Vasoactive Intestinal Peptide (VIP): Control of Hippocampal Neurogenesis

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To my mum, my dad, my wife and my beloved kids...I dedicate this work.

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Abstract

Faculty of Medicine, Health and Life Sciences

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Neurogenesis; the generation of new neurons, is persistent in the brain throughout life of mammals, including humans. The two well-defined neurogenic areas in the adult brain are: the subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus in the hippocampus. Hippocampal neurogenesis is involved in learning and memory, and is influenced by many pathological states, such as epilepsy, stress, and mood disorders. The response of stem cells to different stimuli is organised by the surrounding microenvironment (niche), which extends to include endothelial cells, astrocytes and neurons. Neurogenesis is neuronally driven by projection input into the dentate gyrus and modulated by local circuit activity via GABAergic interneurons. Hippocampal interneurons respond to specific patterns of input activity by secreting neuropeptides; principal among them is the vasoactive intestinal polypeptide (VIP). A considerable amount of research has focused on factors which control the proliferation of neuronal precursor cells. However, it has been recently emphasised that cell survival rather than proliferation is the key determinant of net hippocampal neurogenesis and thus learning and memory acquisition.

VIP is a 28 amino acid neuropeptide that has potent trophic and proliferative effects on neuronal precursor cells during neurodevelopment. VIP and its binding sites are expressed at high levels in the adult dentate gyrus of the hippocampus. While reduction in VIP level is associated with learning and memory impairments, it peaks on P10 in the rat hippocampus; the critical period during which the granule cell layer of the DG is largely formed. Therefore, and in an attempt to understand the mechanisms underlying trophic control of hippocampal neurogenesis, we are investigating the hypothesis that VIP modulates the survival, proliferation and differentiation of postnatal hippocampal stem cells and their progeny.

We have shown that VIP at nanomolar concentrations is generally trophic to hippocampal cells *in vitro* with a prominent trophic and self-renewal effect on nestin cells, particularly the amplifying cell population, through enhanced symmetrical cell division. This effect is specific for dentate gyrus progenitor cells. Using immunohistochemistry and PCR techniques, we demonstrated the expression of VPAC1 and VPAC2 receptors and their mRNAs in hippocampal progenitor cell cultures. Pharmacologically, we have shown that VIP survival and self-renewal effects *in vitro* are VPAC2 mediated. We confirm that VPAC2 knockout adult mice have a reduction in the area of and the number of newly born cells in the granule cell layer of the dentate gyrus. We have also found that VIP interacts with FGF-2 and NPY to enhance neurogenesis and nestin cell survival, respectively.

We conclude that VIP is a trophic and self-renewal factor that may play a key role in the process of hippocampal neurogenesis. The importance of this work is our demonstration of a putative mechanism by which neuronal activity can influence trophism and symmetric/asymmetric division of precursor cells in the postnatal dentate gyrus.

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Abbreviations:

ABC	Avidin-Biotinylated enzyme Complex
ADNF	Activity-dependent neurotrophic factor
ADNP	Activity-dependent neuroprotective protein
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BrdU	5.Bromo-2-deoxyUridine
CA	Corno Ammons
DAB	3'3-Diamino-benzidine
DAPI	4'6-Diamidino-2-phenylindole
DIV	Days *in vitro*
EGF	Epidermal growth factor
GABA	Gamma aminobutyric acid
GCL	Granule cell layer
GDGF	Glia-derived growth factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Hippo	Hippocampus
HSVZ	Hippocampal subventricular zone
IGF-I	Insulin like growth factor-I
LTP	Long term ptentiation
NMDA	N-methyl-D-Aspartate
NT	neurotrophin
PBS	Phosphate buffer solution
PFA	Paraformaldehyde
PI	Propidium Iodide
PSA-NCAM	Poly-silyated neural cell adhesion molecule
SE	Standard error
SGZ	Subgranular zone
SVZ	Subventricular zone
TBS	Tris buffered saline
TBS-T	Tris buffered saline plus Triton
TUNEL	TdtT mediated dUTPbiotin nick end labelling
VEGF	Vascular endothelial growth factor

Chapter One

Introduction and Background

1.1 Neurogenesis during periods of development: Change of a dogma

It has been believed throughout history that once the brain is damaged, it is incapable of repair. However, this dogma has been successfully challenged over the last 40 years and has been replaced by the realisation that the brain changes throughout life and perhaps can repair itself through the process of neurogenesis (i.e. the birth of new neurons) (Altman and Das, 1965; Kolata, 1984; Barinaga, 1998; Gould and Gross, 2002) under specific conditions. Moreover from a physiological perspective; it is now well accepted that new neurons are continuously added to certain neural circuits in the adult vertebrate brain. In mammals, including humans, it has been revealed that neurons are generated throughout life in restricted areas; *the subventricular zone* (SVZ) of the lateral ventricles and the *subgranular zone* (SGZ) of the dentate gyrus (DG) (Kaplan and Hinds, 1977; Suhonen et al., 1996; Cameron and McKay, 1998; Eriksson et al., 1998). In order for these new neurons to develop, neural stem cells proliferate and generate their progeny which then differentiate to give neuronal or glial cells. Some of these cells survive, get integrated, start connections with the nearby neurons and become functional; many others die (Ninkovic et al., 2007). These successive events in the process of neurogenesis are apparently tightly regulated by a wide range of factors that can affect the proliferation, the final fate of these cells as well as their survival afterwards (Lowenstein and Parent, 1999). In our group of researchers, we are interested in stu the control mechanisms of hippocampal neurogenesis, particularly the role of interneurons and their corresponding neuropeptides in the regulation of these mechanisms.

1.2 Physiological and pathological functions of hippocampal neurogenesis

Hippocampal neurogenesis dynamically responds to a variety of physiological/ environmental events such as, enriched environment, exercise, learning and memory, and aging, as well as to pathological states like, stress, epilepsy, ischemia and depression. I will briefly address each one of them in this section.

Enriched Environment:

Enriched environment, includes expanded learning opportunities, increased interaction, larger housing and physical activity, has been shown to enhance neurogenesis in the adult mouse dentate gyrus (van Praag et al., 1999). Furthermore, recent studies have demonstrated voluntary exercise, particularly running, to enhance cell proliferation and recruitment of granule cells in the dentate gyrus. In fact, it has been found that trophic factors, such as vascular endothelial growth factor (VEGF), are necessary for the effects of running on adult hippocampal neurogenesis (Fabel et al., 2003).

Learning and Memory:

The interrelationship between learning and memory and hippocampal neurogenesis has been extensively studied as it is of major physiological and pathophysiological importance. Certain learning tasks have been found to be hippocampal-dependent such as trace conditioning, during which an animal learns to associate events that are distant from one another, and allocentric spatial memory; a sophisticated process which involves the creation of an internal map of the surrounding environment (Bangasser et al., 2006; Leuner et al., 2006). The hippocampus is thought to facilitate learning and memory through its unique synaptic plasticity which contributes to the acquisition and retention of memories (Martin et al., 2000). Part of this neural plasticity in the hippocampus may be mediated by neurogenesis; which may be the mechanism behind learning and memory in hippocampal dependent tasks. Clinically, over half of patients with chronic temporal lobe epilepsy, a neurodegenerative disorder which affects the hippocampus, displayed severe progressive memory deficits (Helmstaedter et al., 2003), which further emphasises the importance of establishing role of hippocampal neurogenesis in learning and memory. While Altman and Dayers have been the first to suggest a role for postnatally born neurons in learning, subsequent work has led to the discovery that adult neurogenesis is important for learning and memory of spatial information, too (Altman and Das, 1965; Bayer et al., 1973; Barnea and Nottebohm, 1996; Carlen et al., 2002). Indeed, several factors and conditions have been shown to affect the number/ survival of new neurons in the dentate gyrus of adult vertebrates; many of these have also been shown to influence certain types of learning and memory (for a review see (Leuner et al., 2006)). For instance, the exposure to enriched environment while it enhances

hippocampal neurogenesis, it also improves the animals performance in several learning tasks, including hippocampal-dependent spatial learning (Kempermann, 2002; Fabel et al., 2003; Gerd Kempermann, 2004; Bruel-Jungerman et al., 2005). In fact, hippocampal –dependent associative learning tasks increase survival of new neurons in the DG whilst training on hippocampal non-dependent learning tasks, does not influence hippocampal neurogenesis (Gould et al., 1999). Furthermore, it has recently been shown that while learning, and not mere exposure to training, enhances the survival of cells that are generated 1 week before training, learning over an extended period of time induces a more persistent memory, which then relates to the number of cells that reside in the hippocampus (Sisti et al., 2007).

While these studies indicate that learning enhances the survival of newly-born neurons in the hippocampus, thereby indicating an associational relationship, a causal relationship has been strongly suggested by a study showing that reducing neurogenesis impairs hippocampal dependent trace conditioning, a task in which the animal must associate stimuli that are separated in time (hippocampal dependent) (Shors et al., 2001). In a hippocampal independent task when the same stimuli are not separated, Shores has shown that a similar reduction in newly-born neurons did not affect learning. Similarly, in another study, it has been elegantly shown that adult neurogenesis is involved in specific aspects of hippocampal function, particularly the acquisition of new information (Kempermann and Gage, 2002). In this study Kempermann and his co-team used ten strains of recombinant inbred mice, based on C57BL/6, which are good learners and show high baseline levels of neurogenesis, and DBA/2, which are known to be poor learners and which exhibit low levels of adult neurogenesis. Two of these strains, BXD-2 and BXD-8, displayed a 26-fold difference in the number of newly generated neurons per hippocampus. Over all strains, there was a significant correlation between the number of new neurons generated in the dentate gyrus and parameters describing the acquisition of the water maze task. Interestingly, it has further been demonstrated that there is no correlation between adult hippocampal neurogenesis and, performance on the rotarod, overall locomotor activity, and baseline serum corticosterone levels (Kempermann and Gage, 2002). Taken together, Shores and Kempermann observations may indicate that newly generated neurons in the adult hippocampus are not only affected by hippocampal-dependent learning, but also participate in it. This has also been confirmed by Kee and

co-workers, where they have shown that as newly-born granule cells mature, they are increasingly likely to be incorporated into neuronal circuits supporting spatial memory (Shors, 2004). Indeed, by the time these cells are four or more weeks of age, they are more likely than existing granule cells to be recruited into circuits supporting spatial memory. This preferential recruitment again supports the idea that new neurons contribute to memory processing in the DG.

Aging:

It is known that advanced age, as another physiological state, is associated with decreased hippocampal neurogenesis (Kuhn et al., 1996). In rats, it has been shown that the net neurogenesis over the first year of life declines profoundly (94%) (McDonald and Wojtowicz, 2005). Similar observations were made in monkeys, in which the number of BrdU cells decreased in the DG with aging (Gould et al., 2001). This decrease in neurogenesis throughout lifespan has been attributed to a declining proliferation rather than a general age-related metabolic impairments (Kuhn et al., 1996). In some studies, age-associated decline in hippocampal proliferation and neurogenesis has been linked to the changes in the levels of circulating hormones, including corticosteroids (Cameron et al., 1998).

Pathological conditions (brain disease)

Brain Ischemia:

Increased neurogenesis in the dentate subgranular zone has been found after brain ischemia and strokes (Jin et al., 2001). In fact, the increase in hippocampal neurogenesis after focal ischemic insult is mediated by activation of NMDA receptor; through the involvement of growth factors (Arvidsson et al., 2001). It has been suggested that this ischemia-associated enhanced neurogenesis may be an adaptive process that contribute to recovery and repair after stroke (Mackowiak et al., 2004).

Epilepsy:

It is now well-accepted that acute seizures produced by kindling or kainate increase cell proliferation in the DG of the hippocampus (Cameron and McKay, 1998; Parent et al., 1998; Wunderlich et al., 2000; Parent, 2002; Scharfman and Gray, 2006).

However, while acutely, seizure is associated with enhanced cell proliferation, the net hippocampal neurogenesis is decreased in chronic epilepsy (Parent et al., 1998; Shetty and Hattiangady, 2007). Yet, whether this enhanced cell proliferation is part of the pathology or the repair is not known.

Stress:

Stress is another factor that has been found to exert potent effects on hippocampal neurogenesis (Abrous et al., 2005). Indeed, stress decreases hippocampal neurogenesis where the exposure of non-human primates to psychosocial stressor decreases the rate of cell proliferation in the GCL of the DG (Gould et al., 1997). Similar findings were found in rodents, in which acute or chronic restraint stress suppressed neurogenesis in the DG (Pham et al., 2003). This decreased hippocampal neurogenesis appears to result from stress-induced corticosteroids elevation (Mackowiak et al., 2004).

Mood disorders:

Hippocampal neurogenesis is involved in mood disorders as well, where failure of adult hippocampal neurogenesis is associated with depression and the effect of antidepressants is dependent on the integrity of SGZ of the dentate gyrus (Gerd Kempermann, 2004). Not only that, but long term antidepressant treatment has also been shown to enhance cell proliferation and neurogenesis in the DG (Santarelli et al., 2003; Encinas et al., 2006). Moreover, it has been shown that the disruption of anti-depressant treatment by genetic or radiological methods blocks behavioural response to antidepressants (Santarelli et al., 2003). Taken together, the current findings suggest that hippocampal cell proliferation and neurogenesis may be involved in the pathophysiology of depression and may modulate the effects of antidepressants (Mackowiak et al., 2004).

Brain inflammation:

Brain inflammation, a hallmark of many diseases in the CNS, has also been shown to inhibit the basal generation of new neurons in the hippocampus. This detrimental action of inflammation has always been attributed to the brain resident immune cells; microglia (Ekdahl et al., 2003). The deleterious effects of microglia on neurogenesis are mediated by the actions of cytokines, such as interleukin-6 and tissue necrosis

factor (Vallieres et al., 2002). Furthermore, the selective inhibition of microglia by, minocycline (Ekdahl et al., 2003), and the nonsteroidal anti-inflammatory drug indomethacin, has been found to restore hippocampal neurogenesis during inflammation (Monje et al., 2003).

To sum up, the functional significance of hippocampal neurogenesis is still under investigation. However, several evidences outlined in this section suggest involvement of hippocampal neurogenesis in cognitive processes. In addition, there are several findings which implicate involvement of hippocampal neurogenesis in the pathophysiology of some brain diseases. These findings strongly suggest that the changes in hippocampal neurogenesis rate may have functional and therapeutic implications.

The control of hippocampal neurogenesis, under these variable conditions, has led scientists to look at the way neural stem cells are organised in the SGZ. Indeed, neural stem cells in the SGZ as well as in the SVZ have been found organised in well-controlled microenvironments; stem cell niches (See **Section 1.3** below). These microenvironments have been found to support the life-long survival and self-renewal of neural stem cells and their generation of differentiated neurons (Riquelme et al., 2007).

1.3 Neural stem cell niches at the areas of neurogenesis in the adult brain

It has been reported that in all mammals including humans, endogenous neural stem cells (including both multipotent and restricted progenitors) exist not only in the developing, but also in the adult nervous system (Gage et al., 1995). They exist in neurogenic areas, particularly in the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles, but also in non-neurogenic sites including, cerebral cortex, septum, striatum, corpus callosum and spinal cord, where they are quiescent (Gage, 1995). Neural stem cells in permissive neurogenic areas, including the SGZ, are organised in specialised microenvironments (niches) which suppot their self-renewal and differentiation (Palmer et al., 1997; Watts et al., 2005). These niches are basically composed of the stem cells and their progeny, the endothelial cells of the surrounding blood vessels, the basal Lamina/ stromal cells which anchored stem cells and the extracellular matrix (Watts et al., 2005; Conover

and Notti, 2007). The stem cell niche acts as a dynamic structure that alters the location and characteristics of neural stem cells whilst, more importantly, allowing them to interact with each other (Riquelme et al., 2007). Indeed, stem cell niches are well organised structures in which cell-cell interactions and diffusible signals allow feedback control of stem cell self-renewal and differentiation (Conover and Notti, 2007). Neural stem cells have been isolated from the different niches and have been described loosely as the cells that are derived from the nervous system or generate neural tissue, including neurons, astrocytes, and oligodendrocytes, have the capacity of self-renewal (symmetrical division) , and can give rise to cells other than themselves through asymmetric cell division (Gage, 2000). The progeny of neural stem cells are progenitor cells that have been shown to self-replicate, but have a limited life span (Gage, 1995). Upon division, progenitor cells have been found to be further lineage restricted and to give rise to progeny that generate either neuroblasts or glioblasts, but not both (Gage, 1995). In the light of the debate about the definition of CNS stem cells, I will use the term "precursor cells" to refer to any cell type that has not yet terminally differentiated.

As I stated above, the two principal regions (niches) with high density of dividing (precursor/progenitor) cells within the adult mammals CNS are the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Kaplan and Hinds, 1977; Cameron and McKay, 1998; Smith and Luskin, 1998; Rietze et al., 2001). The SVZ is a layer of dividing cells that extends along the lateral walls of the lateral ventricles (**Figure 1.1**) (Doetsch, 2003). In this stem cell niche, the identity of neural stem cells and the lineage of new neurons have been nearly defined. Cells in this region have been classified as A, B, and C cells (**Figure 1.1**). B cells are considered as the primary precursors (Doetsch, 2003). This cell subpopulation has been described to be astrocytic in nature and capable of self-regeneration. B cells have been shown to proliferate and generate the transit-amplifying C cells (Doetsch, 2003). C cells in turn divide to give rise to neuroblasts (A cells) (**Figure 1.1**) (Doetsch, 2003). New neuroblasts born throughout the SVZ have been shown to migrate in chains to form the rostral migratory stream leading to the olfactory bulb. In the olfactory bulb these neuroblasts differentiate to their final fate as granule and periglomerular cells (interneurons) (Doetsch, 2003).

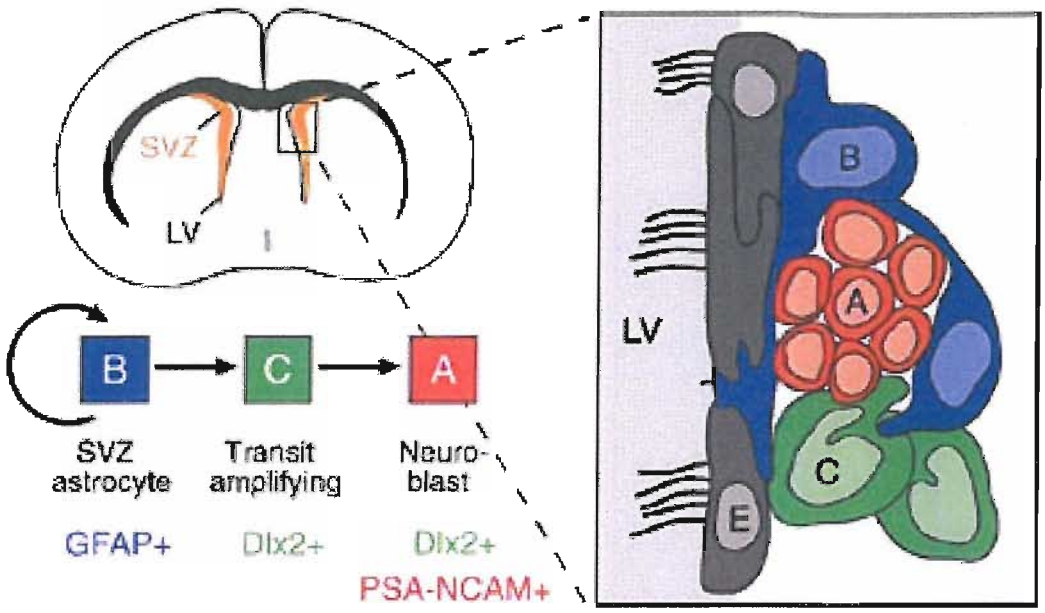


Figure 1.1: Frontal schema of the adult mouse brain showing the SVZ (orange), adjacent to the lateral ventricle (LV), and the SVZ cell types. Multi-ciliated ependymal cells (E, gray) line the LV. The chains of neuroblasts (A, red) migrate through glial tunnels formed by SVZ astrocytes (B, blue). Rapidly dividing transit-amplifying cells (C, green) are scattered in small foci along the network of chains. Occasionally an SVZ astrocyte extends a process between ependymal cells to contact the lateral ventricle. These SVZ astrocytes have a single cilium. SVZ astrocytes ($GFAP^+$) are stem cells in this region and generate neuroblasts ($GFAP^-$, $Dlx2^+$, $PSA\text{-}NCAM^+$) via the C cells ($GFAP^-$, $Dlx2^+$, $PSA\text{-}NCAM^-$). See text for details (adopted from (Doetsch, 2003)).

In the dentate gyrus of the hippocampus, cell proliferation was first demonstrated in the SGZ of rodents 40 years ago by autoradiography (Altman and Das, 1965). This stem cell niche is, in contrast to the SVZ, at the interface between the granule cell layer (GCL) and the hilus of the dentate gyrus, deep within the parenchyma (Abrous et al., 2005). Cells in the SGZ have been classified into B, D, and G cells (**Figure 1.2**) (Doetsch, 2003). B cells the same as those in the SVZ have been described as astrocytes (GFAP positive) and considered as the primary precursor cells (Doetsch, 2003). A few days after the birth of these GFAP-expressing B cells, their number starts to decrease while the proportion of actively dividing GFAP-negative cells increases (amplifying cell population). These small dark cells are called D cells (**Figure 1.2**). Chronic treatment with the anti-mitotic Ara-C resulted in elimination of most B and D cells. However, soon after cessation of the treatment, surviving B cells

begin to divide, whereas D cells reappear only at after 4 days (Seri, 2004). Using techniques of lineage tracking, these B cells have been demonstrated to give neurons (G cells) through the intermediate progeny (D cells) (**Figure 1.2**) (Doetsch, 2003; Seri, 2004). These neurons in contrast to SVZ neurons migrate only a short distance into the granule cell layer where they differentiate to granule neurons with mossy fibres reaching the CA3 subfield (Doetsch, 2003).

Kemperman's group has adopted an attractive approach to study the phenotype of neural stem cells, using nestin-promotor GFP transgenic mice. In these studies, two classes of progenitor/ stem cells were identified: Type 1 cells (nestin+, GFAP+, PSA-NCAM -), which correspond to B cells and are considered the putative stem cells, and type 2 cells (nestin+, GFAP-, PSA-NCAM +), supposed to be D cells (Seri, 2004; Riquelme et al., 2007). This cell subpopulation (D cells) is described as the amplifying cell population and has been shown to be more actively dividing compared to B cells. D cells in their turn give rise to a neuronal restricted progeny cells (G cells), which are Nestin-, GFAP-, Dcx+/TuJ1+) (Riquelme et al., 2007).

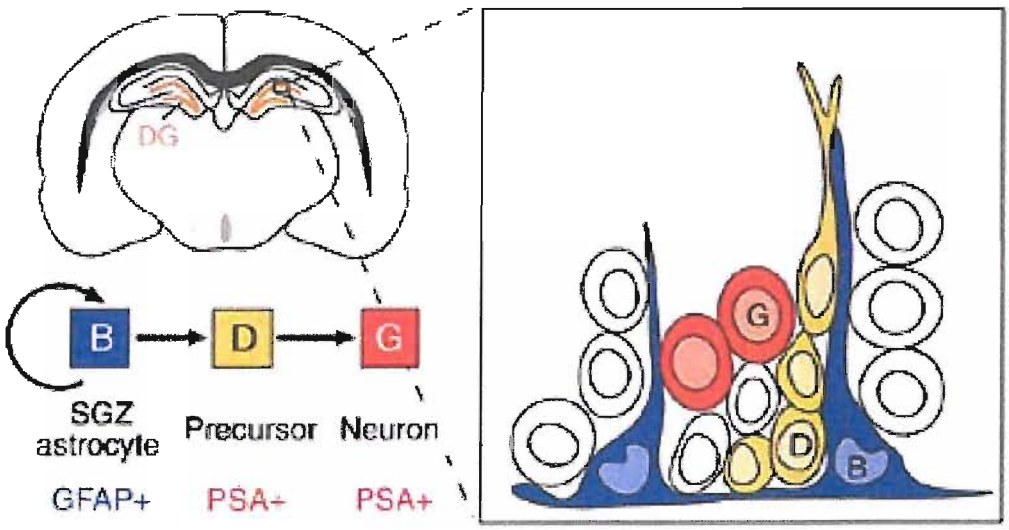


Figure1.2: (a) Frontal schema of the adult mouse brain showing the dentate gyrus (orange) and the cell types in the SGZ. SGZ astrocytes (B, blue, $GFAP^+$) are the *in vivo* primary precursors and give rise to an intermediate precursor (D, yellow, $GFAP^-$, $PSA\text{-}NCAM^+$) which in turn generates new granule neurons (G, red, $GFAP^-$, $PSA\text{-}NCAM^+$) (adopted from (Doetsch, 2003)).

1.4 Regulation of hippocampal neurogenesis within the stem cell niche

Although various factors that modulate the proliferation, survival and differentiation of neural progenitor/ stem cells have been isolated, the precise mechanisms that control neuronal stem cell fate remain largely unknown. Within the stem cell niche, both intrinsic programs (within the stem cells), and extracellular factors delivered by the circulation or secreted locally by nearby neuronal, glial, and endothelial cells, tightly regulate neurogenesis.

Intrinsic Control

Intrinsic programs controlling neurogenesis involve cell autonomous cues that direct stem cells toward a particular fate (i.e. self-renewal, mitotic arrest and /or differentiation). These control programs are divided into two groups. Firstly, those that control cell proliferation, such as the cell cycle factors retinoblastoma (Rb) and their related proteins (p107, p130, necdin and E2F) (Abrous et al., 2005). Indeed, these factors regulate the transition of dividing cells from G1 phase to S phase of the cell cycle. In the dentate gyrus, Rb immunoreactivity is high in proliferating neuronal precursors and low during terminal differentiation (Okano et al., 1993). E2F has also been found to be a key regulator of neurogenesis, where E2F knockout mice displayed reduced cell proliferation and neurogenesis in the hippocampus (Cooper-Kuhn et al., 2002). In contrast, transgenic mice lacking p27 have been found to display increased rates of cell proliferation and reduced cell differentiation in the SVZ (Doetsch et al., 2002).

Secondly, factors controlling cell fate, such as mammalian achaete-scute homolog (Mash1), mammalian atonal homolog (Math1), Neurogenins (Ngns), NeuroD and NeuroD2; which generally enhance neural stem cell differentiation towards neuronal lineage (for a review see (Mehler, 2002). These proneuronal genes/ factors are components of lateral inhibition, where through cell-cell signalling mechanisms, a cell that becomes committed to a neuronal fate inhibits its neighbours from doing so (Abrous et al., 2005). Moreover, it has been recently discovered that these proneuronal genes are downstream effectors of Pax6, a transcription factor that promotes neurogenesis (Heins et al., 2002).

Extrinsic Control

The list of extrinsic factors regulating neurogenesis comprises hormones, growth factors, glial cells, interneuron-released neurotransmitters and neuroregulators and many others. I will briefly describe each category below.

Circulating factors:

Hormones delivered by the blood circulation, including adrenal corticosteroids and oestrogen's, have been identified over the past few years as potential regulators of hippocampal neurogenesis. While adrenal steroids decrease production of new neurons, suppression of corticosterone secretion after bilateral adrenalectomy increases cell proliferation and birth of neuronal and glial cells in the DG (Harrelson and McEwen, 1987; Conrad and Roy, 1993; Montaron et al., 1999). However, the survival of these newly-born cells was independent from corticosteroids' effects (Abrous et al., 2005). In contrast to adrenal steroids, estrogens have been shown to increase but then decrease granule cell proliferation in the dentate gyrus of adult female rats in a time-dependent effect (Ormerod et al., 2003). Consistently, removal of circulating estrogens by bilateral ovarictomy results in significant decrease in hippocampal granule cell layer precursors (Mackowiak et al., 2004).

Growth Factors:

The other important group of neurogenesis-regulators is growth factors, which includes fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), Insulin-like growth factor-I (IGF-I) and many others, (For review see (Cameron et al., 1998; Abrous et al., 2005). Fibroblast growth factors (FGFs) including FGF-2 and their receptors constitute an elaborate signaling system that participates in many developmental and repair processes of virtually all mammalian tissues (Reuss et al., 2003; Reuss and von Bohlen und Halbach, 2003). In the CNS, FGFs are widely expressed; while FGF-2 is predominantly synthesized by astrocytes, the other FGF family members, e.g., FGF-5, FGF-8, and FGF-9, are primarily synthesized by neurons (Reuss and von Bohlen und Halbach, 2003). In the dentate gyrus, FGF-2 over-expression has been shown to upregulate cell proliferation (Mackowiak et al., 2004). Moreover; endogenous FGF-2 has been shown to stimulate proliferation and differentiation of progenitor cells in the adult hippocampus post-injury (Yoshimura et

al., 2001). However, FGF-2 has been reported to have no effect on the birth of new neurons in the hippocampus and the basal hippocampal neurogenesis is "normal" in mice lacking FGF-2 (Abrous et al., 2005). For EGF, it has been found that heparin-binding EGF, but not EGF by its own, enhances cell proliferation in the DG, through interactions with EGF receptors (Mackowiak et al., 2004). The other factor that has been investigated is the growth promoting peptide hormone; Insulin-like growth factor-I (IGF-I). Peripheral administration of IGF-I has been demonstrated to enhance cell proliferation and neurogenesis in the GCL and the hilus of the DG (Aberg et al., 2000). Further roles in the regulation of hippocampal neurogenesis of some of these factors are considered again, with other factors that are classified as neurotrophic factors in **Section 1.5** below.

Glial Control:

Hippocampal neurogenesis has also been shown to be regulated by glial cells (Song et al., 2002). Astrocytes residing in the stem cell niche of the dentate gyrus, which play an important role as sensors of the extracellular environment, may regulate neurogenesis by secreting local signals (Song et al., 2002). These signals extend to involve ionic fluxes, neurosteroids, cytokines, growth factors and glutamate metabolites (Riquelme et al., 2007). Indeed, it has been demonstrated that proliferating cells in the DG express S100β; a small acidic calcium binding neurotrophic protein released by astrocytes (Abrous et al., 2005). Many of these factors act on progenitor cells to regulate their cell pool maintenance and self-renewal (Hagg, 2005). This suggests that astrocytes are key regulators of cell proliferation in these neurogenic areas and it would be of major importance to determine whether these growth factors are released by mature astrocytes and/or a subpopulation of progenitor cells that are GFAP positive.

Neurotransmitters and Neuronal Control:

The involvement of the neuroregulator nitric oxide and neurotransmitters such as glutamate and serotonin (5-HT) and more importantly γ-aminobutryic acid (GABA) in the regulation of neurogenesis has also been investigated. Nitric oxide (NO), as a gaseous messenger molecule, is involved in learning and memory formation as well as synaptogenesis and morphogenesis (Reif et al., 2004). In fact, the role of NO as a neuroregulator in hippocampal neurogenesis has been emphasised, where the

administration of DETA/NONOate, a NO donor, to young adult rats significantly increases cell proliferation and migration in the dentate gyrus (Abrous et al., 2005). In another study, two independent approaches were used to investigate NO effects on hippocampal neurogenesis; pharmacological approach, where the NO production was suppressed in the rat brain by intraventricular infusion of a NO synthase inhibitor and a genetic approach of a null mutant neuronal NO synthase knockout mouse line. In both models, the number of new cells generated dentate gyrus, was strongly augmented, which indicates that division of neural stem cells in the adult brain is negatively regulated by NO (Packer et al., 2003).

In most of the studies while the stimulation of the NMDA glutamate receptor subtype by NMDA administration decreased cell proliferation in the hippocampus, the administration of the NMDA receptor antagonist has been shown to enhance proliferation (Mackowiak et al., 2004). In addition, lesion of the entrohinal cortex, which provides a major glutamatergic input to the hippocampus, increases hippocampal cell proliferation (Cameron and McKay, 1998). For the 5-HT system, a role in hippocampal neurogenesis has been demonstrated. Lesion in 5-HT system or inhibition of 5-HT synthesis has been shown to decrease cell proliferation in the hippocampus (Mackowiak et al., 2004). Moreover, administration of 5-HT releasing agents, such as d-fenfluramine, increased the number of proliferating cells in the hippocampus (Brezun and Daszuta, 1999).

Investigating the role of GABA in hippocampal neurogenesis gained a lot of interest as GABAergic interneurons sub-serve a major part of the neuronal inputs to the DG. In fact, the temporal organisation of these neuronal inputs on developing neural progenitor cells recapitulates patterns observed in development (Conover and Notti, 2007). In addition, newly-born neurons respond tonically to ambient extracellular GABA, where it stimulates their differentiation and migration, but as the neurons mature they receive inputs from GABAergic neuronal synapses (Conover and Notti, 2007). GABAergic interneurons in the DG modulate granule cell excitability mainly via feedback circuits from massy fibres but also via forward circuits from entrohinal cortex (Conover and Notti, 2007). Thus, it seems fitting electrophysiologically, pharmacologically and anatomically that GABAergic interneurons play a role in regulating hippocampal neurogenesis.

In our lab, we are interested in stu the role of neuropeptides in the control of hippocampal neurogenesis. In this regard, previous work conducted by our group has shown that the putative neuropeptide; NPY is proliferative for postnatal nestin-positive, sphere-forming hippocampal precursor cells and beta-tubulin-positive (TuJ1+) neuroblasts, and that the neuroproliferative effect is mediated via its Y1 receptor subtype (Howell et al., 2003). In agreement, it has been also shown that adult NPY receptor knock-out mice have significantly reduced cell proliferation and significantly fewer immature doublecortin-positive neurons (reduced neurogenesis), specifically in the dentate gyrus (Howell et al., 2005). Furthermore, the effects of NPY on DG precursors were evaluated in normal conditions and after status epilepticus. In fact, while potentially distinct NPY-responsive precursors were identified, not only of the DG, but also the caudal subventricular zone (cSVZ) and subcallosal zone (SCZ), where seizures modulate glial precursors, a proliferative effect of NPY on multipotent nestin-expressing stem cells from both the DG and the cSVZ/SCZ has been demonstrated *in vitro* (Howell et al., 2007). These studies suggest that the proliferation of hippocampal neuroblasts and precursor cells is increased by neuropeptide Y and, hence, hippocampal learning and memory may be modulated by neuropeptide Y-releasing interneurons. Notably, in response to different stimuli, while a subset of GABAergic interneurons release NPY, others release the neuropeptide vasoactive intestinal peptide (VIP) (Kohler, 1982; Sloviter and Nilaver, 1987; Jinno and Kosaka, 2003). In this project, we are interested in determining the roles of VIP in the regulation of hippocampal neurogenesis (See **Sections 1.6 and 1.7** for details).

In summary, my review of hippocampal neurogenesis regulators implies that the normal regulation of neural stem activity depends on the interaction between a variety of signals within the niche microenvironment and influences from the external macro-environment. The complexity of these interactions further suggests that there is no one single factor will be able to support stem cell behaviour in the absence of the other factors.

1.5 Neurogenesis and cell survival: role for trophic factors

Accumulating evidence suggests that the maintenance of hippocampal neurogenesis, both under physiological and pathological conditions, is largely dependent on cell survival rather than proliferation (Dayer et al., 2003). Indeed, Death of new cells in the granule cell layer occurred at a steady rate between 6 and 28 days after labeling, resulting in loss of 50% of BrdU-labeled cells over this 22-day period (Dayer et al., 2003). This has led scientists to study extensively the different factors affecting neurogenesis-associated cell death; trophic factors. For a long time trophic (survival) factors have been considered to act only on post-mitotic cells (DiCicco-Bloom, 1996). Trophic factors are defined as a subset of molecules that act to maintain cell viability (Morrison et al., 1987). More precisely, neurotrophic molecules act on neurons to enhance survival and induce neurite outgrowth (Morrison et al., 1987).

It has been suggested that during developmental neurogenesis a large number of cells are found in areas of cell proliferation suggesting a link between cell death and cell division (Thomaidou et al., 1997). This cell death which is mostly apoptotic in nature has been viewed traditionally as a fate reserved for differentiating and postmitotic neurons (Blaschke et al., 1998). However, it has been shown that 71% of the TUNEL-labelled cells in the subventricular zone of newborn rats have incorporated BrdU as a marker of the S-phase of the cell cycle (Thomaidou et al., 1997). This in agreement with other studies (Blaschke et al., 1998) suggesting that cell death is prominent during cell proliferation and is related to the progression in the cell cycle. In addition, it has been indicated that dividing stem/ precursor cells require trophic molecules to maintain their survival (Lu et al., 1996). Moreover; it has been suggested that the early expression of trophins receptors in the CNS during development sub-serves the trophic requirement of dividing precursors during the period of neurogenesis (Lu et al., 1996). In addition, for neurogenesis to be completed, progenitor cells have to stop proliferating, differentiate into neuroblasts or glioblasts and then survive. A large number of the cells produced in the adult hippocampus after neurogenesis have a transient existence and a short life span and for survival they need trophic factors (Gould et al., 2001).

Trophic factors list extends to involve brain-derived growth factor (BDNF), vascular endothelial growth factor (VEGF), glial-derived neurotrophic factor (GDGF), nerve growth factor (NGF), neurotrophin-3 (NT-3), VIP and others. There is a growing body of evidence suggesting that, trophic factors are more instrumental with a survival instead of proliferation effect in the modulation of hippocampal neurogenesis, which is induced by hippocampal-dependent learning tasks and enriched environment (Bruel-Jungerman et al., 2005). Indeed, the levels of BDNF, VEGF, GDGF, NGF, NT-3, VIP and many of their corresponding receptors alter in the hippocampus after enriched environment and voluntary exercise (Olson et al., 2006). This strongly suggests that these trophic factors may play a role in the modulation of hippocampal neurogenesis by enriched environment.

Among the most studied trophic factors is BDNF, which has been shown to enhance neuroblast differentiation and survival by reducing proliferation (Hagg, 2005). In addition, whilst exercise increases BDNF mRNA and protein in the hippocampus, BDNF directly injected to the hippocampus enhanced neurogenesis in the DG (Olson et al., 2006). BDNF has also been shown to enhance the survival of neuronal cells in micro-dissected dentate cell cultures (Abrous et al., 2005). In addition, it has been shown that exercise, as a main component of enriched environment, through increases in BDNF can enhance the survival of newborn cells in the hippocampus (Olson et al., 2006). VEGF is an angiogenesis factor that has been shown to enhance cell survival in the dentate gyrus (Sun et al., 2003). In addition, VEGF has been shown to enhance the proliferation of endothelial cells, which are important components of the stem cell niche, and the formation of new blood vessels (Abrous et al., 2005). This may indicate that VEGF is an important factor that induces angiogenesis and survival of new neurons in the hippocampus that occurs with enriched environment. Finally, and in relation to hippocampal neurogenesis, GDGF, NGF and NT-3 have been reported to enhance neuronal cell survival and maturation in the DG (Olson et al., 2006). This again may suggest roles of these trophic factors in the modulation of enriched environment- associated neuronal survival in the hippocampus.

Stress has also been shown to affect cell survival during hippocampal neurogenesis; recent studies have demonstrated that while stress leads to neuronal cell loss (death)

in the hippocampus and decrease in the level of trophic factors, antidepressant treatment reverses or at least blocks these effects of stress (Olson et al., 2006). Moreover, the levels of BDNF and other neurotrophic factors have been found to be decreased in the hippocampus of patients with major depressive disorders as reflected by post-mortem tissue studies (Prickaerts et al., 2004). This finding has been attributed to hippocampal neuronal cell loss seen in these patients.

In summary, trophic factors are important regulators of cell survival during stem cell development. In fact, in stu hippocampal neurogenesis, cell survival is gaining more interest than cell proliferation, as it is believed to be the key regulator of the net neurogenesis. Indeed, it is the cell survival with or without cell proliferation that is suggested as an important contributor to learning-associated hippocampal neurogenesis (Prickaerts et al., 2004; Sisti et al., 2007).

1.6 Neuronal control of the SGZ's stem cell niche in the hippocampus

As I have stated in **Section 1.3**, within the dentate gyrus of the hippocampus, the SGZ is maintained as a stem cell niche. Critical to the stem cell development and differentiation is the contribution of hippocampal astrocytes, endothelial cells lining the blood vessels, stromal cells and interneurons (Riquelme et al., 2007). Despite an increasing knowledge of the structure of the SGZ neurogenic cell niche, our knowledge of the control mechanisms underlying hippocampal neurogenesis remains far from complete. Although neurogenesis is clearly a neuronal activity driven process, precise details of the neuronal control remains to be elucidated. In fact, hippocampal formation forms a trisynaptic network composed of: multimodal inputs which are transferred from neocortical regions to the DG via the entorhinal cortex; information is then transferred from the DG to CA3 through the mossy fibers, onward through the Schaffer collaterals to CA1 (and the subiculum) and then via the entorhinal cortex back out to the cortical associative areas (Kempermann, 2002). Therefore, the SGZ receives inputs originating from distant brain regions, including the entrohinal cortex, and basal forebrain/ septum, and locally from interneurons within the hippocampus, which influences neurogenesis directly or indirectly (see **Figure 1.3**). These neuronal inputs involve the glutamatergic, serotoninergic as well as GABAergic systems. Glutamatergic afferent input negatively regulates dentate neurogenesis (Deisseroth et al., 2004) and acts downstream of steroid control while

serotonin has been demonstrated to enhance SGZ cell proliferation (Brezun and Daszuta, 1999).

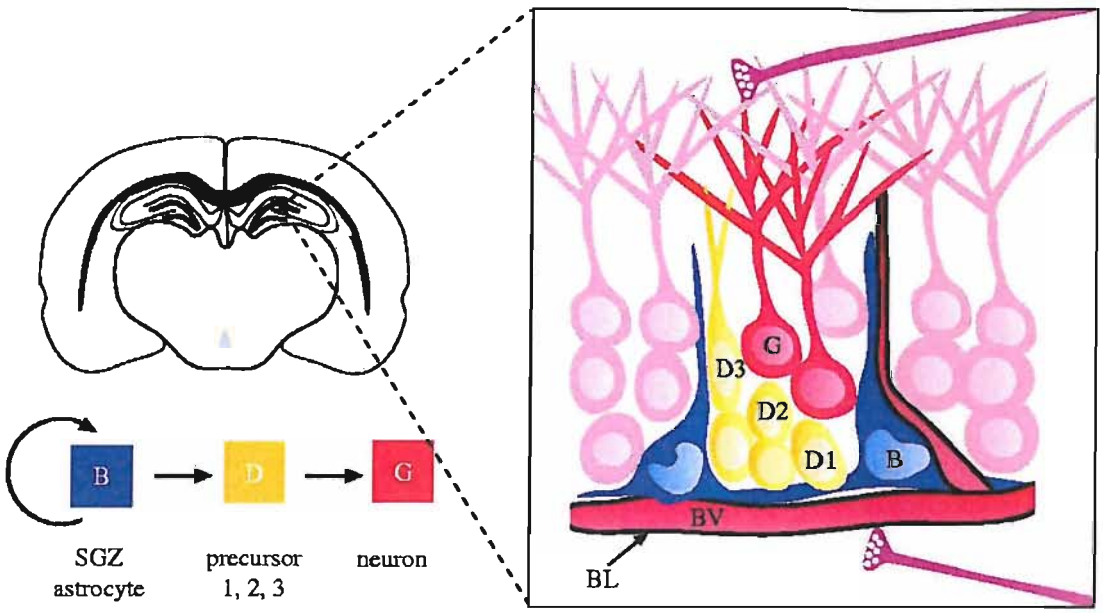


Figure 1.3: Cell types and anatomy of the adult SGZ niche. Schema of frontal section of the adult mouse brain showing the SGZ at the interface between the hilus (area below blood vessel) and the granule cell layer (light pink cells) of the dentate gyrus. SGZ astrocytes (B, blue) divide to generate intermediate precursors (type D cells; nomenclature according to (Seri, 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 (GABAergic) interneurons project to the SGZ [adopted from (Riquelme et al., 2007).

