death." Development **121**(8): 2385-95.

Hollmann, M. and S. Heinemann (1994). "Cloned glutamate receptors." Annu Rev Neurosci **17**: 31-108.

Hollmann, M., A. O'Shea-Greenfield, et al. (1989). "Cloning by functional expression of a member of the glutamate receptor family." Nature **342**(6250): 643-8.

Howell, O. W., K. Doyle, et al. (2005). "Neuropeptide Y stimulates neuronal precursor proliferation in the post-natal and adult dentate gyrus." J Neurochem **93**(3): 560-70.

Howell, O. W., H. E. Scharfman, et al. (2003). "Neuropeptide Y is neuroproliferative for post-natal hippocampal precursor cells." J Neurochem **86**(3): 646-59.

Howell, O. W., S. Silva, et al. (2007). "Neuropeptide Y is important for basal and seizure-induced precursor cell proliferation in the hippocampus." Neurobiol Dis **26**(1): 174-88.

Huettner, J. E. (2003). "Kainate receptors and synaptic transmission." Prog Neurobiol **70**(5): 387-407.

Hunt, S. J., J. I. Morrow, et al. (2007). "Are we failing those with 'The Falling Sickness'? Time to modernise the approach to epilepsy care." Ulster Med J **76**(1): 3-5.

Huttmann, K., M. Sadgrove, et al. (2003). "Seizures preferentially stimulate proliferation of radial glia-like astrocytes in the adult dentate gyrus: functional and immunocytochemical analysis." Eur J Neurosci **18**(10): 2769-78.

Ihrie, R. A. and A. Alvarez-Buylla (2008). "Cells in the astroglial lineage are neural stem cells." Cell Tissue Res **331**(1): 179-91.

Ino, H. and T. Chiba (2000). "Expression of proliferating cell nuclear antigen (PCNA) in the adult and developing mouse nervous system." Brain Res Mol Brain Res **78**(1-2): 163-74.

Jakubs, K., A. Nanobashvili, et al. (2006). "Environment matters: synaptic properties of neurons born in the epileptic adult brain develop to reduce excitability." Neuron **52**(6): 1047-59.

Janssens, N. and A. S. Lesage (2001). "Glutamate receptor subunit expression in primary neuronal and secondary glial cultures." J Neurochem **77**(6): 1457-74.

Jessberger, S. and G. Kempermann (2003). "Adult-born hippocampal neurons mature into activity-dependent responsiveness." Eur J Neurosci **18**(10): 2707-12.

Jessberger, S., K. Nakashima, et al. (2007). "Epigenetic modulation of seizure-induced neurogenesis and cognitive decline." J Neurosci **27**(22): 5967-75.

Jessberger, S., B. Romer, et al. (2005). "Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells." Exp Neurol **196**(2): 342-51.

Jessberger, S., C. Zhao, et al. (2007). "Seizure-associated, aberrant neurogenesis in adult rats characterized with retrovirus-mediated cell labeling." J Neurosci **27**(35): 9400-7.

Jiang, X., F. Tian, et al. (2005). "The excitoprotective effect of N-methyl-D-aspartate receptors is mediated by a brain-derived neurotrophic factor autocrine loop in cultured hippocampal neurons." J Neurochem **94**(3): 713-22.

Jin, K., M. Minami, et al. (2001). "Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat." Proc Natl Acad Sci U S A **98**(8): 4710-5.

Jin, K., A. L. Peel, et al. (2004). "Increased hippocampal neurogenesis in Alzheimer's disease." Proc Natl Acad Sci U S A **101**(1): 343-7.

Jin, K., X. Wang, et al. (2006). "Evidence for stroke-induced neurogenesis in the human brain." Proc Natl Acad Sci U S A **103**(35): 13198-202.

Jin, K., Y. Zhu, et al. (2002). "Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo." Proc Natl Acad Sci U S A **99**(18): 11946-50.

Jinno, S. and T. Kosaka (2003). "Patterns of expression of neuropeptides in GABAergic nonprincipal neurons in the mouse hippocampus: Quantitative analysis with optical disector." J Comp Neurol **461**(3): 333-49.

Johansen, T. N., J. R. Greenwood, et al. (2003). "Stereostructure-activity studies on agonists at the AMPA and kainate subtypes of ionotropic glutamate receptors." Chirality **15**(2): 167-79.

Joo, J. Y., B. W. Kim, et al. (2007). "Activation of NMDA receptors increases proliferation and differentiation of hippocampal neural progenitor cells." J Cell Sci **120**(Pt 8): 1358-70.

Joza, N., G. Kroemer, et al. (2002). "Genetic analysis of the mammalian cell death machinery." Trends Genet **18**(3): 142-9.

Jung, K. H., K. Chu, et al. (2004). "Continuous cytosine-b-D-arabinofuranoside infusion reduces ectopic granule cells in adult rat hippocampus with attenuation of spontaneous recurrent seizures following pilocarpine-induced status epilepticus." Eur J Neurosci **19**(12): 3219-26.

Kaufmann, S. H. and M. O. Hengartner (2001). "Programmed cell death: alive and well in the new millennium." Trends Cell Biol **11**(12): 526-34.

Kawabe, T., M. Suganuma, et al. (2002). "Cdc25C interacts with PCNA at G2/M transition." Oncogene **21**(11): 1717-26.

Ke, Z. J. and G. E. Gibson (2004). "Selective response of various brain cell types during neurodegeneration induced by mild impairment of oxidative metabolism." Neurochem Int **45**(2-3): 361-9.

Kee, N., S. Sivalingam, et al. (2002). "The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis." J Neurosci Methods **115**(1): 97-105.

Kee, N., C. M. Teixeira, et al. (2007). "Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus." Nat Neurosci **10**(3): 355-62.

Keij, J. F., C. Bell-Prince, et al. (2000). "Staining of mitochondrial membranes with 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green is affected by mitochondrial membrane potential altering drugs." Cytometry **39**(3): 203-10.

Keinanen, K., W. Wisden, et al. (1990). "A family of AMPA-selective glutamate receptors." Science **249**(4968): 556-60.

Kempermann, G. (2002). "Why new neurons? Possible functions for adult hippocampal neurogenesis." J Neurosci **22**(3): 635-8.

Kempermann, G., D. Gast, et al. (2002). "Neuroplasticity in old age: sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment." Ann Neurol **52**(2): 135-43.

Kempermann, G., D. Gast, et al. (2003). "Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice." Development **130**(2): 391-9.