Although the principle neural inputs into the dentate gyrus have been well defined, there is much evidence to suggest that local networks, largely sub-served by GABAergic releasing interneurons, significantly modulate activity. Thus GABA has emerged as another candidate for excitation neurogenesis coupling (Ge et al., 2007a). Progenitors in the dentate gyrus receive GABAergic inputs (Wang et al., 2005), and non-synaptic GABA enhances the neuronal differentiation and integration in the SGZ (Tozuka et al., 2005). In the dentate gyrus, GABAergic interneurons modulate granule cell excitability mainly via feedback circuits from mossy fibres collaterals (Freund and Buzsaki, 1996), but also via feed forward circuits from entorhinal cortex

and contralateral hippocampal inputs (Basket Cell Interneurons) (Freund and Buzsaki, 1996). They are thus ideally positioned to sample neuronal activity and signal it to the local stem cell niche. A subset of GABAergic neurons co-releases neuropeptides, usually under specific firing conditions. Principle among these neuropeptides are the neuropeptide Y (Colmers and Bleakman, 1994) and the vasoactive intestinal peptide (VIP) (Leranth et al., 1984; Sloviter and Nilaver, 1987; Sloviter, 1994). In fact, while 40% of the VIPergic interneurons are GABAergic, their fibres course through all the layers of the hippocampus, including the DG (**Figure 1.4**) (Kohler, 1982; Leranth et al., 1984). NPY, which is a powerful modulator of the granule cell excitability, has been studied previously in our lab and shown to mediate the proliferation of progenitor and neuronal precursor cells in the dentate gyrus with no effect on cell survival (Howell et al., 2003; Howell et al., 2005) (see **Section 1.4** for details). While VIP is, like NPY, another key modulator of the excitability of the hippocampus (Wang et al., 1997), it has rather been shown to be a potent trophic and proliferative factor for neuronal precursor cells during embryonic brain development (Brenneman et al., 1998). Although the majority of studies on hippocampal neurogenesis have focused on proliferative factors, such as NPY, the accumulating evidence indicates that cell survival is the key parameter that regulates net hippocampal neurogenesis in-vivo and perhaps in certain forms of hippocampal dependant learning and memory (Prickaerts et al., 2004; Sisti et al., 2007). Indeed, the death of new cells in the granule cell layer occurred at a steady rate between P6 and P28 days after BrdU labeling, resulting in loss of 50% of BrdU-labeled cells over this 22-day period (Dayer et al., 2003); new granule cells that survived this first month lived for at least 5 additional months. Similarly, in another study in adult monkeys, Gould and her co-workers have shown that the number of newly-born neurons in the dentate gyrus declined by 9 wk after BrdUrd labeling, suggesting that some of the new cells have died (Gould et al., 2001). These observations, all together, emphasize the importance of trophism in the control of neurogenesis. Therefore, in an attempt to identify and study neuronal trophic mechanisms controlling hippocampal neurogenesis; we decided to investigate the possible involvement of the trophic neuropeptide VIP in the modulation of hippocampal stem cell survival, proliferation and differentiation during the postnatal period.

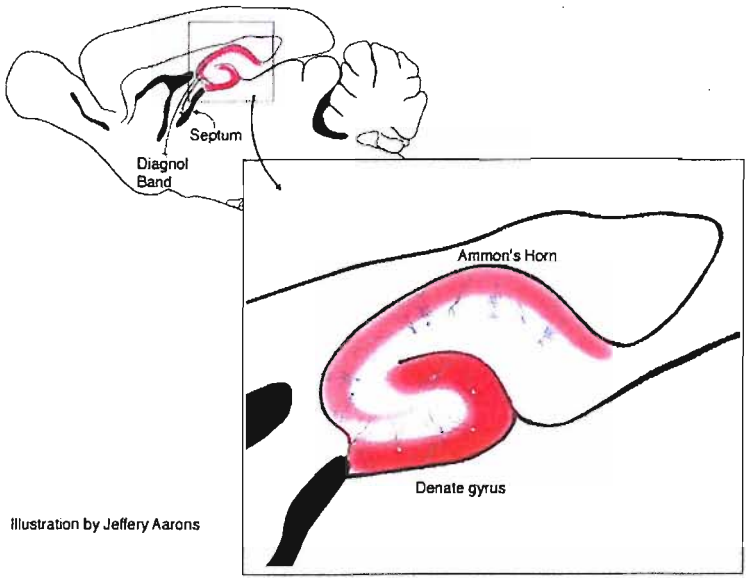


Figure 1.4: Diagrammatic representation of a parasagittal view of the rat brain illustrating the VIP chemistry of the hippocampal formation. Blow-up insert shows that VIP neurons (blue) are small, polymorphic interneurons found in many layers. VIP binding (red) is shown, with the intensity of the color representing the density of VIP binding (adopted from (Douglas E. Brenneman)).

1.7 VIP a neuromodulator during development: role in neurogenesis

In the embryonic period, VIP has been reported as an important player in embryonic development in general, and in the development of the CNS in particular (Hill et al., 1996b). During CNS development, it is believed that progenitor cells proliferate and expand rapidly in a restricted region called the ventricular zone to give all the neuronal and glial cells of the mature CNS (Jaworski and Proctor, 2000). In addition, VIP receptors are all expressed in different regions of the developing embryonic brain at variable levels (see **Section 1.12** for details). VIP itself has been found to be present in the developing central nervous system at critical periods of cell proliferation and differentiation which are corresponding to neuronal cell generation and differentiation (DiCicco-Bloom, 1996). Furthermore, although VIP expression in the CNS was not evident in the CNS during the early embryonic periods, VIP has been demonstrated to present in both human maternal blood and foetal circulation and to cross the rodents placenta (Hill et al., 1996b). Therefore, VIP role during embryonic development of the brain has been extensively explored.

In whole postimplantation embryo cultures, a 4hr treatment of 0.1-100nM VIP has been demonstrated to stimulate embryonic mice growth dramatically as indicated by the increase in the number of somites, and the cellular protein and DNA contents in comparison to control conditions (Gressens et al., 1993). It has been indicated that VIP induces growth through large increases in mitotic activity and/or in survival of dividing precursors (DiCicco-Bloom, 1996). Consistently, In another study using BrdU incorporation to study cell proliferation, VIP has been shown to shorten the S phase and G1 phase of neuroepithelial cells by 50% (4.8-2.4 h) and 58% (1.9-0.8 h), respectively, compared with controls (Gressens et al., 1998). Prenatal administration of VIP receptors antagonist has been shown to produce microcephaly as indicated by the low brain weight and the marked decrease in the cellular content of DNA and protein (Gressens et al., 1994). VIP receptors blockade during E17-E18 has been shown to increase cell death in the murine neocortex and the majority of cells have been found to be astrocytic and neuronal in the upper and deep layers, respectively (Zupan et al., 2000). In embryonic sympathetic ganglia, VIP has been shown to directly regulate neurogenesis as nonneuronal cells were absent. The dose-response analysis has indicated that maximal VIP effects were observed at 1-10μM peptide (DiCicco-Bloom, 1996). Collectively, these observations strongly suggest crucial roles for VIP in the modulation of neurodevelopment.

While VIP is largely involved during the embryonic period, a number of studies have shown its implication in neuromodulation during adulthood. For instance, in human hippocampal tissue surgically removed from patients with medically intractable temporal lobe epilepsy, high VIP binding has been shown especially in the dentate molecular layer (de Lanerolle et al., 1995). Therefore, it has been suggested that VIP may modulate neuronal survival in the face of increased excitation and the potential for injury in a seizure focus (de Lanerolle et al., 1995). In this regard, VIP has been shown to increase nitric oxide (NO) accumulation in the extracellular milieu of rat cerebral cortical cultures (Ashur-Fabian et al., 2001). On the other hand, it has been indicated that epileptic seizures increased cell proliferation in the adult rat dentate gyrus through NO-dependent mechanisms (Jiang et al., 2004). This indirectly supports the idea that VIP may be playing a role in seizures-accompanied neurogenesis.

Stress is a condition that is known to suppress the birth of new cells in the hippocampus. Indeed, both acute and chronic stress have been shown to decrease the number of proliferating cells in the dentate gyrus of the hippocampus (Heine et al., 2004; Heine et al., 2005). In addition, chronic stress has been shown to affect apoptosis in hippocampal sub-regions (Lucassen et al., 2001). Depletion of norepinphrine has been shown to increase cell proliferation with no effect on the cell survival and differentiation of granule cell progenitors in the adult rat hippocampus (Kulkarni et al., 2002). It has been reported that, both adrenalectomy and hypophysectomy (HYPOX) increase VIP level in hippocampal slice culture (Harrelson and McEwen, 1987). These findings implicate VIP in the modulation of stress-mediated hippocampal neurogenesis and may justify stu the possible roles of this neuropeptide in hippocampal neurogenesis.

More evidence on the involvement of VIP in hippocampal neurogenesis-dependent event coming from studies carried out on adult rats, which showed that chronic administration of Met-Hybird, a potent inhibitor of VIP function *in vivo*, results in impairment of associated learning abilities (Gressens et al., 1997). It has also been shown that the administration of HIV envelope protein gp120 or VIP antagonist is associated with learning impairment whilst HIV envelope protein gp120-mediated neuronal cell death is blocked by VIP (Gozes et al., 1991). Similarly, VIP antagonist treatment during embryogenesis has been demonstrated to cause permanent effects on adult brain chemistry and impaired social recognition behavior in adult male mice (Hill et al., 2007a; Hill et al., 2007b). Furthermore, this VIP antagonism resulted in male offspring exhibited deficits in cognitive function, as assessed through cued and contextual fear conditioning (Hill et al., 2007a). In addition, while hippocampal neurogenesis declines with age (Maslov et al., 2004), the levels of the neurons expressing VIP along with other neurons expressing NPY and smatostatin severely drop in the aged rat hippocampus (Cha et al., 1997). This may indicate VIP involvement in hippocampal neurogenesis and the support of neuronal survival thereafter.

To sum up, while VIP is a neuropeptide, which is involved in the generation of new neurons during embryonic development, it is involved in many physiological and pathological events which influence hippocampal neurogenesis in adulthood. The

transition period, between embryonic and adult neurogenesis in the hippocampus, is the postnatal neurogenesis. Analysis of postnatal neurogenesis (as shown by (Namba et al., 2005; Namba et al., 2007) in rats is of major importance, as it provides key information about how neurogenesis continues from the embryonic to the adult stages. Moreover, the formation of the GCL of DG can be divided roughly into two stages. In the first stage, progenitor cells proliferate in the periventricular zone, and then migrate to the prospective dentate regions (outer shell of the GCL and the hilus) during perinatal periods (Namba et al., 2005). In the second stage (P5-P20), the newborn cells generated in hilus and the SGZ are added to the inner part of the GCL and form the inner shell of the GCL (Namba et al., 2005). Thus, more than half of the granule cells are born postnatally. In relation, binding studies in rats (from embryonic day 19 to postnatal day 20) have revealed that an uniformly dense VIP binding throughout the brain with markedly higher binding in germinal zones (Hill et al., 1994a). Indeed, VIP mRNA exhibited a marked increase by P2, became statistically significant by P4 and peaked by P10 (Lopez-Tellez et al., 2004). The expression then decreased gradually towards adult levels by P20 (Lopez-Tellez et al., 2004). It is worth noting that these dramatic changes in VIP mRNA expression during the first 10 postnatal days are accompanied by the formation and maturation of the GCL. Therefore, and as these observations suggest a role for VIP in postnatal hippocampal neurogenesis, we used postnatal hippocampal cell culture to investigate VIP effects on hippocampal stem cell proliferation, survival and differentiation during this critical period of neurodevelopment.

1.8 VIP as a neurotrophic and neuroprotective factor in the CNS

VIP has been shown to have neurotrophic, growth regulating and neuroprotective effects in the CNS (Brenneman et al., 1998). These effects have been shown to be mediated through the release of mediators (**Figure 1.5**) such as activity-dependent neurotrophic factor (ADNF) (Zemlyak et al., 2000; Zusev and Gozes, 2004), activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999), Insulin Growth fatorI (IGF-I) (Servoss et al., 2001), cytokines (Brenneman et al., 2003), and protease nexin I (Dejda et al., 2005), the potentiation of BDNF expression as well as through interactions with NGF (Brenneman et al., 1998). ADNF is a glia-derived protein that has been shown to be released from astroglia after treatment with 0.1 nM VIP (Gozes et al., 1997). It has been shown that VIP regulates embryonic growth, at least in part,

through ADNF (Hill et al., 1999). In a pheochromocytoma cell line (PC12), VIP and one of its derivatives, Stearyl-Nle17-VIP (SNV), have been shown to be protective against oxidative stress. This process has been demonstrated to be ADNF mediated (Steingart et al., 2000). In addition, VIP at a very low concentration in neuronal cultures has been found to be neuroprotective against a wide range of neurotoxins including dopamine, 6-hydroxydopamine (6-OHDA) and Buthionine sulfoximine (BSO), a selective inhibitor of glutathione synthesis (Offen et al., 2000). VIP has been reported as a neuroprotective factor against excitotoxic lesions in mice during the embryonic period as well as neonatally (Gressens, 1999). Ro 25–1553 and steary-norleucine-VIP are two VIP analogues that have been shown to protect the mice developing brain against the excitotoxic toxin ibotenate (Rangon et al., 2005). This expression of VIP in the CNS is regulated by the BDNF as demonstrated in cultured fetal rat cerebrocortical (Villuendas et al., 2001). Once VIP was co-administered with ibotenate neonatally, VIP has been found to prevent white matter lesions (Gressens et al., 1999). However, the co-treatment with ibotenate, VIP and anti-ADNF antibodies has been shown not to abolish VIP neuroprotection (Gressens, 1999); indicating that VIP neuroprotective effects are ADNF independent, at least in this model. Moreover; these neuroprotective effects of VIP against neonatal excitotoxic brain lesions have been demonstrated to be VPAC2 receptor mediated (Rangon et al., 2005).

ADNP as a neuroprotective peptide that shares homology with the ADNF is regulated by VIP in the mouse brain (Bassan et al., 1999). NAP is a smaller peptide that has been derived from ADNP that has been shown to have neuroprotective activity and tosharing structural and immunological homologies with the previously reported, activity-dependent neurotrophic factor (ADNF) (Gozes et al., 1999) (for more details see **Section 6.1.5**).

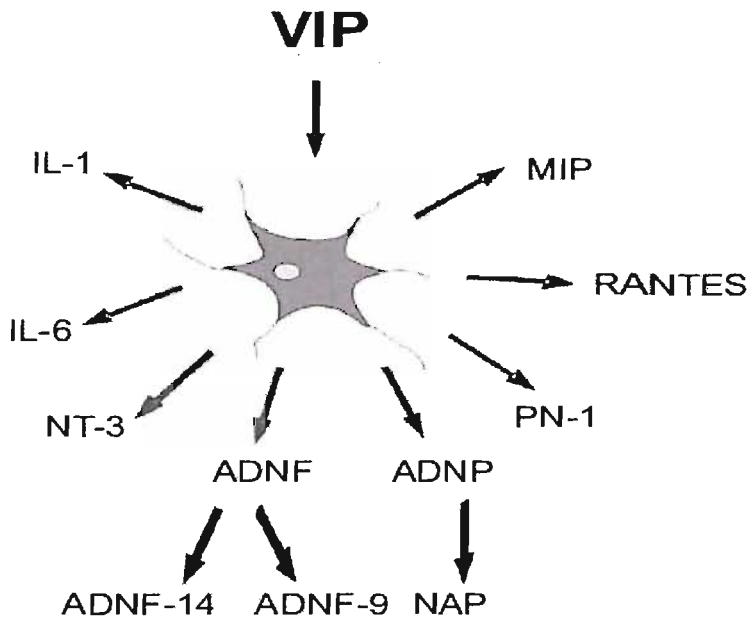


Figure 1.5: Mechanism of VIP action. VIP promotes release of astroglia-derived factors that provide neuroprotection, such as: interleukin-1 (IL-1), interleukin-6 (IL-6), neurotrophin-3 (NT-3) protease nexin-1 (PN-1), RANTES and MIP chemokines and the most potent – activity-dependent neurotrophic factor (ADNF) and activity dependent neuroprotective protein (ADNP). ADNF-14, ADNF-9 and NAP peptides are active fragments of parent proteins: ADNF and ADNP, respectively. Adopted from (Dejda et al., 2005).

1.9 VIP Molecular Biology

VIP belongs to a structurally related family of polypeptides, comprising also Pituitary adenylate cyclase activating polypeptide (PACAP), peptide histidine-isoleucine (PHI), peptide histidine-methionine (PHM), secretin, glucagon, glucagon-like peptide (GLP), glucose-dependent insulinotropic polypeptide (GIP), growth hormone releasing hormone (GHRH) and helodermin. Human VIP gene has been identified in late eighties (Gozes et al., 1987). It is expressed widely in peripheral and central nervous systems. The structure of the VIP gene has been determined and shown to contain 8837 base pairs and seven introns (Gozes et al., 1987). In rats, VIP mRNA was isolated and found to have 2000-2100 bases. The amount of this RNA varies and appears to precede peptide synthesis by 3-4 days (Gozes et al., 1987). In the rat hippocampus, VIP-mRNA peaks at of age postnatal day 8 (Gozes et al., 1987). VIP as a neuropeptide is synthesized as a high molecular weight precursor protein of 170 amino acids and undergoes a multi-step cleavage and metabolism to produce a 28

amino acid peptide. This form which was originally isolated from the porcine small intestine is the active form that is present in the body (Dejda et al., 2005).

The primary structure of VIP is conserved across most mammalian species, except for the guinea pig and opossum. Among the rest vertebrate species, the differences concern 4–5 amino acid residues (see **Table 1.1**). VIP is distributed widely in the brain and has been shown to be present in the brain, particularly in the cerebral cortex, hippocampus and suprachiasmatic nuclei of the hypothalamus (Moody et al., 2003). In addition, VIP has been found in the pituitary body, adrenal glands, nerve endings of the respiratory system, gastrointestinal tract, reproductive system, and the immune system (Moody et al., 2003).

Immunohistochemistry in combination with radioimmunoassay was used to study the distribution of immuno-reactive VIP cell bodies and fibres in the rat brain. The distribution of VIP-immunoreactive fibers has been fount to be in parallel with the distribution of VIP-immunoreactive cell bodies. The highest concentrations (60 pmol/g wet wt) of immuno-reactive VIP has been found in the cerebral cortex and in certain limbic structures, whereas the concentrations in the basal ganglia, thalamus, lower brain stem, cerebellum and spinal cord were low (<15 pmol/g) (Loren et al., 1979). In the hippocampal complex VIP-containing cell bodies and fibers have been demonstrated in the CA1 and CA3 subfields as well as the dentate gyrus (Loren et al., 1979). Most of the cells had the morphology of interneurons, some of them probably being identical to basket cells (Loren et al., 1979). Hippocampal VIP containing interneurons have been studied extensively and 40% of them have been found to be containing GABA (GABAergic) (Acsady et al., 1996; Calegari et al., 2005).

Species	VIP primary structure (sequence)
Human, cow, rat, pig, dog, goat, sheep, mouse and monkey	HSDAVFTDNYTRLRKQMAVKKYLNSILN
Guinea pig	HSDA***L***FTD***T***YTRLRKQMA***M***KKYLNS***V***LN
Opposum	HSDAVFTD***S***YTRL**L**KQMA***MR***KYL***D***SILN
Chicken, turkey, frog and alligator	HSDAVFTDNY***S***R***P***RKQMAVKKYLNS***V***L***T***
Cod	HSDAVFTDNY***S***R***P***RKQMA***A***KKYLNS***V***L***A***
Trout	HSDA***I***FTDNY***S***R***P***RKQMAVKKYLNS***V***L*T*

Table 1.1: VIP primary structure in different species. Italic bold fonts refer to differences in particular residues in amino acid sequences of the peptide. Adopted from (Dejda et al., 2005).

1.10 VIP Receptors in the CNS: structure and distribution

The actions of VIP in the CNS are mediated by at least three receptors. These are VPAC1, VPAC2, and PAC1. VIP binds with high affinity to VPAC1 and VPAC2 receptors but with 1000-fold lower affinity to the PAC1 receptor (DiCicco-Bloom et al., 2000). VPAC1, VPAC2 and PAC1 receptors are all members of a G-protein-coupled seven transmembrane superfamily. Human, rat and mouse VPAC1 receptors have been described to contain 457, 459, 459 amino acids, respectively with the mouse and rat receptors having an 83% homology with the human one (Moody et al., 2003). During embryonic development, the expression of VPAC1 receptor mRNA has been demonstrated to be consistently high in most of the regions of the brain including the hippocampus (Basille et al., 2000). Postnatally, it has been shown that the expression of VPAC1 is markedly declined in most neuroepithelia (Basille et al., 2000). In the adult rat brain, VPAC1 receptor mRNA has been found to predominate in the brain especially the cerebral cortex and the dentate gyrus of the hippocampus (Usdin et al., 1994; Moody et al., 2003). When expressed in cell lines, the recombinant rat VPAC1 receptor recognises VIP at IC50 of 1nM (Anthony et al., 1998).

Two highly selective brain VPAC1 receptor agonists have been described. The VIP/GRF hybrid [Lys15, Arg16, Leu27]VIP(1–7)GRF(8–27)-NH2 (IC50, 1 nM) and the [Arg16] chicken secretin (IC50, 2nM) (Moody et al., 2003). On the other hand,

[Acetyl-His1, D-Phe2, Lys15, Arg16]VIP (3– 7)GRF(8–27)-NH2 has been shown to behave as a selective antagonist of rat and human VPAC1 receptors (IC50, 1 to 10 nM) (Moody et al., 2003).

The rat VPAC2 receptor, which has been reported to contain 459 amino acids, has an 87% homology with the human VPAC2 receptor (Moody et al., 2003). Human VPAC2 and VPAC1 receptors have 49% homology (Moody et al., 2003). It has been demonstrated that the distribution of VPAC2 mRNA in the rat brain varies throughout development. In the early embryonic stages, particularly from E17 onward, intense expression of VPAC2 receptor mRNA has been detected in various diencephalic nuclei of the rat brain but not in neuroepithelia (Basille et al., 2000). In the adult rat hippocampus, VPAC2 mRNA distribution has been found to predominate in the CA1-CA3 subfields and in the granular layer of the dentate gyrus and overlaps with VPAC1 mRNA distribution (W.J. Sheward, 1995). When expressed in cell lines, the recombinant rat and human VPAC2 receptors have been demonstrated to recognize VIP with IC50 of 3 to 4 nM (Gourlet et al., 1997c). Two highly selective VPAC2 receptor agonists have been described: Ro 25–1553 and Ro 25–1392 (Moody et al., 2003).

Human PAC1 receptor contains 467 amino acids, and throughout embryonic development, PAC1 receptor mRNA has been found widely distributed in the rat brain, predominantly in the subventricular zone (Moody et al., 2003). However, it has been shown that the expression of PAC1 receptor mRNA (like VPAC1 mRNA) declines markedly between birth and P12 (Basille et al., 2000). Other studies have demonstrated that the expression of PAC1 receptor mRNA is maintained postnatally in many regions especially the dentate gyrus of the hippocampus (Shioda et al., 2006). Interestingly, it has been reported that while PAC1 receptor mRNA expression is high in the hippocampus, PACAP expression was detected at a low level. This may suggest that other neuropeptides, such as VIP may bind PAC1 to modulate postnatal hippocampal neurogenesis (Moody et al., 2003). When expressed in cell lines, the recombinant rat and human PAC1 receptor has been reported to recognize VIP at IC50 of 1μM. Maxadilan, a 61 amino acid peptide was described as a high affinity agonist for PAC1 (IC50, 1-3nM) with no significant affinity for either VPAC1 or VPAC2 receptors (Moody et al., 2003). PACAP(6-38) has been suggested as a potent

antagonist of PAC1 receptors with no affinity to VPAC1 and a significant affinity for VPAC2 (Moody et al., 2003). At the cellular level, VIP receptors have been shown to be expressed by the different cell phenotypes at different stages of development. In the adult rat brain, the expression of the three VIP receptors has been demonstrated to be confined to the neurolgia and the neurons. They all were found to be expressed by astrocytes, and PAC1 receptor was also expressed in oligodendrocytes (Joo et al., 2004). In the hippocampus, it is been reported that VPAC1 and PAC1 are unlike VPAC2 in being mainly localized in the neuropil (Joo et al., 2004).

Nomenclature of receptors for PACAP and VIP

Receptor subtype		Gene name (HUGO)	Human chromosome location	Selective agonists	Selective antagonist
IUPHAR nomenclature	Previous nomenclature				
PAC_1	PACAP type I PVR1	ADCYAP1R1	7p14	Maxadilan	PACAP(6–38)[a]
$VPAC_1$	VIP VIP_1 PACAP type II PVR2	VIPR1	3p22	[Arg^{16}]chicken secretin[b] [$K^{15}R^{16}L^{27}$]VIP(1–7)/GRF(8–27)-NH_2	[Ac-His^1, D-Phe^2, Lys^{15}, Arg^{16}] VIP(3–7)/GRF(8–27)-NH_2
$VPAC_2$	VIP_2 PACAP-3 PVR3	VIPR2	7q36.3	Ro 25-1553 Ro 25-1392	–

[a] Displays significant affinity for $VPAC_2$ receptors.
[b] Selective only in rodent tissues (e.g., brain) that do not express the secretin receptor.

Table 1.2: The nomenclature of receptors for VIP. Taken from "The International Union of Pharmacology. XVIII. Nomenclature of Receptors for Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide". Adopted from (Anthony et al., 1998).

1.11 Aims of the project

In an attempt to provide a better understanding of how trophism mediated via neuronal activity plays a role in the regulation of postnatal hippocampal neurogenesis, we aim in this project to define the effects of the neuropeptide VIP on postnatal hippocampal neurogenesis *in vitro*. In addition, we aim to study any possible interactions with other factors that are known to affect hippocampal neurogenesis and the SGZ stem cell niche, such as NPY, FGF-2 and galanin. In addition we are attempting to understand the pharmacology and the receptor interaction through which these effects are elucidated. Our *in vitro* model is going to be primary hippocampal neuronal cultures generated from P7-10 Wistar rats.

Chapter 2

General Experimental Methods and Materials

2.1.1 Introduction

Neural stem cells and their progenitors have been isolated from many areas of the embryonic CNS including, basal forebrain, cerebral cortex, hippocampus and spinal cord (Gage, 1995). In the adult nervous system, stem cells have been found to reside in two neurogenic zones: the sub ventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus (Gage, 1995). When isolated under the appropriate conditions, neural stem cells have the ability to renew and produce a clinical amount of cells that may be well-characterized *in vitro* and *in vivo* or be ready for therapeutic use. In our particular study, primary hippocampal neuronal cultures are going to be our method to study the effects of different growth factors including neuropeptides, particularly VIP, that control proliferation, survival and differentiation of stem cells and their progeny in the postnatal hippocampus.

2.1.2 Reliability of primary hippocampal neuronal culture as a paradigm to study hippocampal neurogenesis

The mammalian CNS is a complex structure composed of a variety of cell types, including neuronal cells, glial cells, their progenitors, and many others. This complexity added together to the poor accessibility to the brain makes it difficult to accurately define the effect of different growth factors on these cells (Wheal, 1991). Therefore, in quantitative experiments on the main cell lineages of the CNS, it is of importance to minimize the number of variables, and the dissociated primary neuronal cultures enable us to overcome issues of major importance to our study. *Firstly*, cells can be isolated from an intertwined network of thousands of adhesive contacts without irreversibly damaging them. This is particularly possible with both embryonic and postnatal tissues. *Secondly*, growth inhibitory factors can be separated away. *Thirdly*, different cell types can be identified to study experimental effects on specific cell types. *Fourthly*, adequate substrate for attachment and adequate nutrients are provided for continued viability in culture and for further investigations(Brewer, 1997, 1999). In laboratories, including ours, these technical concerns have been overcome and the ability to expand CNS stem cells *in vitro* was defined.

In rodents, the postnatal hippocampus is a known area of continuous neurogenesis through out life and dentate gyrus is a site where neural stem cells reside (Fred H. Gage, 1995). In fact, more than half of the GCL is formed during postnatal life (Namba et al., 2005; Namba et al., 2007). In addition, the postnatal period is a transition state which may provide information of how neurogenesis continues from embryonic life to adulthood (Namba et al., 2005; Namba et al., 2007). Moreover, It is well-known that the immature CNS is generally less sensitive to anoxia and therefore withstands the procedure of cell culturing (Wheal, 1991). Therefore, we have chosen to generate primary hippocampal neuronal cultures from postnatal rat brains. This model is going to be our paradigm to define the effects of different neuropeptides and growth factors that control neural stem cell proliferation, survival and fate; an issue that has become the focus of many laboratories worldwide.

2.1.3 Methods for stu cell proliferation

Quantitative studies on hippocampal neurogenesis and proliferation have shown that in adult rodents, about 9000 new neuronal cells are generated per day in the dentate gyrus, contributing to about 3.3% per month or 0.1% per day of the granule cell population (Kempermann et al., 1997; Cameron and McKay, 2001). These quantifications were primarily performed by Bromodeoxyunidine (BrdU) labeling as a strategy to measure cell proliferation. Another strategy to study cell proliferation is the evaluation of the expression of markers of the cell cycle, like proliferating cell nuclear antigen (PCNA) and Ki-67 (Taupin, 2007). PCNA, a cofactor of DNA polymerase, is expressed during the S-phase of cell cycle, but can also be expressed in cell undergoing DNA repair, and some non-proliferating neurons (Taupin, 2007). On the other hand, Ki-67 is expressed in all phases of the cell cycle except the resting phase and a short period at the beginning of the phase G1 (Scholzen and Gerdes, 2000). Therefore, Ki-67 is considered a more reliable marker to study cell proliferation and to identify cells that reenter the cell cycle than PCNA (see **Section 2.1.5** for more details) (Taupin, 2007).

2.1.4 BrdU as a marker of the S-phase of the cell cycle

Cell proliferation is defined as the total number of cells dividing. Once cells decide to divide, they enter the cell cycle which can be broken down into four phases: G1 phase, S Phase, G2 phase and M phase. It is the S phase during which a cell

undergoes DNA synthesis and replicates its genome. Thymidine analogs including BrdU incorporate into the DNA of dividing cells during the S-phase of the cell cycle, thus used for birth dating and monitoring of cell proliferation (Taupin, 2007). As a thymidine analog, BrdU has been used to study proliferation, migration and time of origin of cells in the cerebral cortex (Miller and Nowakowski, 1988). An advantage of BrdU is that it can be visualized with immunocytochemical techniques. While BrdU is a marker for new DNA synthesis, it is unlikely that cells with DNA strand breaks incorporate enough BrdU to be detected by immunohistochemistry. Indeed, in a nice study on the effects of ischemia in hippocampal neurogenesis, TUNEL, which detects DNA nicks and strand breaks that indicate DNA damage, was used to label cell death (Liu et al., 1998). The pattern of TUNEL labeling was found dramatically different from the pattern observed with BrdU (Liu et al., 1998). To confirm that BrdU labeling reflected an increase in cell birth, we also performed experiments using an antibody that recognizes Ki-67, which is expressed during cell division (See below for details). Similar to what Sharp et al observed for BrdU labeling, all BrdU incorporated cells were Ki-67 after a short pulse of BrdU. In addition, we make use of double immuno-staining to quantify BrdU and specific cell-phenotype colabelling, such as nestin, class III ß-tubulin (TuJ1) or GFAP co-labelling cells (proliferating). In agreement with other studies which used this method (Wilson et al., 1996), we were able to study the proliferation of each cell subpopulation under control and treatment conditions.

2.1.5 Ki-67 as a marker of cell proliferation

The other method to evaluate cell proliferation is the use of polyclonal antibody against the proliferation antigen Ki-67 in combination with BrdU (Wilson et al., 1996; Kee et al., 2002). Ki-67 is a nonhistone protein that is strictly associated with human cell proliferation (Gerdes et al., 1984; Duchrow et al., 1995). Its name is derived from the city of origin (Kiel) and the number of the original clone in the 96-well plate (Scholzen and Gerdes, 2000). Cloning of Ki-67 cDNA revealed a large mRNA with 15 exons. Exon 13 encodes the core of the protein containing the 16 tandem repeats recognized by most of the commercially available antibodies. Ki-67 protein contains ATP/GTP-binding sites at the C-terminus as well as a large number of phosphorylation sites. Phosphorylation and dephosphorylation of these sites are controlled by key regulatory structures of the cell cycle and occur at the breakdown and reorganization of the nucleus during mitosis. Therefore, Ki-67 protein plays a

pivotal role in maintaining cell proliferation and the removal of Ki-67 protein using anti-sense nucleotides prevents cell proliferation (Sasaki et al., 1987; Schluter et al., 1993; Duchrow et al., 1995). Moreover, Ki-67 protein has been reported to influence progression of S-phase and mitosis of the cell in a self-regulated manner and mediate an anti-apoptotic effect on the cells (Schmidt et al., 2002).

Ki67 protein (antigen) is expressed by cells in the active phases of the cell cycle: lateG1, G2, and M phase, but not detectable in quiescent cells (G0 phase) (Scholzen and Gerdes, 2000). Concerning early G1 phase, experimental results have shown that Ki-67 expression varied: cells passing the early events of transition from G0 to G1 lacked Ki-67 nuclear antigen, whereas G1 cells after mitosis were constantly Ki-67 positive (Kee et al., 2002; Schmidt et al., 2002).

Raising monoclonal and polyclonal antibodies that can detect Ki-67 antigen in many species including, rat, mouse and rabbit has paved the way for another immunohistochemical method of cell proliferation assessment (Scott et al., 1991; Schmidt et al., 2002). Ki-67 protein expression has been used to assess the growth fraction (see below) of a given cell population (Gerdes et al., 1984; Schluter et al., 1993; Duchrow et al., 1995).In rats, ki-67 has been found to be highly expressed within the proliferative zone of the dentate gyrus and its expression pattern mimicked that of BrdU after exogenous BrdU introduction. Ki-67 has been shown to be a reliable marker to measure cell proliferation during adult neurogenesis (Kee et al., 2002).

2.1.6 Phenotyping: markers of progenitor cells and their progeny

Markers of precursor cells, particularly nestin, Class III ß-tubulin, GFAP and NG2 have been extensively studied in both the developing and the postnatal CNS (**Figure 2.1**). These markers as well as many other cell-surface markers that clearly distinguish neural stem cells and their restricted progenitors have enabled researchers to isolate these cells and to study many mechanisms that control neurogenesis. Nestin which is named so as it is expressed specifically in the neuroepithelial stem cells, is a class VI intermediate filament protein (Lendahl et al., 1990). Nestin expression has been described to correlate with proliferating progenitor cells in the rodent and the human CNS (Messam et al., 2000; Chen et al., 2005). Nestin expression was not only

reported in the normal developing CNS tissue but also in primary human CNS tumors (Dahlstrand et al., 1992; Tohyama et al., 1992). Nestin has been used as a marker to study precursor cell development in the subventricular zone and the hippocampus (Roy et al., 2000; Chen et al., 2005) (**Figure 2.1**) . In addition, nestin has been demonstrated to be expressed by cells grown in hippocampal neuronal cultures generated from both postnatal and adult rat brains (Roy et al., 2000; Howell et al., 2003). Moreover; It has been described that nestin is expressed in both CNS progenitor cells as well as the cells which are in transition from a progenitor stage to glial and neuronal differentiation (Reynolds and Weiss, 1992; Messam et al., 2000). In another set of studies, it has been revealed that, in the postnatal hippocampus, the majority of nestin positive cells are GFAP expressing as well (Wei et al., 2002).

Astroglial cells are usually recognized depending on their morphology and the expression of the intermediate filament (IF) Glial fibrillary acidic protein (GFAP). GFAP has been reported as one of the most important and reliable GFA protein , which is widely used for the immunohistochemical labelling of astrocytes and in the diagnosis of tumors of the glioma group (Bonnin and Rubinstein, 1984; Gould et al., 2001). This IF is a 53kDa protein that constitutes the major component of the cytoskeleton of the astrocyte and is subjected to sequential remodeling during development (Sergent-Tanguy et al., 2006). At birth and during the postnatal period, GFAP progressively replaces vimentin, another IF that is expressed by immature astrocytes (Sergent-Tanguy et al., 2006).

Class III ß-tubulin (also called TuJ1) is a marker that has been used to study the process of neurogenesis as a marker of immature neurons (Parent et al., 1997; Gould et al., 2001) (**Figure 2.1**). Class III ß-tubulin belongs to the ß-tubulins family of which seven isotypes have been identified in mammals and birds (Katsetos et al., 2003). In the brain, Class III ß-tubulin isotype has been found highly expressed and demonstrated to exhibit a distinctive neuronal specificity in most chordates, including human and rodents (Katsetos et al., 2003). During mammalian development, Class III ß-tubulin is considered one of the earliest neuronal markers to be expressed (Katsetos et al., 2003). In fact, it is expressed either immediately before or during terminal mitosis, in all but cerebellar Purkinje neurons (Lee et al., 1990; Katsetos et al., 2003). Furthermore; Class III ß-tubulin has been demonstrated to be expressed in the

proliferative zones of the developing cortex. Precisely; it was found to mark neuronal populations that remain in these proliferative zones as well as those that leave it after being generated (Menezes and Luskin, 1994).

During the postnatal period, nestin expressing precursor cells exist in at least two immunohistochemically distinguished subpopulations according to the co-expression of the astrocytic marker GFAP (Wei et al., 2002). The majority (77%) of nestin precursor cells co-express the astrocytic marker. However, there is another subpopulation (23%) of these nestin expressing progenitor cells are GFAP negative (Wei et al., 2002). Another subpopulation nestin expressing cells has been reported with regard to their expression of the neuronal marker TuJ1 (Seri et al., 2001; Seri, 2004; Encinas et al., 2006; Seri et al., 2006). In our group we have also demonstrated the existence of a subpopulation of hippocampal nestin cells that co-express TuJ1 (Howell et al., 2007).

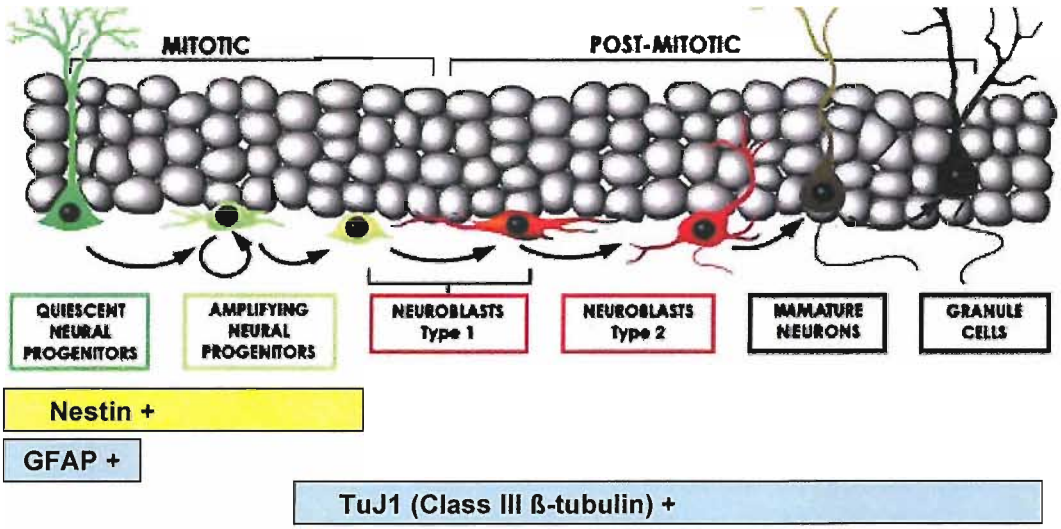


Figure 2.1: A schematic summary of the neuronal differentiation cascade in the DG. Quiescent neural progenitors generate, through asymmetric divisions, the amplifying neural progenitors that, after several rounds of symmetric divisions, exit the cell cycle within 1–3 days and become postmitotic neuroblast type 1 cells. Within next 15–21 days, neuroblast type 1 cells mature into neuroblast type 2 cells and then into immature neurons with apical processes and basal axons and the soma located in the GCL. 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 fibers. Adopted / developed from (Encinas et al., 2006).

NG2 is a high-molecular-weight type 1 membrane proteoglycan that was first characterized in rats (Karram et al., 2005). Then it was found to be present in humans as a melanoma chondroitin sulphate proteoglycan (Karram et al., 2005). Antibodies against NG2 have been used to identify and characterize oligodendrocyte precursor cells in the developing and adult CNS including the hippocampus (Dawson et al., 2000; Lytle et al., 2006; Thallmair et al., 2006). In both the developing and the mature CNS, NG2 cells have been demonstrated to express markers that persist in immature oligodendrocytes, such as O4 and PDGF-αR, suggesting that they are oligodendrocyte lineage restricted cells (Dawson et al., 2000). Although NG2 cells are believed to be oligodendrocyte precursor cells, recent studies have expanded their role by showing that early postnatal NG2 cells in culture are capable of giving neurons (Aguirre et al., 2004). Furthermore, NG2 cells have been reported to be the largest proliferating cell population in the adult rat CNS, suggesting that they have stem cell-like properties (Dawson et al., 2000; Thallmair et al., 2006). Although NG2 proteoglycan expression has been shown to influence the fate-restriction of CNS progenitors, mice lacking NG2 cells have been shown to have unaltered adult hippocampal neurogenesis or hippocampal-dependent behavioral tasks compared to their counteract wild type animals (Thallmair et al., 2006).

2.1.7 Methods of stu cell fate of neural stem cells

In the process of neurogenesis, neural stem cells have been proposed to self-renew via symmetric cell divisions and differentiate unipotently or multipotently via asymmetrical divisions (Huttner and Kosodo, 2005; Encinas et al., 2006). There are several mechanisms that have been found to determine the neural stem cell fates during development, most importantly the plane of cell division, and the asymmetric segregation of NUMB (see (Cayouette and Raff, 2002) for a review).

Throughout neurodevelopment, neural stem cells divide symmetrically to give two similar neural stem daughter cells, or asymmetrically to give one neuronal precursor cell that will differentiate into a neuron and a stem cell daughter cell. In studies on Drosophilae, the orientation of the plane of cell division plays a critical part in segregating cell fate determinants and thereby in cell-fate choices (Cayouette and Raff, 2002). During cerebral development, it has been found that early in development, most cells divide with their mitotic spindle aligned horizontally to the

plane of neuroepithelium, and later in development, more of the cells divide with their mitotic spindle aligned vertically to the plane of neuroepithelium (Chenn and McConnell, 1995). Using videomicroscopy, Chenn and McConnell found that in divisions with a horizontal spindle, both cells stay in the neuroepithelium, suggesting that they remain neuroepithelial cells (symmetric cell division). In divisions with a vertical spindle, by contrast, the basal daughter cell tends to migrate away, suggesting that it has committed to differentiation; the apical daughter of such divisions tends to stay in the epithelium, suggesting that it remains a neuroepithelial cell (asymmetric cell division) .

On the other hand, NUMB is a necessary protein for the adoption of different daughter cell fates, which has been used to study the mode of cell division of stem cells *in vitro* and *in vivo* (Petersen et al., 2002; Shen et al., 2002). The mammalian NUMB (m-NUMB) has been shown to be located in the apical cortex of mitotic neuroepithelial cells in both the developing mouse cortex and rat retina (for simplicity, I will refer to it by NUMB) (Castaneda-Castellanos and Kriegstein, 2004). Therefore, when a neural progenitor cell divides horizontally (symmetric cell division) both cells inherit NUMB, whereas when it divides vertically (asymmetric cell division), only the apical daughter cell inherits NUMB (Cayouette and Raff, 2002). For instance, in cortical development, when a nestin positive cell divides symmetrically to give two daughter cells that are nestin positive, NUMB segregates equally to the two cells (Shen et al., 2002) (**Figure 2.2**). However, when a nestin cell divides asymmetrically to give a nestin positive cell and a neuronal progeny cell (TuJ1 positive), NUMB preferentially segregates to the neuronal daughter cell (Shen et al., 2002). In simple terms, the number of nestin cells that are NUMB negative may predict the frequency of asymmetrical cell divisions of the putative /stem nestin-expressing cells.

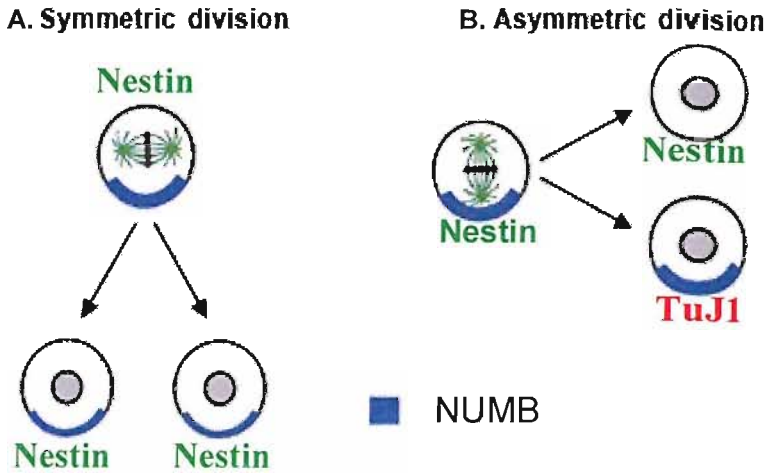


Figure 2.2: Schematic diagram of NUMB segregation during neural stem cell division. A) A nestin positive neural stem cell divides symmetrically giving two nestin positive stem cells; NUMB segregates equally to the two daughter cells. B) Asymmetric cell division of a nestin positive cell giving one nestin putative/ stem cell and one TuJ1 positive neuronal precursor cell; NUMB preferentially segregates to the neuronal daughter cell (Cayouette and Raff, 2002).

2.2 Experimental Methods

2.2.1 Generation of primary hippocampal neuronal culture:

All animal work was performed according to the animal (scientific procedures) Act 1986. The generation of primary neuronal hippocampal culture was carried out in a multi-step procedure that involves hippocampal dissection, cell release and dissociation and finally, cell plating.

2.2.2 Hippocampal dissection:

For each experiment, 2-8 Wistar rats of 7-10 post-natal day age were killed by atlanto-axial dislocation (a schedule I method) followed by quick decapitation. Using scissors the skin was cut, the skull and the meninges were removed and the brain was swiftly removed and placed in a petri dish containing Gey's balanced salt solution (Life Technologies, Paisley, U.K.) supplemented with 4.5 mg/ml glucose at 4°C. Under sterile techniques, hippocampi were rapidly dissected and transferred onto a sterile melinex strip with a few drops of Gey's solution on the stage of a MacIlwain tissue chopper. Slices of 400μm thickness were cut perpendicular to the long axis of

the hippocampus and immediately transferred into a petri dish containing 4ml of Gey's solution and stored at 4°C for 5 minutes pending for the steps of cell release.

2.2.3: Cell release and dissociation:

Gey's solution was then replaced with pre-warmed Neurobasal A, supplemented with 2% B27 (Life Technologies) and 0.5mM Glutamine (Sigma, UK) for approximately 5mins to wash the Gey's solution. Meanwhile, a pre-warmed papain solution was filtered and sterilized through a 0.2um filter (0.22μm pore, Millex-GV, Millipore, USA) and then applied to the hippocampal slices for 30 min at 37°C to aid in the digestion of the connective tissue and to free the cells from their surrounding connections. Papain (22.0 U/mg, Sigma) was prepared at a concentration of 2mg/ml in NB/B27 plus Glutamine at least 30 mins before filter sterilizing and application to tissue slices. After 30 minutes papain solution was aspirated and replaced by 2ml-4ml NB/B27 plus Glutamine and cell release was initiated by trituration for about 10 times after which the suspension was transferred into a 15ml centrifuge tube where the sediment was suspended in a further 2mls of NB/B27 and glutamine for more reliable trituration. This procedure was repeated 2-3 times until the cell pellet was fully dissociated, resulting in a cell suspension in 6mls NB/B27 and Glutamine. For partial purification of cells from debris, cells released by trituration were applied gently to the top of a of 2-step density OptiPrep gradient and then centrifuged for 15mins at room temperature at 1900rpm. OptiPrep gradient was made in two 1ml steps of adding 10% on top of 20% Optiprep in NB/B27 and Glutamine. Optiprep (Axa-shields, Oslo, Norway) is a ready-made sterile solution of Iodixanol. The fraction containing the cells was collected with the trituration pipet and diluted into 2ml NB/B27 and Glutamine to remove the optiprep and again centrifuged for 2 mins at room temperature at 1100rpm. The medium above the pellet was aspirated and discarded and the cell pellet was re-suspended in 1ml NB/B27 and Glutamine. Viable cells were determined by mixing 30μL cell suspension with 50μL Trypan blue (Sigma), live (dye-excluding) cells were counted in a haemocytometer. Thereafter cell suspension was diluted to have a cell density of 100,000 viable cells per ml ready for plating.

2.2.4 Cell plating:

Viable cells were plated in 500μL NB/B27 and Glutamine at the indicated density (100,000cell/ml) directly onto poly-L-lysine coated 24-well plates or on glass cover slips (13mm diameter) previously coated with poly-L-lysine (for confocal images or receptor studies). Coating involved the application of 300μL of 50μg/ml poly-L-lysine (sigma) for about 1hr then it was aspirated, allowed to dry and rinsed with NB/B27 and Glutamine to wash off excess poly-L-lysine as it is toxic to cells. Cover slips were sterilized in 70% alcohol before being coated. At 2 hrs after plating the medium was aspirated to remove any non-adherent cells or debris. Then cells were further rinsed with NB/B27 and Glutamine to remove residual debris. Medium was replaced by fresh medium of NB/B27 and Glutamine under control condition and the addition of other factors to be studied under other conditions. All media included a combined antibiotic/antimycotic (Penicillin/ Streptomycin and Fungizone, Life Tech, USA). And cells were incubated under (5%CO2/ to remove 9%O2/ 37°C) incubator conditions. For cultures longer than three days, 2/3rds of the growth media was replaced every 3 days.

2.3 BrdU incorporation as a measure of cell proliferation

In our cell culture system, we used (BrdU) as a marker of S-phase entry (Kee et al., 2002). The substitution of an endogenous DNA base, Thymidine, with the BrdU analogue ensures specific labeling of only the dividing cells (Kee et al., 2002). Where we added BrdU directly to cells in culture for four hours (or as indicated) to a final concentration of 20μM before cells were rinsed once in PBS and fixed in PFA 4% for 30 mins at 4°C. It has been shown that the length of the cell cycle of progenitor cells is 12-14hrs (Hayes and Nowakowski, 2000, 2002). Because the S-phase is estimated to correspond to a third or a half of the cell cycle length, we added BrdU to cell cultures only for 4-6hrs to measure the number of cells in the S-phase of the cell cycle. Using immunohistochemistry, BrdU incorporation was quantified and DAPI was used to assess total number of cells in culture (**Figure 2.4**). While BrdU is a marker for new DNA synthesis, it is unlikely that cells with DNA strand breaks incorporate enough BrdU to be detected by immunohistochemistry. In a nice study on

the effects of ischemia in hippocampal neurogenesis, TUNEL, which detects DNA nicks and strand breaks that indicate DNA damage, was used to label cell death (Liu et al., 1998). The pattern of TUNEL labeling was found dramatically different from the pattern observed with BrdU(Liu et al., 1998). To confirm that BrdU labeling reflected an increase in cell birth, we also performed experiments using an antibody that recognizes Ki-67, which is expressed during cell division (See below for details). Similar to what Sharp et al observed for BrdU labeling, all BrdU incorporated cells were Ki-67 after a short pulse of BrdU. Making use of double immuno-staining we were able to quantify BrdU and specific cell-phenotype, such as nestin, class III ß-tubulin (TuJ1) or GFAP co-labelling cells (proliferating) (Wilson et al., 1996).

2.4 The labelling index and growth fraction: assessment of cell proliferation

Making use of two different strategies, BrdU labelling and colabeling for the cell cycle endogenous marker, we were able to determine (as detailed below) the number of cycling cells in as well as the number of actively dividing cells in the S-phase of the cell cycle. At the indicated time, cells in culture were pulsed with 20μM BrdU for the final 6 hrs (or as indicated) before fixation to mark the dividing cells in the S-phase of the cell cycle. Using anti-BrdU antibodies and immunocytochemical techniques, BrdU positive cells were quantified. It has been questioned over the last 10 years whether a factor that has increased BrdU incorporating cell number is purely proliferative or trophic to dividing cells (Lu et al., 1996; Nicot et al., 2002). In addition, as the cells are exposed to the factor over the whole period since 2 hrs after plating, we raised the question whether these factors increase the number of BrdU incorporating cells directly or indirectly through the release of other mediators from their target cells. To address this issue with VIP we exposed the cells to the indicated peptide concentration in conjunction with 20μM BrdU for only 6 or 8 hrs (Lu et al., 1996; Nicot et al., 2002). During this short period of time cell death effect is minimized and thus trophic activity can be ruled out. Also the likely hood of synthesis and release of secondary mediators in such a short time is much less. Moreover; as we used BrdU to label cells in the S-phase of the cell cycle, we extended our experimental set up to mark all cycling precursors at that particular time in culture using Ki-67 as a marker (Hodge et al., 2004). Then cells were counterstained with the nuclear stain DAPI. This enables us to calculate the labelling index which corresponds to the percentage of BrdU positive cells with respect to Ki-67 positive

cells. An increase in the labelling index indicates that the factor facilitates the transition of cells from the G1 phase of the cell cycle to the S-phase and/or it shortens G1 phase of the cell cycle (i.e. increased rate of cell proliferation). Also this set up enables us to work out the growth fraction which is expressed as the proportion of cycling precursors (Ki-67 positive) with respect to the total number of cells (DAPI positive). An increase in the growth fraction indicates an increase in the number of dividing cells through the recruitment of quiescent cells to divide. To indicate which cell specific phenotype VIP drives to proliferate we used the same set up of short (6hrs) VIP and BrdU pulse. Then we stained the cells for BrdU and either nestin, as a marker of putative progenitor cells, class-III ß-tubulin as a marker of neuroblasts or GFAP as a marker of mature astrocytes. We then calculated the proportion of cells that co-labeled for BrdU and each specific cell phenotype marker with respect to the total number of cells of that specific phenotype. An increase in this proportion indicates a direct proliferative effect of our treatment (factor) on that particular phenotype of cells.

2.5 Quantification of post-mitotic cells and cell cycle exit

We have designed a paradigm, in which we could determine the number of cells that decide to exit the cell cycle and become postmitotic. Cells in culture were grown for 5 days. BrdU (20μM) was added to the medium under different conditions at 2hrs after plating. This time point is considered 0hr as it is the same time at which different conditions are applied. Because BrdU gets incorporated to the daughter cells as cells proliferate, it gets diluted, but only after 4 cell-cycles (Dayer et al., 2003). Therefore, BrdU was loaded again at the time of change of 2/3rds of the medium on day two to minimize the likelihood of BrdU dilution over time. At 5DIV, cells were fixed in 4% PFA. Then cells were dual-immuno-labeled for BrdU and Ki-67. We identified cells that had exited the cell cycle as those cells that are immuno-positive for BrdU, but not Ki-67 (BrdU+/Ki67-). Then we worked the proportion of cells exiting the cell cycle by dividing the number of cells that are BrdU+/Ki67- by the total number of cells (DAPI positive).

2.6 Cell death quantification:

Cell death in culture was quantified using the cell death marker Propidium Iodide (PI) and the nuclear stain 4',6-diamidino-2-phenylindole (DAPI). PI is a phenanthridinium derivative, which has been shown to be a reliable method of cell death quantification (Noraberg et al., 1999; Noraberg, 2004; Noraberg et al., 2005). PI a highly polar fluorescent dye which is normally excluded from healthy cells, enters dead or cells with leaky membranes. Once inside the cells PI binds to nucleic acids in both the cytoplasm and nucleus, making the cells highly fluorescent at light wave lengths of 490nm excitation and 590nm emission (Wilson et al., 1996; Sullivan et al., 2002). The disadvantage of PI is that it cannot be applied to non-living fixed tissue (Eyupoglu et al., 2003). On the other hand DAPI is a nuclear cell marker that binds to double-stranded DNA by forming a stable fluorescent complex with a blue stain. However, DAPI a non-polar dye that enters both healthy (intact cell membrane) and dying cells (damaged cell membrane). Therefore, the number of cells stained with DAPI indicates the total number of cells in culture.

PI was added to living cells in cultures at 5μg/ml (Eyupoglu et al., 2003) for 40 mins at 37°C. Cells were (while still alive) then incubated in NB/B27 and Glutamine containing DAPI (20μg/ml) for another 40 mins. Finally, DAPI containing medium was removed and live cells (not fixed) were maintained in fresh culture medium (NB/B27 and Glutamine) while being imaged 5-6 images per well. The proportions of non-viable cells (PI stained) of the total (DAPI stained) cell population were then calculated.

2.7 Immunocytochemistry protocol

At the indicated times for each experiment, cells were rinsed free of medium with phosphate buffer solution (PBS) and immediately fixed in 4% paraformaldehyde (PFA) for a minimum of 30 mins at 4°C. Fixed cells were then washed 3 times in PBS to remove the PFA. For BrdU detection, cells were incubated for 30 mins with 2M HCl in a water bath at 37°C to unfold the double stranded DNA. This was followed by rinsing in PBS 3 times to remove any residual HCl. After rinsing, non-specific binding sites were blocked with 5% donkey blocking serum in 0.1%Triton-X in Tris-buffered saline (TBS) for 30 minutes at room temperature. Primary antibodies in TBS-0.1% Triton-X including rat anti-BrdU diluted 1:200 , mouse anti-rat nestin

diluted 1:200, mouse anti–GFAP diluted 1:200 (Sigma), rabbit anti-GFAP diluted 1:500, rabbit anti NG2 chondroitin sulphate proteoglycan dilute 1:500 (Chemicon), mouse anti-Class III ß-tubulin diluted 1:500 (Sigma), rabbit anti- Class III ß-tubulin diluted 1:500 (Chemicon) and/or rabbit anti-Ki67 diluted 1:500 were incubated with cells overnight at 4°C (see **Table 2.1** for details).

Cells were then rinsed once with PBS and incubated with TBS-0.1% Triton-X containing Cy2 or Cy3-conjugated anti-rat diluted 1:500, anti-mouse diluted 1:500 and/or anti-rabbit diluted 1:200 secondary antibodies for 2 hrs at room temperature. After that cells were washed once with PBS and counterstained with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI;5 µg/mL) (Sigma) for 6 min. Optimum antibody concentrations were determined by testing each antibody on cultures, and these dilutions mentioned above for each antibody are the concentrations of antibody that provided optimal signal. For each experiment, negative controls (the addition of secondary in the absence of primary antibodies) were obtained to rule out any non-specific secondary antibody binding (see **Table 2.1** for details).

Primary Antibody	Working dilution	Company
Mouse anti-TuJ1	1:500	Cambridge Bioscience
Rabbit anti-TuJ1	1:500	Chemicon
Mouse anti-rat nestin	1:200	Pharminogen
Mouse anti-GFAP	1:200	Sigma
Rabbit anti-GFAP	1:500	DAKO
Rabbit anti-NG2	1:500	Chemicon
Rabbit anti-ki67	1:500	Novocastra
Rat anti-BrdU	1:200	Insite Biotechnology
Goat anti-VPAC1	1:50	Santa Cruz
Goat anti-VPAC2	1:50	Santa Cruz
Rabbit anti-NUMB	1:200	Santa Cruz
Secondary antibodies	-------	----------
Donkey Cy2-conjugated anti-rat	1:500	Jackson ImmunoResearch
Donkey Cy3 conjugated anti-rat	1:500	Jackson ImmunoResearch
Donkey Cy2-conjugated anti-mouse	1:500	Jackson ImmunoResearch
Donkey Cy3 conjugated anti-mouse	1:500	Jackson ImmunoResearch
Donkey Cy2-conjugated anti-rabbit	1:500	Jackson ImmunoResearch
Donkey Cy3 conjugated anti-rabbit	1:500	Jackson ImmunoResearch
Donkey Cy3-conjugated anti-goat	1:500	Jackson ImmunoResearch

Table 2.1 The antibodies used to stain and study hippocampal cells *in vitro*. This table shows the different antibodies used with the working dilution as worked out from the dilution experiments and the company from which each antibody was purchased. TuJ1 is a marker of neuronal precursor cells, nestin is a marker of putative/ progenitor cells, GFAP is a marker of glial cells, NG2 is a marker of oligodendrocyte precursor cells, BrdU is a marker of the S-phase of the cell cycle, Ki-67 is a marker of cycling cells, VPAC1 and VPAC2 are the high affinity receptors for VIP and NUMB is a protein that determines the fate of a stem cell.

2.8 Immunocytochemistry for VIP receptors:

For confocal microscopy imaging, cells were grown on cover slips. At day 5 cells were fixed in 4% PFA for 30 minutes. Cells were then washed three times in PBS free of PFA. Non specific binding sites were blocked with 10% donkey blocking serum (DBS) in PBS for 20 minutes at room temperature. Then cells were incubated in 1:50 dilution of goat anti-VPAC1 or anti-VPAC2 primary antibodies in PBS containing 1.5% DBS for 60 minutes at room temperature. Cells were then washed three times in PBS. After that, cells were incubated in Cy3-conjugated anti-goat secondary antibody diluted at 1:500 in PBS containing 1.5% DBS for 45 minutes at room temperature in a dark chamber. Finally, cells were washed again three times with PBS and then counterstained with DAPI.

2.9 Measuring the cell-fate of hippocampal progenitor cells

Using a rabbit polyclonal antibody specifically recognizing NUMB, we examined NUMB expression in the in postnatal hippocampal cell cultures at 3DIV. To quantify NUMB expression in the hippocampal progenitor cell population, cultured P7-10 hippocampal cells were fixed in 4% PFA and stained for nestin and NUMB. The proportions of nestin cells that were NUMB negative with respect to the total number of cells were determined to quantify the frequency of asymmetrical cell divisions under each condition.

2.10 Elimination of cell proliferation in culture

(β-D-Arabinofuranosyl) cytosine (ara-C) is a compound that has been reported to block the activity of DNA ligases and polymerases and therefore inhibits DNA recombination and replication (Colon-Cesario et al., 2006). Ara-C has been used in stu neurogenesis to eliminate actively dividing cells (Ma et al., 2006b). This anti-mitotic drug has been demonstrated to eliminate rapidly dividing type A and type C cells but not type B cells in the SVZ (Ma et al., 2006b).

I used ara-C (Sigma) to abolish cell proliferation and to eliminate the actively dividing stem/precursor cells to study if VIP has any trophic activity on lineage restricted progeny non mitotic cells. Ara- C was applied to cells at 0.2μM on the second day as the cells have adapted to the *in vitro* environment. This concentration was determined in dose response curve experiments where the number of BrdU

incorporated cells after a 4hr pulse was determined. Indeed, 0.2μM Ara-C was enough to abolish cell proliferation (no BrdU positive cells) in culture without killing all the cells.

2.11 Imaging, cell Counting and statistical analysis:

Imaging was performed on an inverted DM IRB microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). The area of a 20× field was measured using a 255 μm grid graticule slide (Microbrightfield, Williston, USA). Cell counting was performed on 5-6 random 20× fields per well using the Open Lab image-capturing system version 2.1 (Improvision, Lexington, MA, USA). Raw data from the 20× field counts were averaged and plotted ± SEM and expressed as cells/mm² per well, based on a sample of four to eight wells per condition per repeat. All experiments were repeated at least two to three times. One experiment consisted of four hippocampi from two animals, pooled and prepared as described above. Data points were plotted using GraphPad Prism data analysis software (GraphPad inc. USA). The statistical significances between the means was assessed by either *Student's-t test* for single comparisons and by ANOVA followed by *post-hoc* tests for multiple comparisons, with $p<0.05$ considered significant.

2.12 Investigating cell growth and proliferation over time in culture

Hippocampal cells were grown in culture under standard control conditions. Cells in one third of the wells were fixed at three days, cells in the second third of wells were fixed at 5 days and cells in the last third of wells were fixed at seven days. Cells in the three groups were pulsed with BrdU 4 hrs before fixation. Cells were then immuno-labeled for BrdU incorporation, as a marker of proliferation, and were counterstained with DAPI to assess total cell counts in culture at each time point.

2.13 Comparing cell counts in cultures grown under control or bFGF conditions:

To characterize bFGF effects *in vitro* on the total number of cells and on cell proliferation. Cells were grown in culture under control or 20ng/ml FGF-2 conditions for 5 days and then fixed in 4% Para formaldehyde (PFA) for a minimum of 30 minutes at 4°C after being pulsed with BrdU to a final concentration of 20μM for the last 24 hrs. On the 3rd day, two thirds of the media were replaced with fresh aliquots.

Then cells were stained for BrdU incorporation to assess cell proliferation and counter stained with DAPI to indicate total cell counts ready for counting and analysis.

2.14 NPY effects on cell counts in primary hippocampal neuronal cultures

To study NPY effects on the total number of cells in culture and the number of cells which have incorporated BrdU as a marker of the cells entering the S-phase of the cell cycle? Cells were grown under control or 1μM NPY for 5 days. Cells were pulsed with BrdU for the last 24hrs before being fixed in 4% Para formaldehyde (PFA) for a minimum of 30 minutes at 4°C. Then we measured cell proliferation by staining for BrdU and determined total cell count by counterstaining with DAPI. Then imaging, counting and analysis were carried out.

2.15 Stu Cell death in primary hippocampal culture

2.15.1 Cell death in culture and the effect of washing on fixed cells

To quantify cell death in culture, cells were grown under control conditions for three days. At day three while the cells were still alive, they were incubated in a pre-warmed medium plus 20μg/ml DAPI for 40 minutes after which medium was replaced by fresh medium (to remove the excess DAPI which increases the background as it binds to debris at the bottom of the well) and the cells were imaged quickly. Counts of DAPI positive cells were determined as an indicator of the total number of cells. Then counts of DAPI cells that have pyknotic morphology (small, shrunken bright nucleus) (see arrowed nuclei in **Figure 2.8**) were determined as a measured dead cell count. To address the question whether we are losing cells through the process of washing and fixation, cells were then washed once with PBS before being fixed in 4% PFA. After 30 minutes in PFA, cells were washed three times in PBS and stained with DAPI. Again cell counts of the total number of cells (DAPI positive) and DAPI pyknotic cell were determined.

2.15.2 Quantifying effects of VIP on cell death

To study VIP effects on hippocampal cells, cells were plated at the indicated density under control or varying concentrations of VIP ranging between 3nM and 1μM peptide concentration. At 2 hrs after plating, PI at 5μg/ml was added to the medium and left there for the whole time of cells in culture. Cells were taken out of the incubator and imaged quickly under the fluorescent microscope at 1, 2, 3 DIV. The

number of cells that were PI positive were determined and compared under different conditions at the three time points. At day three, cells were further fixed and counterstained with DAPI and total number of cells was determined at that particular time point.

2.16 Results

2.16.1 Cells grow and proliferate over time in culture

Hippocampal cells were grown under standard control conditions (Neurobasal A/ B27 plus glutamine) for 3, 5 and 7 days. To assess proliferation (S-phase entry), cells were pulsed with 20μM BrdU 4 hrs before fixation. Total numbers of cells and number of cells, which were BrdU immuno-positive, were determined. Our results indicate that cells in culture grow with time as indicated by the gradual increase in the total number of cells with time. The number of cells at day 3 was 172.7 ± 4.9 cells/mm² grew to 241.7 ± 6.7 cells/mm² at day 5 and to 266.5 ± 10.6 cells/mm² at day7 (Figure 2.3 A). At the three time points, considerable numbers of cells incorporated BrdU after a 4 hrs pulse as a marker of proliferating cells in the S-phase of the cell cycle (**Figure 2.3 B**). See also (**Figure 2.4 B**) For BrdU staining.

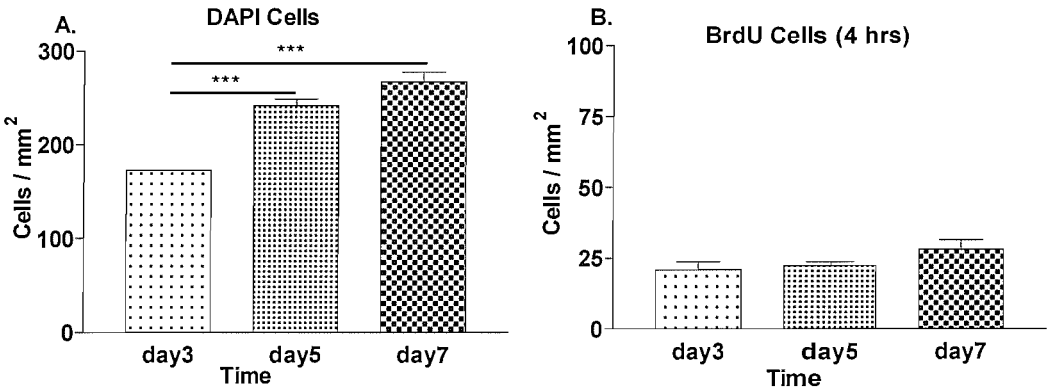


Figure 2.3: Time course proliferation and growth under control conditions. Cells were grown in culture for 3, 5 or 7 days. Cells were pulsed with BrdU for the last 4 hrs before fixation at each time point. (A) Total number of cells, (B) Counts of BrdU incorporated cells. Data represent mean ± SE based on a sample that represent at 6 wells per condition from one experiment. Comparisons between different conditions are one way ANOVA with Dunnetts' multiple comparison test (***, $p < 0.001$).

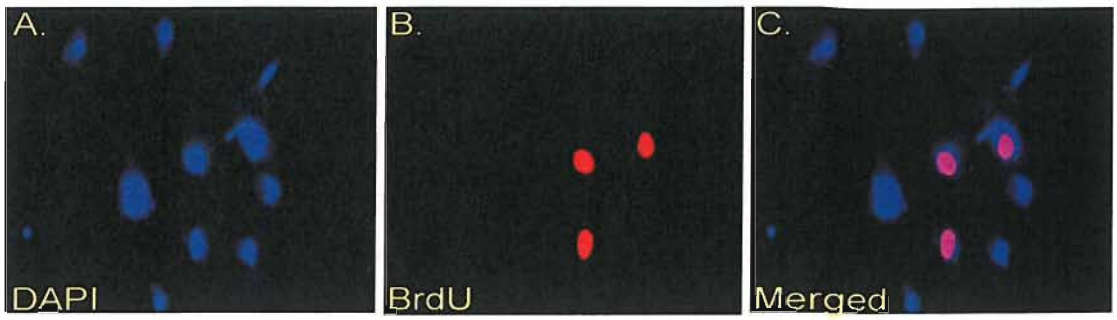


Figure 2.4: BrdU staining. Cells were grown for 3 days in culture. At day 3 cells were pulsed with BrdU 4 hrs before fixation. Cells were then processed for BrdU and counterstained with DAPI. (A) DAPI stained cells, (B) BrdU incorporated Cells, (C) merged image.

2.16.2 bFGF increased the total number and the number of proliferating cells in culture

To characterize the effects of bFGF as a peptide growth factor on hippocampal stem cells, hippocampal cultures were generated from (P7-10) Wistar rats. Cells were grown under standard control or control plus 20ng/ml FGF-2. Cells were pulsed with BrdU (20μM) 24 hr before being fixed in 4% PFA. Then total cell density and proliferating cell numbers were quantified. The total number of cells as indicated by the number of DAPI positive cells was 81.2 ± 12.5 cells / mm^2 under control condition compared to 206.5 ± 22.3 cells /mm^2 under 20ng/ml bFGF (**Figure 2.5 A**). This dramatic increase in the total number of cells was statistically significant as indicated by Students, *t*-test. bFGF has a potent effect on cell division during the last 24 hrs before fixation. The number of cells that incorporated BrdU over the last 24 hrs was 57.4 ± 10 cells /mm^2 under control condition significantly increased to160.3 ± 6.2 cells/ mm^2 under 20ng/ml bFGF treatment(**Figure 2.5 B**).

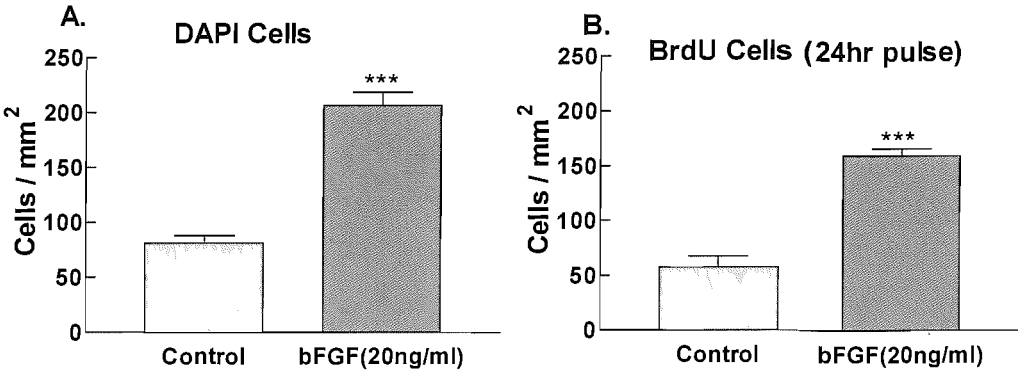


Figure 2.5: Comparing cell growth under control (Neurobasal A/ B27) or bFGF conditions. Total and BrdU incorporating cell counts were quantified. BrdU was added for the last 24 hrs to cells under the two different conditions. Data is means ± SEM for 8 wells per condition. Students *t*-test; ***, $p < 0.001$.

2.16.3 NPY is proliferative to hippocampal cells in culture

Hippocampal cultures grown in the presence of NPY at a concentration of 1μM displayed increase in the total number of cells (131±5.3 cells / mm² compared to 86.5±3.2 cells / mm²). BrdU incorporating cells were 76.2±7.7 cells / mm² versus 33.9±2.3 cells / mm² for NPY and control, respectively (see **Figure 2.2**). Data points are expressed as absolute numbers under different conditions (**Figure 2.6 A & B**).

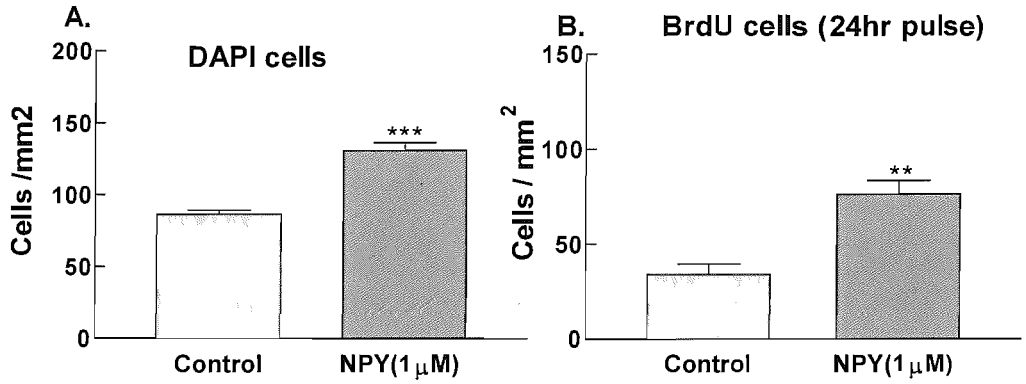


Figure 2.6: NPY increased both the total number of cells and the number of BrdU incorporating cells after five DIV. BrdU was added for the final 24 hrs. Data is the means ± SEM from 6 wells per condition. Students'*t*-test; **, $p < 0.01$ and ***, $p < 0.001$.

2.16.4 Cell death in culture and the effect of washing on fixed cells

The total number of cells as indicated by DAPI positive cells at three days was 107.5±1.2 cells/ mm² while the cells were still alive. This number of cells has dropped significantly to 86.8±3.4 cells/ mm² after fixation and washing. The number of pyknotic DAPI cells (dead) before fixation was 31.1±5.4 cells/ mm². However, only 4.6± 2 pyknotic cells per millimeter square were found after fixation and washing (**Figure 2.7 & 2.8**).

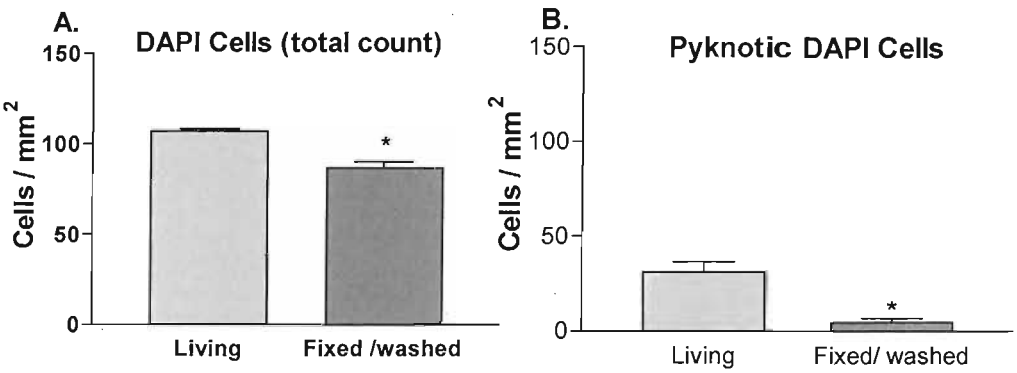


Figure 2.7: Effects of washing on cell death quantification. After three days in culture, cells were incubated with DAPI for 40 minutes and imaged while still alive. Cells were then fixed and washed and re-stained with DAPI. Counts of total DAPI cells and pyknotic DAPI cells before and after fixation were determined. Data is the means ± SEM from 20 random images from 2 wells per condition. Students' *t*-test; ; *, $p < 0.05$.

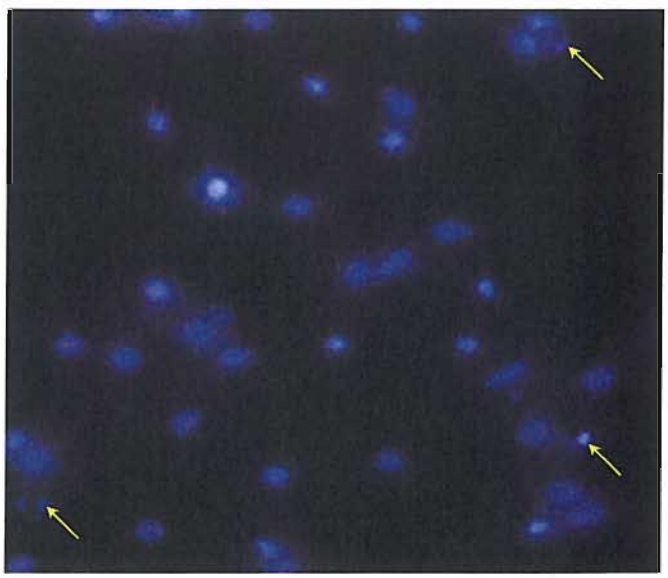


Figure 2.8: **DAPI stained cells with some pyknotic nuclear staining**. Cells were grown for 3 days. Cells were then fixed and washed three times free of PFA before being stained with the nuclear stain DAPI. Counts of pyknotic cells (yellow arrowed) as opposed to healthy cells were determined.

2.16.5 VIP decreased cell death in culture as early as 48hrs

As demonstrated in (**Figure 2.7**), a large number of cells under different conditions underwent cell death during the first 24hrs after plating (**Figure 2.5A**). In contrast to control conditions, cells allowed to grow in the presence of VIP, showed a gradual decrease in cell death over the next 2 days in culture (**Figure 2.9 B & C**). Cell death at 2 and 3 DIV is significantly different between control and different VIP concentrations (one way ANOVA with Dunetts' multiple comparisons post hoc test) (**Figure 2.9 B & C**). At day 3 and after the cells had been fixed, cells were further counterstained for DAPI to assess total cell density at that stage and to the proportion of cells with respect to the total number of cells. By analyzing the data, we found that the total number of cells under different VIP concentrations has gone up in comparison to control conditions (**Figure 2.9 D**). However as the fixative destroyed the PI fluorescence, it was not possible to determine the counts of PI positive cells after fixation and the signal from the red filter of the fluorescent microscope was so faint to pick up. Consequently, we could not determine proper cell death proportions after fixation and therefore the proportions of cells were calculated by dividing the number of PI positive cells before fixation by the total number of cells that remain after fixation (**Figure2.9 E**).

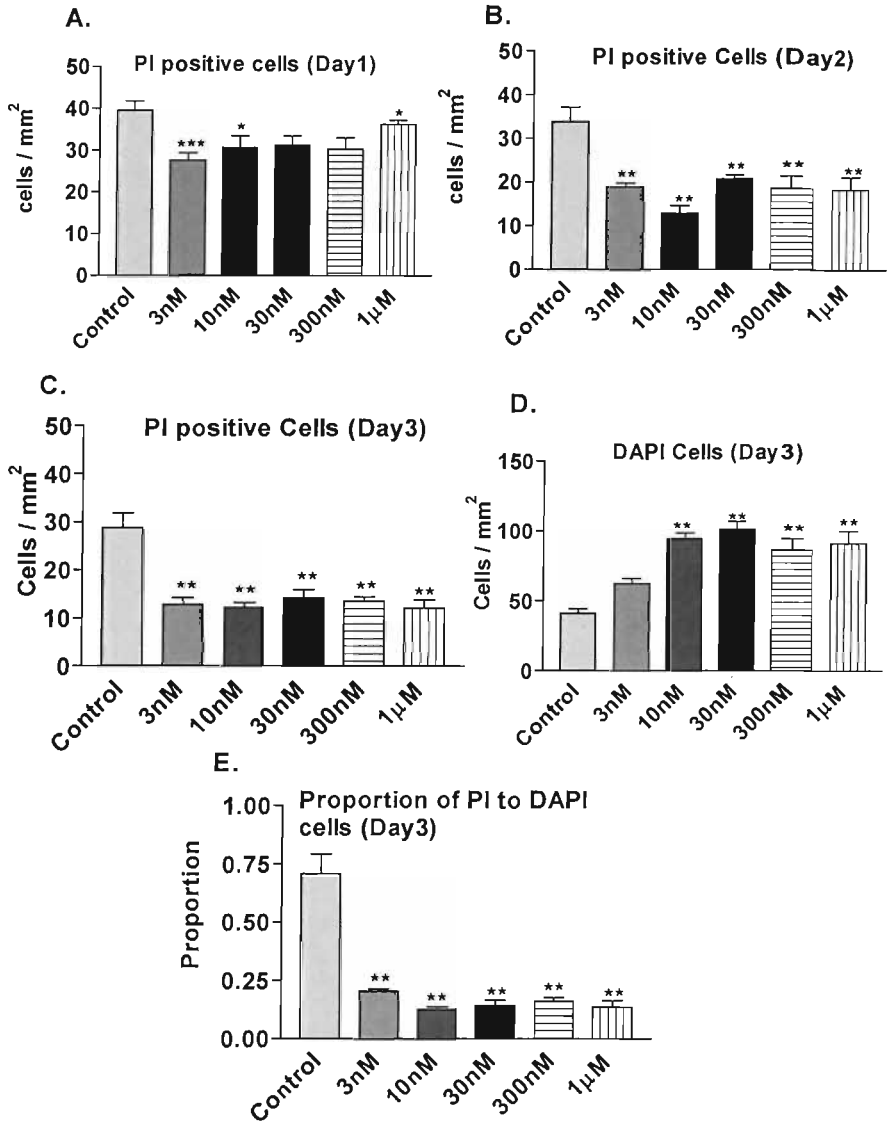


Figure 2.9: **VIP reduced cell death in culture within 48 hrs**. Hippocampal cells were grown for three days in culture under standard control or different concentrations of VIP. Cells were exposed to PI for the whole time in culture. Cells imaged at day 1, 2 and 3 before being fixed and counterstained with DAPI. Counts of PI positive cells were determined at each time point. DAPI counts were determined at day three.(A) PI positive cells at day1, (B) PI positive cells at day2, (C) PI positive cells at day3, (D) DAPI positive cells at day3, (E) Proportion of PI to DAPI positive cells at day3. Data represent mean ± SE based on a sample that represent at least 4 wells per condition from one experiment. Comparisons between different conditions are one way ANOVA with Dunnetts' multiple comparison test (*, $p < 0.05$ and **, $p < 0.01$).

2.16.6 Postnatal hippocampal cell cultures contained the major cell-specific phenotypes populations

It is one of our aims to define the effects of different neuropeptides, such as VIP and other growth factors on specific cell types in culture. Therefore, it was of major importance to define the exact proportions of different cell phenotypes under control conditions. For this purpose, cells were grown in cultures generated from postnatal rats for 5 days. Cells were then immuno-labeled for the neural stem cell marker nestin, the neuronal marker class III ß-tubulin, the astrocytic marker GFAP and the oligodendrocyte cell marker NG2. In agreement with other studies (Howell et al., 2003; Howell et al., 2005), our primary hippocampal cultures, grown for five days under control conditions (Neurobasal A/ B27 and glutamine) contained cells immunoreactive for the major cell specific phenotypes: the stem/precursor cell marker nestin (**Figure 2.10 B**), the neuron specific marker class III ß-tubulin (**Figure 2.10 C**), the astrocytic marker GFAP (**Figure 2.9 A**) and the oligodendrocyte precursor cell marker NG2 (**Figure 2.10 D**). Analysis of our results has shown that 41.3 ± 1.0 % of the cells are nestin positive, 38.7± 2.5 % of the cells are class III ß-tubulin positive, 46±2.8 % of the cells are GFAP positive and 9.3 ± 2.6 % of the cells are NG2 positive.