Kempermann, G., S. Jessberger, et al. (2004). "Milestones of neuronal development in the adult hippocampus." Trends Neurosci **27**(8): 447-52.

Kempermann, G., H. G. Kuhn, et al. (1997). "More hippocampal neurons in adult mice living in an enriched environment." Nature **386**(6624): 493-5.

Kidd, F. L. and J. T. Isaac (1999). "Developmental and activity-dependent regulation of kainate receptors at thalamocortical synapses." Nature **400**(6744): 569-73.

Kim, J. B., J. Y. Ju, et al. (2004). "Dexamethasone inhibits proliferation of adult hippocampal neurogenesis in vivo and in vitro." Brain Res **1027**(1-2): 1-10.

Komuro, H. and P. Rakic (1993). "Modulation of neuronal migration by NMDA receptors." Science **260**(5104): 95-7.

Kornack, D. R. and P. Rakic (1999). "Continuation of neurogenesis in the hippocampus of the adult macaque monkey." Proc Natl Acad Sci U S A **96**(10): 5768-73.

Kornack, D. R. and P. Rakic (2001). "Cell proliferation without neurogenesis in adult primate neocortex." Science **294**(5549): 2127-30.

Kornyei, Z., V. Szlavik, et al. (2005). "Humoral and contact interactions in astroglia/stem cell co-cultures in the course of glia-induced neurogenesis." Glia **49**(3): 430-44.

Kuan, C. Y., K. A. Roth, et al. (2000). "Mechanisms of programmed cell death in the developing brain." Trends Neurosci **23**(7): 291-7.

Kuhn, H. G., H. Dickinson-Anson, et al. (1996). "Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation." J Neurosci **16**(6): 2027-33.

Kuhn, H. G., J. Winkler, et al. (1997). "Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain." J Neurosci **17**(15): 5820-9.

Kwon, Y. K. (1997). "Expression of brain-derived neurotrophic factor mRNA stimulated by basic fibroblast growth factor and platelet-derived growth factor in rat hippocampal cell line." Mol Cells **7**(3): 320-5.

Lagace, D. C., M. C. Whitman, et al. (2007). "Dynamic contribution of nestin-expressing stem cells to adult neurogenesis." J Neurosci **27**(46): 12623-9.

Laywell, E. D., P. Rakic, et al. (2000). "Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain." Proc Natl Acad Sci U S A **97**(25): 13883-8.

Ledergerber, D., J. M. Fritschy, et al. (2006). "Impairment of dentate gyrus neuronal progenitor cell differentiation in a mouse model of temporal lobe epilepsy." Exp Neurol.

Lee, J., W. Duan, et al. (2002). "Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice." J Neurochem **82**(6): 1367-75.

Lee, M. K., L. I. Rebhun, et al. (1990). "Posttranslational modification of class III beta-tubulin." Proc Natl Acad Sci U S A **87**(18): 7195-9.

Lees, G. J. (2000). "Pharmacology of AMPA/kainate receptor ligands and their therapeutic potential in neurological and psychiatric disorders." Drugs **59**(1): 33-78.

Leite, J. P., N. Garcia-Cairasco, et al. (2002). "New insights from the use of pilocarpine and kainate models." Epilepsy Res **50**(1-2): 93-103.

Lendahl, U., L. B. Zimmerman, et al. (1990). "CNS stem cells express a new class of intermediate filament protein." Cell **60**(4): 585-95.

Lerma, J. (1997). "Kainate reveals its targets." Neuron **19**(6): 1155-8.

Lerma, J. (2003). "Roles and rules of kainate receptors in synaptic transmission." Nat Rev Neurosci **4**(6): 481-95.

Lerma, J. (2006). "Kainate receptor physiology." Curr Opin Pharmacol **6**(1): 89-97.

Lerma, J., A. V. Paternain, et al. (1993). "Functional kainate-selective glutamate receptors in cultured hippocampal neurons." Proc Natl Acad Sci U S A **90**(24): 11688-92.

Lerma, J., A. V. Paternain, et al. (2001). "Molecular physiology of kainate receptors." Physiol Rev **81**(3): 971-98.

Leuner, B., E. Gould, et al. (2006). "Is there a link between adult neurogenesis and learning?" Hippocampus **16**(3): 216-24.

Lewis, P. D. (1978). "Kinetics of cell proliferation in the postnatal rat dentate gyrus." Neuropathol Appl Neurobiol **4**(3): 191-5.

Li, P., A. A. Calejesan, et al. (1998). "ATP P2x receptors and sensory synaptic transmission between primary afferent fibers and spinal dorsal horn neurons in rats." J Neurophysiol **80**(6): 3356-60.

Lidow, M. S. and P. Rakic (1995). "Neurotransmitter receptors in the proliferative zones of the developing primate occipital lobe." J Comp Neurol **360**(3): 393-402.

Lie, D. C., H. Song, et al. (2004). "Neurogenesis in the adult brain: new strategies for central nervous system diseases." Annu Rev Pharmacol Toxicol **44**: 399-421.

Liem, R. K. (1993). "Molecular biology of neuronal intermediate filaments." Curr Opin Cell Biol **5**(1): 12-6.

Linnarsson, S., C. A. Willson, et al. (2000). "Cell death in regenerating populations of neurons in BDNF mutant mice." Brain Res Mol Brain Res **75**(1): 61-9.

Lipski, J., C. K. Wan, et al. (2007). "Neuroprotective potential of ceftriaxone in in vitro models of stroke." Neuroscience **146**(2): 617-29.

Lipton, P. (1999). "Ischemic cell death in brain neurons." Physiol Rev **79**(4): 1431-568.

Litt, B., R. Esteller, et al. (2001). "Epileptic seizures may begin hours in advance of clinical onset: a report of five patients." Neuron **30**(1): 51-64.

Liu, J., K. Solway, et al. (1998). "Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils." J Neurosci **18**(19): 7768-78.

Liu, X., H. Zou, et al. (1997). "DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis." Cell **89**(2): 175-84.

Liu, Z., C. E. Stafstrom, et al. (1997). "Seizure-induced glutamate release in mature and immature animals: an in vivo microdialysis study." Neuroreport **8**(8): 2019-23.

Lois, C. and A. Alvarez-Buylla (1994). "Long-distance neuronal migration in the adult mammalian brain." Science **264**(5162): 1145-8.

Lossi, L., C. Cantile, et al. (2005). "Apoptosis in the mammalian CNS: Lessons from animal models." Vet J **170**(1): 52-66.

LoTurco, J. J., D. F. Owens, et al. (1995). "GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis." Neuron **15**(6): 1287-98.

Love, S., R. Barber, et al. (2000). "Neuronal death in brain infarcts in man." Neuropathol Appl Neurobiol **26**(1): 55-66.

Lowenstein, D. H. and L. Arsenault (1996). "The effects of growth factors on the survival and differentiation of cultured dentate gyrus neurons." J Neurosci **16**(5): 1759-69.

Lowenstein, D. H. and J. M. Parent (1999). "Brain, heal thyself." Science **283**(5405): 1126-7.

Lowenstein, D. H., M. S. Seren, et al. (1993). "Prolonged increases in neurotrophic activity associated with kainate-induced hippocampal synaptic reorganization." Neuroscience **56**(3): 597-604.

Lu, N., I. B. Black, et al. (1996). "A paradigm for distinguishing the roles of mitogenesis and trophism in neuronal precursor proliferation." Brain Res Dev Brain Res **94**(1): 31-6.

Luzzati, F., S. De Marchis, et al. (2006). "Neurogenesis in the caudate nucleus of the adult rabbit." J Neurosci **26**(2): 609-21.

Mackowiak, M., A. Chocyk, et al. (2004). "Neurogenesis in the adult." Pol J Pharmacol **56**(6): 673-87.

Madsen, T. M., A. Treschow, et al. (2000). "Increased neurogenesis in a model of electroconvulsive therapy." Biol Psychiatry **47**(12): 1043-9.

Malberg, J. E., A. J. Eisch, et al. (2000). "Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus." J Neurosci **20**(24): 9104-10.

Mandyam, C. D., G. C. Harburg, et al. (2007). "Determination of key aspects of precursor cell proliferation, cell cycle length and kinetics in the adult mouse subgranular zone." Neuroscience **146**(1): 108-22.

Marks, N. and M. J. Berg (1999). "Recent advances on neuronal caspases in development and neurodegeneration." Neurochem Int **35**(3): 195-220.

Marksteiner, J., G. Sperk, et al. (1989). "Differential increases in brain levels of neuropeptide Y and vasoactive intestinal polypeptide after kainic acid-induced seizures in the rat." Naunyn Schmiedebergs Arch Pharmacol **339**(1-2): 173-7.

Marshall, C. A. and J. E. Goldman (2002). "Subpallial dlx2-expressing cells give rise to astrocytes and oligodendrocytes in the cerebral cortex and white matter." J Neurosci **22**(22): 9821-30.

Martin, S. J., P. D. Grimwood, et al. (2000). "Synaptic plasticity and memory: an evaluation of the hypothesis." Annu Rev Neurosci **23**: 649-711.

Masuda, R., J. W. Monahan, et al. (2005). "D-beta-hydroxybutyrate is neuroprotective against hypoxia in serum-free hippocampal primary cultures." J Neurosci Res **80**(4): 501-9.

Mathern, G. W., P. D. Adelson, et al. (2002). "Hippocampal neuron damage in human epilepsy: Meyer's hypothesis revisited." Prog Brain Res **135**: 237-51.

Mathern, G. W., T. L. Babb, et al. (1996). "The pathogenic and progressive features of chronic human hippocampal epilepsy." Epilepsy Res **26**(1): 151-61.

Mathern, G. W., J. K. Pretorius, et al. (1995). "Influence of the type of initial precipitating injury and at what age it occurs on course and outcome in patients with temporal lobe seizures." J Neurosurg **82**(2): 220-7.

Matsuzaki, M., G. C. Ellis-Davies, et al. (2001). "Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons." Nat Neurosci **4**(11): 1086-92.

Matsuzaki, M., N. Honkura, et al. (2004). "Structural basis of long-term potentiation in single dendritic spines." Nature **429**(6993): 761-6.

Matthew Tremblay, S. J. (2003). "Epilepsy and Neurogenesis

A profile of hippocampal neurogenesis after induced temporal lobe epilepsy.

." jur **Volume** (2 Issue 1): 20-26.

Mattson, M. P. and S. B. Kater (1987). "Calcium regulation of neurite elongation and growth cone motility." J Neurosci 7(12): 4034-43.

Mayo, W., V. Lemaire, et al. (2005). "Pregnenolone sulfate enhances neurogenesis and PSA-NCAM in young and aged hippocampus." Neurobiol Aging **26**(1): 103-14.

Mazer, C., J. Muneyyirci, et al. (1997). "Serotonin depletion during synaptogenesis leads to decreased synaptic density and learning deficits in the adult rat: a possible model of neurodevelopmental disorders with cognitive deficits." Brain Res **760**(1-2): 68-73.

Mazza, M., F. Orsucci, et al. (2004). "Epilepsy and depression: risk factors for suicide?" Clin Ter **155**(10): 425-7.

McCloskey, D. P., T. M. Hintz, et al. (2006). "Stereological methods reveal the robust size and stability of ectopic hilar granule cells after pilocarpine-induced status epilepticus in the adult rat." Eur J Neurosci **24**(8): 2203-10.

McIntyre, D. C., M. O. Poulter, et al. (2002). "Kindling: some old and some new." Epilepsy Res **50**(1-2): 79-92.

McKeown, M. J. and J. O. McNamara (2001). "When do epileptic seizures really begin?" Neuron **30**(1): 1-3.

Menezes, J. R. and M. B. Luskin (1994). "Expression of neuron-specific tubulin defines a novel population in the proliferative layers of the developing telencephalon." J Neurosci **14**(9): 5399-416.

Merkle, F. T., A. D. Tramontin, et al. (2004). "Radial glia give rise to adult neural stem cells in the subventricular zone." Proc Natl Acad Sci U S A **101**(50): 17528-32.

Meshi, D., M. R. Drew, et al. (2006). "Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment." Nat Neurosci **9**(6): 729-31.

Mikati, M. A., S. Tarif, et al. (2001). "Time sequence and types of memory deficits after experimental status epilepticus." Epilepsy Res **43**(2): 97-101.

Miller, M. W. and R. S. Nowakowski (1988). "Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system." Brain Res **457**(1): 44-52.

Mino, M., H. Kamii, et al. (2003). "Temporal changes of neurogenesis in the mouse hippocampus after experimental subarachnoid hemorrhage." Neurol Res **25**(8): 839-45.