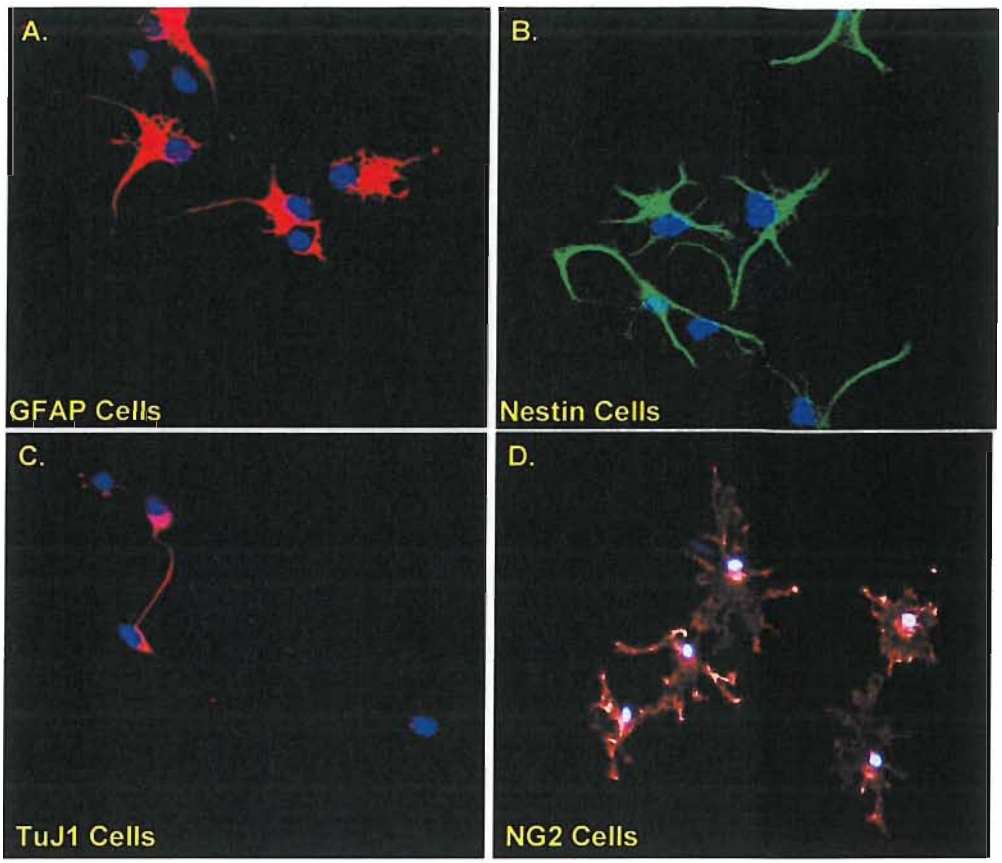


Figure 2.10: Hippocampal cells in culture express the major cell phenotypes. Primary hippocampal cultures were grown for 5DIV under standard control conditions. Cells were fixed and subsequently processed for the expression on the astrocytic marker GFAP (A), the stem cell marker nestin (B), the neuronal cell marker TuJ1 (C) and the oligodendrocyte precursor cell marker NG2 (D). Scale bar; 20μm.

2.16.7 The majority of hippocampal cells in culture are cycling and proliferating cells

Hippocampal cells were grown for 5 days under standard control conditions. Cells were labeled for the cycling cell marker Ki-67 and the S-phase marker BrdU. Our results revealed that 83.8±3.4 % of the cells are Ki-67 positive (cycling) (**Figure 2.11 C**) and 5.0±2.4 % of the cells are BrdU positive (in the S-phase of the cell cycle) (**Figure 2.11 B**) after short (4hrs) BrdU pulse. It is also very important to point that all of the cells in the S-phase of the cell cycle were Ki-67 positive which strongly indicates that these cells were cycling at the time of BrdU incorporation (DNA synthesis) but not undergoing DNA repair (**Figure 2.11 D**). Total cells in cultures were quantified using the nuclear stain DAPI (**Figure 2.11 A**).

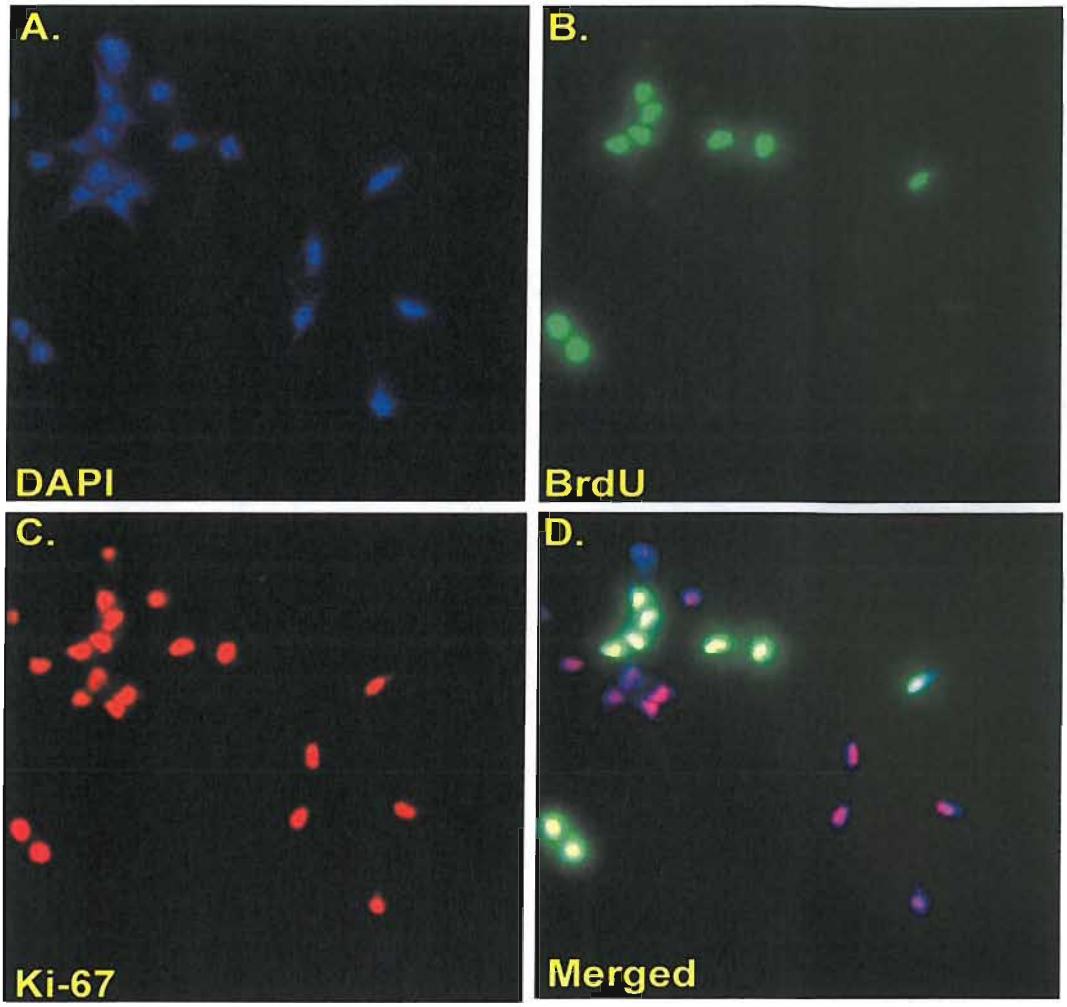


Figure 2.11: Markers of cycling and proliferating cells *in vitro*. Hippocampal cells were grown for 5 days under standard control conditions. At day 5 cells were pulsed with BrdU to a final concentration of 20μM. After being fixed, cells were then processed for BrdU incorporation (B) and Ki-67 expression. Cells were then counterstained with DAPI (A). (D) is the merged image for the three stainings together.

2.16.8 Ara-C at 0.2μM abolished cell proliferation and reduced the total number of different cell specific phenotypes in culture

To characterize the optimal concentration of ara-C to abolish cell proliferation in culture, cells were pulsed at 2DIV with different concentrations of ara-C between 0.02-10μM and left to grow for 5 days under control, 1μM or bFGF-2. At day 5 cells were pulsed with BrdU (20μM) for the last 4hrs before fixation in 4%PFA. Cells then were stained for BrdU incorporation and counterstained with DAPI. Under the

inverted fluorescent microscope a thorough search was accomplished for BrdU immuno-labelled cells. At as low as 0.2μM ara-C concentration, there was no one single BrdU positive cell under any of the three conditions. Therefore, 0.2μM is the recommended optimal ara-C concentration to eliminate cell proliferation without killing all the cells in culture.

Moreover; the addition of 0.2μM on the second day resulted in a massive loss of the total number of cells compared to control conditions. Phenotyping of the remaining cells indicated that almost 50% of nestin positive precursor cells had been lost. In terms of the effects of ara-C on progeny cells, the number of class III ß-tubulin and GFAP positive cells has dropped by another 50% compared to control condition (**Figure 2.12 A-D**).

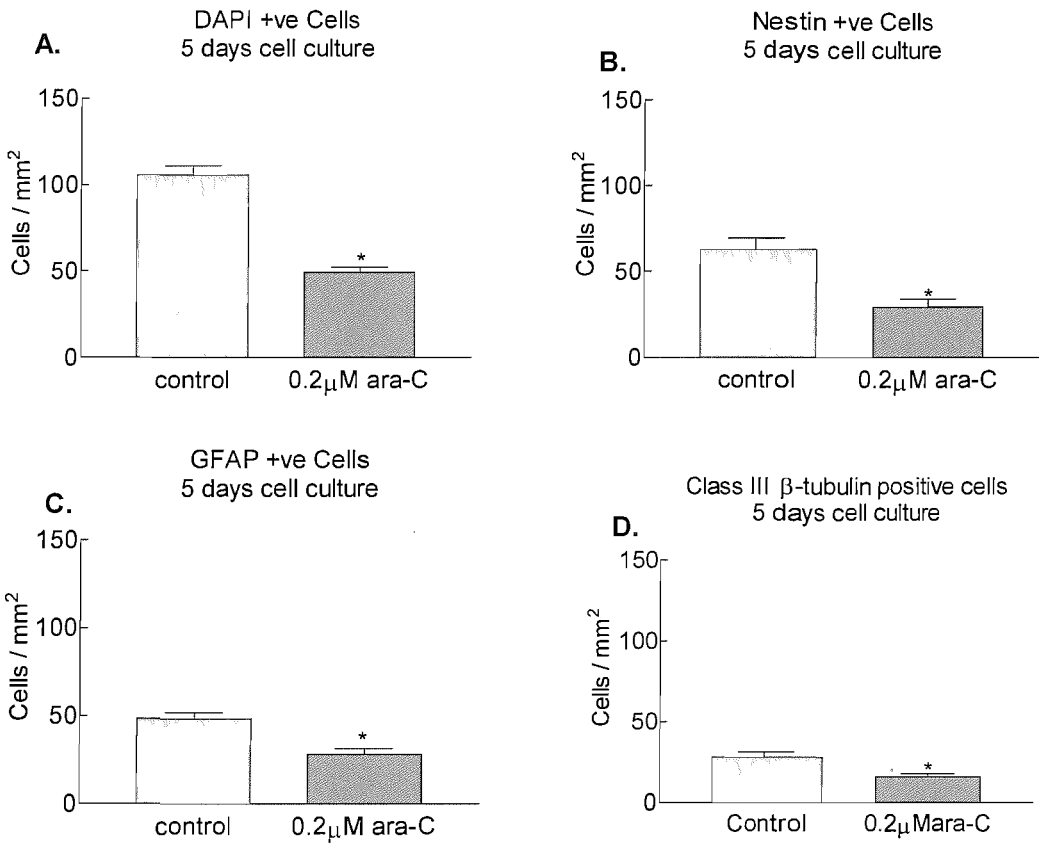


Figure 2.12: **Elimination of cell proliferation in culture**. Cells were grown under control conditions for 5 days. At 2DIV, cells in half of the wells were pulsed with 0.2μM ara-C. (A) total number of cells, (B) nestin-expressing cells, (C) GFAP-expressing cells, (D) class III ß-tubulin-expressing cells. All data points are means ± SEM for 8-10 values of each condition. Students' *t*-test; ; *, $p < 0.05$.

2.17 Discussion

2.17.1 Establishment of cells in culture

To generate hippocampal neuronal culture, I used an already existing protocol in our laboratory (Howell et al., 2003; Howell et al., 2005). However, I adjusted a few things and developed others with regards to immunohistochemistry and cell death quantification (see **Section 2.6** for details). According to our protocol (**Section 2.2**) cells ready for plating were generated from P7-10 Wistar rat hippocampi. The whole procedure from killing the animal until plating the cells onto the coated 24-well plates takes less than 2 hrs, this relatively short time combined with the use of papain as a proteolytic enzyme and the removal of debris by using optiprep gradient has enabled us to generate a viable and reproducible paradigm.

The hippocampal cultures we are generating contained the major cell-specific phenotypes: nestin, class III ß-tubulin and GFAP positive cells. In addition, the majority of the cells in our culture system are proliferating as indicated by the expression of the proliferative marker Ki-67 by more than 80% of the total number of cells in culture. These facts make this model a suitable paradigm to investigate the effects of putative growth peptides, like VIP. These results are in agreement with Howells' findings using the same cell culture setup (Howell et al., 2003).

2.17.2 Effects of bFGF on hippocampal cells in culture

bFGF is a cytokine that has been shown to have a dose dependent effect in culture ranging from trophic activity at doses as low as 30pg/ml, neurite outgrowth (200-500pg/ml) to mitogenic effects at doses of 1-20ng/ml . In our culture system, we followed the total cell counts and the rates of BrdU incorporation between control conditions and bFGF (20ng/ml) (Gage, 1998). We observed an increase in the total number of cells and the number of BrdU incorporated cells under bFGF conditions over control conditions. This data implies that bFGF is proliferative to hippocampal cells in culture and has increased cell density through this enhanced cell proliferative activity. These results while are consistent with others observations (Lowenstein and Arsenault, 1996), indicate that our culture provides a suitable model to investigate the effects of other growth factors on cell survival and proliferation.

2.17.3 NPY effects on hippocampal cells in culture

NPY is a neuropeptide that has been shown to have proliferative effects on hippocampal cells in culture at 1μM peptide concentration. As demonstrated in (**Figure 2.2**), NPY produced a significant increase in the total number of cells (DAPI) as well as the number of BrdU incorporating cells over standard control conditions. These results were in agreement with the results of published work conducted in our laboratory (Howell et al., 2003; Howell et al., 2005). Therefore, these results strongly suggest that the primary cell cultures I am generating is a suitable paradigm to study the effects of other neuropeptides on cell survival and proliferation in culture.

2.17.4 VIP effects on cell death in culture

There were many methods to assess cell death *in vitro*. One of these methods was described using DAPI nuclear staining. Dead cells were defined as those cells with small/ shrunken nuclei (pyknotic). At 3DIV, cells were pulsed with DAPI and imaged while still alive. Then cells were fixed and re-stained with the nuclear stain DAPI again. Total cell counts and counts of pyknotic DAPI cells were determined before and after fixation. We found two important findings: firstly, about 30% of DAPI cells were pyknotic (dead) at three days in culture which is consistent with other studies using this method to study cell death (Howell et al., 2003). Secondly, a certain proportion of cells have been lost after washing once and thrice with PBS before and after fixation, respectively. Interestingly, the majority of the lost cells were pyknotic (dead). This may be because these cells have lost cell membrane integrity and become less sticky to the poly-L-lysine coating. This method of stu cell death is dependent on the quality of DAPI staining and there is no precise definition of the size below which cells are considered pyknotic. Therefore, we designed the protocol detailed in section 2.6 to solve all these odds and to study cell death using PI and DAPI while the cells are still alive to get a more accurate measure of cell death in culture.

The other method of stu cell death which has been widely used is the application of the cell death marker PI to mark dead/ cells in culture. To try this method, and in an initial protocol, cells were grown for 3 days and exposed to the cell death marker PI from the early start at 2 hrs after plating. Then cells were imaged successively at day1, 2 and 3 under fluorescent microscopy using the red filter for PI emission signal. Positive PI cells were counted under different conditions. Analysis of the data

revealed that the number of dead/ cells under VIP condition was reduced by more than 66% compared to control conditions. This was in parallel with the increase that was observed at 3DIV in the total number of cells under VIP conditions over control conditions. This strongly suggests that VIP increased cell density through enhanced cell survival. Also this data implies that our culture system is a robust to assess cell death *in vitro* and to further study the effects of novel neuropeptide on ongoing cell death in culture. However this protocol to evaluate cell death has some limitations: (i) as cells die, PI bound to fragments of nucleic acids leaks to the cell medium. This has led to a very bright background at the time of imaging and made it more difficult to count. Therefore a shorter time of exposure to PI is to be considered. (ii) At the time of counting it was difficult to distinguish cellular from non-cellular PI staining. Therefore it is important to counter-stain the cells with DAPI while still alive to differentiate cellular from non cellular ones. (iii) As the cells were fixed, PI leaks out of the cells and disappears, therefore it was not possible to determine cell proportions after fixation. Interpreting the proportion of cells that were labeled for PI before fixation with respect to the total number of DAPI stained cells after fixation possible. However, we have shown that we are losing cells through fixation and after cell washing. Therefore, this interpretation may be criticized as it may be overestimating proportions of cells. These limitations have been considered and all sorted out in our protocol that we used consistently in this study (**for details see section 2.6**).

2.17.5 Consequences of ara-C treatment of cells in culture

Ara-C is a compound that has been used widely to block cell proliferation and to eliminate actively dividing cells in areas of neurogenesis in the CNS (Seri et al., 2001). Our results indicate that 0.2μM ara-C is enough to entirely block cell proliferation in culture without a massive fatal effect on non-dividing cells. This is consistent with other studies in which they have shown that as low as 0.5μM is enough to eliminate proliferating cells in culture (Ma et al., 2006b). Ara-C is toxic to replicating cells because when it gets incorporated to the DNA, it inhibits DNA replication. However, terminally differentiated cells do not actively divide and synthesize DNA and are therefore resistant to ara-C at this concentration. Terminally differentiated cells in granular cerebellar and cerebral cortical cultures were observed to die when exposed to 10 and 60 μM ara-C, respectively (Dessi et al., 1995). Therefore the addition of 0.2μM is unlikely to affect the survival of these cells. This

will enable us to rule in/out any activity of our growth factor of interest on this particular population of cells. Ara-C has been shown to induce cell death in many neuronal cell culture systems including, sympathetic ganglion and cerebellar neuronal cultures (Takano et al., 2006). In agreement, our results imply that ara-C reduced the general cell survival in culture. Indeed, it reduced the survival of the three major cell phenotypes in culture. The treatment of cells with this anti-mitogenic agent on the second day has led to reduction in the total number of cells of more than 50%. In parallel the cell count of the major cell phenotypes has been reduced by the same percentage. This indicates that our cell culture represents a model in which the expansion of cell density over time occurs through successive cell proliferation through which these cells replicate and give their progeny. This cell culture system with the introduction of ara-C forms a proper paradigm to test the effects of novel neuropeptides like VIP on the non-dividing cell population and to rule in/out any direct effect on precursor cells and their progeny.

2.18 Prospective and conducting remarks

Herein, I have demonstrated the primary hippocampal culture as an *in vitro* system that supports the growth and proliferation of hippocampal cells. This robust also contains the major cell specific phenotypes including putative precursor cells, neuronal and astrocytic cells. Cells in culture have shown a good response to the mitogenic factor bFGF and the proliferative neuropeptide NPY. Cell death was demonstrated using the classical methods to assess cell death *in vitro* (PI and DAPI). These characteristics all together put this paradigm at a position to be our robust to study the effect of novel neuropeptides like VIP and galanin with regards to their ability to modulate the proliferation of stem cells and the survival and differentiation of their progeny as important events to define the process of neurogenesis *in vitro*.

Chapter 3

VIP enhances the survival and the proliferation of progenitor cells in postnatal hippocampal cell culture

3.1 Introduction

Vasoactive intestinal polypeptide (VIP) a neuropeptide which is widely distributed in the mammalian nervous system has potent growth-related actions that influence cell division, neuronal survival, and neurodifferentiation (Gressens et al., 1997). VIP receptors are expressed at a high level in the central nervous system, particularly in the hippocampus (Vaudry et al., 2000). These levels have been found to be affected by a number of physiological and pathological conditions, such as exercise, stress, memory, learning and epilepsy (Glowa et al., 1992; de Lanerolle et al., 1995; Gozes et al., 1996). These conditions are also well-known to affect hippocampal neurogenesis (van Praag et al., 1999; Parent, 2002; Bruel-Jungerman et al., 2005; Heine et al., 2005). Therefore, we have designed this study to investigate the hypothesis that VIP modulates the survival, proliferation, and differentiation of postnatal hippocampal progenitor cells and their progeny.

3.1.1 VIP involvement in neuron generation in the CNS

While VIP receptors have been found prenatally, VIP mRNA was not detected in the rat CNS before birth (Hill et al., 1996a; Hill et al., 1996b). A peak in VIP concentration of rat maternal serum has been found at days E10-E12 of pregnancy, with VIP levels 6- to 10-fold higher than later during pregnancy. Radio-labeled VIP, administered intravenously to pregnant female mice, was found in the E10 embryo (Hill et al., 1996b). These results suggest that VIP produced by extraembryonic tissues may regulate embryonic growth during the early postimplantation stage of development in the rodent (Hill et al., 1996a; Hill et al., 1996b). This expression of VIP in the CNS is regulated by the BDNF as demonstrated in cultured fetal rat cerebrocortical (Villuendas et al., 2001).

Binding studies in rats (from embryonic day 19 to postnatal day 20) have revealed that an uniformly dense VIP binding throughout the brain with markedly higher binding in germinal zones (Hill et al., 1994a). The VIP mRNA has been evaluated from birth through the postnatal and adulthood in the rat brain. VIP mRNA exhibited

a marked increase by P2, became statistically significant by P4 and peaked by P10 (Lopez-Tellez et al., 2004). The expression then decreased gradually towards adult levels by P20 (Lopez-Tellez et al., 2004). Interestingly, these dramatic changes in VIP mRNA expression during the first 10 postnatal days were accompanied by a gradient maturation in the different subfields of the hippocampus (CA2-3 firstly then the CA1 and dentate gyrus) (Lopez-Tellez et al., 2004). Another study on the measurement of VIP protein concentration in mice rats has revealed a 10-fold increase by P7, but in contrast to the mRNA pattern in rats, VIP protein levels continued to increase hitting 50-fold increase in adulthood (Girard et al., 2006).

From physiological and behavioral point of view, VIP antagonist treatment during embryogenesis has been demonstrated to cause permanent effects on adult brain chemistry and impaired social recognition behavior in adult male mice (Hill et al., 2007a; Hill et al., 2007b). Furthermore, this VIP antagonism caused deficits in cognitive function, as assessed through cued and contextual fear conditioning in the male offspring (Hill et al., 2007a).

3.1.2 Growth factors' role in the fate-determining of neural stem cells

The molecular machinery controlling cell fate during neurogenesis is still a long way from being completely characterized. Many growth factors, including trophic and proliferative factors, have been studies. FGF-2 as a trophic and proliferative factor has been commonly linked to neural stem cell biology. FGF-2 has been shown recently to enhance neural stem cell self-renewal via symmetric cell divisions through FGFR1 and FGFR3 receptor subtypes (Maric et al., 2007). Indeed, knocking these two FGF-2 receptors down, has resulted in enhanced neuronal differentiation via asymmetric divisions (Maric et al., 2007). Stem cell-derived neural stem/progenitor cell supporting factor (SDNSF) is another trophic factor that has been shown to enhance self-renewal and multipotency of hippocampal neural stem cells without altering their mitotic activity (Toda et al., 2003). Epidermal growth factor (EGF) treatment has been shown to increase the number of newborn glial cells and reduced the number of newborn neurons, both in the hippocampus as well as in the olfactory bulb (Kuhn et al., 1997). Platelet-derived growth factor is another growth factor that has been shown to induce neuronal differentiation of embryonic stem cells, where the newly born neurons showed less mature morphology and continued to proliferate (Johe et al.,

1996). These embryonic clones, has been shown to exclusively differentiate into astrocytes once treated by ciliary neurotrophic factor or leukemia inhibitory factor (Johe et al., 1996; Lillien, 1998). In rat embryonic hippocampal neuronal cultures VIP has been demonstrated to promote neuronal differentiation through activity-dependent neurotrophic factor (ADNF) (Blondel et al., 2000). These findings may suggest that the regulation of progenitor cell fate depends, at least in part, on spatially and temporally regulated environmental signals that involve endogenous and exogenous growth factors.

3.1.3 VIP effects on neuronal cells in primary neuronal cell cultures

VIP effects in primary cell cultures generated from different areas of the brain have been evaluated at different developmental stages in rodents. VIP protective effects against toxicity of dopamine, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium ion (MPP+) have been investigated in neuronal cultures including rat pheochromocytoma (PC12), human neuroblastoma (SH-SY5Y) and rat cerebellar granular cells (Offen et al., 2000). Remarkably, VIP at femtomolar concentrations protected neurons against dopamine and 6-OHDA toxicity in PC12 and neuroblastoma cells. At nanomolar concentrations, VIP protected cerebellar granule neurons against 6-OHDA. In contrast, VIP did not rescue neuronal cells from death associated with MPP+. VIP has been also investigated in dissociated spinal cord-dorsal root ganglion (SC-DRG) cultures. Previous studies demonstrated that VIP increased neuronal survival in SC-DRG cultures when synaptic transmission was blocked with tetrodotoxin (TTX) (Brenneman et al., 1987). This effect has been reported to be dependent on the presence of nonneuronal cells (astroglial cells) which secret survival-promoting substances (Brenneman et al., 1987). In the same culture system, VIP has also been found to be an astroglial mitogen (Brenneman et al., 1990). In sympathetic neuroblast cell cultures, serial time-lapse photography was employed to evaluate cell survival and revealed that VIP combined with nerve growth factor virtually rescued all newly born neuroblasts (Pincus et al., 1994). In rat astrocyte culture, VIP has been shown to stimulate cAMP accumulation in a dose-dependent manner (Masmoudi-Kouki et al., 2007). In whole embryonic cultures, a 4-hr incubation, VIP stimulated growth by increasing somite number, embryonic volume, DNA and protein content, and number of cells in S-phase of the cell cycle (Gressens

et al., 1993). VIP has been reported to stimulate the expression of BDNF mRNA in primary cultures of cortical neurons and astrocytes (Pellegri et al., 1998).

In contrast to this abundance in VIP studies in different cell culture types, very few studies were interested in VIP effects on neural stem cell cultures. In one of these studies, VIP at concentrations of not less that 5μM, was found to reduce apoptosis in hippocampal stem cell culture generated from adult female mice (Antonawich and Said, 2002). In another model of adult mice cell culture, VIP at 300nM was found to enhance the proliferation of the precursor cells (Mercer et al., 2004). However, this study did not deal with VIP survival effects on these progenitor cells and was on embryonic hindbrain, but not postnatal hippocampal cells.

3.2 Aims and objectives

Our main goal in this study is to investigate systematically VIP effects on stem cell survival, proliferation and/or differentiation in the postnatal rat hippocampal cell cultures.

In this regard we will be using the methods outlined in chapter two to study VIP effects on the survival and proliferation of nesting expressing cells, astrocytic cells expressing GFAP, neuronal cells expressing TuJ1 and oligodendrocyte precursor cells expressing NG2. We will also investigate any possible VIP effects on cells fate. Primary rat hippocampal cultures were used as our paradigm in this study as it represents a relatively controlled system and as it helps to minimise the number of variables. Primary cultures give us the chance to elucidate the effects of different factors on specific target cells. Also it helps us to adhere to home office guidelines to reduce animal number and use *in vitro* experiments instead of *in vivo* ones.

3.3 Methods

3.3.1 Introduction

In this section, I will point to some important points in cell culture generation, cell proliferation measurement, cell death quantification, immunohistochemistry and cell counting as these procedures detailed in **chapter 2**. Rather I will detail here how the individual experiments were designed and carried out.

3.3.2 Generating primary hippocampal culture

In each experiment, primary hippocampal cultures were generated from 2-8 Wistar rats of P7-10 age. Viable cells were plated in 500μL NB/B27 and glutamine at a density of 100,000 cells directly onto poly-L-lysine coated 24-well plates or on glass cover slips for confocal images. At 2 hrs after plating cells were rinsed free of debris and replenished with fresh medium of NB/B27 and glutamine under control conditions and the addition of the indicated VIP concentration for each experiment under VIP conditions.

In most of our experiments, VIP was added at 30nM or 1μM concentration to study trophic and proliferative effects of VIP, respectively. VIP from human, porcine and rat powder was supplied by Sigma-Aldrich. A working stock solution was prepared from 1ml aliquots of 1mM in sterile water and being stored as 10μL aliquots at -20C in the freezer.

3.3.3 Examining VIP effects on total cell number in culture

In an initial set of experiments, cells were treated with either fresh medium (control conditions) or fresh medium containing a range of VIP concentrations between 1pM-10μM. Cells were grown for 5 days before being fixed in 4%PFA for 30 minutes at 4°C. Two thirds of the medium were changed on the third day. Fixed cells were then washed three times in PBS before being stained with the nuclear stain DAPI. Then six images were taken per well and cultures were processed for DAPI positive cells as it represents the total number of cells in culture under different conditions.

3.3.4 Characterising proliferative effects of VIP in culture

In another set of experiments, cells were grown under standard control conditions or different VIP concentrations 3nM-1μM for 3 or 5 days. Two thids of the medium were changed on the third day for 5 days cultures. Cells were pulsed with 20μM BrdU for the last 4 hrs before fixation. Cells were then immuno-stained for BrdU incorporation and counterstained with the nuclear stain DAPI. Cultures were then processed for BrdU incorporation and the numbers as well as the proportions of cells incorporated BrdU were calculated.

3.3.5 Examining VIP effect on cell death in culture

To examine VIP effects on cell death in culture, cells were grown under standard control conditions or under a range of VIP concentrations between 3nM -1μM. At 5 DIV, cells were pulsed with 5μg/ml PI for 40 minutes. Then cells were incubated in fresh medium containing 20μg/ml DAPI for another 40 minutes. Cells were then rinsed free of DAPI and maintained in fresh pre-warmed medium while being imaged (while still alive before being fixed). Proportions of PI positive cells (dead/ cells) with respect to the total number of cells (DAPI positive) were calculated (see **Section 2.6** for details).

3.3.6 Characterising cell-specific phenotypes that VIP affects

In a set of experiments, cells were grown under control conditions or control plus 30nM VIP. At 5 DIV, cells were fixed and immuno-stained for the putative progenitor cell marker nestin, the neuronal marker class-III ß-tubulin, or the astrocytic marker GFAP. Cultures were processed for the immunochemistry of these markers and the numbers as well as the proportions of each cell specific marker were determined.

3.3.7 Examining VIP effects on oligodendrocyte precursor cells

Hippocampal cells were grown for 5 days under control condition, control plus 30nM VIP or control plus 1μM VIP. At day 5, cells were processed for the expression of oligodendrocyte precursor cell marker NG2. Cells were then counterstained with DAPI to measure total cell counts. The absolute number of NG2-expressing cells and their proportions under each condition were determined and compared.

3.3.8 Examining VIP effects on nestin-precursor cell subpopulations

To further define VIP trophic activity on precursor cells, 5 day-old cell cultures were generated from 7-10 day age postnatal rat hippocampi. Cells were grown in the presence or absence of 30nM VIP. After tissue fixation cells were processed for the expression of nestin and GFAP markers or nestin and TuJ1. Proportions of nestin immuno-reactive cells that co-expressed or did not express GFAP or TuJ1 with respect to the total number of nestin positive cells were determined and compared under control and 30nM VIP.

3.3.9 Examining VIP effects on the self-renewal of nestin-expressing cells

In two different but related sets of experiments, hippocampal precursor cells were grown for 5 days under control conditions or control plus 30nM VIP. In the first set of experiments, cells were given a pulse of BrdU for the last 24hrs before fixation. Cells were then processed for BrdU incorporation and colabeled for nestin expression. Within this proliferating/growing cell population the proportion of nestin cells was determined by dividing the number of nestin cells that incorporated BrdU by the total counts of BrdU cells.

In the second set of experiments, in order to check whether VIP enhanced the expression of nestin in the cycling cell population or not, cells at 5 DIV, were fixed and processed for Ki-67 and nestin expression. Then the proportion of nestin cells that co-labelled for ki-67 with respect to the total population of cycling cells was worked out.

3.3.10 Examining VIP effect on symmetric vs. asymmetric cell division of nestin-expressing cells

Cells were grown for three days under control conditions or 30nM VIP. At day three, cells were fixed and then immuno-histochemically processed for the expression of NUMB and nestin. The proportion of nestin cells that were NUMB negative with regard to the total number of nestin cells was determined under each condition.

3.3.11 Examining VIP effects in the absence of cell proliferation on cells expressing different cell-specific phenotypes

Early on experiments in our study have shown two important facts: firstly, VIP is a trophic factor to postnatal precursor cells (**Figure 3.2**); secondly, VIP increased the

numbers of GFAP and class III ß-tubulin labeled cells in addition to a prominent increase in the numbers and proportions of nestin positive precursor cells (**Figure 3.7**). These observations naturally raised two important questions: (i) Is VIP trophic to mitotic, non-mitotic cells or both? (ii) Is this increase in the number of GFAP and class III ß-tubulin labeled cells a direct effect of VIP on these cells or a secondary effect through the enhanced survival of nestin positive precursor cells? The generation of these lineage restricted progeny cells occurs through successive cell proliferation. In order to address these two questions, it was important to abolish cell proliferation in culture in which case progression from nestin to either GFAP or class III ß-tubulin cells is no longer going to happen and any increase in the number of these cells is a direct effect of VIP on these cells. In addition, as long as dividing cells are no longer present in our culture, this will enable us to look at the effects of VIP on non-mitotic cells. To achieve this aim, cells were cultured as mentioned before under control or 30nM VIP and pulsed with 0.2μM of the anti-mitogenic agent ara-C 48 hrs after plating. Cells were then processed for the expression of the major cell specific phenotypes and counterstained with DAPI to assess the total number of cells in culture. Total cell number, counts and proportions of each cell specific phenotype were determined under both conditions.

3.3.12 Defining VIP effects on the labelling index and growth fraction

In one set of experiments, Cells were grown under control conditions for three or five days. At day 3 or 5, cells in half of the wells were exposed to 1μM VIP and 20μM BrdU for 6 hrs before being fixed. The other half of the wells were exposed to BrdU for the same period of time and maintained under control conditions. In another set of experiments, cells were again grown for 5 days in culture. At 5 DIV cells in half of the wells were pulsed with 30nM VIP and 20μM BrdU for the last 10hrs and 4hrs, respectively before fixation. Again the other cells in the other half of wells were exposed to BrdU only for 4 hrs. In the two sets of experiments, cells were immuno-stained for BrdU incorporation and Ki-67 expression. Finally cells were counterstained with DAPI. The labelling index and the growth fraction were then calculated.

3.3.13 Fluorescence microscopy: Imaging, cell Counting and statistical analysis:

Imaging was performed on an inverted DM IRB microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). The area of a 20× field was measured using a 255 μm grid graticule slide (Microbrightfield, Williston, USA). Cell counting was performed on 5-6 random 20× fields per well using the Open Lab image-capturing system version 2.1 (Improvision, Lexington, MA, USA). Raw data from the 20× field counts were averaged and plotted ± SEM and expressed as cells/mm² per well, based on a sample of four to eight wells per condition per repeat. All experiments were repeated at least two to three times. One experiment consisted of four hippocampi from two animals, pooled and prepared as described above. Data points were plotted using GraphPad Prism data analysis software (GraphPad inc. USA). The statistical significances between the means was assessed by either Student's test for single comparisons and by ANOVA followed by post hoc tests for multiple comparisons, with $P<0.05$ considered significant.

3.3.14 Time-lapse microscopy: imaging, counting and analysis

For time-lapse light microscopy, hippocampal progenitor cells were again plated on poly-L-lysine coated plastic wells. Cells were incubated in a custom-made chamber at 37°C in a humidified atmosphere (5.0% CO2 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 48 hrs. An automatic shutter was used to minimize phototoxicity. The Cell^P 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 studies for newly born cells. 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 were then compared under control conditions with those under VIP conditions. The statistical significances between the means was assessed by Chi square test, with $P<0.05$ considered significant.

3.4 Results

3.4.1 VIP enhanced the survival of hippocampal cells in culture

Primary hippocampal cell cultures were generated from postnatal 7-10 day-old rats and cultured under standard control conditions (Neurobasal A/ B27/ Glutamine) or under a wide range of concentrations of VIP between 1pM and 10μM. The total counts of cells in culture under all VIP concentrations were higher than those under control conditions (**Figure 3.1 A & B**). As low as 1pM VIP was sufficient to increase the total number of cells significantly over control conditions. At a range of VIP concentrations between 3nM and 1μM the increase in the total number of cells was the same. It was not until 10μM till we observed an additional significant increase in the total number of cells **(Figure 3.1 B)**.

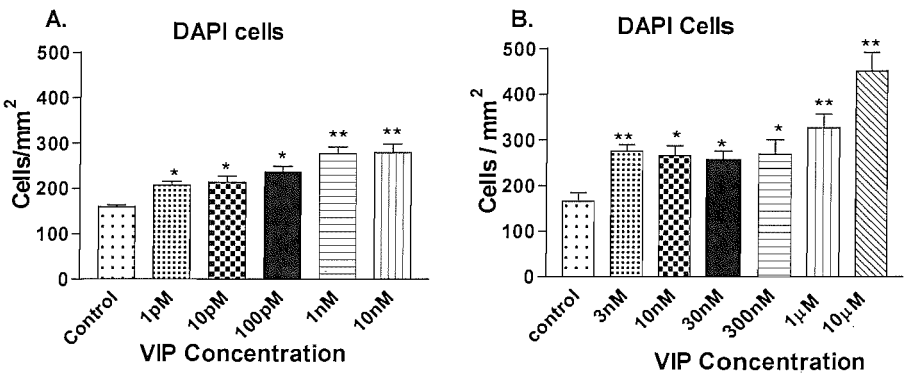


Figure 3.1: Comparing total cell counts under control or different concentrations of VIP. Cells were grown in culture for 5 days before being fixed. All the data points are mean ± SEM for 8-15 values from 2 to 3 different experiments. Data represent mean ± SE based on a sample that represent at least 12 wells per condition from three different experiments. Comparisons between control and different conditions are one way ANOVA with Dunnetts' multiple comparison test (*, $p < 0.05$, **, $p < 0.01$).

3.4.2 VIP is not proliferative at concentrations of less than 1μM peptide

In a set of experiments, cells were grown for 3 or 5 days under standard control conditions or VIP of 3nM-1μM peptide concentration. The rate of cell proliferation at day 3 and day 5 was measured using a 4 hr pulse of BrdU prior to tissue fixation. Three and five days growth under 3-300nM VIP yielded no increase in the total

number of BrdU incorporated cells or in the proportions of BrdU positive cells with respect to the total number of cells (**Figure 3.2 A-D**). It was up until 1μM VIP when a significant increase in the total number of BrdU positive cells and their proportions with respect to the total number of cells was observed (23.3 ± 3.4 % vs. 12.0 ± 2.5 % under control conditions at 3 days) (**Figure 3.2 B**). At 5 DIV, the proportion of BrdU positive cells were only 5.5 ± 1.5 % under control conditions increased significantly to 19.4 ± 3.4 % under 1μM VIP conditions (**Figure 3.2 D**).

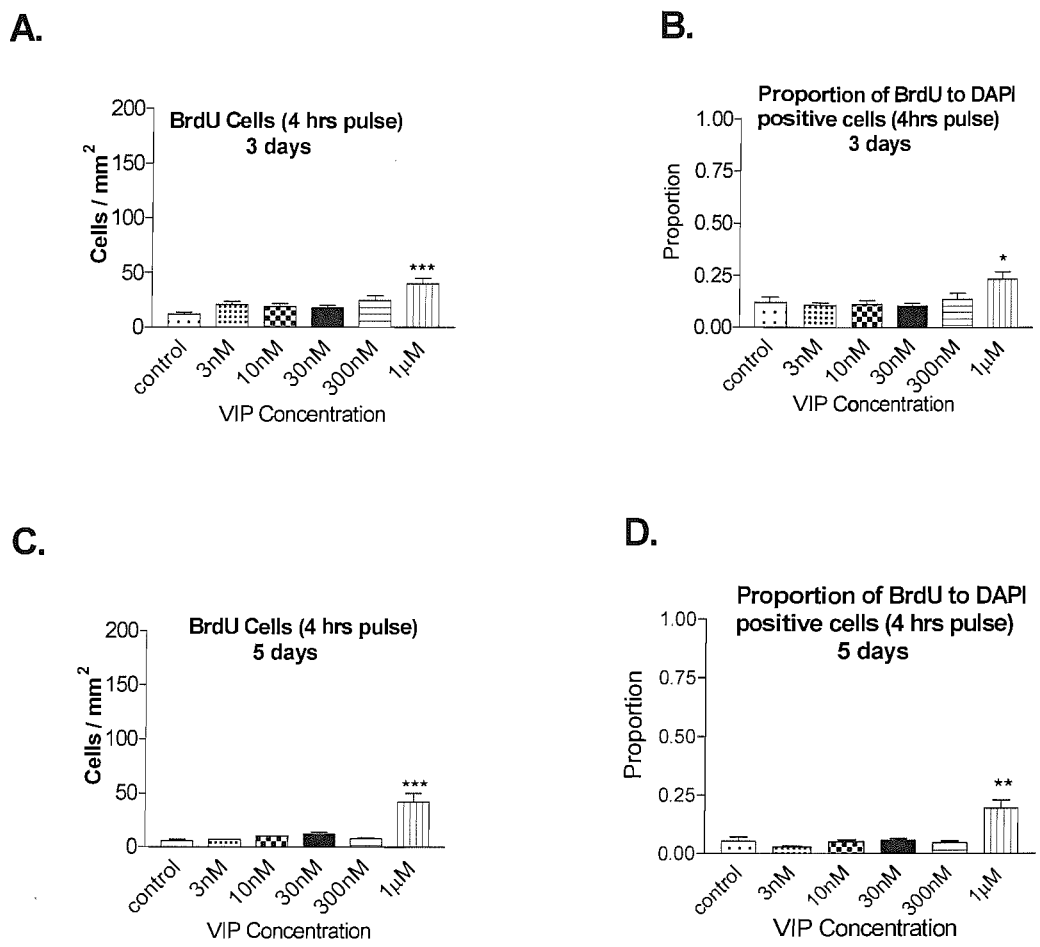


Figure 3.2: VIP is not proliferative at concentrations of less than 1μM. Hippocampal cells were grown for 3 (A & B) or 5 (C & D) days under control or control plus VIP (3nM-1μM). BrdU was added to culture medium 4 hrs before fixation. Cells were then immuno-stained for BrdU incorporation. (A)Counts of BrdU incorporating cells (B)Proportions of BrdU incorporating cells with respect to the total number of cells in three days cultures. (C) Counts of BrdU incorporating cells and (D) Proportions of BrdU incorporating cells with respect to the total number of cells in five days cultures. Data represent mean ± SE based on a sample that represent at least 12 wells per condition from three different experiments. Comparisons between control and different VIP conditions are one way ANOVA with Dunnetts' multiple comparison test (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

3.4.3 VIP at 3nM to 1μM decreased cell death in culture

Having shown that VIP increased the total number of cells in culture at concentrations between 3nM and 1μM with no significant proliferative activity at nanomolar concentrations has led us to examine VIP effects on cell death in culture. To address this issue, cells were cultured for 3 or 5 days in the absence or presence of a range of VIP concentrations (3nM-1μM) and exposed to the cell death marker PI. DAPI stain was used to quantify the total cell number. Cell death under different conditions was assessed as detailed in **Section 2.6.** Cell death at day 3 and 5 decreased under different VIP concentrations significantly compared to control conditions. The proportion of cells dropped from 44 ± 6 % and 38 ± 6 % under control conditions to 17 ± 2 % and 8 ± 1 % under as low as 3 nM at day 3 and day 5, respectively (**Figure 3.3 B & C**). This decrease in cell death under nanomolar VIP concentrations with no increase in the number of BrdU incorporated cells suggests that VIP increased cell density at these low concentrations mainly through enhanced cell survival (**Figure 3.3 A & B**).