Minturn, J. E., D. H. Geschwind, et al. (1995). "Early postmitotic neurons transiently express TOAD-64, a neural specific protein." J Comp Neurol **355**(3): 369-79.

Mirescu, C. and E. Gould (2006). "Stress and adult neurogenesis." Hippocampus **16**(3): 233-8.

Mitchell, J., L. E. Sundstrom, et al. (1993). "Microglial and astrocytic cell responses in the rat hippocampus after an intracerebroventricular kainic acid injection." Exp Neurol **121**(2): 224-30.

Mochizuki, N., N. Takagi, et al. (2007). "Effect of NMDA receptor antagonist on proliferation of neurospheres from embryonic brain." Neurosci Lett **417**(2): 143-8.

Mohapel, P., C. T. Ekdahl, et al. (2004). "Status epilepticus severity influences the long-term outcome of neurogenesis in the adult dentate gyrus." Neurobiol Dis **15**(2): 196-205.

Monaghan, D. T., D. Yao, et al. (1984). "Distribution of [3H]AMPA binding sites in rat brain as determined by quantitative autoradiography." Brain Res **324**(1): 160-4.

Monyer, H., N. Burnashev, et al. (1994). "Developmental and regional expression in the rat brain and functional properties of four NMDA receptors." Neuron **12**(3): 529-40.

Morshead, C. M., A. D. Garcia, et al. (2003). "The ablation of glial fibrillary acidic protein-positive cells from the adult central nervous system results in the loss of forebrain neural stem cells but not retinal stem cells." Eur J Neurosci **18**(1): 76-84.

Morshead, C. M., B. A. Reynolds, et al. (1994). "Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells." Neuron **13**(5): 1071-82.

Mulle, C., A. Sailer, et al. (1998). "Altered synaptic physiology and reduced susceptibility to kainate-induced seizures in GluR6-deficient mice." Nature **392**(6676): 601-5.

Nacher, J., G. Alonso-Llosa, et al. (2003). "NMDA receptor antagonist treatment increases the production of new neurons in the aged rat hippocampus." Neurobiol Aging **24**(2): 273-84.

Nacher, J. and B. S. McEwen (2006). "The role of N-methyl-D-asparate receptors in neurogenesis." Hippocampus **16**(3): 267-70.

Nacher, J., E. Varea, et al. (2007). "N-methyl-d-aspartate receptor expression during adult neurogenesis in the rat dentate gyrus." Neuroscience **144**(3): 855-64.

Nadler, J. V. (1979). "Kainic acid: neurophysiological and neurotoxic actions." Life Sci **24**(4): 289-99.

Nadler, J. V. (1981). "Minireview. Kainic acid as a tool for the study of temporal lobe epilepsy." Life Sci **29**(20): 2031-42.

Nadler, J. V. and G. J. Cuthbertson (1980). "Kainic acid neurotoxicity toward hippocampal formation: dependence on specific excitatory pathways." Brain Res **195**(1): 47-56.

Nakagawa, E., Y. Aimi, et al. (2000). "Enhancement of progenitor cell division in the dentate gyrus triggered by initial limbic seizures in rat models of epilepsy." Epilepsia **41**(1): 10-8.

Nakahira, E. and S. Yuasa (2005). "Neuronal generation, migration, and differentiation in the mouse hippocampal primoridium as revealed by enhanced green fluorescent protein gene transfer by means of in utero electroporation." J Comp Neurol **483**(3): 329-40.

Namba, T., H. Mochizuki, et al. (2005). "The fate of neural progenitor cells expressing astrocytic and radial glial markers in the postnatal rat dentate gyrus." Eur J Neurosci **22**(8): 1928-41.

Namba, T., H. Mochizuki, et al. (2007). "Postnatal neurogenesis in hippocampal slice cultures: early in vitro labeling of neural precursor cells leads to efficient neuronal production." J Neurosci Res **85**(8): 1704-12.

Nath, R., A. Probert, Jr., et al. (1998). "Evidence for activation of caspase-3-like protease in excitotoxin- and hypoxia/hypoglycemia-injured neurons." J Neurochem **71**(1): 186-95.

Ni, B., X. Wu, et al. (1998). "Transient global forebrain ischemia induces a prolonged expression of the caspase-3 mRNA in rat hippocampal CA1 pyramidal neurons." J Cereb Blood Flow Metab **18**(3): 248-56.

Nijhawan, D., N. Honarpour, et al. (2000). "Apoptosis in neural development and disease." Annu Rev Neurosci **23**: 73-87.

Nilsson, M., E. Perfilieva, et al. (1999). "Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory." J Neurobiol **39**(4): 569-78.

Noonan, M. A., K. H. Choi, et al. (2008). "Withdrawal from cocaine self-administration normalizes deficits in proliferation and enhances maturity of adult-generated hippocampal neurons." J Neurosci **28**(10): 2516-26.

Nowakowski, R. S., S. B. Lewin, et al. (1989). "Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population." J Neurocytol **18**(3): 311-8.

Okano, H. J., D. W. Pfaff, et al. (1993). "RB and Cdc2 expression in brain: correlations with 3H-thymidine incorporation and neurogenesis." J Neurosci **13**(7): 2930-8.

Okuyama, N., N. Takagi, et al. (2004). "Phosphorylation of extracellular-regulating kinase in NMDA receptor antagonist-induced newly generated neurons in the adult rat dentate gyrus." J Neurochem **88**(3): 717-25.

Olariu, A., K. M. Cleaver, et al. (2007). "Decreased neurogenesis in aged rats results from loss of granule cell precursors without lengthening of the cell cycle." J Comp Neurol **501**(4): 659-67.

Owens, D. F. and A. R. Kriegstein (2002). "Is there more to GABA than synaptic inhibition?" Nat Rev Neurosci **3**(9): 715-27.

Palmer, T. D., J. Takahashi, et al. (1997). "The adult rat hippocampus contains primordial neural stem cells." Mol Cell Neurosci **8**(6): 389-404.

Palmer, T. D., A. R. Willhoite, et al. (2000). "Vascular niche for adult hippocampal neurogenesis." J Comp Neurol **425**(4): 479-94.

Parent, J. M. (2002). "The role of seizure-induced neurogenesis in epileptogenesis and brain repair." Epilepsy Res **50**(1-2): 179-89.