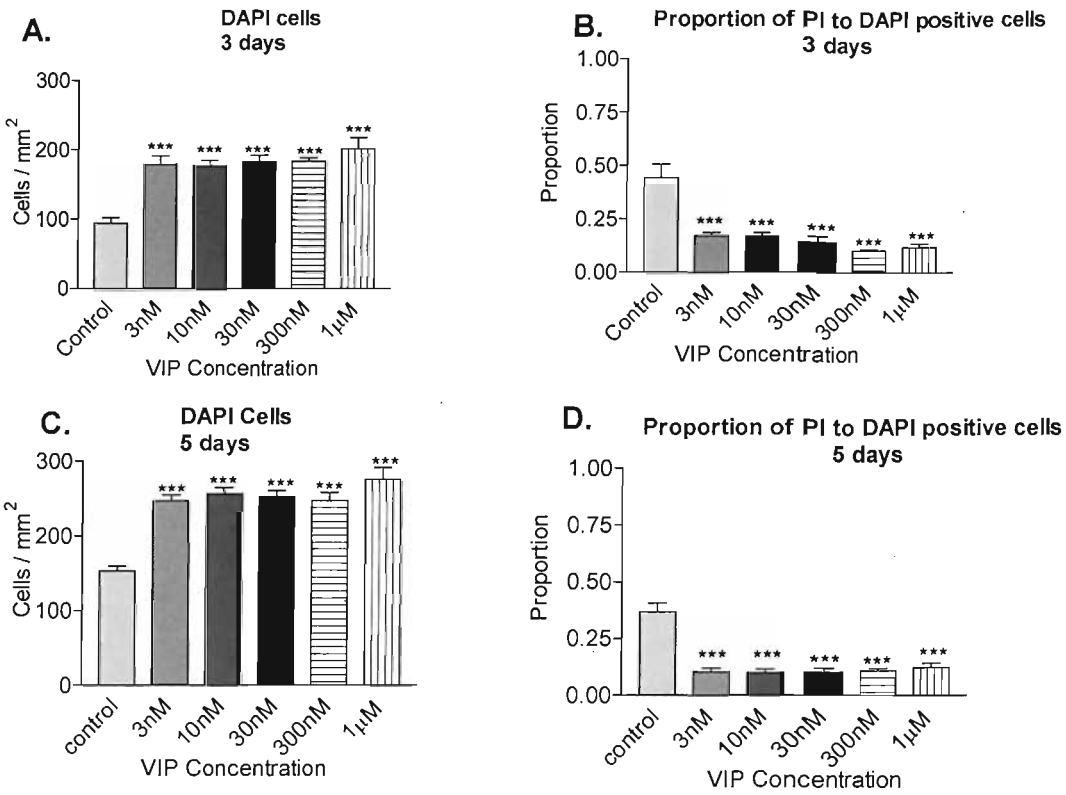


Figure 3.3: **Assaying cell death in the presence or absence of 3nM-1μM VIP.** DAPI positive cells indicate total number of cells in culture. Dead cells represent the proportion of cells incorporating PI with respect to the total number of cells. (A & C) Total cell counts at 3 and 5 days, respectively. (B & D) proportions of dead/ cells to the total number of cells at 3 and 5 days, respectively. Data represent mean ± SE based on a sample that represent at least 12 wells per condition from three different experiments. Comparisons between control and different VIP conditions are one way ANOVA with Dunnetts' multiple comparison test (***, $p < 0.001$).

Using the protocol detailed in **Section 2.6**, we assessed cell death in culture and quantified total cell counts as cells were exposed to DAPI before fixation. This enabled us to work out the proportions of dead/dying cells with respect to total cell numbers (**See Figure 3.4: images A, B and C**) in live prefixed cultures.

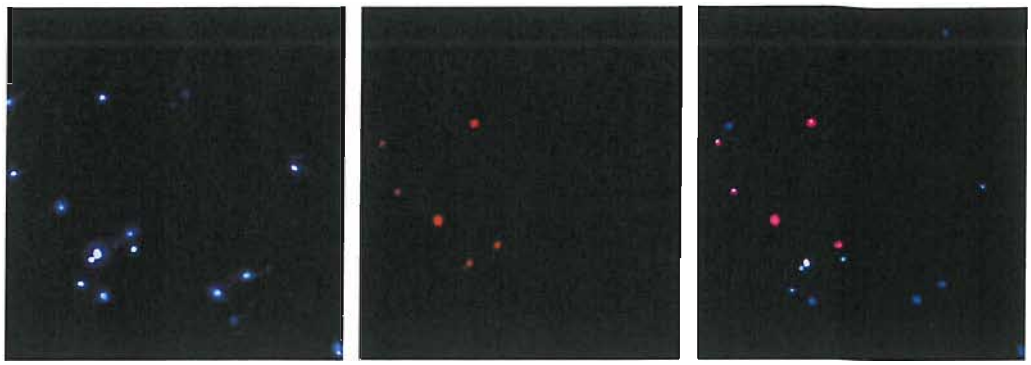


Figure 3.4: DAPI and PI staining of living cells in culture. Hippocampal cells were grown for 3 and 5 days in culture. At the indicated time, cells were pulsed with PI for 40 minutes followed by DAPI for another 40 minute. Cells were then washed free of DAPI and maintained in warm medium while being imaged. (A) DAPI positive cells = total number of cells in culture, (B) PI positive cells = dead or cells, (C) merged image of DAPI and PI. Those cells that are DAPI positive but PI negative are the viable cells in culture.

3.4.4 VIP at 30nM concentration has no effect on the labelling index and growth fraction

Cells were grown for 3 or 5 days under control conditions. At day 3 or 5, cells in half of the wells were pulsed with 30nM VIP (the physiological concentration) and BrdU for the last 10 and 4 hrs, respectively. At the same time, control cells in the other half of the wells were given a pulse of BrdU for the last 4 hrs before fixation. Cells were processed for BrdU incorporation and the expression of the proliferation marker Ki-67. Total cell counts, and the numbers of BrdU positive cells and Ki-67 expressing cells were determined. The labeling index and the growth fraction were calculated under control and VIP conditions.

At day 3 and 5, the treatment of cultured hippocampal cells with 30nM VIP did not change the total number of cells, BrdU incorporating cells or Ki-67 cells over control conditions (**Figure 3.5 and Figure. 3.6**). Furthermore, the labeling index, the growth fraction and the proportion of BrdU positive cells with respect to the total number of cells were not different from control conditions (**Figures 3.5 and 3.6**). These results in agreement with our previous results presented in **Sections 3.4.2 and 3.4.3** suggest that VIP has a purely trophic rather than proliferative effect at 30nM peptide concentration.

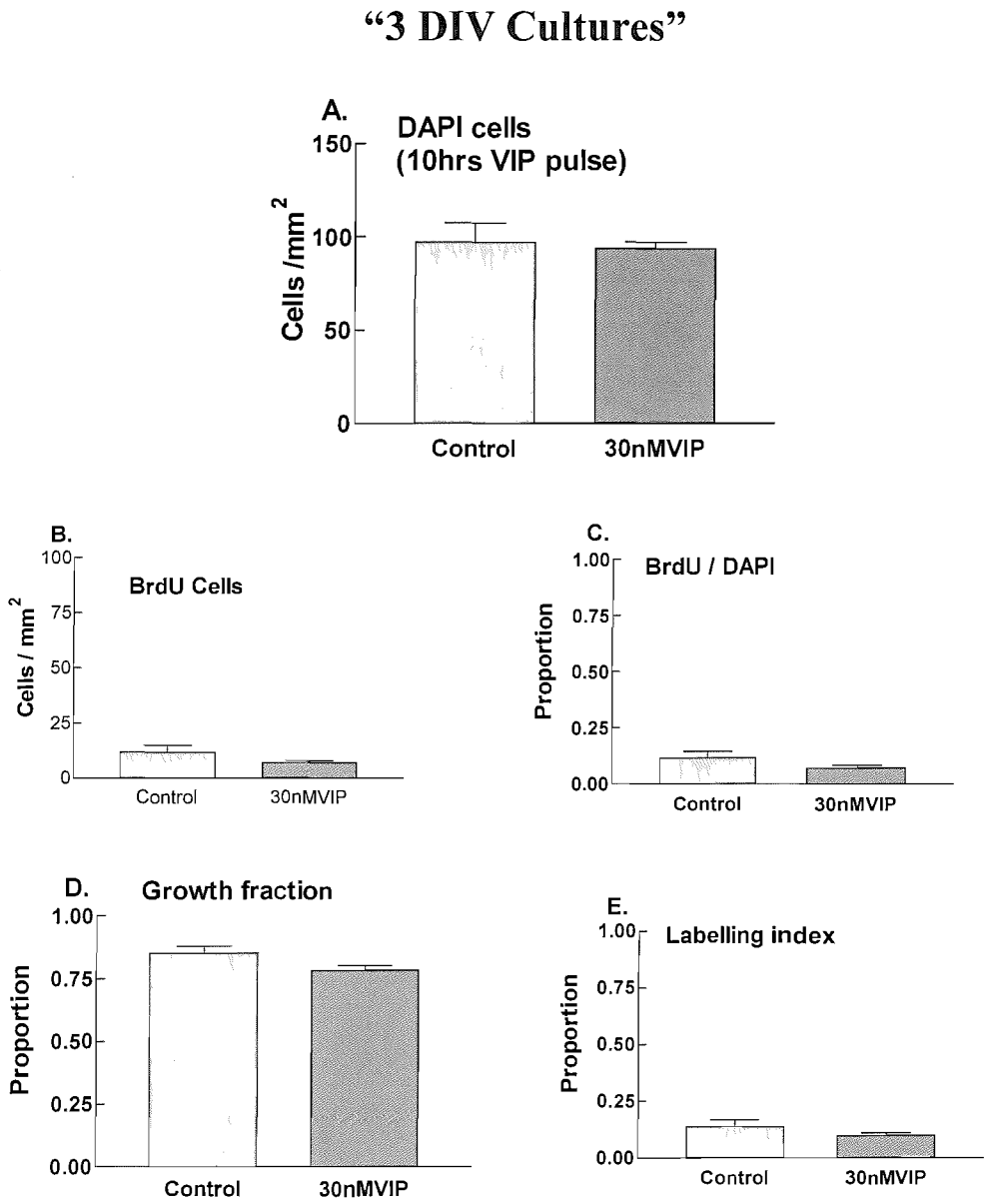


Figure 3.5: **30nM VIP pulse for 10 hrs has no effect on cell proliferation at day 3**. Culture hippocampal cells were pulsed in half of the wells with 30nM VIP for 10 hrs before fixation. Cell proliferation was assessed with a combination of Ki-67 expression and BrdU incorporation after a 4 hr pulse before fixation. (A) total cell counts, (B) Counts of cells expressing Ki-67, (C) Counts of cells incorporated BrdU, (D)the labelling index, (E) the proportions of BrdU positive cells, and (F) the growth fraction. Data represent mean ± SE based on a sample that represent at least 12 wells per condition from three different experiments. Comparisons between control and VIP conditions are simple *t*-student test.

"5 DIV cultures"

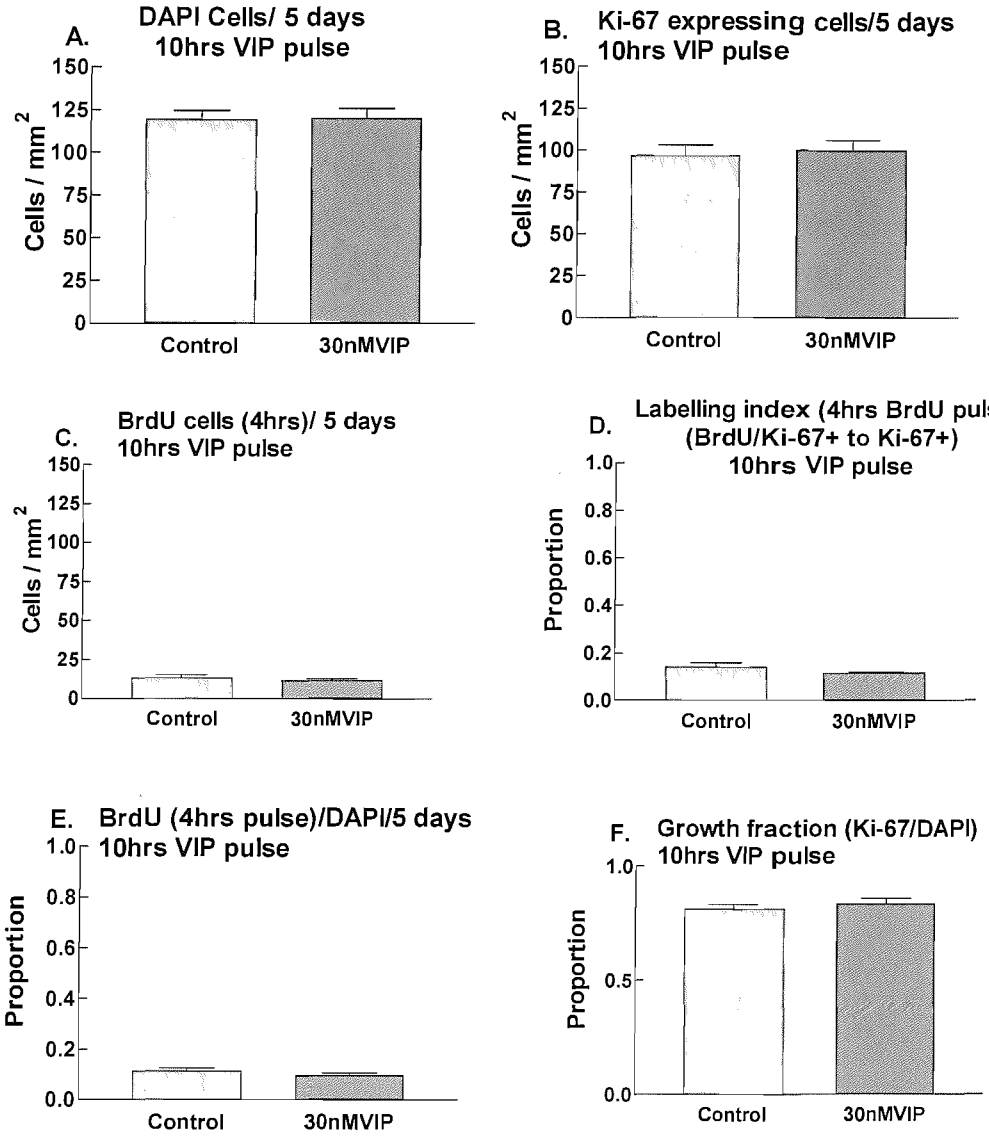


Figure 3.6: **30nM VIP pulse for 10 hrs has no effect on cell proliferation at day 5.** Culture hippocampal cells were pulsed in half of the wells with 30nM VIP for 10 hrs before fixation. Cell proliferation was assessed with a combination of Ki-67 expression and BrdU incorporation after a 4 hr pulse before fixation. (A) total cell counts, (B) Counts of cells expressing Ki-67, (C) Counts of cells incorporated BrdU, (D)the labelling index, (E) the proportions of BrdU positive cells, and (F) the growth fraction. Data represent mean ± SE based on a sample that represent at least 12 wells per condition from three different experiments. Comparisons between control and VIP conditions are simple *t*-student test.

3.4.5 VIP enhanced the survival of different cell-specific phenotypes

Hippocampal cells dissociated from postnatal rat hippocampi were cultured for 5 days. Cells were then fixed and immunocytochemically processed for the expression of the progenitor cell marker nestin, the neuronal marker class-III ß-tubulin and the glial marker GFAP. Our results indicate that, in parallel to the increase in the total cell numbers of cells under VIP conditions, VIP (30nM) increased the numbers of nestin-, TuJ1- and GFAP- expressing cells (**Figure 3.7 A-F**). Nestin-expressing cells' numbers increased from 78.9 ± 13.4 cells /mm^2 under control conditions to 233.4 ± 3.4 cells/mm^2 under VIP treatment (**Figure 3.7 A**). Class III ß-tubulin also increased to 110.4 ± 12.1 cells/ mm^2 under 30nM VIP compared to 62.0 ± 2.3 cells/ mm^2 under control conditions (**Figure 3.7 C**). Further more, our data showed that the number s of GFAP labelled cells increased from 62.8 ± 3.2 cells /mm^2 under control conditions to 104.5 ± 4.3 cells/mm^2 under 3nM VIP (**Figure 3.7 E**).

By analysing the proportions of each cell-specific phenotype with respect to the total number of cells, we found that VIP induced a significant proportional increase in nestin positive cells (0.64 ± 0.04 vs. 0.44 ± 0.03) under control conditions (**Figure 3.7 B**). However, no change in the proportions of class-III ß-tubulin and GFAP immuno-reactive cells was observed (**Figure 3.7 D & F**).

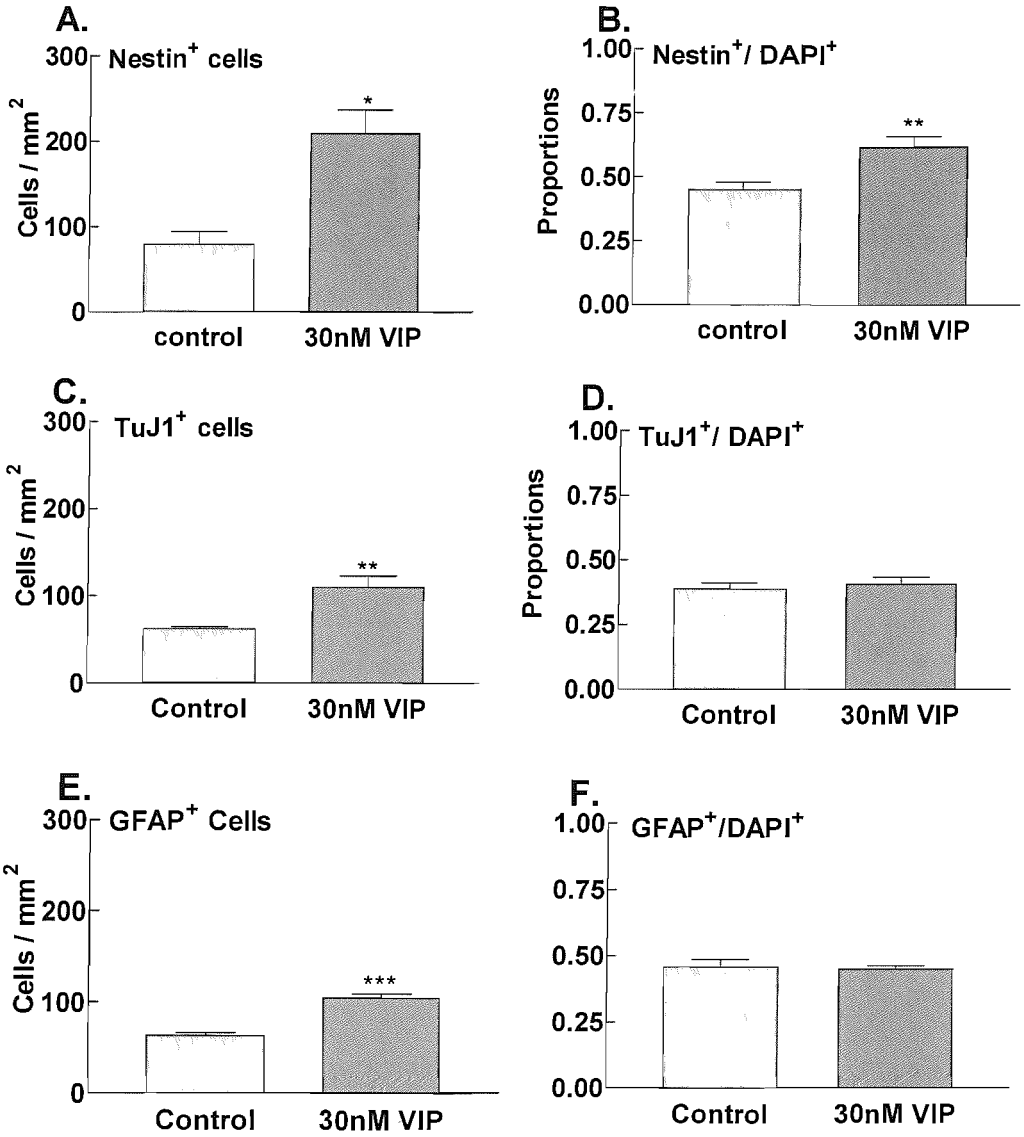


Figure 3.7: Effects of VIP on the putative progenitor cell marker nestin, the neuronal marker class-III ß-tubulin and the mature astrocyte marker GFAP positive cells' numbers and proportions. Data represent mean ± SE based on a sample that represent at least 12 wells per condition from three different experiments. Comparisons between control and VIP conditions simple *t*-student test with *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

3.4.6 VIP enhanced the survival of the progeny cells mainly through a trophic effect on progenitor cells

Our forgoing data and some of the literature cited indicate that progenitor cells undergo successive proliferation to give their progeny (Seri, 2004). Also we found that VIP at trophic concentrations increased the numbers and proportions of nestin cells and the numbers of GFAP and class III ß-tubulin cells but not their proportions. To address whether VIP acts on these cells directly to enhance their survival or whether it does so indirectly through an effect on the progenitor cells which in their turn give these progeny, we abolished cell proliferation in culture and exposed cells to VIP. Cultured cells were then processed for the expression of nestin, GFAP and class III ß-tubulin.

By analysing total number of cells under different conditions (**Figure 3.8 A**), we found that the addition of 0.2 μM ara-C resulted in a significant drop in total number of cells (39.8 ± 2.7 cells/mm² vs. 63. ± 3.3 cells/ mm² under control conditions). Interestingly, treatment with 30nM of VIP to cells exposed to ara-C restored the number of cells nearly back to control conditions (61.4 ± 2.3 cells/mm²). Consistent with our data before, total cell counts increased under 30nM VIP over control conditions (97.7± 4.3 cells/mm² compared to 63. ± 3.3 cells/ mm²).

Quantification of nestin expressing cells (**Figure 3.8 B**) revealed that the number of nestin expressing progenitor cells dropped biologically but did not reach the statistical significance once cells were treated with the anti-mitogenic agent ara-C with relative to control conditions (17.3 ± 1.9 cells/ mm² compared to 29.9 ± 3.6 cells/ mm²). The addition of 30nM VIP to cells treated with ara-C failed to rescue nestin positive cells. However, as I showed before the addition of 30nM VIP to control conditions increased significantly the number of nestin labelled cells from 29.8 ± 3.6 cells/ mm² to 61.6 ± 5.9 cells/mm².

In addition, our results indicated that the addition of ara-C again resulted in a biologically but not statistically significant decrease in the number of cells expressing the glial marker GFAP with relative to control conditions (20.0 ± 2.6 cells/ mm² vs. 27.2 ± 4.4 cells/ mm²). Co-treatment of cells with VIP restored the number of GFAP

cells back to control counts (26.5 ± 2.7 cells/mm²) (**Figure 3.8 C**). However this effect has not reached the statistical significance. Treatment of control cells with VIP resulted in a significant increase in the number of GFAP cells over control conditions.

In contrast, counts of cells expressing the neuronal marker class III ß-tubulin showed a significant drop in the number of these cells once cells were exposed to ara-C (22.2 ± 1.4 cells/mm² vs. 39.5 ± 4.2 cells/mm² (**Figure 3.8 D**). The addition of VIP failed to rescue class III ß-tubulin expressing cells from the neurotoxic effects of ara-C. As I showed before 30nM VIP increased the numbers of class III ß-tubulin expressing cells over control conditions (64.7 ± 5.2 cells/ mm² compared to 39.5 ± 4.2 cells /mm²).

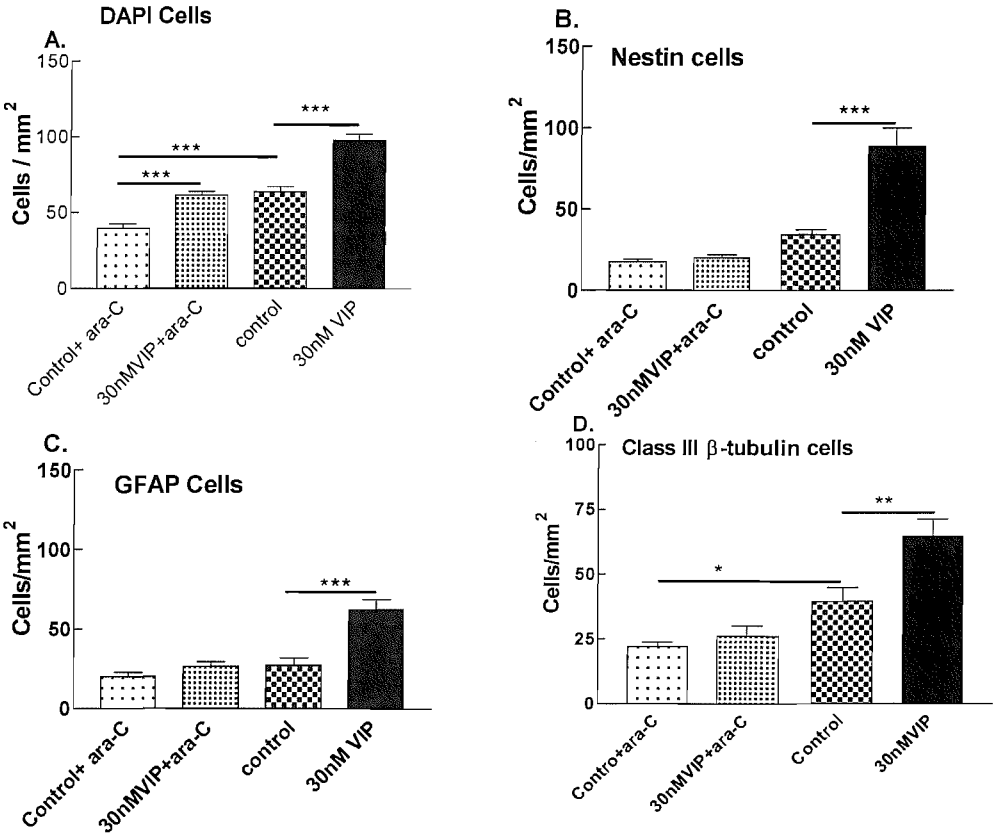


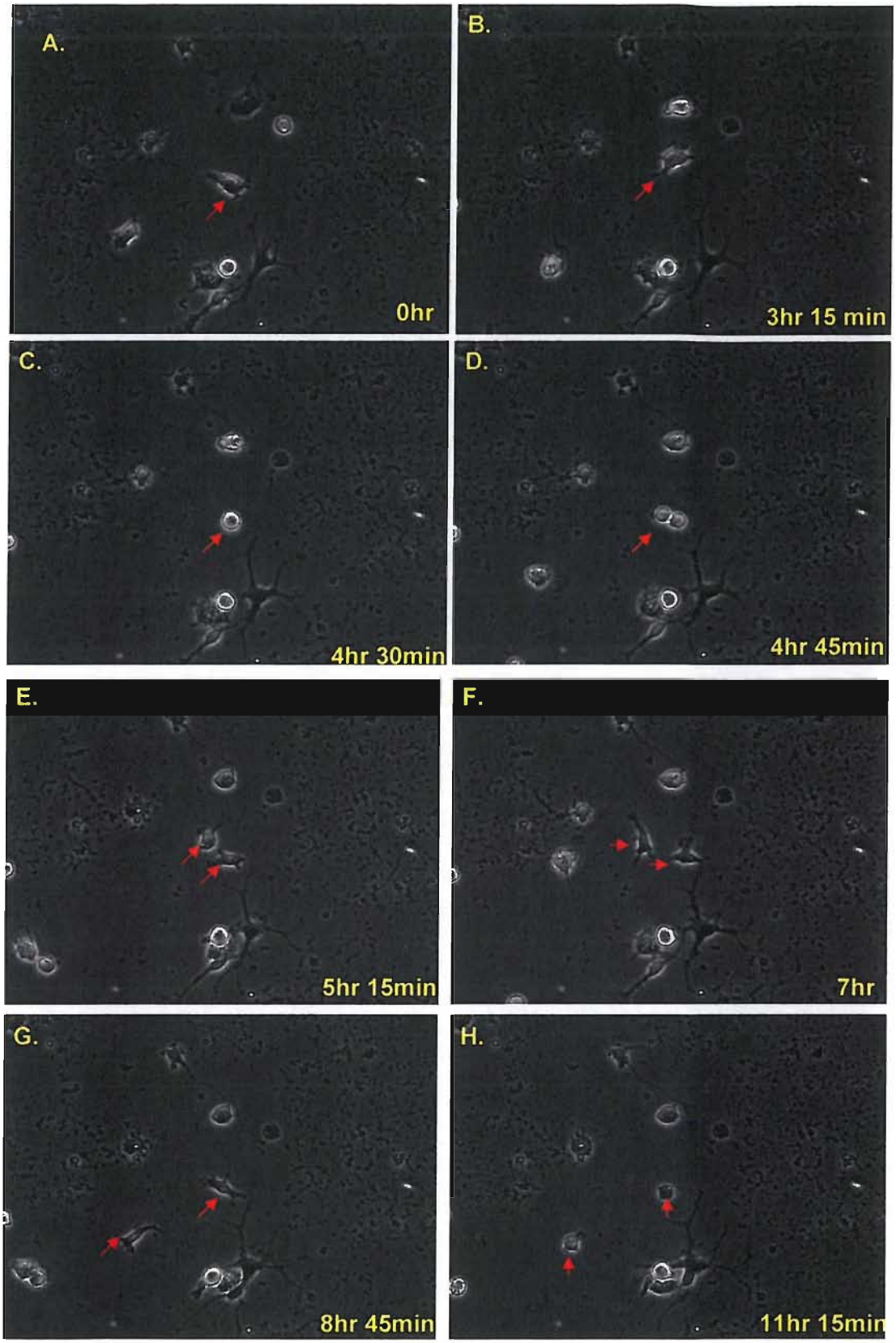
Figure 3.8: VIP effects in the absence of cell proliferation on the major cell phenotypes. Hippocampal cells were grown for 5 days under control plus ara-C, control plus ara-C and VIP, control or control plus VIP conditions. Ara-C was added to cultured cells after 46 hrs in culture while VIP was added at 2 hrs after intial plating. (A) total cell counts (DAPI), (B) counts of nestin expressing cells, (C) counts of GFAP expressing cells, and (D) the numbers of class III ß-tubulin expressing cells. Values represent mean ± SE based on a sample that represent at least 8 wells per condition from 3 experiments. Comparisons between different conditions, one way ANOVA with Bonferronis multiple comparison test (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

3.4.7 VIP enhanced the survival of newly-born cells

Hippocampal progenitor cells were grown in the presence and absence of 30nM VIP for three days. On day three, cells were staged on the time lapse microscopy for the next 48 hrs, where an image was acquired every 15 minutes. Time-lapse movies were then processed and studies. Each newly-born cell was morphologically followed up to predict its survival state over the 48hrs, with cell membrane burst and disintegration and subsequent size shrinkage as signs of cell death (**Figure 3.9 A-J**). In 9 fields from three different wells for each condition (controls and VIP), the number of newly born cells (42 cells) under control was not significantly different from those grown under VIP treatment (46 cells). However, 29% of the newly-born cells died over the period of follow up under control conditions compared to only 5% under VIP treatment. This difference is statistically significant (**Table 3.1**).

	Newly-born cells	
	Survived	**Died**
Control	**30 ± 2.5**	**12 ± 1.2**
VIP(30nM)	**44 ± 1.8**	**2 ± 0.4**

Table 3.1: VIP effects on the survival of newly born cells in hippocampal cell culture as measured by time-lapse microscopy. Hippocampal cells were grown for 3 days before being staged and imaged on time lapse microscope. One image was acquired from each field per 15 minute for 48 hrs. Three fields per well were selected from three wells per condition per repeat. Values represent mean ± SE based on a sample that represent at 9 fields per experiment from three different expriments. Comparisons between different conditions, Degrees of freedom: 1, Chi-square = 9.63 and $p \leq 0.01$.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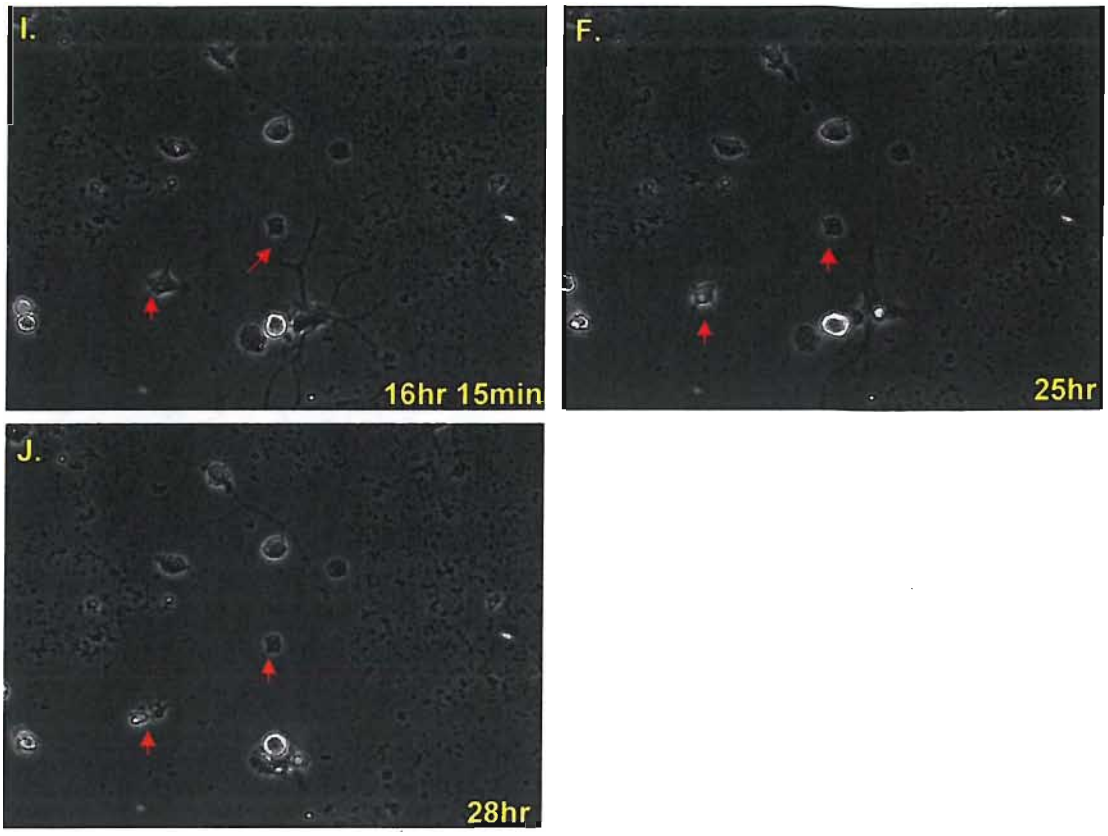


Figure 3.9: VIP enhanced the survival of newly-born cells in hippocampal cell culture. Hippocampal cells were harvested from postnatal pups (P7-10) and grown for three days under standard conditions in the incubator. Cells were then staged on an inverted microscope for serial time lapse imaging. An image was acquired every 15 minute for 48hrs. Red arrows indicate the cell (s) of interest. (A) the reference image at the beginning of the experiment, (B) the arrowed cell has become brighter (C) the cell in the bright phase and adopted a around shape, (D) the cell is in late phases of cell division (cytokinesis), (E) the newly-born daughter cells have adopted a flat multi-process morphology, (F) while one daughter cell stayed in place the other one has started to migrate downwards, (G) the daughter cell that has migrated has completed its journey, (H) Both daughter cells adopted round shape with the upper one showing irregular edges, (I) the upper cell is becoming darker and its boundaries are becoming more irregular, (F) both daughter cells are becoming smaller in size (pyknotic), and (J) lower daughter cell has burst and the upper one has become very pyknotic indicating cell death. The time on each image indicates the time at which each serial image was acquired from the start of the experiment.

3.4.8 VIP increased the proportions of nestin positive cells that co-express neither GFAP nor TuJ1 subpopulation of cells in culture

We have demonstrated that VIP at nanomolar concentrations applied to hippocampal cells increased the numbers and proportions of nestin-positive precursor cells after 5 days in culture. However, nestin positive cells in the postnatal hippocampus have been divided into two subpopulations based on the co-expression of GFAP or TuJ1 (**Figure 3.10 & 3.11**). It is therefore important that we investigate the effects of VIP at nanomolar concentrations on these nestin-positive subpopulations of cells. For this purpose, hippocampal cells were grown in culture for 5 days under standard control or control plus 30nM VIP. Cells were then processed for the co-expression of nestin and GFAP or TuJ1. The proportions of each subpopulation of nestin-expressing cells with respect to total number of cells were determined under different conditions.

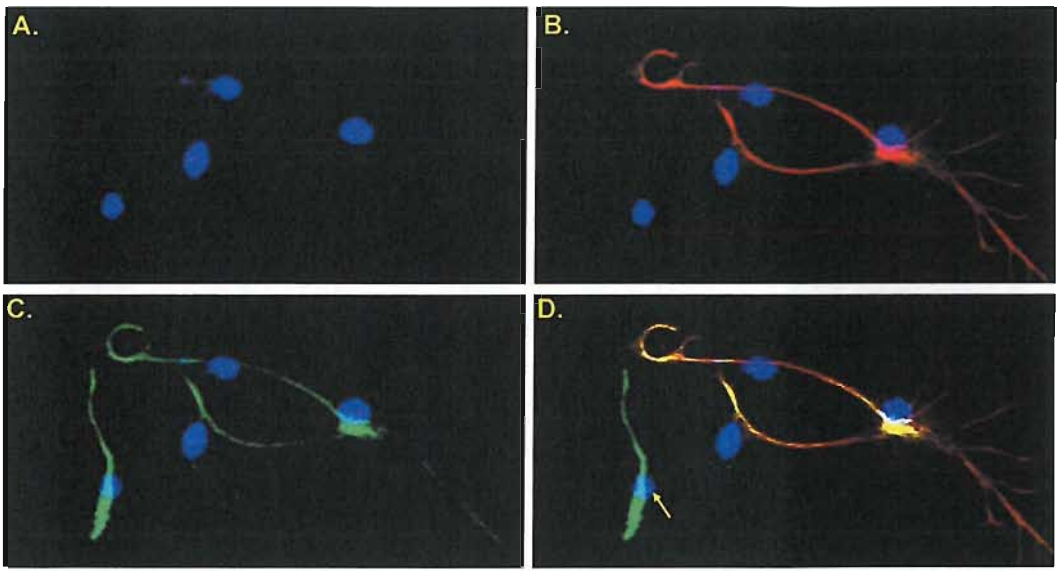


Figure 3.10: VIP enhances the generation of nestin positive GFAP negative cells. After 5 DIV, hippocampal progenitor cells were double-immuno-labeled for the expression of nestin and GFAP and then counterstained with the nuclear stain DAPI. (A) DAPI stained nuclei, (B) GFAP labeled cells, (D) Nestin-expressing cells and (D) the merged image. The yellow-arrowed cell is a representative cell that is nestin positive but GFAP negative (i.e. transient amplifying cell population; the VIP-responsive cell population).

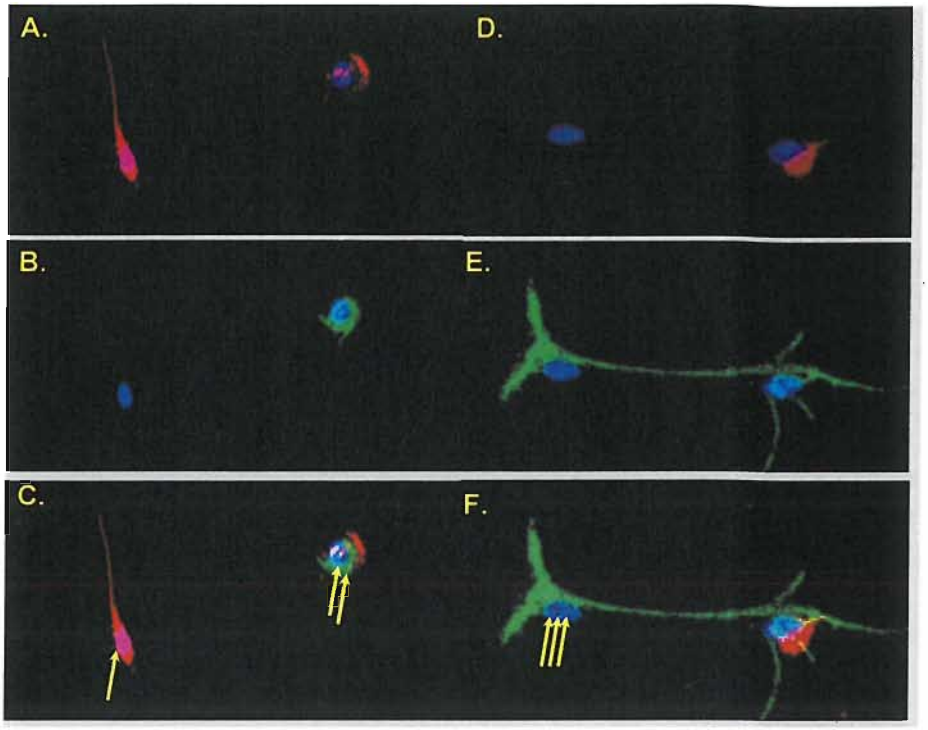


Figure 3.11: Nestin stem/progenitor hippocampal cells coexpress the neuronal marker TuJ1. Hippocampal progenitor cells were grown for 5 days under standard control conditions or control plus 30nM VIP. Cells were then fixed and double-immuno-labeled for nestin and TuJ1. (A & D) The neuronal marker TuJ1-expressing cells, (B & E) nestin-expressing cells, (C & F) the overlay image, **Yellow arrows**: single arrow; mature neuronal cell that does not express nestin, double arrows; nestin+TuJ1+ cells; precursor cells that have committed neuronal lineage, triple arrows; nestin+TuJ1- transient amplification cell population (the VIP-responsive cell population).

VIP at 30nM increased the proportion of nestin positive but GFAP negative cells (**Figure 3.10**) from 0.22 ± 0.02 under control to 0.33± 0.01 **(Figure 3.12 A)**. This increase is statistically significant. However, VIP treatment was devoid from any effect on nestin-expressing cells that co-expressed GFAP **(Figure 3.12 B)**.

In addition, the proportion of nestin-expressing cells that were TuJ1 negative (**Figure 3.11**) increased significantly under VIP treatment to 0.50 ± 0.01 compared to 0.30 ± 0.02 under control condition **(Figure 3.12 C)**. No change was observed in the proportion of nestin cells that co-expressed TuJ1 **(Figure 3.12 D)**.