Parent, J. M., R. C. Elliott, et al. (2006). "Aberrant seizure-induced neurogenesis in experimental temporal lobe epilepsy." Ann Neurol **59**(1): 81-91.

Parent, J. M., E. Tada, et al. (1999). "Inhibition of dentate granule cell neurogenesis with brain irradiation does not prevent seizure-induced mossy fiber synaptic reorganization in the rat." J Neurosci **19**(11): 4508-19.

Parent, J. M., V. V. Valentin, et al. (2002). "Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway." J Neurosci **22**(8): 3174-88.

Parent, J. M., N. von dem Bussche, et al. (2006). "Prolonged seizures recruit caudal subventricular zone glial progenitors into the injured hippocampus." Hippocampus **16**(3): 321-8.

Parent, J. M., T. W. Yu, et al. (1997). "Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus." J Neurosci **17**(10): 3727-38.

Paternain, A. V., M. T. Herrera, et al. (2000). "GluR5 and GluR6 kainate receptor subunits coexist in hippocampal neurons and coassemble to form functional receptors." J Neurosci **20**(1): 196-205.

Paternain, A. V., M. Morales, et al. (1995). "Selective antagonism of AMPA receptors unmasks kainate receptor-mediated responses in hippocampal neurons." Neuron **14**(1): 185-9.

Perera, T. D., J. D. Coplan, et al. (2007). "Antidepressant-induced neurogenesis in the hippocampus of adult nonhuman primates." J Neurosci **27**(18): 4894-901.

Petralia, R. S., Y. X. Wang, et al. (1994). "The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those of NR1." J Neurosci **14**(10): 6102-20.

Petralia, R. S. and R. J. Wenthold (1992). "Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain." J Comp Neurol **318**(3): 329-54.

Pierce, J. P., J. Melton, et al. (2005). "Mossy fibers are the primary source of afferent input to ectopic granule cells that are born after pilocarpine-induced seizures." Exp Neurol **196**(2): 316-31.

Platel, J. C., B. Lacar, et al. (2007). "GABA and glutamate signaling: homeostatic control of adult forebrain neurogenesis." J Mol Histol **38**(4): 303-11.

Pleasure, S. J., A. E. Collins, et al. (2000). "Unique expression patterns of cell fate molecules delineate sequential stages of dentate gyrus development." J Neurosci **20**(16): 6095-105.

Plumpe, T., D. Ehninger, et al. (2006). "Variability of doublecortin-associated dendrite maturation in adult hippocampal neurogenesis is independent of the regulation of precursor cell proliferation." BMC Neurosci **7**: 77.

Pozzo Miller, L. D., N. K. Mahanty, et al. (1994). "Spontaneous pyramidal cell death in organotypic slice cultures from rat hippocampus is prevented by glutamate receptor antagonists." Neuroscience **63**(2): 471-87.

Prickaerts, J., G. Koopmans, et al. (2004). "Learning and adult neurogenesis: survival with or without proliferation?" Neurobiol Learn Mem **81**(1): 1-11.

Pringle, A. K., C. D. Benham, et al. (1996). "Selective N-type calcium channel antagonist omega conotoxin MVIIA is neuroprotective against hypoxic neurodegeneration in organotypic hippocampal-slice cultures." Stroke **27**(11): 2124-30.

Pringle, A. K., F. Iannotti, et al. (1997). "Neuroprotection by both NMDA and non-NMDA receptor antagonists in in vitro ischemia." Brain Res **755**(1): 36-46.

Pringle, A. K., L. E. Sundstrom, et al. (1996). "Brain-derived neurotrophic factor, but not neurotrophin-3, prevents ischaemia-induced neuronal cell death in organotypic rat hippocampal slice cultures." Neurosci Lett **211**(3): 203-6.

Rai, K. S., B. Hattiangady, et al. (2007). "Enhanced production and dendritic growth of new dentate granule cells in the middle-aged hippocampus following intracerebroventricular FGF-2 infusions." Eur J Neurosci **26**(7): 1765-79.

Rakic, P. and H. Komuro (1995). "The role of receptor/channel activity in neuronal cell migration." J Neurobiol **26**(3): 299-315.

Rao, M. S., B. Hattiangady, et al. (2005). "Newly born cells in the ageing dentate gyrus display normal migration, survival and neuronal fate choice but endure retarded early maturation." Eur J Neurosci **21**(2): 464-76.

Rao, M. S., B. Hattiangady, et al. (2006). "The window and mechanisms of major age-related decline in the production of new neurons within the dentate gyrus of the hippocampus." Aging Cell **5**(6): 545-58.

Rao, M. S. and A. K. Shetty (2004). "Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus." Eur J Neurosci **19**(2): 234-46.

Reynolds, B. A. and S. Weiss (1992). "Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system." Science **255**(5052): 1707-10.

Rietze, R., P. Poulin, et al. (2000). "Mitotically active cells that generate neurons and astrocytes are present in multiple regions of the adult mouse hippocampus." J Comp Neurol **424**(3): 397-408.

Riquelme, P. A., E. Drapeau, et al. (2007). "Brain micro-ecologies: neural stem cell niches in the adult mammalian brain." Philos Trans R Soc Lond B Biol Sci.

Rodriguez-Moreno, A., O. Herreras, et al. (1997). "Kainate receptors presynaptically downregulate GABAergic inhibition in the rat hippocampus." Neuron **19**(4): 893-901.

Rodriguez-Moreno, A. and T. S. Sihra (2007). "Metabotropic actions of kainate receptors in the CNS." J Neurochem **103**(6): 2121-35.

Rola, R., S. Mizumatsu, et al. (2006). "Alterations in hippocampal neurogenesis following traumatic brain injury in mice." Exp Neurol **202**(1): 189-99.

Rossi, D. J. and N. T. Slater (1993). "The developmental onset of NMDA receptor-channel activity during neuronal migration." Neuropharmacology **32**(11): 1239-48.

Routbort, M. J., S. B. Bausch, et al. (1999). "Seizures, cell death, and mossy fiber sprouting in kainic acid-treated organotypic hippocampal cultures." Neuroscience **94**(3): 755-65.

Ruano, D., B. Lambolez, et al. (1995). "Kainate receptor subunits expressed in single cultured hippocampal neurons: molecular and functional variants by RNA editing." Neuron **14**(5): 1009-17.

Sadgrove, M. P., J. E. Chad, et al. (2005). "Kainic acid induces rapid cell death followed by transiently reduced cell proliferation in the immature granule cell layer of rat organotypic hippocampal slice cultures." Brain Res **1035**(2): 111-9.