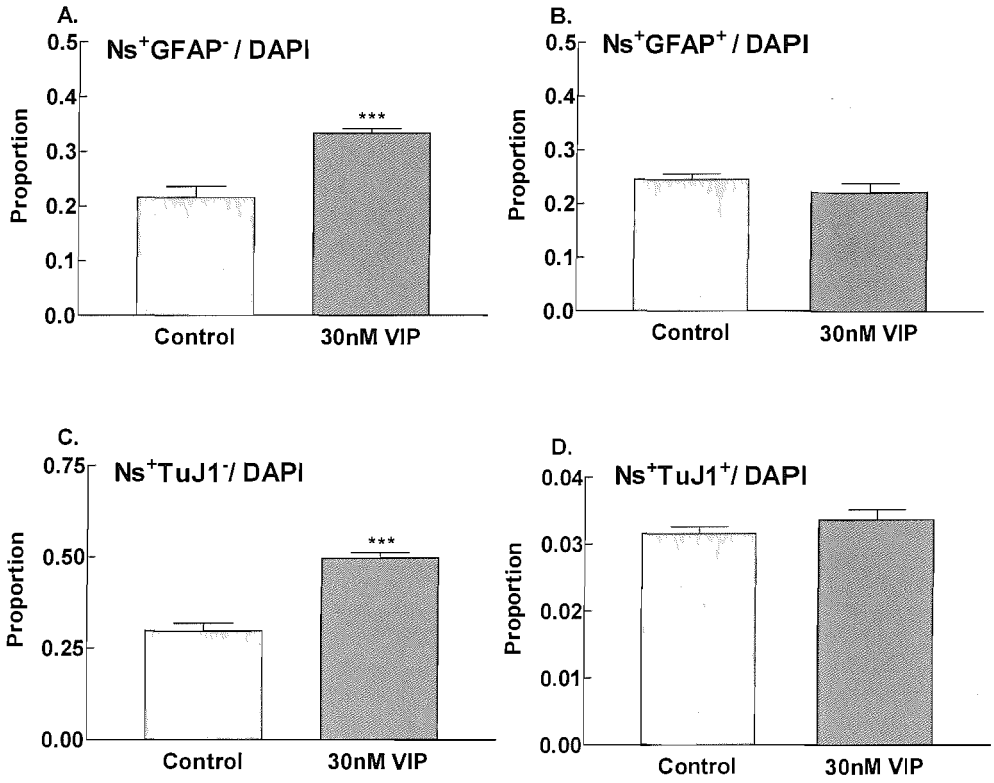


Figure 3.12: **VIP increased the proportion of nestin positive GFAP or TuJ1 negative cells in culture**. Cells were grown for 5 days under control or 30nM VIP condition. Cells were immuno-labelled for nestin and GFAP or TuJ1. (A) proportion of nestin positive but GFAP negative cells, (B) proportions of cells that were nestin and GFAP positive, (C)proportion of nestin cells that did not express TuJ1, (D) proportion of nestin that co-expressed TuJ1. Values represent mean ± SE based on a sample that represent at least 8 well per condition from two different experiments. Comparisons between control and VIP conditions are, Students' *t*-test; (***, $p < 0.001$).

3.4.9 Within the cycling and growing cell population, VIP enhanced the generation of nestin-expressing cells

Hippocampal cells were grown for five days and processed for the expression of the endogenous cell cycle marker Ki-67 and nestin. Under control conditions, the proportion of nestin cells that colabeled with Ki-67 with respect to the total number of Ki-67 cells (cycling cells) was 0.67 ± 0.01. 30nM VIP treatment increased this proportion significantly to 0.80 ± 0.02 **(Figure 3.13 A)**. In parallel, after a relatively long pulse of BrdU (24hrs), the proportion of nestin cells that incorporated BrdU with respect to the total number of BrdU incorporated cells increased significantly under VIP treatment (0.51 ± 0.02) compared to control (0.21 ± 0.05) conditions **(Figure 3.13 B)**.

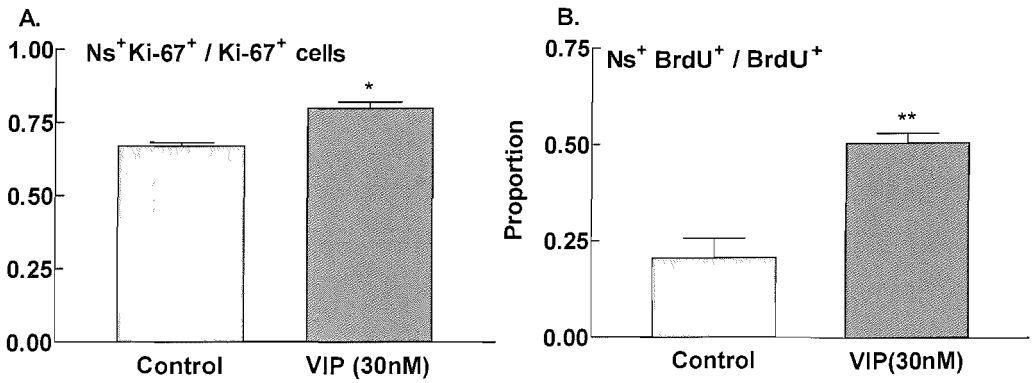


Figure 3.13: VIP enhanced the proportion of nestin-expressing cells within the cycling/ growing cell population. Hippocampal cells were grown for 5 days and then either pulsed with BrdU for the last 24 hr and processed for BrdU and nestin immunoreactivity or fixed and processed for Ki-67 and nestin expression. (A) The proportion of nestin positive cells that colabeled for Ki-67 with respect to total number of Ki-67 cells and (B) the proportion of nestin cells that incorporated BrdU with respect to the total number of BrdU immunoreactive cells. Values represent mean ± SE based on a sample that represent at least 8 well per condition from two different experiments. Comparisons between control and VIP conditions are, Students' *t*-test; (*, $p < 0.05$, **, $p < 0.01$).

3.4.10 VIP enhanced nestin-expressing cells to divide symmetrically

At 3DIV, hippocampal cells in culture were processed for the expression of nestin and NUMB. The proportion of nestin expressing cells that are NUMB negative was 0.189 ± 0.02 under control conditions. This proportion dropped significantly to 0.08 ± 0.02 under 30nM VIP **(Figure 3.14)**.

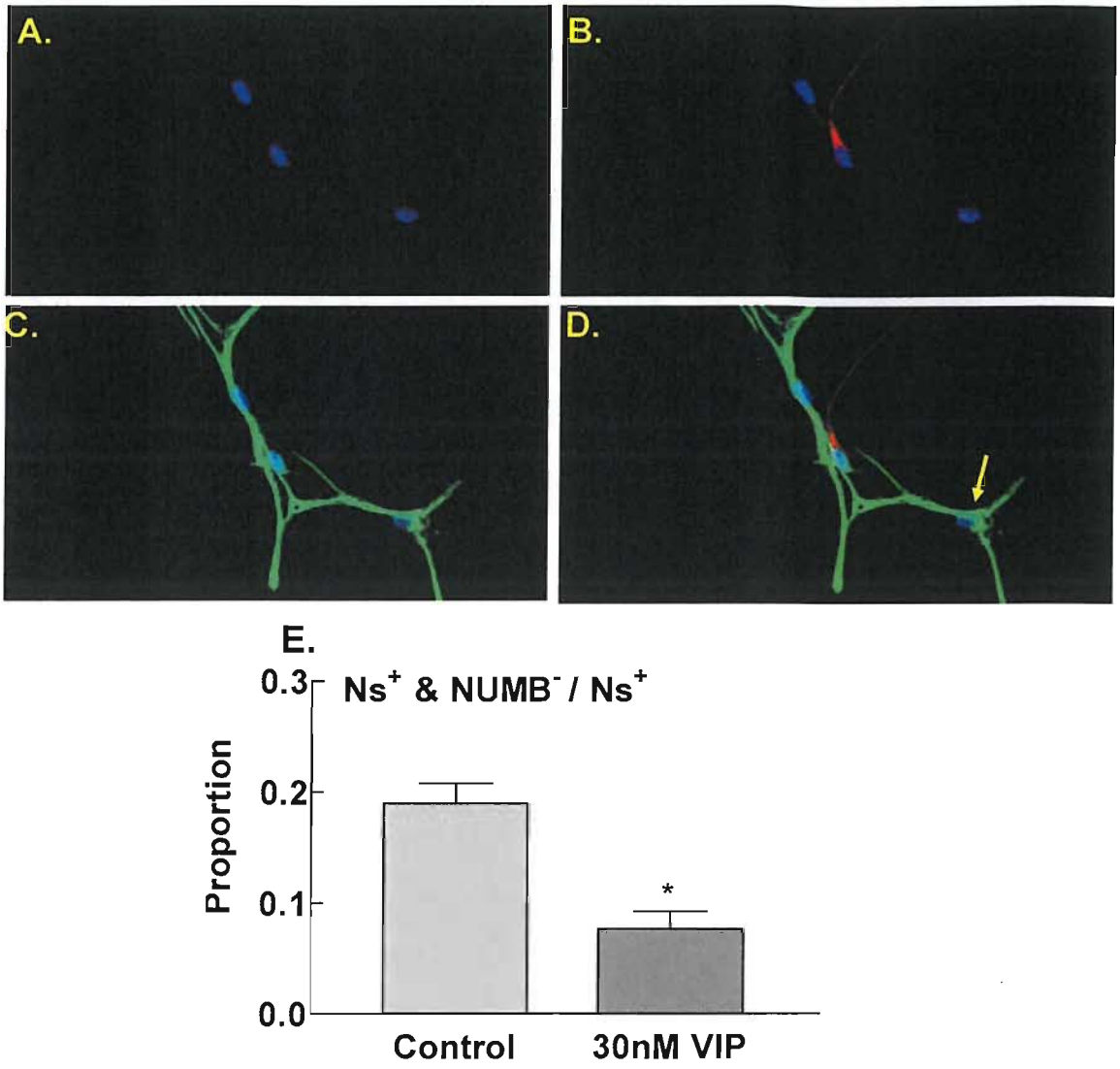


Figure 3.14: VIP enhanced symmetric cell division of nestin-expressing cells. Hippocampal cells were grown for 3 days under control or 30nM VIP. Cells were then processed for the co-expression of nestin and NUMB. (A) DAPI stained nuclei, (B) NUMB expressing cells, (C) Nestin-expresing cells, (D)the overlay image, the **single arrowed** cell is a nestin positive but NUMB negative cell (the second half of asymmetrical cell division), and (E) The proportion of nestin cells that were NUMB negative with respect to the total number of nestin positive cells were then determined. Values represent mean ± SE based on a sample that represent at least 8 well per condition from two different experiments. Comparisons between control and VIP conditions are, Students' *t*-test; (*, $p < 0.05$).

3.4.11 VIP at 1μM, but not at 30nM, increased the number and proportion of NG2 cells

Hippocampal cells were grown for 5 days under control conditions, control plus 30nM or 1μM VIP. Processing the cells in culture for the expression of NG2 revealed that VIP at 1μM significantly increased the numbers and proportions of NG2 cells from 13.7 ± 2.6 cells / mm² and 0.09 ± 0.01 (under control conditions), to 31.5 ± 5.6 cells / mm² and 0.20 ± 0.03, respectively (**Figure 3.15 A & B**). VIP at 30nM resulted in a small but insignificant increase in the number and proportions of NG2 cells in hippocampal cell cultures.

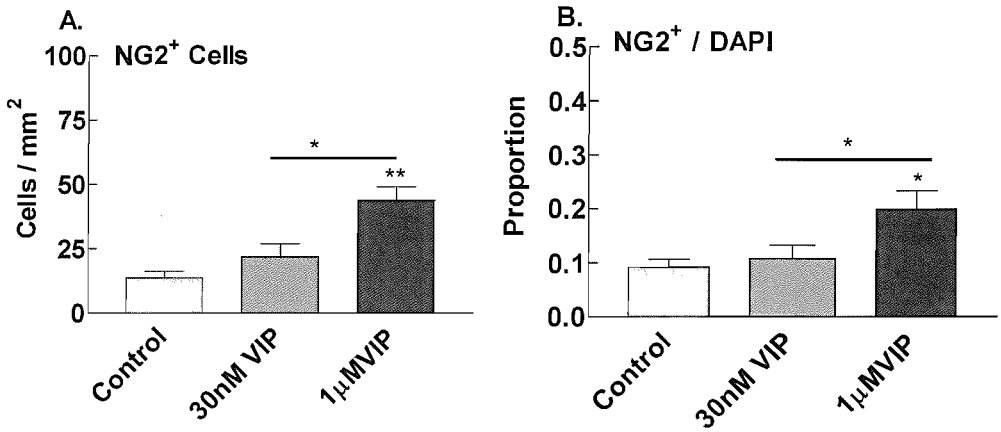


Figure 3.15: 1μM VIP increased the number and proportions of NG2 cells. Hippocampal cells after 5 days in culture under control, 30nM VIP or 1μM VIP, were fixed and processed for the expression of the oligodendrocyte precursor cells expressing NG2. (A) the absolute numbers of NG2-expressing cells, and (B) indicates the proportion on NG2 expressing cells with respect to total number of cells (DAPI). Data represent mean ± SE based on a sample that represent at least 12 wells per condition from three different experiments. Comparisons between control and different VIP conditions are one way ANOVA with Dunnetts' multiple comparison test (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

3.4.12 1μM VIP increased the labelling index but not the growth fraction

To further study VIP effects at micromolar concentrations on the proliferation of postnatal cultured hippocampal cells, we generated three and five days cultures under control conditions. In each experiment, cells in half of the wells were exposed to 1μM VIP and BrdU for the last 6hrs before fixation. Cells were labelled for BrdU to assess proliferating cells in the S-phase of the cells cycle and Ki-67 to determine the total numbers and proportions of cycling cells in culture. To properly measure the

proliferative effects of VIP, the labelling index and the growth fraction were determined at 3 and 5 days and compared to control conditions.

Our results revealed that at 3 and 5 days a significant increase in the labelling index under VIP conditions compared to controls. The labelling index increased from 10.1 ± 1.2 % and 10.7 ± 2 % to 15.8 ± 0.8 % and 15.3 ± 1 % at 3 and 5 days, respectively. Indeed the number of cells in the S-phase at 3 DIV nearly doubled after a 6 hr pulse of 1μM VIP. At 5 days, this number has grown up significantly as well (**Figure 3.16 & 3.17**).

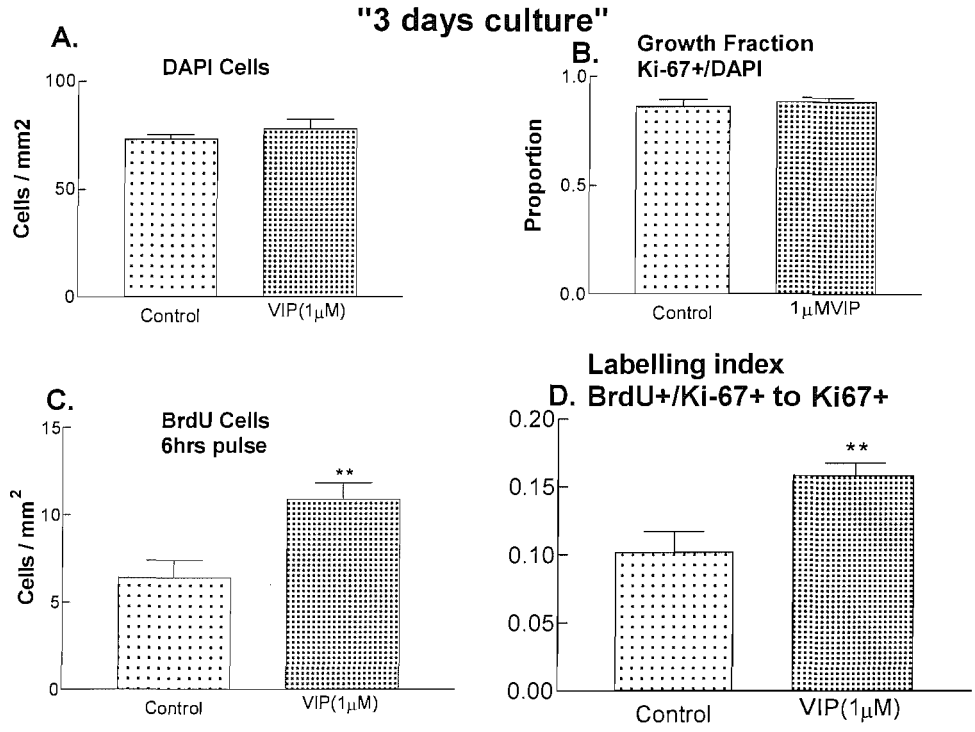


Figure 3.16: 1μM VIP pulse for 6 hrs increased the labelling index but not the growth fraction at day 3. Culture hippocampal cells were pulsed in half of the well with 1μM VIP and 20μM BrdU for the last 6 hrs before fixation. Cell proliferation was assessed with a combination of Ki-67 expression and BrdU incorporation. (A) total cell counts, (B) the growth fraction (C) Counts of cells incorporated BrdU, (D) the labelling index. Data represents mean ± SE based on a sample that represent at least 8 wells per condition from two different experiments. Comparisons between VIP and control conditions are simple *t*-student test ** $p<0.01$.

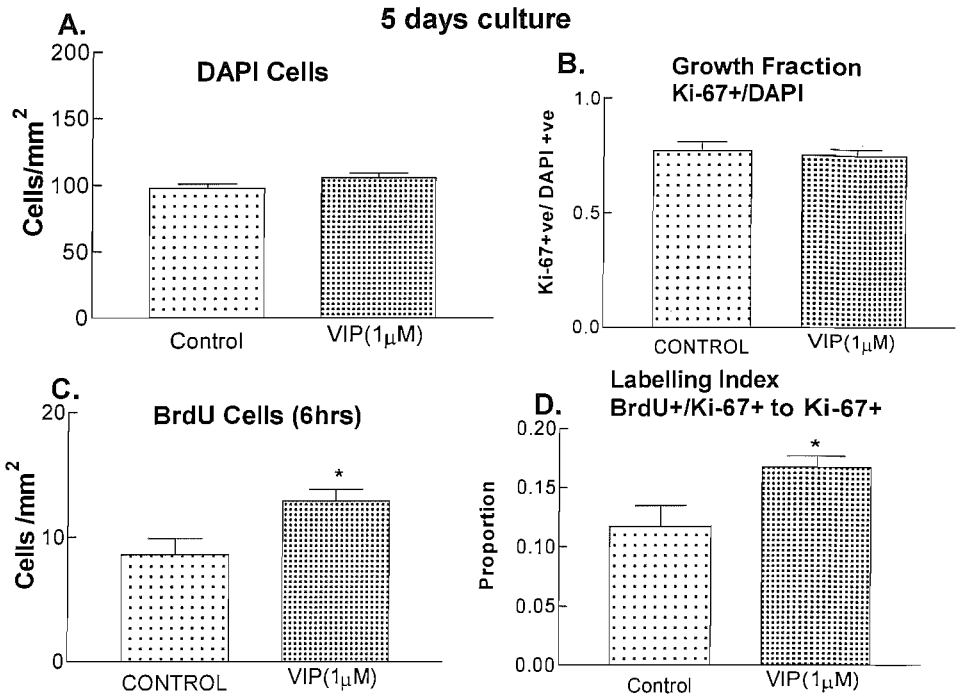


Figure 3.17: 1μM VIP pulse for 6 hrs increased the labelling index but not the growth fraction at day 5. Culture hippocampal cells were pulsed in half of the well with 1μM VIP and 20μM BrdU for the last 6 hrs before fixation. Cell proliferation was assessed with a combination of Ki-67 expression and BrdU incorporation. (A) total cell counts, (B) the growth fraction (C) Counts of cells incorporated BrdU, (D) the labelling index,. Data represents mean ± SE based on a sample that represent at least 8 wells per condition from two different experiments. Comparisons between VIP and control conditions are *Students' t-test* with * $p<0.05$.

3.4.13 VIP is proliferative to hippocampal stem/precursor cells in culture

Hippocampal cells were cultured in the presence or absence of 1μM VIP for 3 days. Cells were pulsed with BrdU 4 hrs prior to fixation. Cells were then processed for BrdU incorporation and nestin expression. Numbers and proportions of proliferating nestin cells (nestin cells incorporated BrdU) were determined under different conditions. The total number of cells increased significantly under 1μM VIP (155.3 ± 7.2 versus 104.1 ± 10.2 under control conditions) (**Figure 3.18 A**). In parallel, the number of cells expressing nestin has grown up from 39.7 ± 3.7 under control conditions to 70 ± 10.5 cells/mm² under 1μM VIP conditions (**Figure 3.18 B**). Also the number of BrdU positive cells has doubled under 1μM VIP conditions (10.2 ± 1.3 under control versus 21 ± 1.7 cells/ mm² under VIP conditions) (**Figure 3.18 C**). Moreover; the addition of 1μM VIP to cells in culture yielded a 5-fold increase in the numbers of nestin positive cells that have incorporated BrdU (entered the S-phase of

the cells cycle) and doubling in their proportions with respect to the number of nestin expressing cell population (**Figure 3.18D**).

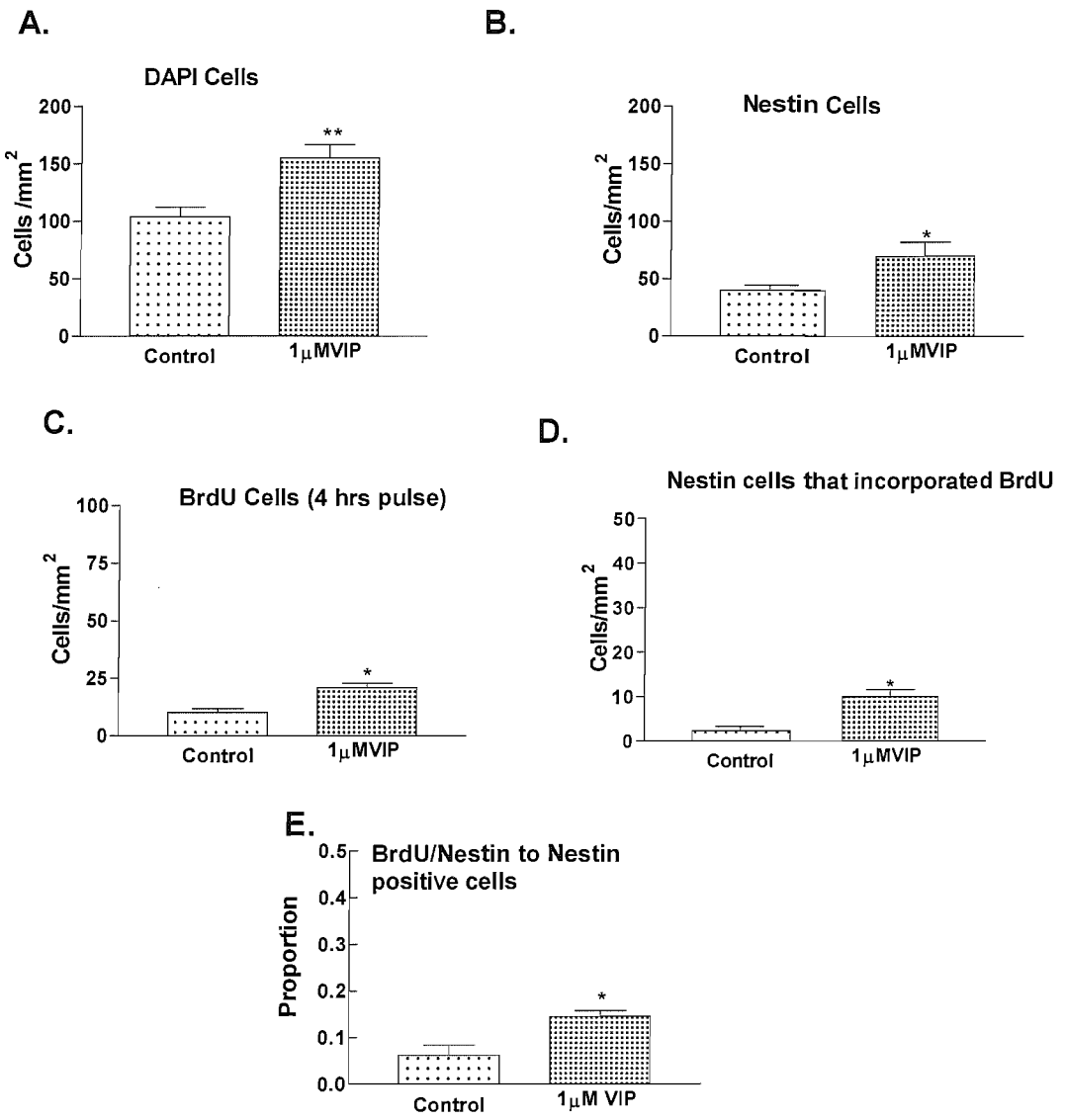


Figure 3.18: **Hippocampal cells were grown under control or 1μM VIP conditions for three days.** Cells were pulsed with BrdU 4 hrs before fixation. Cells were stained for BrdU incorporation and nestin coexpression. (A)total cell counts, (B) counts of nestin expressing cells, (C) numbers of cells incorporated BrdU, (D) counts of nestin expressing cells incorporated BrdU, (E) proportions of cells colabelled for nestin and BrdU to total nestin expressing cells. Data is the means ± SEM from 6 wells per condition. *Students' t-test*; *, $p < 0.05$ and **, $p < 0.01$.

3.4.14 VIP is proliferative to hippocampal neuronal progeny cells in culture

To quantify the proliferation of neuronal progeny cells, cultured hippocampal cells in half of the wells were pulsed with 1μM VIP and BrdU for the last six hrs before fixation. At the same time control cells in the other half of the wells were pulsed with BrdU only. Cells were then processed for class III ß-tubulin and BrdU co-labelling. Our results showed that the proportion of class III ß-tubulin that are newly proliferating with respect to the total number of class III ß-tubulin cells increased from 4 ± 0.8 % under control to 11.4 ± 0.7 % under 1μM VIP (**Figure 3.19 B**).

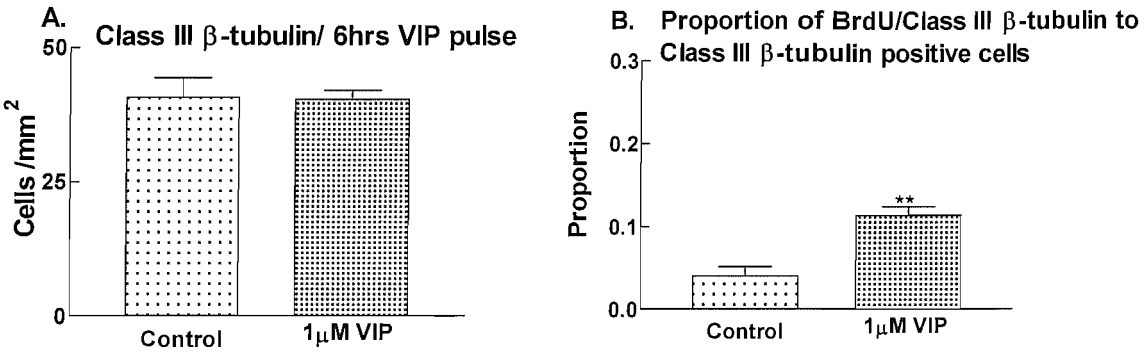


Figure 3.19: Six hrs pulse of VIP is proliferative to class III ß-tubulin cells. Hippocampal cells were cultured under control conditions. At 5 DIV, cells in half of the wells were exposed for BrdU and 1μM VIP for the last six hrs before fixation. Cells in the other half of the wells were maintained under control conditions and pulsed at the same time with BrdU for the last 6hrs before fixation. (A) total counts of class III ß-tubulin cells, (B) proportions of class III ß-tubulin cells that co-labelled with BrdU to the total number of class III ß-tubulin cells. Data is the means ± SEM from 6 wells per condition. *Students't-test*; **, $p < 0.01$.

3.5 Discussion:

3.5.1 VIP at physiological concentrations enhanced the survival of hippocampal precursor cells through trophic mechanisms

VIP effects on cells in cultures varied depending on the type of cell and stage of development. In some systems, VIP has been shown to enhance survival, and/ or induce cell differentiation, while in others, it induces cell proliferation (Waschek, 1996). In dissociated spinal cord cultures generated form foetal rats (E12-14), 100nM VIP has been shown to enhance neuronal survival with no significant effect on thymidine incorporation (Waschek, 1996). In cultures generated from E15.5 rat superior cervical ganglia, VIP increased the survival of both dividing and non-dividing neuroblasts (Pincus et al., 1994). In embryonic rat hippocampal culture, 100nM of VIP enhanced neuronal differentiation (Blondel et al., 2000). To define VIP activity *in vitro*, we used a dissociated hippocampal neuronal cell culture generated from postnatal (P7-10) Wistar rats. In this model, precursor cells undergo sequential proliferation and differentiation to give their progeny and terminally differentiated cells (Howell et al., 2003; Howell et al., 2005; Howell et al., 2007). In this experimental setup, cells were grown for 3 or 5 days under standard control conditions or the indicated VIP concentration.

VIP is a neuropeptide that has trophic and growth-regulating properties in the CNS (Brenneman et al., 1998). The results of the first set of experiments indicate that VIP enhanced the survival and subsequently increased the number of hippocampal precursor cells in culture at a wide range of concentrations (1pM-10μM). This expansion in the precursor cell pool could be a result of VIP mediated- (i) increase in cell proliferation rates, (ii) trophism (decrease in cell death), (iii) recruitment of quiescent cells to divide or (iv) any combination of the three. To address these issues we addressed VIP effects on cell proliferation *in vitro* using the S-phase marker BrdU. BrdU has been reported as a suitable marker for examining cell proliferation in the CNS (Morstyn et al., 1986; Trent et al., 1986). Our results demonstrated that VIP at nanomolar concentrations has no significant effects on the total numbers nor on the proportions of BrdU labelled cells. It was up until 1μM when a significant increase in the numbers and proportions of BrdU incorporated cells was observed. These findings suggest that VIP at physiological concentrations has no proliferative activity on

hippocampal precursor cells. This naturally raised the question regarding the second possible mechanism (trophism) through which VIP enhanced cell survival in culture. To answer this question, I designed a new protocol to study death in primary hippocampal cell culture using PI and DAPI (**Section 2.6**). PI has been shown to be a reliable marker to study cell death in many *in vitro* models, including hippocampal slice cultures and hippocampal dissociated cell cultures (Bevensee et al., 1995; Noraberg et al., 1999; Brana et al., 2002). After strictly applying our protocol, proportions of nonviable cells under different conditions were determined by dividing the number of PI positive cells by the total number of cells (DAPI cells). In this experimental paradigm, VIP reduced cell death in culture by more than 66%. In parallel, the number of viable cells increased under different VIP concentrations used. These VIP effects at nanomolar peptide concentrations suggest that VIP enhanced cell survival in culture through trophic mechanisms rather than proliferative ones. In a broad definition, neurotrophic factors are defined as secreted proteins that promote neurite outgrowth, neuronal differentiation and survival both *in vivo* and *in vitro* (Pollack and Harper, 2002). Trophism has been described to play a role early during ontogeny and neurogenesis (DiCicco-Bloom, 1996). As dividing precursor cells and their progeny may undergo death, they need necessary trophic (survival) factors to support their survival and consequently permit their accumulation to continue proliferating. This explains how VIP through trophism possibly on proliferating precursor cells and their progeny has led to the expansion of the cell pool in comparison with standard control conditions.

To investigate further the trophic effects of VIP at these nanomolar concentrations, we studied VIP effects at 30nM (physiological concentration) on the recruitment of quiescent cells to proliferate or on the rates of cell proliferation (cell kinetics). Therefore, we designed a set of experiments in which we tested the effects of 30nM VIP on the labelling index and the growth fraction. From methodological standpoint, the labelling index and growth fraction provide useful approach to distinguish mitogenic from trophic effects in cell cultures (Nowakowski et al., 1989; Lu et al., 1996). Indeed, Lu used a similar paradigm where he pulsed sympathetic neuroblasts in culture with the proliferative factor EGF and the trophic factor NT-3 for 6hrs and evaluated cell proliferation by measuring the labelling index using thymidine as a marker of the S-phase of the cell cycle. In these experiments, it has been shown that

EGF expanded the labelling index significantly, whereas NT-3 did not. In our setup cells were grown for 5 days, and then pulsed with 30nM VIP and 20μMBrdU for the last 8 and 4 hr before fixation, respectively. Cells were then dual-immuno-stained for BrdU incorporation and Ki-67 expression. Labelling index was worked out by dividing the number of BrdU and Ki-67 co labelled cells by the number of Ki-67 positive cells (cycling cells). However, the growth fraction (which represents the proportion of cells that comprise the cycling/ proliferating population) was calculated by dividing the total number of Ki-67 positive cells by the total number of cells. The analysis of the results of this set of experiments revealed that an 8hr pulse of 30nM VIP has increased neither the labelling index nor the growth fraction. In other terms, VIP at 30nM has no effect neither on the recruitment of quiescent cells to divide nor proliferation rate. The results of this set of experiments are supportive evidence that VIP at nanomolar concentration has increased the total number of cells in culture only through trophic mechanisms. Further evidence for VIP trophic effect for proliferation cells and their newly born daughters emerged from our time-lapse experiments, where VIP did not change the rate of cell proliferation, but enhanced the survival of proliferating cells and their progeny.

Recently, while neural stem cell survival has become an essential concern in the aging brain and many diseases of the central nervous system, trophic factors are gaining more interest (Kwon, 2002). Kwon and his workers have shown that trophic factors, like platelet-derived growth factor, are important for the long survival of neural stem cell and the subsequent integration to the GCL when transplanted in the hippocampus. Similarly, the results of this set of experiments indicate clearly that VIP supports cell proliferation by enhancing the survival of newly born cells which continue to divide expanding the cell pool. Interestingly, high levels of VIP have been found in the rat hippocampus (Lopez-Tellez et al., 2004), when more than half of the GCL of the dentate gurus is formed (Namba et al., 2005). Therefore, this putative neuropeptide may act as an important trophic factor that modulates the survival of hippocampal precursor cell during this critical period of development.

3.5.2 VIP enhanced the survival of precursor cells and their progeny with a prominent effect on nestin positive cells

To characterise further VIP effects on each particular cell-specific phenotype *in vitro*, we used histoimmunochemistry to stain for the major cell phenotypes: nestin the precursor cell marker, class III ß-tubulin as a neuroblasts marker, GFAP as an astrocytic marker and NG2 as an oligodendrocyte precursor cell marker. Our observations indicate that the addition of 30nM VIP to cells in culture resulted in about 3-fold increase in nestin positive cell, and doubling of both class III ß-tubulin and GFAP positive cells compared to control conditions but no effect on the number of NG2 cells. Looking at the proportions of each cell phenotype with respect to the total number of cells, it was only the proportion of nestin positive cells under different VIP concentrations that has increased significantly over control conditions. It was not until 1μM VIP till we observed an increase in the numbers and proportions of NG2 cells. This finding is in agreement with observations of Lee, where he showed that VIP enhances the proliferation of NG2 cells at 1μM in pure oligodendrocyte precursor cell culture generated from the SVZ of postnatal rat (Lee et al., 2001). In fact, this study has shown that NG2 oligodendrocyte precursor cells, as opposed to mature oligodendrocytes, do not express the high affinity VIP receptors; in accordance, we did not observe an effect of VIP at 30nM. Therefore, the effect of VIP on this cell subpopulation appears to be mediated by the PACAP receptor; PAC1.

Further investigations on cell phenotyping showed that VIP increased the proportions of cells that are nestin positive but GFAP and TuJ1 negative. This subpopulation of cells has been reported recently to be the second most primitive and the amplifying cell population during neurogenesis in the hippocampus (Encinas et al., 2006). This strongly supports the idea that VIP is trophic to precursor cells, particularly the amplifying subpopulation and, hence, the proportional increase; we observed in nestin expressing cells under VIP treatment. In this regard, we sought to further investigate VIP effects on the expression of nestin within the cycling and or growing cell populations. Our findings indicate that VIP preferentially enhanced nestin cell generation (self-renewal) with no effect on cell differentiation (**Figure 3.12**). In terms of cell-fate studies, this implies that VIP may be driving the cells to divide symmetrically without affecting their mitotic activity. To validate this, we looked at the number of nestin cells that are NUMB negative (i.e. the resultant of asymmetric division as NUMB preferentially segregates to the differentiated cell). This

subpopulation of nestin positive NUMB negative cells dropped by about 50% under VIP treatment, indicating that the proportion of nestin cells that underwent asymmetric division under VIP treatment is much less than those of control conditions (**Figure 3.14**). All together, these finding support the hypothesis that VIP increased the proportion of nestin cells through enhanced trophic as well as self-renewal effects on these cells.

In agreement with our findings, using nestin-GFP transgenic mice, two subpopulation of nestin cells have been identified; GFP-bright cells express GFAP but not TuJ1 and GFP-dim cells which are GFAP negative (Mignone et al., 2004). We identified these two cell subpopulations. We furthermore demonstrated that VIP enhances the self-renewal of the nestin positive GFAP negative cells by potentiating symmetrical cell divisions. This cell population has been described by Encinas to be as the amplifying cell population (Encinas et al., 2006). Interestingly, the selective serotonin reuptake inhibitor antidepressant fluoxetine has been demonstrated to mirror VIP effects in the hippocampus. Indeed, fluoxetine does not affect the rate of stem cell proliferation in the dentate gyrus, but increases symmetric divisions of nestin positive GFAP negative cells which results in enhanced neurogenesis (Encinas et al., 2006).

Like VIP, many trophic factors have recently been shown to enhance the self-renewal of neural stem cells. Among these, soluble factors released by endothelial cells, which have been demonstrated to stimulate the self-renewal of neural stem cells and to inhibit their differentiation, both in embryonic and adult neural stem cells cultures (Shen et al., 2004). In addition, FGF-2, is a trophic factor that has been shown to enhance the self-renewal of neuronal precursor cells in isolated and cultured olfactory bulb core cells and cortical neural stem cells (Liu and Martin, 2003; Maric et al., 2007). Moreover, stem cell-derived neural stem/progenitor cell supporting factor (SDNSF) is a another trophic factor that has been described to enhance the survival and self-renewal of adult hippocampal neural stem/progenitor cells (Toda et al., 2003).

In broad terms, these findings imply that VIP is generally trophic to nestin precursor cells and their progeny cells. However, this effect may be mainly on precursor cells and the effect we observed on their progeny is a secondary effect. Knowing that

precursor cells in culture give their progeny through consecutive cell divisions, we addressed this issue through the use of the cell proliferation inhibitor ara-C (to eliminate actively dividing cells). Likewise, ara-C was used by other studies to inhibit cell proliferation of neural stem cells and their progeny in the SVZ (Maslov et al., 2004). In embryonic mice, ara-C administration has been shown to cause neural cell precursor apoptosis, particularly the nestin positive glial cells, leading to microcephaly (Takano et al., 2006). The hypothesis we made was that, if we abolish cell proliferation, we can terminate cell progression from precursor to progeny state. Therefore, if VIP is trophic to progeny cells it will enhance their survival and consequently increase their number in culture. To test this hypothesis, cells were grown under control and 30nM VIP as a trophic concentration. At 2DIV some cells were pulsed with 0.2μM. At day 5, cells were fixed and stained for the major cell-specific phenotypes under ara-C, 30nMVIP plus ara-C, control and 30nM VIP. Our results presented in **Section 3.4.6** have shown that the addition of ara-C has lead to the loss of VIP effects on the survival of progeny cells. This again supports the idea that VIP is trophic for proliferative progenitor cells. Moreover; this may suggest that VIP enhanced the survival of progeny cells indirectly through its effects on progenitor cells which in their turn proliferate and, hence give more progeny cells compared to control conditions.

3.5.3 VIP is proliferative to precursor cells at 1μM peptide concentration

Dicicco-Bloom and co-workers demonstrated that VIP is a proliferative neuropeptide to precursor cells in embryonic sympathetic ganglion cultures (DiCicco-Bloom, 1996). In cultured neuroblasts generated from rat sympathetic ganglia, 1-10μM VIP has been demonstrated to enhance thymidine incorporation (Pincus et al., 1990b). In cultured neural stem cells generated from the lateral ventricle wall of adult mice, 300nM VIP has been shown to be proliferative to these cells (Mercer et al., 2004). Our data indicates that VIP at micromolar concentrations has increased the number of BrdU incorporating cells after a 4 hrs pulse both in three and five days cultures. Moreover, exposure of hippocampal precursor cells in culture to a short time (6hrs) pulse of 1μM VIP has increased the proportion of cells in the S-phase of the cell cycle and has doubled the labelling index. In a similar model of sympathetic neuroblasts cell cultures, VIP at 3μM displayed a proliferative effect as indicated by the increase in the labelling index after 6hr exposure (Lu et al., 1996). Taking together these

results indicates that VIP is also a proliferative neuropeptide for hippocampal precursor cells *in vitro*. Indeed, VIP has a direct proliferative effect on these cells as indicated by the significant increase in the labelling index within a short time. The extended interpretation of these results indicates that VIP increased the proliferation rates of these cells either through the shortening of the cell cycle time or facilitation of the transition from the G1 to the S-phase of the cell cycle. An effect on the recruitment of quiescent cells to divide is highly unlikely as the growth fraction has not changed under VIP conditions compared to control conditions.

Having shown that VIP is proliferative to hippocampal precursor cells in culture, we have become interested in defining the phenotypes of VIP responsive cells. In three days cultures, VIP increased the number of nestin cells that co-label with BrdU after a 4 hr pulse of BrdU. This implies that the addition of VIP to cells in culture yielded an increase in the proliferation rates of nestin stem / progenitor cells. However, this effect could be a direct or indirect effect of VIP on nestin progenitor cells through other mediators that may have been released from VIP target cells after long time incubation with VIP (3 days). To address this question, cells at day 3 in culture need to be exposed to a short time pulse of VIP and BrdU. Then the proportion of nestin cells that have incorporated BrdU is to be determined. Only an increase in these proportions would suggest a direct proliferative effect of VIP on nestin cells (DiCicco-Bloom et al., 2000).

Notably, VIP at nanomolar concentration has no effect on the number of cells incorporated BrdU or the labelling index neither at 3 days nor at 5 days (as discussed in **Section 3.5.1**). This indicates that VIP enhanced cell proliferation through the low affinity receptor (PAC1) rather than the low affinity receptors (VPAC1 and VPAC2). This is likely as PAC1 receptor along with other VIP receptors is expressed in the dentate gyrus of the rat and mice hippocampi (Mercer et al., 2004). In agreement, PAC1 has been suggested to mediate the VIP proliferative effects on neuroblasts cultures generated from the embryonic sympathetic ganglion (DiCicco-Bloom et al., 2000). In addition, PAC1 receptor subtype has been shown to be expressed by mitotic cortical precursors during embryonic development (DiCicco-Bloom et al., 2000).

VIP proliferative effect at high concentrations on hippocampal progenitor cells may have a clinical relevance. For instance, VIP levels are elevated acutely for the first three days in the rat hippocampus after kainate injection; as a model of acute epilepsy (Marksteiner et al., 1989). Concomitantly, acute seizures enhance cell proliferation in the dentate gyrus (Jiang et al., 2003). This may also become more relevant, if we take in consideration our observations that elevated levels of VIP are also associated with enhanced survival of NG2 cells, which are important for remyelination and repair after neuronal injury (Reynolds et al., 2002); another pathology that occurs in the kindling hippocampus (Mazarati et al., 2004a).

3.6 Chapter summary

The data presented in this chapter indicate that VIP has pleiotropic functions in hippocampal progenitor cell cultures generated from the postnatal rat hippocampus (P7-10). VIP at 30 nM enhanced the survival of hippocampal cells in culture including nestin-expressing putative/stem cells, TuJ1-expressing neuronal cells and GFAP-expressing astrocytic cells. Ongoing cell proliferation is essential for VIP to enhance the survival the three cell phenotypes. VIP, at physiological concentrations, increased the proportions of only nestin-expressing cell population in culture. Characterizing this population further revealed that the transient amplifying cell population is the VIP-responsive cell subpopulation (nestin positive, but GFAP and/ or TuJ1 negative). Our results imply that while VIP had no effect on the rate of cell proliferation, it enhanced nestin cell generation within the cycling / growing cell population. Furthermore, VIP appears to enhance symmetric cell division as indicated by the studies on NUMB expression.