Sairanen, M., G. Lucas, et al. (2005). "Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation, and survival in the adult dentate gyrus." J Neurosci **25**(5): 1089-94.

Sakimura, K., T. Morita, et al. (1992). "Primary structure and expression of the gamma 2 subunit of the glutamate receptor channel selective for kainate." Neuron **8**(2): 267-74.

Santarelli, L., M. Saxe, et al. (2003). "Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants." Science **301**(5634): 805-9.

Sarkisian, M. R., P. Tandon, et al. (1997). "Multiple kainic acid seizures in the immature and adult brain: ictal manifestations and long-term effects on learning and memory." Epilepsia **38**(11): 1157-66.

Sastry, P. S. and K. S. Rao (2000). "Apoptosis and the nervous system." J Neurochem **74**(1): 1-20.

Schanzer, A., F. P. Wachs, et al. (2004). "Direct stimulation of adult neural stem cells in vitro and neurogenesis in vivo by vascular endothelial growth factor." Brain Pathol **14**(3): 237-48.

Scharfman, H., J. Goodman, et al. (2005). "Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats." Exp Neurol **192**(2): 348-56.

Scharfman, H. E., J. H. Goodman, et al. (2000). "Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis." J Neurosci **20**(16): 6144-58.

Scharfman, H. E., J. H. Goodman, et al. (2002). "Spontaneous limbic seizures after intrahippocampal infusion of brain-derived neurotrophic factor." Exp Neurol **174**(2): 201-14.

Scharfman, H. E. and W. P. Gray (2006). "Plasticity of neuropeptide Y in the dentate gyrus after seizures, and its relevance to seizure-induced neurogenesis." EXS(95): 193-211.

Schmahl, W. (1983). "Developmental gradient of cell cycle in the telencephalic roof of the fetal NMRI-mouse." Anat Embryol (Berl) **167**(3): 355-64.

Scholzen, T. and J. Gerdes (2000). "The Ki-67 protein: from the known and the unknown." J Cell Physiol **182**(3): 311-22.

Scott, B. W., S. Wang, et al. (1998). "Kindling-induced neurogenesis in the dentate gyrus of the rat." Neurosci Lett **248**(2): 73-6.

Scott, B. W., J. M. Wojtowicz, et al. (2000). "Neurogenesis in the dentate gyrus of the rat following electroconvulsive shock seizures." Exp Neurol **165**(2): 231-6.

Seaberg, R. M. and D. van der Kooy (2002). "Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors." J Neurosci **22**(5): 1784-93.

Segal, R. A. and M. E. Greenberg (1996). "Intracellular signaling pathways activated by neurotrophic factors." Annu Rev Neurosci **19**: 463-89.

Segovia, G., A. G. Yague, et al. (2006). "Environmental enrichment promotes neurogenesis and changes the extracellular concentrations of glutamate and GABA in the hippocampus of aged rats." Brain Res Bull **70**(1): 8-14.

Selden, J. R., F. Dolbeare, et al. (1994). "Validation of a flow cytometric in vitro DNA repair (UDS) assay in rat hepatocytes." Mutat Res **315**(2): 147-67.

Selden, J. R., F. Dolbeare, et al. (1993). "Statistical confirmation that immunofluorescent detection of DNA repair in human fibroblasts by measurement of bromodeoxyuridine incorporation is stoichiometric and sensitive." Cytometry **14**(2): 154-67.

Seri, B., J. M. Garcia-Verdugo, et al. (2004). "Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus." J Comp Neurol **478**(4): 359-78.

Seri, B., J. M. Garcia-Verdugo, et al. (2001). "Astrocytes give rise to new neurons in the adult mammalian hippocampus." J Neurosci **21**(18): 7153-60.

Shapiro, L. A., S. Figueroa-Aragon, et al. (2007). "Newly generated granule cells show rapid neuroplastic changes in the adult rat dentate gyrus during the first five days following pilocarpine-induced seizures." Eur J Neurosci **26**(3): 583-92.

Shapiro, L. A. and C. E. Ribak (2006). "Newly born dentate granule neurons after pilocarpine-induced epilepsy have hilar basal dendrites with immature synapses." Epilepsy Res **69**(1): 53-66.

Sheardown, M. J., E. O. Nielsen, et al. (1990). "2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia." Science **247**(4942): 571-4.

Shetty, A. K. and B. Hattiangady (2007). "Concise review: prospects of stem cell therapy for temporal lobe epilepsy." Stem Cells **25**(10): 2396-407.

Shetty, A. K., B. Hattiangady, et al. (2005). "Stem/progenitor cell proliferation factors FGF-2, IGF-1, and VEGF exhibit early decline during the course of aging in the hippocampus: role of astrocytes." Glia **51**(3): 173-86.

Shetty, A. K., M. S. Rao, et al. (2004). "Hippocampal neurotrophin levels after injury: Relationship to the age of the hippocampus at the time of injury." J Neurosci Res **78**(4): 520-32.

Shetty, A. K., V. Zaman, et al. (2003). "Hippocampal neurotrophin levels in a kainate model of temporal lobe epilepsy: a lack of correlation between brain-derived neurotrophic factor content and progression of aberrant dentate mossy fiber sprouting." J Neurochem **87**(1): 147-59.

Shingo, T., C. Gregg, et al. (2003). "Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin." Science **299**(5603): 117-20.

Shirai, K., T. Mizui, et al. (2006). "Differential effects of x-irradiation on immature and mature hippocampal neurons in vitro." Neurosci Lett **399**(1-2): 57-60.

Shneker, B. F. and N. B. Fountain (2003). "Epilepsy." Dis Mon **49**(7): 426-78.

Shors, T. J. (2004). "Learning during stressful times." Learn Mem **11**(2): 137-44.

Shors, T. J., G. Miesegaes, et al. (2001). "Neurogenesis in the adult is involved in the formation of trace memories." Nature **410**(6826): 372-6.

Shors, T. J., D. A. Townsend, et al. (2002). "Neurogenesis may relate to some but not all types of hippocampal-dependent learning." Hippocampus **12**(5): 578-84.

Simon, D. K., G. T. Prusky, et al. (1992). "N-methyl-D-aspartate receptor antagonists disrupt the formation of a mammalian neural map." Proc Natl Acad Sci U S A **89**(22): 10593-7.