At 1μM concentrations, VIP effect is mainly proliferative as indicated by our studies on BrdU cells, the labeling index and the growth fraction. This enhanced cell proliferation is through increased rates of cell proliferation with no effect on recruitment of quiescent cells. The phenotyping of cells indicates that this proliferative effect extends to involve both progenitors as well as neuroblast cells. At this elevated concentration, VIP also enhanced the survival of NG2 cells; the oligodendrocyte precursor cells.

VIP effects, at nanomolar concentrations, are mediated by the high affinity receptors; VPAC1 and VPAC2. In the next chapters we will be interested in stu the expression of VIP receptors in the DG and in hippocampal cell cultures. In addition, we will seek to identify the contribution of each receptor subtype in the mediation of VIP activity on hippocampal stem cells. Furthermore, we will address the specificity of VIP on dentate nestin stem cells as opposed to the rest of the hippocampus nestin-expressing cells.

Chapter 4

Investigating VIP effects on hippocampal progenitor cells and their neuronal progeny in the dentate gyrus and the hippocampal subventricular zones

4.1 Introduction:

In **Chapter 2**, we have described and characterised primary hippocampal progenitor cell cultures generated from postnatal pups (P7-10). Using this type of cell culture, we have shown (**Chapter 3**) that VIP has novel trophic as well as proliferative effects on hippocampal progenitor cells and their progenies.

Within the adult CNS, the SGZ of the dentate gyrus is an area in which new neurons are generated (Lie et al., 2004). The hippocampal formation contains in addition to the subgranular zone (SGZ) of the dentate gyrus, a number of neural stem cell niches that may contribute to neuronal cell input. These niches include the *caudal subventricular zone*, the *hippocampal subventricular zone*, located under the posterior lateral ventricle, and the adjacent *subcallosal zone* between the hippocampus and the corpus callosum collectively referred to as hippocampal subventricular zones (HSVZ) (Howell et al., 2007). For example, within the SGZ of the dentate and SVZ of the lateral ventricle the generation and integration of new neurons occur through sequential steps that are well controlled. These steps include neural stem cell proliferation and amplifying progenitor cell generation, differentiation into immature neuron, migration to the final location, growth of axon and synapse and formation of synapses and ultimately mature into fully functional neurons (**Figure 4.1**) (Lie et al., 2004). The control of these steps is as yet incompletely characterised.

The nature and responsiveness to growth factors of stem cells from the periventricular/subventricular areas have been reported to be different from those in the SGZ of the dentate gyrus. Indeed, while both, FGF-2 and EGF expanded the SVZ progenitor population after 2 weeks of intracerebroventricular administration, the proliferation of hippocampal progenitors was not affected by either growth factor (Kuhn et al., 1997; Wagner et al., 1999). In addition, FGF-2 induced an increase in

the number of newborn neurons, in the olfactory bulb, the normal destination for neuronal progenitors migrating from the SVZ (Kuhn et al., 1997). In contrast, EGF reduced the total number of newborn neurons reaching the olfactory bulb and substantially enhanced the generation of astrocytes in the olfactory bulb. Similarly, EGF increased the number of newborn glia and reduced the number of newborn neurons in the hippocampus, but FGF had no effect on cell differentiation (Kuhn et al., 1997).

Therefore, and in an attempt to provide a better understanding of the control mechanisms of hippocampal neurogenesis, we are interested, in this chapter, to address the question whether the neuropeptide VIP responding-nestin-expressing cells reside in the dentate gyrus and or the rest of the hippocampus, including HSVZ. Using real time PCR and immunocytochemistry, we will also assess the expression levels of VPAC1 and VPAC2 receptors and their mRNAs by dentate micro-dissected as opposed to HSVZ cultures.

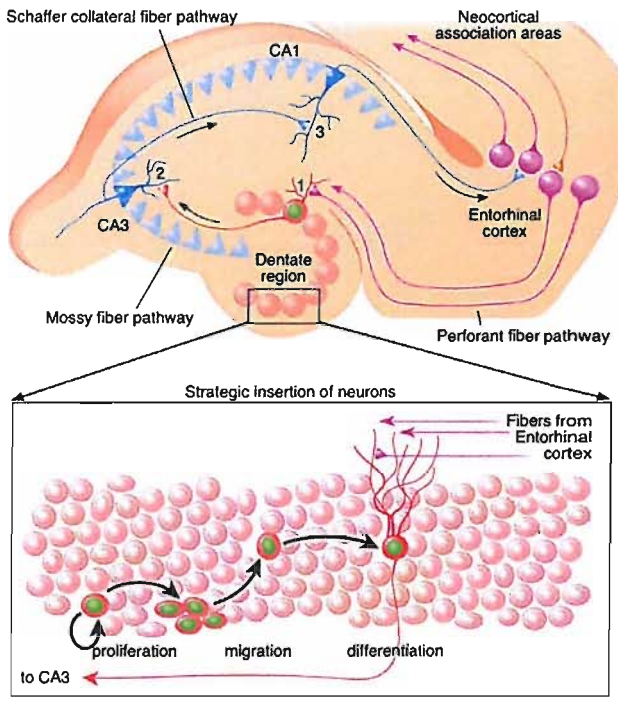


Figure 4.1: Neurogenesis in the adult hippocampal dentate gyrus. 1. Proliferation and fate determination : Stem cells (beige) in the subgranular zone of the dentate gyrus give rise to transit amplifying cells that differentiate into immature neurons. 2. Migration: Immature neurons migrate into the granule cell layer of the dentate gyrus. 3. Integration: Immature neurons mature into new granule neurons, receive inputs from the entorhinal cortex, and extend projections into CA3 .Adopted from (Lie et al., 2004).

4.1.1 Identity of stem cells in the HSVZ as opposed to those in the SGZ of the dentate gyrus:

The nature of stem cells residing within each niche has been found to vary from one area to the other. A considerable amount of work conducted by Seaberg (Seaberg and van der Kooy, 2002, 2003) and her colleagues has shown that cultured cells from the SVZ and the periventricular margin of the hippocampus alveus display stem cell characteristics like, the capability to self-renew and multipotency (Seaberg et al., 2005). Whereas, under the same experimental setup, stem cells from the SGZ of the dentate gyrus were found to be lineage restricted precursors with a limited self-renewal capacity (Seaberg et al., 2005). In contrast, other work done by Palmer et al and many others has demonstrated similarities in self-renewal and multipotency between stem cells from the two regions (Palmer et al., 1995; Palmer et al., 1997; Lie et al., 2004; McKay, 2004). Not only that, but these hippocampal stem cells, grown under favourable conditions, have been found to give the derivatives of the neural crest cells, including melanocytes, chondrocytes and smooth muscle cells (Alexanian and Sieber-Blum, 2003). Although the identity of these cells is not completely defined, it is clear that there are at least two distinct cell populations of putative/progenitor cells within these neurogenic pools of the adult CNS.

In agreement with that, our group's published work has indicated differences in the proportions of progenitor cells and neuroblasts in cultures grown from the dentate compared to the HSVZ. These neural stem cells and their progeny responded to the neuropeptide Y (NPY) in the dentate cultures in a different manner compared to the HSVZ cell cultures (Howell et al., 2007). In fact, while NPY has been shown to enhance the proliferation of the nestin-expressing cells both in the dentate and the HSVZ cultures, only the neuroblasts within the dentate gyrus responded to NPY (Howell et al., 2005). Furthermore, mice lacking Y1 receptor have been shown to have reduced cell proliferation and neurogenesis in the dentate gyrus (Howell et al., 2003).

Taken together, Howell and Kuhn observations (detailed in the previous section) suggest variable responses of neural stem cells and their neuronal progeny in different areas of the brain. Therefore, and having shown a prominent effect of VIP on nestin-expressing hippocampal stem cells (Chapter 3), we need to validate whether this

nestin-expressing population of cells can be generated from dentate only cell cultures and / or cultures containing the rest of the hippocampus including the HSVZ.

4.1.2 The expression of VIP high affinity receptors' mRNAs in the CNS

Vasoactive intestinal peptide (VIP) is a prominent neuropeptide that is widely distributed in the peripheral and central nervous systems and also secreted by immune cells (Delgado et al., 2004b). VIP is involved in a large array of physiological as well as pathological processes related to growth and development, cancers, circadian rhythm and the control of neuronal and endocrine cells (Laburthe et al., 2007). In development, VIP is a very important regulator of rodent embryogenesis, particularly during the period of neural tube closure (Hill et al., 2007a; Hill et al., 2007b). In fact, VIP blockade has resulted in deficits in the development of the CNS and permanent effects on the adult brain chemistry (Hill et al., 2007b). In a nice experimental setup conducted by Hill and his colleagues, they have shown that after the treatment of the pregnant mice with VIP antagonist, the male offspring exhibited reduced social activity and more importantly deficits in cognitive function (Hill et al., 2007a). In another study the application of this antagonist impaired VIP-associated learning abilities (Gozes et al., 1995). VIP also has an excitatory effect on the CA1 pyramidal neurons and enhances γ-aminobutyric acid (GABA)-ergic synaptic transmission in cultured hippocampal neurons (Joo et al., 2004; Cunha-Reis et al., 2005). The hippocampal formation, including the dentate gyrus is the key area of the brain that is implicated in memory, learning and cognitive functions (Shors et al., 2001; Kempermann, 2002).

VIP exerts its effects through two families of receptors. One family includes VPAC1 and VPAC2 receptors which recognise VIP and PACAP with the same high affinity; and hence the name (Moody et al., 2003). The other family contains PAC1 receptor which is a very low affinity receptor for VIP (Moody et al., 2003). VPAC1 and VPAC2 mRNAs have been studied extensively to define their distribution of expression within the rat brain. VPAC1 mRNA receptor is widely distributed in the rat brain and highly expressed in the cerebral cortex, hippocampus, cerebellar nuclei and thalamus. VPAC2 mRNA is generally less abundant in the rat brain than VPAC1 (Joo et al., 2004). However, it is highly expressed in the cerebral cortex, the hippocampus and brain stem. Within the granular layer of the dentate gyrus, VPAC2

receptor is abundantly expressed, even more than VPAC1 and PAC1 receptors (Joo et al., 2004).

As it is quite clear that VIP has vital roles within the hippocampal formation, especially if we take in consideration our findings with its role on hippocampal neurogenesis (**Chapter 3**). In addition, VIP receptors including, VPAC1 and VPAC2 are highly expressed within the hippocampus. Therefore, it can be hypothesised that VPAC1, VPAC2 and probably PAC1 participate in the modulation of memory and learning. However, and in the light of VIP involvement in postnatal hippocampal neurogenesis, it would be interesting to study if VIP receptors are expressed in the GCL of the DG or not.

4.1.3 Aims of this chapter

In this chapter, we plan to address the VIP effects upon nestin-expressing cells grown from the dentate proper of the hippocampus as opposed to nestin cells from the HSVZ. We are particularly interested in whether the VIP responsive sub-population of nestin-expressing cells is generated from the dentate gyrus and/or the HSVZ. Furthermore, and having shown (**Chapter 3**) that a subpopulation of nestin cells represents a neuronal restricted precursors (as they co-express TuJ1), we would like to find out the identity (in terms of stem cells developmental markers) of the subpopulation of nestin stem cells as progenitor cells (nestin positive but TuJ1 negative) and/ or neuronal restricted precursors. We also aim to know whether there is a difference in the expression of VIP receptors' mRNA in dentate compared to HSVZ cultures at the time we apply VIP to the cells.

4.2 Methods:

In this section, I will again point to some important points in cell culture generation Immunohistochemistry, cell counting and statistical analysis, as these processes are detailed in **Chapter 2**. However, I will detail here how the individual experiments were designed and carried out and PCR protocol.

4.2.1 Micro-dissection and primary culture generation from the dentate gyrus and HSVZ

The generation of primary neuronal hippocampal cell cultures was carried out as detailed below (**Sections 2.4.2-2.4.4**) and as mentioned elsewhere, (Reynolds and Weiss, 1992; Lowenstein and Arsenault, 1996; Howell et al., 2007)). This protocol involved a multi-step procedure that includes hippocampal dissection, dentate micro-dissection, cell release and dissociation and finally, cell plating.

4.2.2 Dentate gyrus micro- dissection:

For each experiment, 2-8 Wistar rats of 7-10 post-natal day age were killed by atlanto-axial dislocation (schedule I of animal killing; act 1986) followed by quick decapitation. Using scissors the skin was cut, the skull and the meninges were removed and the brain was swiftly removed and placed in a petri dish containing GEY's balanced salt solution (Life Technologies, Paisley, U.K.) supplemented with 4.5 mg/ml glucose at 4°C. Under sterile techniques, hippocampi were rapidly dissected and transferred onto a sterile melinex strip with a few drops of Gey's solution on the stage of a MacIlwain tissue chopper. Slices of 100μm thickness were cut perpendicular to the long axis of the hippocampus and immediately transferred into a petri dish containing 4ml of Gey's solution. Under a Zeiss dissecting microscope, the dentate gyrus was dissected free from the hippocampus using tungsten needle and fine scalpel blade. A slice of tissue was held in place by the needle and a wedge cut was drawn along the hippocampal fissure separating the dentate gyrus from the rest of the hippocampus. To include the SGZ of the dentate and effectively exclude the ependymal stem cells, a cut was then placed between the dentate gyrus and the ventricular surface on one side and the CA3 region on the other (**Figure 4.2**). Dissected dentate gyri from 8 animals per preparation were pooled. The rest of each dissected tissue slices containing the HSVZ was also separately pooled.

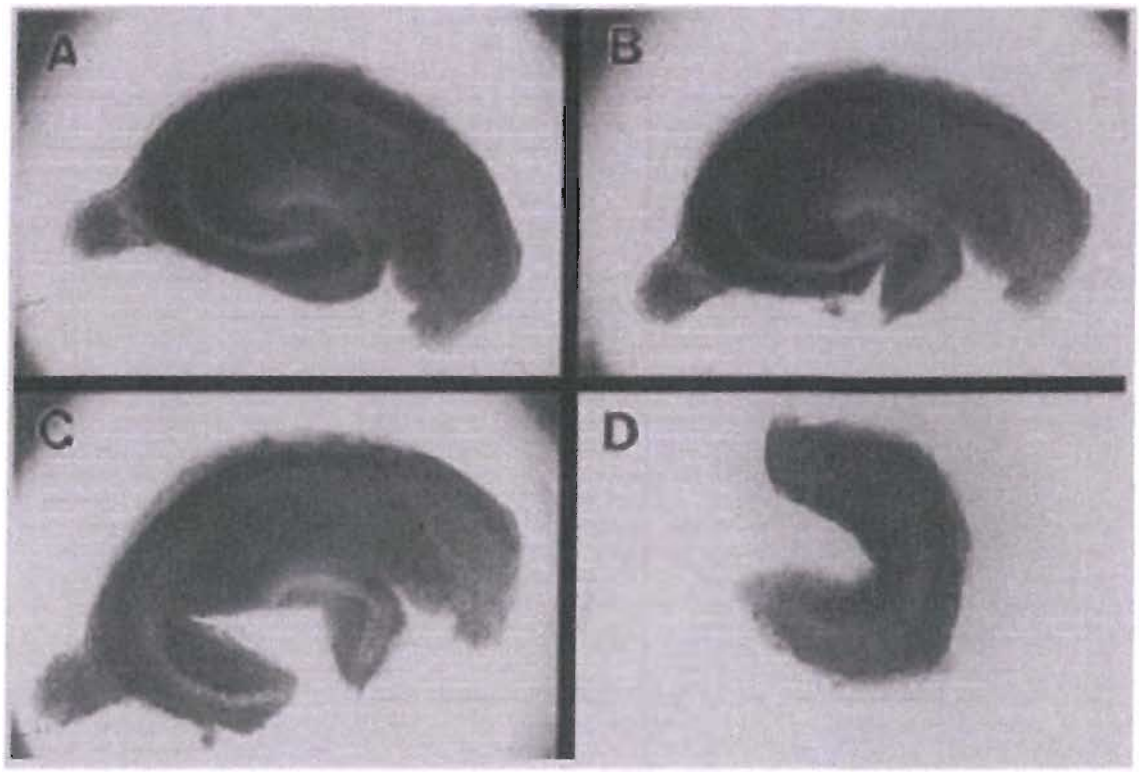


Figure 4.2: Dissection technique for isolating dentate gyrus progenitor cells. .A, 1 mm of hippocampal tissue slice from a P7-10 Wistar rat. B, Using one tungsten needle to hold the slice in place and the other to make cuts, we made the initial cut along the lower half of the hilus to isolate the inferior DG blade from the CA3c pyramidal layer. C, A second cut was made at right angles to the first to isolate the superior DGC blade. D, this is followed by a cut along the hippocampal fissure to free the DG. Magnifications: A-C, 10X; D, 13X (adapted from (Lowenstein and Arsenault, 1996)).

4.2.3 Cell release and dissociation:

Gey's solution was then replaced with pre-warmed Neurobasal A, supplemented with 2% B27 (Life Technologies) and 0.5mM Glutamine (Sigma, UK) for approximately 5mins to wash the Gey's solution. Meanwhile, a pre-warmed papain solution was filtered and sterilized through a 0.2um filter (0.22μm pore, Millex-GV, Millipore, USA) and then applied to the dentate / hippocampal slices (to aid enzymatic digestion and cell release) for 30 min at 37°C to aid in the digestion of the connective tissue and to free the cells from their surrounding connections. Papain (22.0 U/mg, Sigma) was prepared at a concentration of 2mg/ml in NB/B27 plus Glutamine at least 30 mins before filter sterilizing and application to tissue slices. After 30 minutes papain solution was aspirated and replaced by 2ml-4ml NB/B27 plus Glutamine and cell release was initiated by trituration for about 10 times after which the suspension was transferred into a 15ml centrifuge tube where the sediment was suspended in a further

2mls of NB/B27 and glutamine for more reliable trituration. This procedure was repeated 2-3 times until the cell pellet was fully dissociated, resulting in a cell suspension in 6mls NB/B27 and Glutamine. For partial purification of cells from debris, cells released by trituration were applied gently to the top of a of 2-step density OptiPrep gradient and then centrifuged for 15mins at room temperature at 1900rpm. OptiPrep gradient was made in two 1ml steps of adding 10% on top of 20% Optiprep in NB/B27 and Glutamine. Optiprep is a ready-made sterile solution of Iodixanol. The fraction containing the cells was collected with the trituration pipet and diluted into 2ml NB/B27 and Glutamine to remove the optiprep and again centrifuged for 2 mins at room temperature at 1100rpm. The medium above the pellet was aspirated and discarded and the cell pellet was re-suspended in 1ml NB/B27 and Glutamine. Viable cells were determined by mixing 30µL cell suspension with 50µL Trypan blue (Sigma), live (dye-excluding) cells were counted in a haemocytometer. Thereafter cell suspension was diluted to have a cell density of 100,000 viable cells per ml ready for plating.

4.2.4 Cell plating and VIP treatment

Viable cells were plated in 500µL NB/B27 and Glutamine at the indicated density (100,000cell/ml) directly onto poly-L-lysine coated 24-well plates or on glass cover slips (13mm diameter) previously coated with poly-L-lysine (for confocal images or receptor studies). Coating involves application of 300µL of 50µg/ml poly-L-lysine (sigma) for about 1hr then it was aspirated, allowed to dry and rinsed with NB/B27 and Glutamine to wash any excess poly-L-lysine as it is toxic to cells. For the cover slips they were sterilized in 70% alcohol before being coated. At 2 hrs after plating cells were rinsed free of debris and replenished with fresh medium of NB/B27 and glutamine under control conditions and the addition of 30nM VIP under VIP conditions. VIP from human, porcine and rat powder was supplied by Sigma-Aldrich. A working stock solution was prepared from 1ml aliquots of 1mM in sterile water and being stored as 10µL aliquots at -20C in the freezer. All media included a combined antibiotic/antimycotic (Pen/Strep and Fungizone, Life Tech, USA). And cells were incubated under (5%CO2/ to remove 9%O2/ 37°C) incubator conditions. For all cultures, 2/3rds of the growth media was replaced at day three.

4.2.5 Fluorescence Immunochemistry

At 5 days for each experiment, cells were rinsed free of medium with phosphate buffer solution (PBS) and immediately fixed in 4% Para formaldehyde (PFA) for a minimum of 30 mins at 4°C. Fixed cells were then washed 3 times in PBS to remove the PFA. After rinsing, non-specific binding sites were blocked with 5% donkey blocking serum in 0.1%Triton-X in Phosphate-buffered saline (PBS.T). Primary antibodies in PBS-0.1% Triton-X including mouse anti-rat nestin diluted 1:500 and rabbit anti-Class III ß-tubulin diluted 1:500 were incubated with cells overnight at 4°C.

Cells were then rinsed once with PBS and incubated with TBS-0.1% Triton-X containing Cy2 or Cy3-conjugated, anti-mouse diluted 1:500 and/or anti-rabbit diluted 1:500 secondary antibodies for 2 hrs at room temperature. After that cells were washed once with PBS and counterstained with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI;5 µg/mL) (Sigma) for 6 min. Optimum antibody concentrations were determined by testing each antibody on cultures, and these ratios mentioned above for each antibody are the concentrations of antibody that provided optimal signal. For each experiment, negative controls (the addition of secondary in the absence of primary antibodies) were obtained to rule out any non-specific secondary antibody binding.

4.2.6 Immunohistochemistry:

Postnatal rats were sacrificed by schedule I of animal killing. Brains were then swiftly removed and the corpus callosum was bisected dividing the brain into two hemispheres to allow better penetration of the fixative. Each hemisphere was washed once in cold PBS before being fixed in 4% PFA and stored at 4°C for a week. Coronal sections containing hippocampus were cut at 40µM on Leica VT100M vibratome and stored at 4°C in PBS until needed. Intact sections were then stained for VPAC1 and VPAC2 receptors. Pre-mounted sections were treated with 3% H2O2 in water for 30min to block endogenous peroxidase activity. Sections were washed three times in TBS before being blocked in TPS,0.1% (TBS.T)Triton and 5% Donkey blocking serum for 30 minutes at room temperature. After blocking the non-specific binding sites, sections were incubated in TBS.T Triton containing goat anti-VPAC1 or goat anti-VPAC2 primary antibody at 1:50 dilution. Following three TBS washes, primary

antibodies were probed by biotinylated donkey anti-goat antibody Secondary antibody in TBS.T for two hours at room temperature. Specifically bound biotin-conjugated secondary antibody was probed using streptavidin ABC/ peroxidase complex (1;200; Dako, Glostrup, Denmark) and visualised by diaminobenzidine (Vector Labs) staining. Mounted sections were rinsed free of salt in distilled water before being dehydrated through a graded series of ethanol solutions (2x 70%, 2x 90% and 2x 100%) cleared in xylene and the cover-slipped in DPX mountant (Fisher). Staining was visualised and pictures were taken using inverted light microscope (Improvision, Lexington, MA, USA).

4.2.7 Cell counting and data analysis:

Imaging was performed on an inverted DM IRB microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). The area of a 20× field was measured using a 255 μm grid graticule slide (Microbrightfield, Williston, USA). Cell counting was performed on 6 random 20× fields per well using the Open Lab image-capturing system version 2.1 (Improvision, Lexington, MA, USA). Raw data from the 20× field counts were averaged and plotted ± SEM and expressed as cells/mm^2 per well, based on a sample of four to eight wells per condition per experiment. All experiments were repeated at least two to three times. One experiment consisted of four hippocampi from two animals, pooled and prepared as described above. Data points were plotted using GraphPad Prism data analysis software (GraphPad inc. USA). The statistical significances between the means was assessed by either Student's test for single comparisons and by ANOVA followed by post hoc tests for multiple comparisons, with $P<0.05$ considered significant.

4.2.8 VPAC1 and VPAC2 mRNAs expression in the dentate and the HSVZ cell cultures:

In this part of **Chapter 4**, we will be investigating (using using real time PCR techniques) the relative expression of VPAC1 and VPAC2 mRNAs in the DG cell cultures as opposed to HSVZ ones (see **sections 4.2.9-4.2.13** below for details).

4.2.9 RNA extraction and cDNA synthesis:

We used (as outlined in **Figure 4.3**) the SuperScript™ III CellsDirect cDNA Synthesis Kit (Invitrogen™ life technologies) to directly synthesize the first-strand

cDNA from our progenitor cell lysate without firstly isolating RNA as detailed below. Progenitor dentate and HSVZ primary cultures were first generated as pointed above (**Sections 4.2.1-4.2.4**). At 2hrs after plating, medium was aspirated and cells were washed once free of culture medium in cold PBS. Cells adherent to each plastic well were then treated with 200µl Resuspension buffer / Lysis Enhancer Solution (1:10) and incubated on ice for 10 minutes. During that the plate was tapped periodically to detach the cells from the poly-L-Lysine. Cells were checked under the light microscope to see whether they had detached or burst. After 10 minutes, the cells in each well were gently pipetted up and down to dislodge the remaining attached cells. After that cell counts were estimated based on the plating density and found to be ~2000cell/ 10µl (the manufacturer recommendation for best results of this kit is a cell count of < 10,000 cell / µl). Then two samples of 10 µl of cell lysate from each well were transferred into a 0.2-ml thin-walled PCR plate well. In order to remove the RNase enzymes and prevent RNA degradation, each tube sample was treated with 1µl of RNaseOUT™ (40U/µl) and transferred to a thermal cycler preheated to 75°C and incubated for 10 minutes. After incubation and before proceeding to the next step of DNase digestion, the plate was centrifuged briefly to collect the condensation. For genomic DNA digestion, each well tube was treated with 5µl DNase I, Amplification Grade (1U/µl) and 1.6µl of 10X DNase I buffer. The mixture was gently pipetted up and down and briefly centrifuged to collect the contents and incubated for 5 minutes at room temperature. The plate was then placed on ice and treated with 1.2µl of 25mM EDTA to each well tube. The contents were mixed again by gently pipetting up and down and immediately incubated at 70°C for 5 minutes in a thermal cycler. After that and before proceeding into the first-strand cDNA synthesis, the plate was briefly centrifuged again to collect the condensation. The first-strand cDNA was synthesized in basically two successive steps. Firstly, each well plate from DNase I digestion was treated with 2µl Oligo(dT)20(50mM) and 1µl of 10mM dNTP Mix. Contents were mixed by pipetting up and down and briefly centrifuged before being incubated at 70°C for 5 minutes. Secondly, to accomplish the step, the following were added to each tube: 6µl 5X RT buffer, 1µl RNaseOUT™ (40U/µl), 1µl SuperScript™ III RT (200U/µl), and 1µl 0.1M DTT. Contents were again mixed and collected by pipetting and centrifuging, respectively. Then the whole mixture in each well plate was incubated at 50°C for 50 minutes in a thermal cycler. After 50 minutes, the reaction was inactivated by incubation at 80°C for 5 minutes. To remove any

residual RNA primers and to allow completion of the newly synthesized cDNA, 1µl of the enzyme RNAse H (2U/µl) was added to each well tube and the mixture was further incubated at 37°C for 20 minutes. After that, the reaction was chilled on ice and the cDNA concentration and purity were determined using a spectrophotometere (NanoDrop® ND-1000 UV-Vis Spectrophotometer). Samples were accordingly either stored at -20°C in the freezer or directly diluted to the indicated concentration (5ng/ml) for RT-PCR essay.

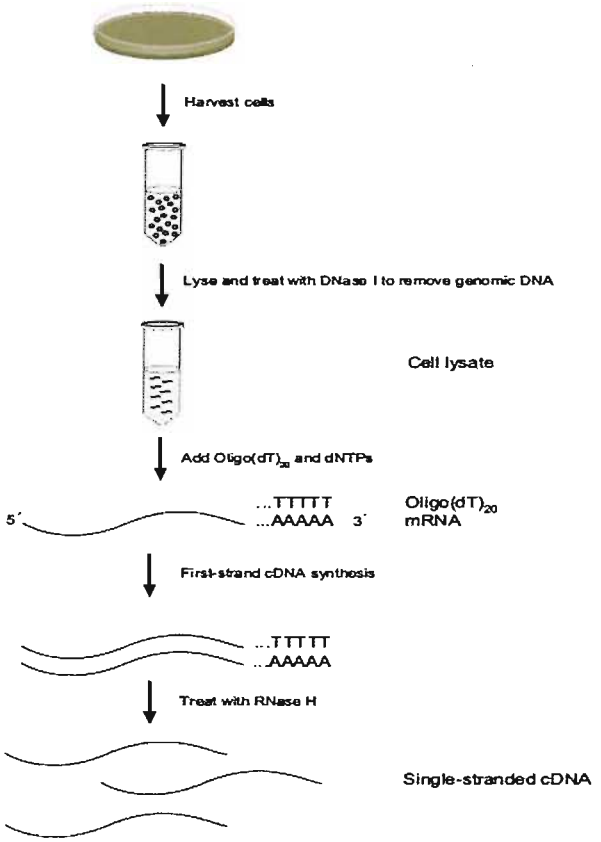


Figure 4.3: Schematic graph to summarise and outline the main steps in the process of cDNA extraction (Invitrogen, life technologies).

4.2.10 Determining cDNA concentration and purity

For all our extracted samples of cDNA, concentration and purity were determined on NanoDrop® ND-1000 UV-Vis Spectrophotometer. For the purpose of cleaning, the pedestals of the machine were thoroughly wiped. The cycle was first blanked using H2O as our buffer and then loaded with 1.5µl of our cDNA solution. Then the concentration of cDNA was measured in ng/µl. Using standard 260nm:280nm spectrophotometric analysis, the purity of our cDNA was examined by calculating the A260/A280 Ratio with a ration of greater than 1.50 indicates good quality cDNA.

4.2.11 Quantitative real time PCR assay:

Real-time (quantitative) PCR has made quantifying mRNA transcripts a simpler automated process (Higuchi et al., 1993). In fact, real time PCR is considered a sensitive technique for mRNA detection and quantitation (Heid et al., 1996). It is particularly useful when compared to other commonly used techniques, such as Northern blot analysis, as it enabled researchers to quantify mRNA from small samples of RNA. It can also be sensitive enough to enable quantification of RNA from one singe cell (Wells, 2007). In this chapter we will be using this technique to quantify the expression of VPAC1 and VPAC2 mRNAs by cultured dentate and HSVZ progenitor cells.

The quantitative realtime-PCR was carried out on our single-stranded cDNA using a one-step PCR kit (Rat Custom real-time PCR assay for use with SYBRgreen chemistry) (PrimerDesign Ltd, Southampton) in a realtime thermocycler (Rotor-Gene 6000, Corbett Robotics. Ltd). PCR reactions were performed according to the kit manufacturer instructions for 40 cycles. For VPAC1, VPAC2 and the housekeeping gene ß-actin mRNAs, primers were designed by PrimerDesign Ltd (Southampton) and provided in a lyophilised form. Each primer tube was first centrifuged to ensure that the primer mix is in the bottom and then reconstituted in RNase free water as indicated by the provider (i.e. 660µl). Forward and reverse primers, sequence, positions on the gene, melting temperatures and expected product lengths are as detailed (**Table 4.1**) below:

Primer	Sequence	Position	Tm	Product length
VPAC1	Forward:TAACTGAAGCGGGTGTGGAT	3,842	57	113
	Reverse:CCTCTCCTAGCCCTCAAACA	3,954	56.4	
VPAC2	Forward:CGGATTTCATAGATGCGTGTG	420	55.8	99
	Reverse:CACTGTAGCCCAAGGTATAAA ATG	518	55.7	
ß-actin	PrimerDesign Ltd ß-actin Kit			

Table 4.1: VPAC1, VPAC2, and ß-actin primers. This table shows the primers' sequences, position on the gene of origin, melting temperature and the amplicon length.

In each PCR reaction tube, the mix contains 1µl of the reconstituted primer mix, 10µl of PrimerDesign 2X Precision™ MasterMix with SYBRgreen and 4µl PCR-Grade water. PrimerDesign 2X Precision™ MasterMix is basically made of 2X reaction buffer, 0.025 U/ µl Taq polymerase, 5 mM MgCl2 and DNTP mix (200µM each dNTP). To this 15µl mix of each tube, 25ng/µl of the indicated (DG vs. HSVZ) cDNA was added to be amplified at a final volume of 20µl. For this purpose the Rotor Gene machine was programmed according to instruction of the Kit provider. The PCR reaction amplification conditions were: enzyme activation for 10min at 95°C followed by 15 second for denaturation at 95°C and then the data was collected in 60 seconds at 60°C.

4.2.12 Data collection and Analysis:

Fluorescent data was collected at least once during each cycle of amplification which allowed us for real time monitoring of the amplification. We were able to determine which samples are amplifying on a cycle-by-cycle basis. And therefore, we determined the relation in the amplification of our genes of interest VPAC1, VPAC2 and ß-actin as a house keeping gene from each sample. As soon as the data was collected, data analysis began. To account for differences in background fluorescence, the Rotor Gene software automatically normalises the data. Once normalization was complete, a threshold at which fluorescence data was analysed was set. This threshold is set at the level where the rate of amplification is the greatest during the exponential phase. The number of cycles it takes for each sample to reach the threshold level is defined as the Ct-value (threshold cycle). Using this set up we attained highly accurate and reproducible results.

As soon as the Ct-values were collected, raw data was processed and analysed using the Comparative Ct method which is also known as 2[-Delta Delta C(t)] method (2[-ΔΔCt])(Schmittgen, 2001). This method involves comparing Ct values of the sample with those of a calibrator or control. Therefore, and before the data was analysed, one of the samples was chosen as a calibrator for each comparison to be made. The average Ct value (avr-Ct) was calculated for the replicates of each particular group. Then the ΔCt-value was calculated according to the equation below. ΔCt-value is defined as the difference between the avr-Ct value for gene of interest and that of the house keeping gene.

ΔCt-value = avrCt(gene of interest) - avrCt(Housekeeping gene).

Having calculated the ΔCt-values we sought to determine the delta-delta Ct value (ΔΔCt) as follows:

ΔΔCt = ΔCt-value (calibrator/Control) - ΔCt-value (Sample of interest)

Then in the last step of quantification the ΔΔCts values were transformed into absolute values of the level of comparative expression according to the formula below:

Comparative Expression Level = 2 ^-ΔΔCt.

For this method of analysis to be successful, a validation experiment is required. This validation experiment was considered successful when the dynamic change of both the target and reference gene was similar (Bustin, 2000). This validation experiment was carried out for each primer by the provider on our cDNA samples before being repeated again in our lab. The protocol for this experiment involved running a RT-PCR in Rotor Gene on a series of dilution of cDNA (10Fold, 50Fold, 100Fold,...). Three replicates of each dilution using primers for the gene of interest and the house keeping gene were amplified. The Ct values were averaged for amplification of each gene from the same initial template amount. The ΔCt-values were determined as the difference between the two avr-Ct values of two amplifications of the same amount of template. The Log (input amount) versus the ΔCt-values was plotted. The slope of the plot was determined for each experiment. If the efficiencies of the two amplicons are approximately equal, the plot will be a nearly horizontal line (slope< 0.1) (Bustin, 2000). This indicates that the two amplicons performed equally efficiently across the range of the initial template amounts, and therefore, the 2[-Delta Delta C(t)] method is valid method of analysis to study our data(Bustin, 2000).

4.2.13 Characterization of dentate and HSVZ cultures:

In an initial set of experiments, we sought to determine the total counts of cultured hippocampal cells to see if it varies between dentate gyrus cultures compared to those harvested from the HSVZ. Furthermore, in order to look at the proportions of progenitor cell population and neuronal progeny, we stained these cells for the stem/putative cell maker nestin and the neuronal marker TuJ1. From raw data, proportions of each phenotype of cells within each cell culture system were then determined and compared.

4.2.14 Quantifying VIP effects on the total counts of cultured progenitor cells in the dentate and the HSVZ:

To determine the effects of VIP on the survival of cultured dentate cells and HSVZ, we grew cultures from these two areas for 5 days. In each set of cultures, cells in half of the wells were grown under standard control conditions (NB/B27 and Glutamine) and in the other half cells were additionally treated with 30nM VIP. At day five, cells were washed once with PBS before being fixed in 4% PFA for 30 minutes at 4°C. Cells were then stained with the nuclear stain DAPI. Total cells counts were then determined under each condition. Raw data was processed and analysed.

4.2.15 Stu VIP effects on nestin-expressing progenitors in the dentate as opposed to the HSVZ

Monolayer cell cultures prepared from dissected dentate and HSVZ were grown either under control conditions or in the presence of 30nM VIP. All cells were grown for 5 days after which they were fixed. Cultures were thereafter processed for the expression of nestin. Cells were then counterstained with DAPI to determine the total cell counts. Absolute counts and proportions of nestin expressing cells in each separate set of cultures were determined.

4.2.16 The role of VIP in dentate and HSVZ neuronal restricted progenitors:

Primary hippocampal culture were established as described earlier (**Sections 4.2.2-4.2.4**) from the dentate and the HSVZ and grown in the absence or presence of 30nM VIP for 5 days. Cells were fixed in 4% PFA, and subsequently immuno-stained for TuJ1 expression and counter stained with 10μg/ml DAPI. As for nestin, absolute counts and proportions of neuronal cells expressing TUJ1 were determined under each condition within each type of cell culture.

4.3 Results:

4.3.1 cDNA was extracted from both dentate as well as HSVZ cell cultures

For all our samples extracted from the dentate and HSVZ progenitor cultures at 2 hrs after plating, the concentration and purity were determined using NanoDrop® ND-1000 UV-Vis Spectrophotometer. After carefully cleaning the upper and lower pedestals of the machine, the cycle was blanked to ensure that the instrument is working well and any non-specific fluorescence is not a concern. After that the machine was wiped again and 1.5µl of our cDNA solution was loaded. The machine was then instructed to measure cDNA concentration and purity. In all our samples, we managed to extract 700-850 ng/µl with a 260/280 ratio of more than 1.5. This last measure indicates pure nucleic acid within our samples. **Figure 4.4** below presents the curves we obtained from the Nano-drop for loaded cDNA samples extracted from cell lysate from dentate micro-dissected (A) and HSVZ cultures (B). Both samples represent a descent amount of high quality cDNA.

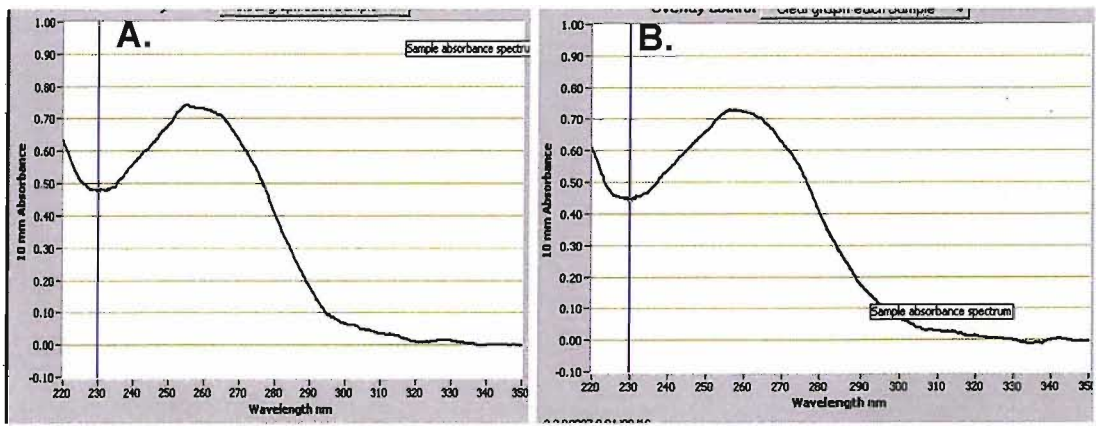


Figure 4.4: Quantity and quality of cDNA extracted from dentate and HSVZ cultures. A. cDNA extracted/synthesised from DG cultures cell lysate with 36.4ng/µl concentration and 1.82 260/280 ratio. B. HSVZ cell lysate was processed to obtain cDNA; concentration is 36 g/µl and 260/280 ratio is 1.80.

4.3.2 VPAC1 and VPAC2 mRNAs are expressed in both dentate as well as HSVZ cultures

Using real time PCR, we quantified the proportion of expression of VPAC1 and VPAC2 mRNAs in dentate gyrus cultures as opposed to HSVZ cultures. The cDNA templates extracted from dentate or HSVZ cultures were used to assess the correlative changes in transcript expression of the VPAC1 and VPAC2 mRNAs. The

amplification primers and parameters for the two receptor subtypes generated a single product with unique melting isotherm to validate the quantitative procedures (**Figure 4.5**). In addition, the amplification conditions did not generate multiple or anomalous products, or primer dimers. As described in **Section 4.2.12**, data was collected and the delta-delta Ct- method of analysis was used. Our calibrator is cDNA purchased by the primers' provider (PrimerDesign, Ltd) which represents a universal cDNA extracted from multiple Wistar rat cell lines. Primers for the ß-actin housekeeping gene were used as our normaliser. VPAC1 and VPAC2 have been tested on this cDNA and shown to be highly expressed; therefore, we used as a positive control. Consistent with this our result indicates that both VPAC1 and VPAC2 mRNAs are highly expressed by this cDNA extract. Using this sample as our calibrator, we found that VPAC1 is twice more expressed in dentate culture compared to HSVZ cultures (0.02 ± 0.002 vs. 0.04 ± 0.02. This difference is statistically insignificant. Our results also indicate that VPAC2 is equally expressed by cells in both types of culture (0.06 ± 0.02 vs. 0.06 ± 0.04) (**Figure 4.6**).

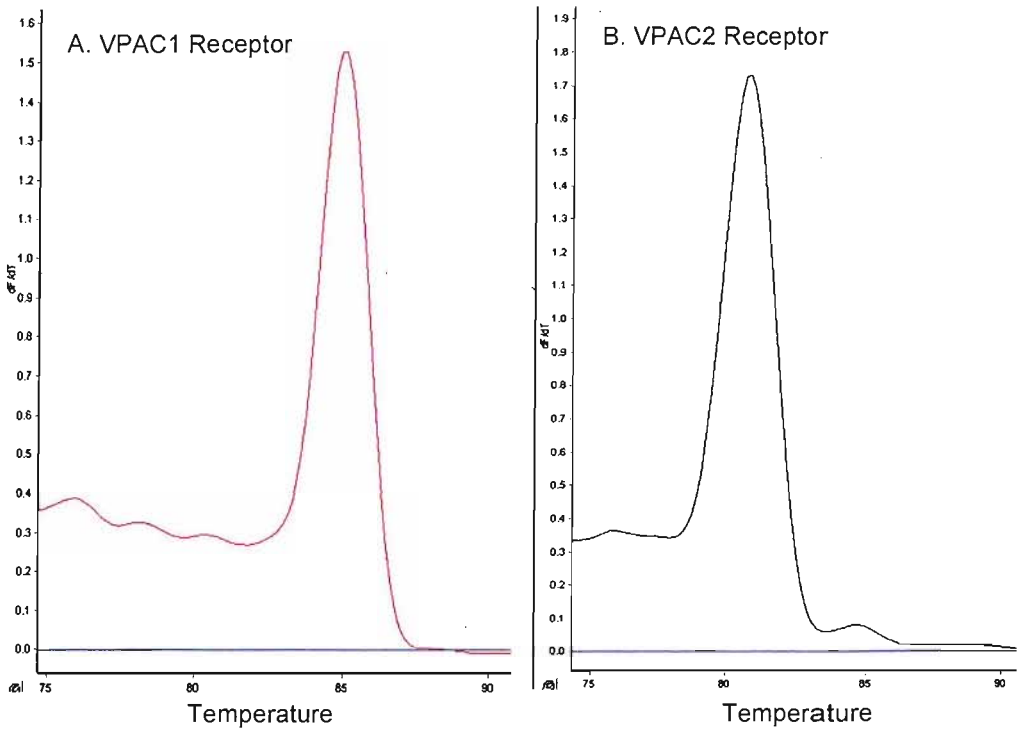


Figure 4.5: Unique quantitative PCR products amplified from VPAC1 or VPAC2 receptor primers. The uniqueness of the amplicons synthesized using the VPAC1 (A) and VPAC2 (B) primers and amplification conditions were assessed by SYBR Green I dye melting analyses. Ramping of the temperature from 65°C to 95°C produced a single unique DNA dissociation curve in each case. The amplification conditions did not generate multiple or anomalous products, or primer dimers.