Simonian, N. A., R. L. Getz, et al. (1996). "Kainic acid induces apoptosis in neurons." Neuroscience **75**(4): 1047-55.

Sisti, H. M., A. L. Glass, et al. (2007). "Neurogenesis and the spacing effect: learning over time enhances memory and the survival of new neurons." Learn Mem **14**(5): 368-75.

Sloviter, R. S., C. A. Zappone, et al. (2003). ""Dormant basket cell" hypothesis revisited: relative vulnerabilities of dentate gyrus mossy cells and inhibitory interneurons after hippocampal status epilepticus in the rat." J Comp Neurol **459**(1): 44-76.

Smith, M. T., V. Pencea, et al. (2001). "Increased number of BrdU-labeled neurons in the rostral migratory stream of the estrous prairie vole." Horm Behav **39**(1): 11-21.

Smithson, W. H. and N. J. Hanna (2002). "Deaths from epilepsy: what next?" Br J Gen Pract **52**(483): 795-6.

Smolders, I., Z. A. Bortolotto, et al. (2002). "Antagonists of GLU(K5)-containing kainate receptors prevent pilocarpine-induced limbic seizures." Nat Neurosci **5**(8): 796-804.

Song, H. J., C. F. Stevens, et al. (2002). "Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons." Nat Neurosci **5**(5): 438-45.

Sperber, E. F., K. Z. Haas, et al. (1991). "Resistance of the immature hippocampus to seizure-induced synaptic reorganization." Brain Res Dev Brain Res **60**(1): 88-93.

Sperk, G., H. Lassmann, et al. (1983). "Kainic acid induced seizures: neurochemical and histopathological changes." Neuroscience **10**(4): 1301-15.

Springer, J. E., R. D. Azbill, et al. (1999). "Activation of the caspase-3 apoptotic cascade in traumatic spinal cord injury." Nat Med **5**(8): 943-6.

Spruston, N., D. B. Jaffe, et al. (1994). "Dendritic attenuation of synaptic potentials and currents: the role of passive membrane properties." Trends Neurosci **17**(4): 161-6.

Squire, L. R. (1982). "The neuropsychology of human memory." Annu Rev Neurosci **5**: 241-73.

Srinivasan, A., K. A. Roth, et al. (1998). "In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system." Cell Death Differ **5**(12): 1004-16.

Stein-Behrens, B. A., W. J. Lin, et al. (1994). "Physiological elevations of glucocorticoids potentiate glutamate accumulation in the hippocampus." J Neurochem **63**(2): 596-602.

Steinhauser, C. and V. Gallo (1996). "News on glutamate receptors in glial cells." Trends Neurosci **19**(8): 339-45.

Suh, H., A. Consiglio, et al. (2007). "In Vivo Fate Analysis Reveals the Multipotent and Self-Renewal Capacities of Sox2(+) Neural Stem Cells in the Adult Hippocampus." Cell Stem Cell **1**(5): 515-528.

Sundstrom, L. E., J. Mitchell, et al. (1993). "Bilateral reorganisation of mossy fibres in the rat hippocampus after a unilateral intracerebroventricular kainic acid injection." Brain Res **609**(1-2): 321-6.

Sutherland, R. J., I. Q. Whishaw, et al. (1983). "A behavioural analysis of spatial localization following electrolytic, kainate- or colchicine-induced damage to the hippocampal formation in the rat." Behav Brain Res **7**(2): 133-53.

Suzuki, M., A. D. Nelson, et al. (2006). "Glutamate enhances proliferation and neurogenesis in human neural progenitor cell cultures derived from the fetal cortex." Eur J Neurosci **24**(3): 645-53.

Takahashi, T. and V. S. Caviness, Jr. (1993). "PCNA-binding to DNA at the G1/S transition in proliferating cells of the developing cerebral wall." J Neurocytol **22**(12): 1096-102.

Tanapat, P., L. A. Galea, et al. (1998). "Stress inhibits the proliferation of granule cell precursors in the developing dentate gyrus." Int J Dev Neurosci **16**(3-4): 235-9.

Tanapat, P., N. B. Hastings, et al. (1999). "Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat." J Neurosci **19**(14): 5792-801.

Tandon, P., Y. Yang, et al. (2002). "Downregulation of kainate receptors in the hippocampus following repeated seizures in immature rats." Brain Res Dev Brain Res **136**(2): 145-50.

Tanious, F. A., J. M. Veal, et al. (1992). "DAPI (4',6-diamidino-2-phenylindole) binds differently to DNA and RNA: minor-groove binding at AT sites and intercalation at AU sites." Biochemistry **31**(12): 3103-12.

Tashiro, A., H. Makino, et al. (2007). "Experience-specific functional modification of the dentate gyrus through adult neurogenesis: a critical period during an immature stage." J Neurosci **27**(12): 3252-9.

Taupin, P. (2006). "Neural progenitor and stem cells in the adult central nervous system." Ann Acad Med Singapore **35**(11): 814-20.

Taupin, P. (2007). "BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation." Brain Res Rev **53**(1): 198-214.

Taupin, P. and F. H. Gage (2002). "Adult neurogenesis and neural stem cells of the central nervous system in mammals." J Neurosci Res **69**(6): 745-9.

Temple, S. (2001). "The development of neural stem cells." Nature **414**(6859): 112-7.

Theodore, W. H. and W. D. Gaillard (2002). "Neuroimaging and the progression of epilepsy." Prog Brain Res **135**: 305-13.

Thornberry, N. A. (1998). "Caspases: key mediators of apoptosis." Chem Biol **5**(5): R97-103.

Toga, A. W., K. Ambach, et al. (1994). "Postmortem anatomy from cryosectioned whole human brain." J Neurosci Methods **54**(2): 239-52.

Tokuhara, D., S. Sakuma, et al. (2007). "Kainic acid dose affects delayed cell death mechanism after status epilepticus." Brain Dev **29**(1): 2-8.

Tooyama, I., J. P. Bellier, et al. (2002). "Morphologic study of neuronal death, glial activation, and progenitor cell division in the hippocampus of rat models of epilepsy." Epilepsia **43 Suppl 9**: 39-43.

Tozuka, Y., S. Fukuda, et al. (2005). "GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells." Neuron **47**(6): 803-15.

Trejo, J. L., E. Carro, et al. (2001). "Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus." J Neurosci **21**(5): 1628-34.

van Praag, H., G. Kempermann, et al. (1999). "Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus." Nat Neurosci **2**(3): 266-70.

van Praag, H., A. F. Schinder, et al. (2002). "Functional neurogenesis in the adult hippocampus." Nature **415**(6875): 1030-4.

van Praag, H., T. Shubert, et al. (2005). "Exercise enhances learning and hippocampal neurogenesis in aged mice." J Neurosci **25**(38): 8680-5.

Vannucci, R. C., R. M. Brucklacher, et al. (1999). "CSF glutamate during hypoxia-ischemia in the immature rat." Brain Res Dev Brain Res **118**(1-2): 147-51.

Vignes, M., D. Bleakman, et al. (1997). "The synaptic activation of the GluR5 subtype of kainate receptor in area CA3 of the rat hippocampus." Neuropharmacology **36**(11-12): 1477-81.

Walker, M. C., P. T. Galley, et al. (1995). "Ascorbate and glutamate release in the rat hippocampus after perforant path stimulation: a "dialysis electrode" study." J Neurochem **65**(2): 725-31.

Walker, M. C., A. Ruiz, et al. (2001). "Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses." Neuron **29**(3): 703-15.

Wang, L. P., G. Kempermann, et al. (2005). "A subpopulation of precursor cells in the mouse dentate gyrus receives synaptic GABAergic input." Mol Cell Neurosci **29**(2): 181-9.

Wang, X., C. Yang, et al. (2002). "Mechanisms of AIF-mediated apoptotic DNA degradation in Caenorhabditis elegans." Science **298**(5598): 1587-92.

Waters, J. and S. J. Smith (2003). "Mitochondria and release at hippocampal synapses." Pflugers Arch **447**(3): 363-70.

Westbrook, G. L. and E. W. Lothman (1983). "Cellular and synaptic basis of kainic acid-induced hippocampal epileptiform activity." Brain Res **273**(1): 97-109.

Westenbroek, C., J. A. Den Boer, et al. (2004). "Chronic stress and social housing differentially affect neurogenesis in male and female rats." Brain Res Bull **64**(4): 303-8.

Wiese, C., A. Rolletschek, et al. (2004). "Nestin expression--a property of multi-lineage progenitor cells?" Cell Mol Life Sci **61**(19-20): 2510-22.

Wilde, G. J., L. E. Sundstrom, et al. (1994). "Propidium iodide in vivo: an early marker of neuronal damage in rat hippocampus." Neurosci Lett **180**(2): 223-6.

Wilding, T. J. and J. E. Huettner (1996). "Antagonist pharmacology of kainate- and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-preferring receptors." Mol Pharmacol **49**(3): 540-6.

Winocur, G., J. M. Wojtowicz, et al. (2006). "Inhibition of neurogenesis interferes with hippocampus-dependent memory function." Hippocampus **16**(3): 296-304.

Wong, L. A., M. L. Mayer, et al. (1994). "Willardiines differentiate agonist binding sites for kainate- versus AMPA-preferring glutamate receptors in DRG and hippocampal neurons." J Neurosci **14**(6): 3881-97.

Wu, X., D. Zhu, et al. (2004). "AMPA protects cultured neurons against glutamate excitotoxicity through a phosphatidylinositol 3-kinase-dependent activation in extracellular signal-regulated kinase to upregulate BDNF gene expression." J Neurochem **90**(4): 807-18.

Xu, G., J. Ong, et al. (2005). "Subventricular zone proliferation after alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor-mediated neonatal brain injury." Dev Neurosci **27**(2-4): 228-34.

Xu, X. and X. Zheng (2007). "Potential involvement of calcium and nitric oxide in protective effects of puerarin on oxygen-glucose deprivation in cultured hippocampal neurons." J Ethnopharmacol **113**(3): 421-6.

Yakovlev, A. G. and A. I. Faden (2001). "Caspase-dependent apoptotic pathways in CNS injury." Mol Neurobiol **24**(1-3): 131-44.

Yan, X. X., J. Najbauer, et al. (2001). "Expression of active caspase-3 in mitotic and postmitotic cells of the rat forebrain." J Comp Neurol **433**(1): 4-22.

Yoshikawa, K. (2000). "Cell cycle regulators in neural stem cells and postmitotic neurons." Neurosci Res **37**(1): 1-14.

Yoshimura, S., Y. Takagi, et al. (2001). "FGF-2 regulation of neurogenesis in adult hippocampus after brain injury." Proc Natl Acad Sci U S A **98**(10): 5874-9.

Yoshimura, S., T. Teramoto, et al. (2003). "FGF-2 regulates neurogenesis and degeneration in the dentate gyrus after traumatic brain injury in mice." J Clin Invest **112**(8): 1202-10.

Young, D., P. A. Lawlor, et al. (1999). "Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective." Nat Med **5**(4): 448-53.

Yuan, J., M. Lipinski, et al. (2003). "Diversity in the mechanisms of neuronal cell death." Neuron **40**(2): 401-13.

Yuan, J. and B. A. Yankner (2000). "Apoptosis in the nervous system." Nature **407**(6805): 802-9.

Zacchetti, A., E. van Garderen, et al. (2003). "Validation of the use of proliferation markers in canine neoplastic and non-neoplastic tissues: comparison of KI-67 and proliferating cell nuclear antigen

(PCNA) expression versus in vivo bromodeoxyuridine labelling by immunohistochemistry." Apmis **111**(3): 430-8.

Zhao, C., E. M. Teng, et al. (2006). "Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus." J Neurosci **26**(1): 3-11.

Zhao, L. R. and S. C. Nam (2007). "Multiphoton microscope imaging: the behavior of neural progenitor cells in the rostral migratory stream." Neurosci Lett **425**(2): 83-8.

Zhu, H. and A. Dahlstrom (2007). "Glial fibrillary acidic protein-expressing cells in the neurogenic regions in normal and injured adult brains." J Neurosci Res **85**(12): 2783-92.

Zigova, T., V. Pencea, et al. (1998). "Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb." Mol Cell Neurosci **11**(4): 234-45.

Zucchini, S., M. Barbieri, et al. (2005). "Alterations in seizure susceptibility and in seizure-induced plasticity after pharmacologic and genetic manipulation of the fibroblast growth factor-2 system." Epilepsia **46 Suppl 5**: 52-8.

Zusso, M., P. Debetto, et al. (2004). "Cerebellar granular cell cultures as an in vitro model for antidepressant drug-induced neurogenesis." Crit Rev Neurobiol **16**(1-2): 59-65.