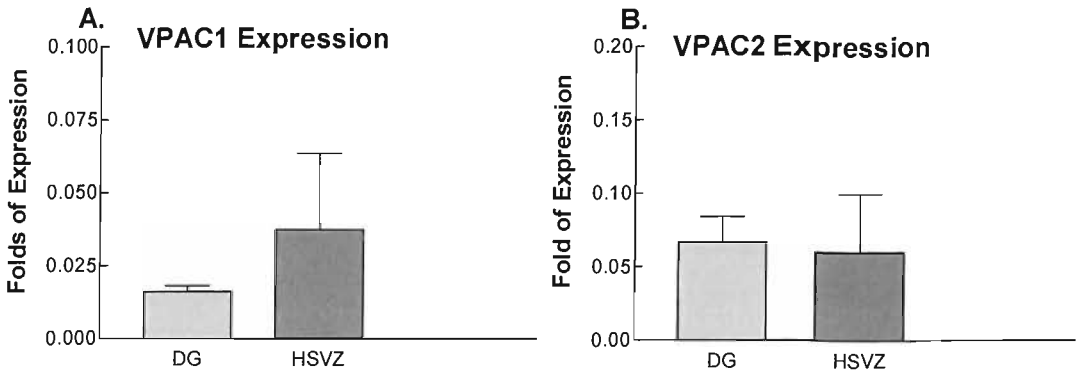
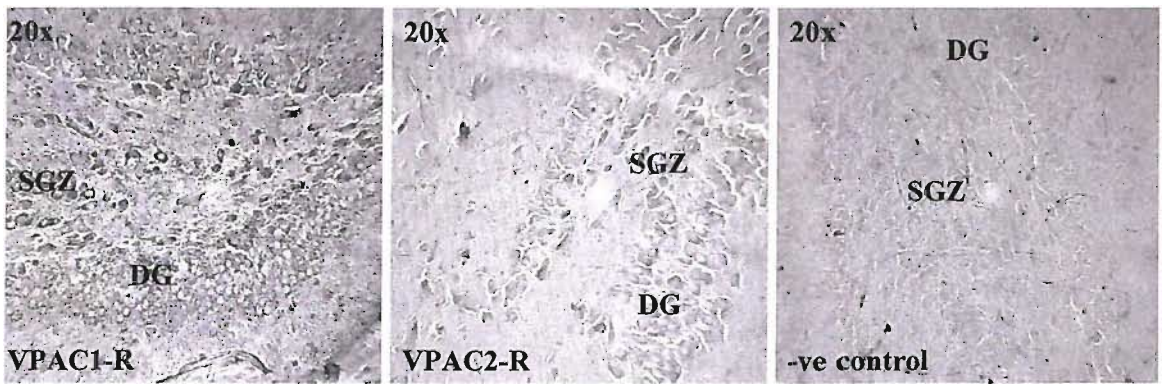


Figure 4.6: VPAC1 and VPAC2 mRNAs expression in DG and HSVZ cell cultures. Total cDNA from rat dentate and HSVZ cultures was extracted cDNA, synthesised from DG cell lysate, HSVZ cell lysate or universal rat tissue, was used as template for PCR reactions containing primers for either VPAC1, VPAC2 or ß-actin. Each reaction contained 40ng/µl cDNA. Three replicates of each reaction were performed. Values are means ± SEM for folds of expression, as calculated by delta-delta Ct, normalized to the expression of each amplicon in the calibrator; universal rat cDNA.

4.3.3 VPAC1 and VPAC2 are expressed in the granular layer of the dentate

We have previously shown that VPAC1 and VPAC2 are expressed by the major cell phenotypes in our primary hippocampal cell culture. Herein, we aimed to look at their expression within the dentate gyrus of the hippocampus in postnatal rat brain sections. Compared to our negative control, it is quite clear that both VPAC1 and VPAC2 are expressed in the area of the dentate gyrus and particularly in the SGZ **(Figure 4.7)**. The intensity of VPAC1 is higher than that of VPAC2.



A. B. C.

Figure 4.7: 20x field images taken on the light inverted microscope for VPAC1 and VPAC2 expression in the dentate gyrus. 40µm postnatal (P7-10) rat brain sections were stained for VPAC1 and VPAC2 expression. A. VPAC1 expression, B. VPAC2 expression and C. negative control; (no primary antibodies were added).

4.3.4 Both micro-dissected dentate and HSVZ cell cultures contain progenitor cells expressing nestin and their neuronal progeny:

Micro-cultures were generated from dentate and remaining of the hippocampus (HSVZ) and grown under standard control (NB/B27 and Glutamine) conditions for 5 days. The total cell count as measured by DAPI cell count was 155.0 ± 4.1 cells /mm^2 in the dentate cultures compared to 175.8 ± 8.1 cells /mm^2 in HSVZ cultures **(Figure 4.8 A)**. The difference in total cell counts between the two types of cultures was statistically significant. Looking at the phenotype of these cells, the proportion of cells expressing the putative/stem cell marker nestin was statistically higher in the HSVZ cultures at 0.63 ± 0.02 compared to only 0.48 ± 0.03 cells /mm^2 in dentate ones **(Figure 4.8 B)**. On the contrary, HSVZ cultures contained a lower proportion of neuronal cells expressing TuJ1 at 0.12 ± 0.01 compared to 0.24 ± 0.01 cells /mm^2 in the dentate micro-dissected cultures **(Figure 4.8 C)**.

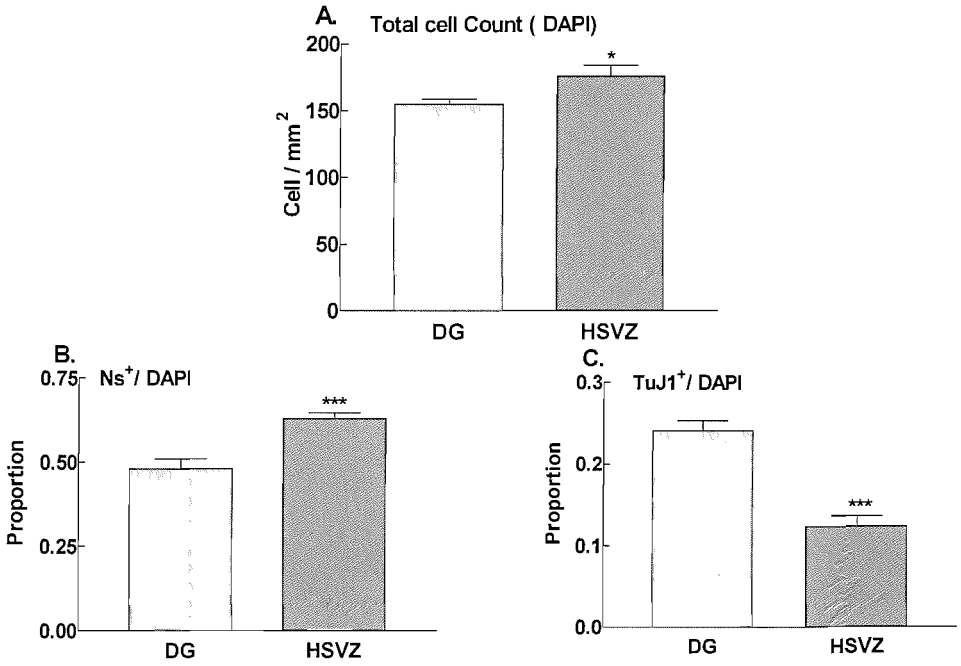


Figure 4.8: Characterization of progenitor cells in dentate micro-dissected as opposed to hippocampal subventricular zone cultures. Cells were grown under control conditions for 5 days. At 5DIV, cells were stained for nestin and TuJ1 before being counterstained with DAPI. (A) Total number of cells, (B) proportion of nestin-expressing cells over DAPI, (C) the proportion of TuJ1- expressing cells with respect to DAPI counts.. All data points are means ± SEM for 8-10 values of each condition. Comparisons are between control and VIP conditions; Students'*t-test*; *, $p < 0.05$ and ***, $p < 0.001$).

4.3.5 VIP increased the absolute number and the proportion of nestin-positive cells from the dentate compared with HSVZ cells

VIP increased the total cell count as measured by DAPI stained cells in both types of cultures significantly compared to control conditions. In dentate gyrus cultures, VIP significantly increased the number of DAPI cells from 155.0 ± 4.1 cells /mm² under control conditions to 226.5 ± 4.7 cells /mm² **(Figure 4.9 A)**. Similarly, in the HSVZ cell culture, the number of DAPI cells under control conditions significantly increased from 175.8 ± 8.1 cells /mm² to 265.3 ± 11.0 cells /mm² in VIP treated cultures **(Figure 4.9 B)**.

In order to find out whether the VIP-responsive nestin expressing cells exist in the dentate or in HSVZ, 5 days micro-dissected dentate and HSVZ cultures were established and grown under standard control conditions or control plus 30nM VIP. Cultures were then processed for nestin expression. Nestin cells from both culture systems responded to VIP. Both dentate as well as HSVZ cultures responded well to VIP with an increase in both the absolute numbers and proportions of nestin-positive cells in the dentate compared to the HSVZ cultures. As far as the dentate cultures are concerned, VIP increased the number and proportion of nestin-expressing cells from 74.0 ± 4.5 cells /mm² and 0.48 ± 0.03 under control conditions to 158.5 ± 5.5 cells /mm² and 0.70 ± 0.02; respectively **(Figure 4.9 C & D)**. On the other hand, VIP expanded the number of nestin-expressing cells in HSVZ cultures from 109.1 ± 6.4 cells /mm² under control conditions to 186.9 ± 8.4 cells /mm² **(Figure 4.9 E)**. The proportion of nestin cells with respect to the total number of cells was 0.63 ± 0.02 under control conditions compared to 0.70 ± 0.02 in the presence of 30nM VIP **(Figure 4.9 F)**.

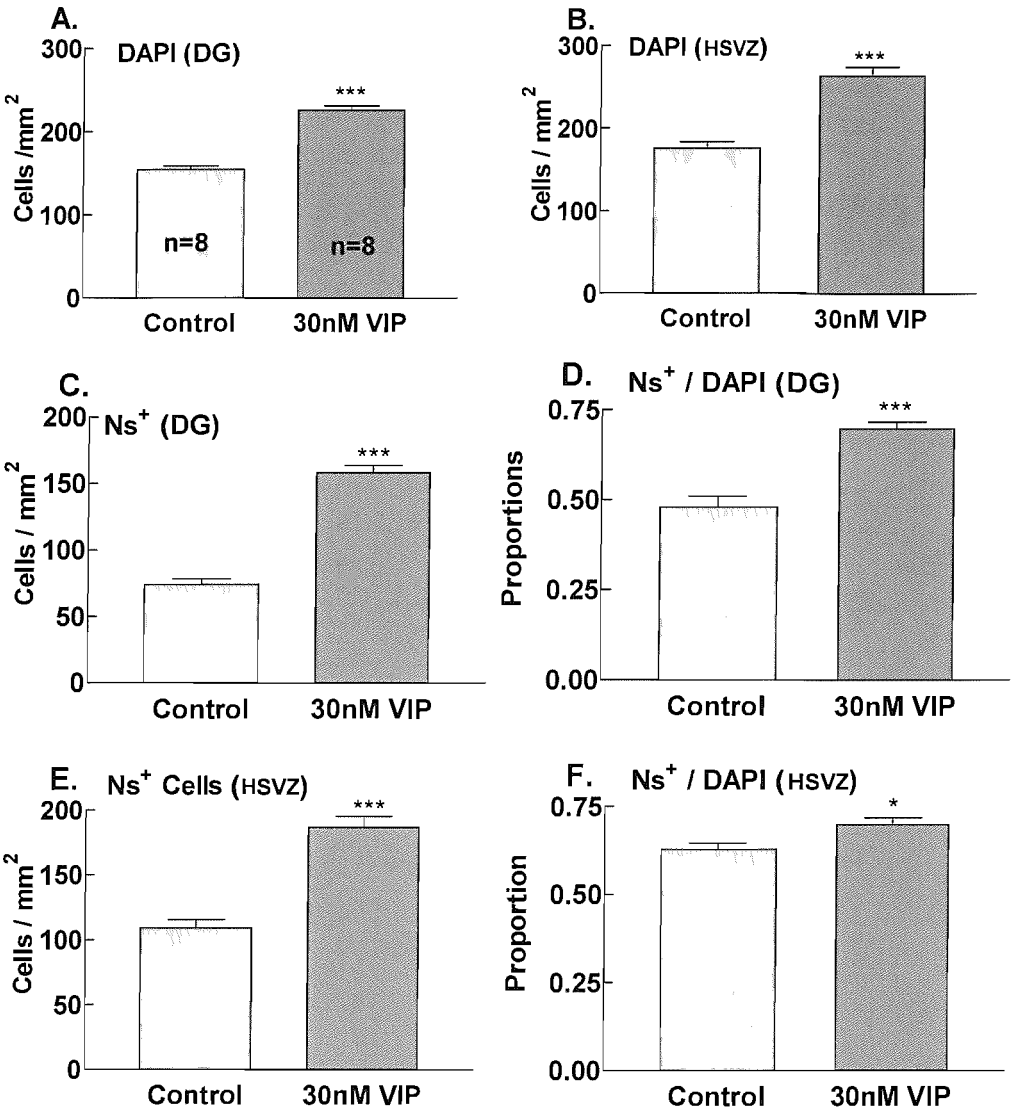


Figure 4.9: A VIP responsive nestin-positive cell population exists in dentate micro-dissected as well as hippocampal subventricular zone cultures (HSVZ). Cells were grown under control conditions for 5 days. At 5DIV, cells were stained for nestin before being counterstained with DAPI. (A) Total number of dentate culture nestin cells, (B) proportion of dentate culture nestin-expressing cells over DAPI, (C)) Total number of HSVZ culture nestin cells, (D) proportion of HSVZ culture nestin-expressing cells with respect to DAPI. Values are means ± SEM for 8-10 values of each condition from three different experiments. Comparisons are between control and VIP conditions; Students'*t-test*; *, $p < 0.05$, and ***, $p < 0.001$).

4.3.6 In both the DG and HSVZ cultures, absolute number but not the proportions of neuronal progeny cells are increased in response to 30nM VIP

Using a simple micro-dissection technique, we sought to examine whether VIP could enhance the survival and or the differentiation of neuronal precursors in precisely

dissected dentate gyrus cell cultures grown in parallel with hippocampal subventricular zone cultures. In dentate gyrus cell cultures, 30nM VIP increased the number of cells expressing the neuronal marker TuJ1 from 39.22 ± 2.825 cells /mm² under control conditions to 62.25 ± 4.347 cells /mm² with no effect on the proportion of these neuronal precursor cells with respect to the total number of cells in culture **(Figure 4.10 A & B)**. Likewise, VIP at 30nM concentration expanded the absolute number of neuronal cells expressing TuJ1 from 21.83 ± 2.716 cells /mm² under standard control conditions to 31.69± 3.674 cells /mm² with no significant change in the proportions of this cell population **(Figure 4.10 C & D)**.

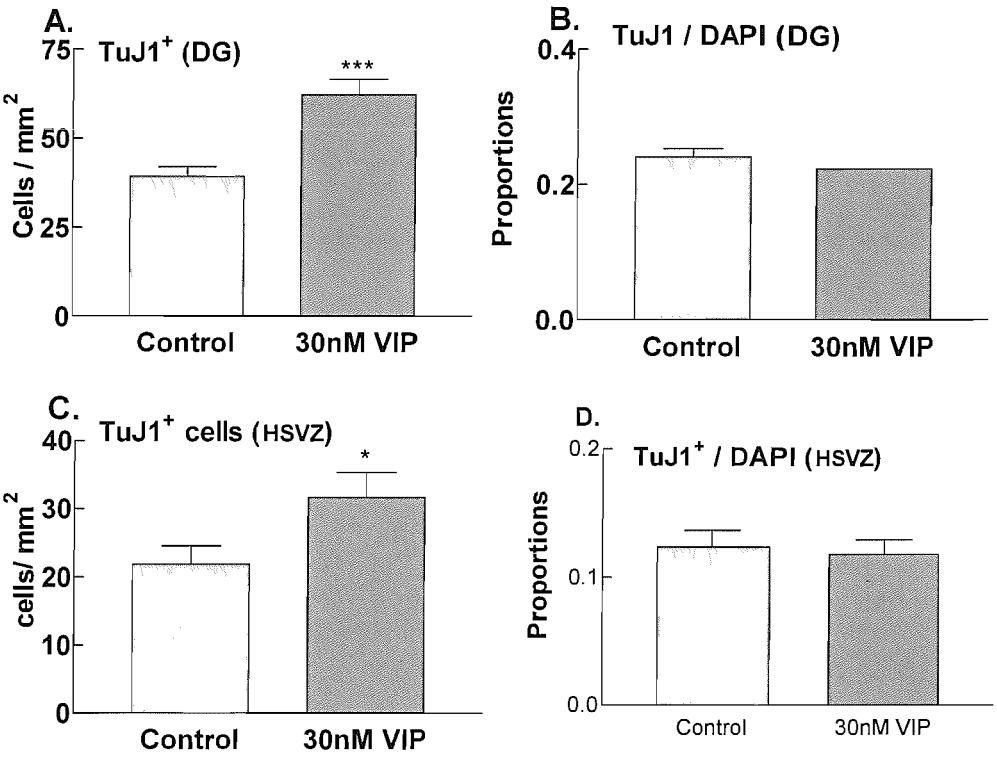


Figure 4.10: VIP increases the number but not the proportion of neuronal cells in dentate micro-dissected as well as hippocampal subventricular zone cultures (HSVZ). Cells were grown under control conditions for 5 days. At 5DIV, cells were stained for the neuronal cell marker TuJ1 before being counterstained with DAPI. (A) Total number of dentate culture neuronal cells, (B) proportion of dentate culture TuJ1-expressing cells over DAPI, (C)) Total number of HSVZ culture neuronal cells, (D) proportion of HSVZ culture TuJ1-expressing cells with respect to DAPI. Values are means ± SEM for 8-10 values of each condition. Comparisons are between control and VIP conditions; Students'*t-test*; *, $p < 0.05$ and ***, $p < 0.001$).

4.3.7 VIP increased the proportion of dentate primitive progenitors but has no effect on the HSVZ cells

In **section 4.3.5**, we have shown that VIP has increased the proportion of nestin-expressing cells slightly in HSVZ culture but dramatically in dentate gyrus cultures. Because we know that a subpopulation in the hippocampus of these cells is neuronal restricted precursors, we sought to investigate whether VIP effects in each culture system is on progenitor cells that express nestin but not TuJ1 or those precursor cells that express both markers. Again and as detailed in (**Sections 4.2.1-4.2.4**), micro-dissected dentate and HSVZ cultures were grown for 5 days in the presence and absence of 30nM VIP. Cultures were double-immuno-stained for TuJ1 and nestin. The proportions of nestin cells that co-express or do not express TuJ1 were determined. In cultures harvested from the dentate but not the HSVZ, VIP significantly increased the proportion of progenitor cell that are nestin-positive, but TuJ1 negative from 0.38 ± 0.03 under control conditions to 0.63 ± 0.03 **(Figure 4.11 A & B)**. However, the nestin cell subpopulation that co-expressed TuJ1 was not different under VIP treatment compared to control conditions in neither dentate not HSVZ cultures **(Figure 4.11 C -D)**.

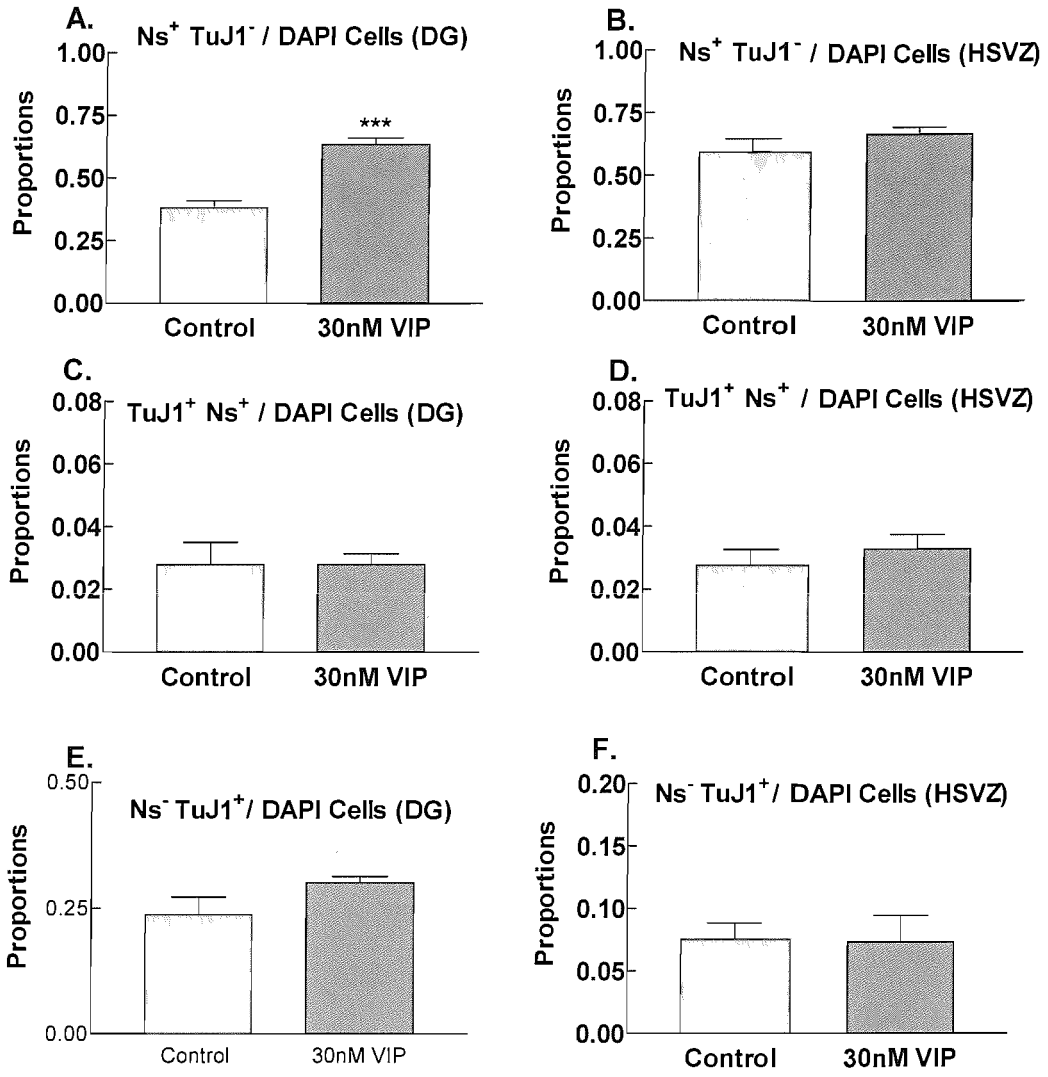


Figure 4.11: VIP increases the proportion of nestin positive/TuJ1 negative cells in dentate micro-dissected but not in the hippocampal subventricular zone cultures (HSVZ). Cells were grown under control conditions for 5 days. At 5DIV, cells were co-labeled for the neuronal cell marker TuJ1 and the putatice/stem cell marker nestin before being counterstained with DAPI. (A) Proportion of dentate culture nestin-expressing cells that are TuJ1 negative, (B) proportion of HSVZ culture nestin-expressing cells that are TuJ1 negative, (C)) proportion of dentate culture nestin-expressing cells that co-expressed TuJ1, (D))proportion of HSVZ culture nestin-expressing cells that co-expressed TuJ1. Values are means ± SEM for 8-10 values of each condition. Comparisons are between control and VIP conditions, Students'*t-test*; ***, $p < 0.001$.

4.4 Discussion

4.4.1 VPAC1 and VPAC2 are expressed in the subgranular layer of the dentate gyrus and their mRNAs are expressed in the dentate as well as HSVZ cultures

Using antibodies against VPAC1 and VPAC2 receptors, we demonstrate their expression in the different subfields of the postnatal hippocampus, including the subgranular cell layer. Furthermore, we show the expression of the mRNA of both VPAC1 and VPAC2 receptors in cultures generated from the dentate as well as those generated from the HSVZ. This mRNA was evident as early as two hours after plating. It is exactly the time when these cultures were treated with VIP. Having shown that both the proteins and the mRNAs of VIP receptors are expressed in the dentate and HSVZ fields and cultures, respectively, would make these cultures a good tool to investigate VIP effects in the modulation of hippocampal neurogenesis in the two areas.

4.4.2 HSVZ cultures contain a greater proportion of nestin- expressing progenitor cells but far smaller proportion of neuronal precursors compared with dentate culture.

In all mammalian organisms including humans, neural stem cells exist not only in embryonic developing CNS, but also in the adults (Gage, 2000). In the adult CNS, the SGZ of the dentate gyrus is an important area where precursor cells proliferate and generate granule cells which get added to the granule cell layer (Reynolds and Weiss, 1992; Gage, 2000). Dentate gyrus' stem cells have been strongly evidenced to be multipotent progenitor cells that have the capability to renew themselves and expand *in vitro* (Palmer et al., 1997; Gage et al., 1998). As pointed in (**Section 4.1**), the hippocampal region contains areas other than the dentate which house stem cells, namely, the caudal subventricular zone, hippocampal subventricular zone and the subcallosal zone to which we collectively have referred as hippocampal subventricular zones (HSVZ) (Gage, 2000; Seri et al., 2006; Howell et al., 2007; Kronenberg et al., 2007). By using an already existing dissection protocol, that had been first described by Lowenstein and Arssenault and was used in our lab (Lowenstein and Arsenault, 1996; Howell et al., 2007), we were able to establish primary cultures from both the dentate gyrus and the remaining hippocampus with attached subependymal margins (HSVZ).

As the results of our early experiments **(Figure 4.10 A & B)** indicate, cultured cells harvested from the HSVZ and the dentate gyrus survived well under our standard control conditions, despite the fact that, the procedure was 2hrs longer (for micro-dissection) than that of primary whole hippocampal cultures. Our total cell counts are comparable and strongly agrees with counts of published work for dentate cultures grown under the same condition and for the same period of time (Lowenstein and Arsenault, 1996; Howell et al., 2005). Phenotyping these cultures, we demonstrated considerable proportions of cells that are expressing the putative/stem cell marker nestin and the neuronal marker TuJ1. The proportion of nestin-expressing putative/stem cells with respect to the total DAPI stained cell in HSVZ cell culture is significantly greater ($p<0.001$) compared to DG cultures, grown at the same plating density and volume. Our results which are in agreement with the results of previous work conducted in our lab are not unexpected (Howell et al., 2007). As detailed earlier, the HSVZ culture was generated from the remaining hippocampus which included at least 3 stem cell niches and, therefore would presumably contain a larger proportion of nestin-expressing stem-putative cells than the dentate gyrus. These nestin cells are an actively dividing and expanding cell population *in vitro* (Encinas et al., 2006), hence, the significant increase($p<0.05$) in the total number of cells we observed in the HSVZ compared to the dentate.

The rest of the data within this section **(Section 4.3.4)** indicates that primary cultures from the micro-dissected dentate tissue demonstrated a significantly larger proportion ($p<0.001$) of neuronal precursor cells expressing TuJ1 compared to HSVZ cultures. This can be explained by the fact that, neurons of the Ammon's horn are born by the time of birth. However, about 85% of neuronal cells are generated by the first three postnatal weeks in the dentate gyrus (Bayer, 1980a, b). Although our HSVZ contains progenitors from the subcallozal (SCZ) and ventricular zone adjacent to the hippocampus, these precursor cells appear not to contribute greatly to the neuroblast population we are investigating. This is in agreement with a newly published work which has demonstrated that, the SVZ precursor cells do not contribute to the cellular plasticity in the CA1; even those cells that have migrated from the SCZ to the CA1 do not give rise to new neurons (Kronenberg et al., 2007).

4.4.3 VIP enhanced the survival / generation of nestin cells in the dentate at a greater proportion than the HSVZ

In **Chapter 3**, we reported that VIP at physiological concentrations has a trophic effect on hippocampal progenitor cells harvested from the whole hippocampus, and that 30nM peptide concentration increased the proportion of nestin-expressing cells, significantly. In this chapter, we were particularly interested to see if similar effect of VIP on these progenitors' cells and their neuronal progeny can be demonstrated in cultures generated from the dentate gyrus proper and/ or the HSVZ. To address this question, primary cultures enriched in dentate gyrus and HSVZ were prepared by micro-dissecting hippocampal slices from postnatal rats (P7-10). Growing monolayer cultures under control conditions or control plus 30nMVIP were then investigated for nestin expression and DAPI staining.

After 5 DIV, VIP significantly ($p<0.001$) increased cell survival as reflected by the total number of DAPI cells in both types of cultures. The phenotyping of cells in both micro-dissected cultures has shown that VIP enhanced a significant increase in the absolute numbers and proportion of nestin-expressing putative/stem cells. However, the proportional change induced by VIP in dentate cultures is nearly three times greater than that on HSVZ cultures. In simple terms, this confirms our prediction that the VIP enhanced the survival and the generation of stem cells of the hippocampus including the developing dentate gyrus. Furthermore, this indicates that VIP effect is prominent on nestin-expressing putative/stem cells from the dentate gyrus rather than those of the other hippocampal regions.

A further important point to consider is that this significant proportional increase in nestin cells with respect to the total cell count is presumably not only due to VIP trophic effects but also due to VIP-driving the cells to divide symmetrically. This is strongly supported by our findings that VIP acting on dentate cultured cells (not HSVZ) enhanced a significant proportional increase in nestin-positive/TuJ1 negative progenitor cells. In this regard, the weak response of nestin cells from the HSVZ does not agree with the abundance of VPAC2 receptor in the HSVZ compared to DG. However, this may be partially explained by the fact that a considerable population of nestin cells isolated from the SVZ and cultured *in vitro* are slow cycling nestin cells (Ma et al., 2006a). Therefore, if these cells respond to VIP, the response will be

slower, and hence poorer. To conclude, we herein present the neuropeptide VIP, which is expressed by interneurons in the dentate as well as in the rest of the hippocampal subfields, to modulate the regeneration and survival of stem cells in this area (Acsady et al., 1996; Eilam et al., 1999).

This finding has an important implication on many physiological and pathological conditions, including learning, cognition, memory and depression (Lie et al., 2004). In the case of depression, it takes two weeks for anti-depressant drugs to have an effect. It is nearly the same period of time a neural stem cell needs to give a fully functional neuron in the hippocampus (Lie et al., 2004). Flouxetine as a selective serotonin reuptake inhibitor is an affective drug treatment for depressive disorders which are known to be associated with reduced hippocampal neurogenesis. Flouxetine has been shown to modulate hippocampal neurogenesis by enhancing dentate gyrus progenitor cells (expressing nestin but not TuJ1) to divide symmetrically to maintain the pool of this important amplifying cell population (Encinas et al., 2006). It is quite clear that VIP mimics Flouxetine effects in the dentate and therefore it can be put on the front for antidepressant drug therapy, especially if we take in consideration that one of the VIP agonists; [stearyl-norleucine17]VIP, is available commercially for intranasal administration (Gozes et al., 1996). Indeed, this potent lipophilic VIP-analogue has not only been shown to protect neurons in rat cerebral cortical cultures against beta-amyloid peptide toxicity, but also to prevent impairments in spatial learning and memory associated with cholinergic blockade in a model of Alzheimer disease (Gozes et al., 1996).

4.4.4 VIP has a pure survival effect on neuroblasts with no effects on neuronal differentiation

Primary hippocampal cultures dissected from dentate tissue and hippocampus-only (HSVZ) tissue responded well to 30nM VIP treatment for 5 days. These cells were then co-stained for nestin and TuJ1. The number of neuroblasts increased under VIP treatment at least two times more in the dentate cultures compared to HSVZ culture. However, the proportion of these neuronal precursors in neither micro-dissected cell culture changed. The survival effect on neuronal progeny agrees again with the same VIP effect we demonstrated on whole hippocampal cultures. These findings are also in agreement with the fact that VIP has a potent trophic effect on CNS neuroblasts

generated from sympathetic ganglia (Pincus et al., 1994). However, this finding adds to our knowledge the fact that VIP is trophic to neuronal precursors derived from the dentate gyrus in parallel to its trophic effect on other hippocampal neuroblasts. The number of neurons under VIP treatment increased in the dentate cultures more than in the HSVZ can be explained by the fact that dentate cultures contained more VIP responsive TuJ1 cells from the start.

As far as neuronal differentiation is concerned, VIP appears to have no effect at 30nM concentration after 5 days exposure on stem cell differentiation towards a neuronal phenotype. This is evidenced (**Section 4.3.6**), in both culture systems, not only by the lack of proportional increase under VIP treatment in neuroblasts, but also by the lack of increase in the proportion of neither cells co-expressing nestin and TuJ1 nor cells expressing TuJ1 but not nestin. This survival effect of VIP on dentate along with HSVZ neuroblasts is an important finding. The mainstream of reseachers now believe that the survival of newly born neurons rather than their proliferation is the key event in the net hippocampal neurogenesis (Gage et al., 1998; Lie et al., 2004).

4.4.5 VIP enhanced the generation of primitive nestin cell that are TuJ1 negative

Consistent with a considerable amount of published work, our dentate and HSVZ cultures contained nestin and TuJ1 expressing cells. Our results (**Section 4.3.7**) indicate that, within the dentate and the HSVZ cultures, these two populations of cells are distinct but variably overlapping. About 30% of the cells are nestin positive, but TuJ1 negative. This means that three fourths of the nestin cells are TuJ1 negative. We have shown that VIP enhanced the proportion of this subpopulation of cells. This strongly suggests that VIP enhanced the self-renewal of the nestin cells within the dentate gyrus as opposed to the HSVZ. Also this explains the VIP prominent effect in the dentate cultures as opposed to HSVZ cultures.

4.5 Summary

Within the CNS, the SGZ of the dentate gyrus is well known to mediate hippocampal adult neurogenesis (Lie et al., 2004). However, little is known about signals that regulate the survival and the self-renewal to maintain the pool of neural progenitor/stem cells within this discrete region. Our results (**Sections 4.3.5 and 4.3.6**) revealed that VIP enhances the survival of the putative progenitor cells expressing nestin, and the neuronal precursor cells, both in the dentate as well as in the HSVZ. However, a prominent proportional (~22%) increase in the cells that are expressing nestin was observed only in the dentate gyrus cell cultures. Our further characterization of these nestin cells (**Section 4.3.7**) revealed that the subpopulation of nestin cells that are TuJ1 negative is the VIP-responsive cells. Although nestin-positive-TuJ1 negative subpopulation existed in both culture systems, it is only the dentate cells that responded to VIP.

Chapter 5

VPAC2 mediates VIP trophic effects on hippocampal progenitor cells *in vitro* and *in vivo*

5.1 Introduction

The actions of VIP in the CNS are mediated by at least three receptors. These are VPAC1, VPAC2, and PAC1. VIP binds equally with high affinity to VPAC1 and VPAC2 receptors but with 1000-fold lower affinity to the PAC1 receptor (DiCicco-Bloom et al., 2000). In **Chapter 3** and **Chapter 4**, we have shown a general trophic effect on the major cell phenotypes and an additional effect on self-renewal of the nestin-expressing cells of VIP at 30nM concentration. However, VIP does not interact with PAC1 at concentrations of less than 500nM (Vaudry et al., 2000). Therefore, we believe that this VIP effect is VPAC1 and/or VPAC2 mediated. In this chapter, I will outline the pharmacology of VIP high affinity receptors and the expression of these receptors in our hippocampal progenitor cell cultures.

VPAC receptors have been called so because they recognise VIP and PACAP with the same affinity. Although they both bind VIP with high affinity (Kd-1nM), they have been further subdivided into VPAC1 and VPAC2 depending on their affinity for secretin (Vaudry et al., 2000). VPAC1 and VPAC2 receptors are expressed in various organs including the lung, duodenum, thymus and the brain.

5.1.1 Distribution of VIP receptors in the CNS

Within the CNS, VPAC1 and VPAC2 receptors have been found to be widely expressed and localised to both neurons and astrocytes (Brenneman, 2007). In the rat CNS, VIP high affinity receptors are mainly located in the olfactory bulb, the cerebral cortex, the dentate gyrus of the hippocampus, the thalamus and the pineal gland (Vaudry et al., 2000). In vitro studies have shown the expression of both VPAC1 and VPAC2 by both cultured neuronal as well as astrocytic cells (Vaudry et al., 2000).
VPAC1 mRNA is mainly expressed in the cerebral cortex and the hippocampus while VPAC2 mRNA is present in many areas within the brain including the hippocampus, the amygdale, the suprachiasmatic nucleus and thalamus (Vaudry et al., 2000). So the

expression of VPAC1 mRNA overlaps with that of VPAC2 in the hippocampus. In the cerebral cortex, while VPAC1 receptor mRNA has been found to be abundant in layers III and V, VPAC2 receptor mRNA was confined to layer VI (Vaudry et al., 2000). At the cellular level, both VPAC1 and VPAC2 mRNAs have been characterised by real-time-PCR on glial cells. In primary cultures generated from rat cortical neurons and type I astrocytes (glial cells that have been shown to give rise to neural stem cells (Itoh et al., 2006)), mRNA-expression of VPAC2 has been confined in neurons, while both VPAC1 and VPAC2 are present in astrocytes (Grimaldi and Cavallaro, 1999). VPAC1 and VPAC2 have been shown to interact with VIP in mediating neuroprotection, directly or affecting the expressing/ release of other survival-promoting substances (Brenneman, 2007).

In mice embryonic stem cell extracts, semiquantitative RT-PCR amplifications have revealed a signal for VPAC2 receptor while VPAC1 mRNA was slightly detected (Cazillis et al., 2004). In another model of embryonic stem cell culture (Hirose et al., 2005) generated in five-step protocol, VPAC2 receptor mRNA has been found to increase in stage 2-4 during which cultures were enriched for stem cells expressing nestin (Hirose et al., 2005). Interestingly, its expression continued at a high level through stage 5 as cells were allowed to differentiate to neuronal precursors. VPAC1 mRNA on the other hand, was expressed all the time (stage 1-5), but only prominently during stage 2, which was just before the enrichment for nestin cells (Hirose et al., 2005). This data may indicate both a functional and molecular diversity of VIP receptors in these cell types and importantly, a vital role for VPAC2 receptor in modulation of nestin cells and their progeny.

5.1.2 VPAC1 and VPAC2 receptors' molecular biology

VPAC1 was first cloned from a rat lung cDNA library (Vaudry et al., 2000). Then it was cloned in human, goldfish, and frog (Vaudry et al., 2000). The rat and human VPAC1 receptor were described to have a 459 and 457-amino acid proteins, respectively, with 84% sequence identity (Vaudry et al., 2000). The VPAC1 receptor gene (22kb) has been found to comprise 13 exons with promoter region containing several binding sites for nuclear factors like, activator protein 2 and protein 1.

VPAC2 was initially cloned from the rat pituitary cDNA library and then from the human placenta. The human and rat VPAC2 receptor' proteins have been found to

exhibit 87% amino acid identity (Vaudry et al., 2000). VPAC2 receptor gene has been located in region q36.3 of chromosome 4 and 7 in rats and humans, respectively (Vaudry et al., 2000). This gene in humans is encoded by 13 exons and spans 117kb (Vaudry et al., 2000).

5.1.3 Structure-activity relationship

Different cell lines including Chinese hamster ovary (CHO) and COS-7 have been used to investigate the binding properties of chimeric VIP receptors. Most of the available VPAC1 and VPAC2 receptors' antagonists are more or less N-terminal truncated or substituted VIP peptides. For VPAC1 receptor, a selective high affinity VPAC1 receptor antagonist [Acetyl-His1, D-Phe2, Lys15, Arg16, Leu17] VIP(3-7)/GRF(8-27) (PG 97-269) has been synthesized by Gourlet et. al. and his colleagues (Gourlet et al., 1997a). The properties of the new peptide have been evaluated on CHO cell membranes expressing either the rat VPAC1 or VPAC2- recombinant receptors. The IC50 values of 125I-VIP binding inhibition by PG 97-269 were 10, 2000 nM on the rat VPAC1 and VPAC2, respectively. PG 97-269 had a negligible affinity for the PAC I receptor type. It did not stimulate adenylate cyclase activity, but inhibited competitively effect of VIP on the VPAC1 receptor mediated stimulation of adenylate cyclase activity (Gourlet et al., 1997a).

On the other hand, the synthesis of a VPAC1 receptor selective agonist GRF was achieved (Gourlet et al., 1997b). The chimeric, substituted peptide [K15, R16, L27]VIP(1-7)/GRF(8-27) has been shown to have IC50 values of binding of 1nM ,10μM, and 30μM for the rat VIP1-, VIP2-, and PAC1 receptors, respectively. Furthermore, it has also been shown to have an IC50 of 0.8 nM for the human VIP1 receptor and a low affinity for the human VIP2 receptor. This analogue has been demonstrated to stimulate maximally the adenylate cyclase activity on membranes expressing each receptor subtype (Gourlet et al., 1997b).

In another set of *in vitro* studies carried on endocrine tissue, and making use of the structure-activity relationship between rat growth hormone-releasing factor (rGFR) and vasoactive intestinal peptide (VIP), a VPAC1/VPAC2 nonselective antagonist has been predicted. It has been named as [D-*p*-Cl-Phe^{6}, Leu^{17}]-Vasoactive Intestinal

Peptide (Pandol et al., 1986; Griffiths et al., 1989). This peptide has been shown to competitively antagonize the action of VIP at micromolar concentrations (Pandol et al., 1986; Griffiths et al., 1989). As detailed below (**Section 5.2.3**) I will be using theVPAC1 receptor agonist [Lys15, Arg16, Leu27]-VIP (1-7) - GRF (8-27), the VPAC1 antagonist [Ac-His1, D-Phe2, Lys15, Arg16, Leu27]-VIP (3-7)-GRF (8-27) and the VPAC1/VPAC2 non selective antagonist [D-*p*-Cl-Phe^{6}, Leu^{17}]-Vasoactive Intestinal Peptide to investigate the pharmacology of VPAC receptors *in vitro*.

5.1.4 VPAC1 KO and VPAC2 KO models

To date we are not aware of the existence of VPAC1 knockout animals. For VPAC2, a knockout strain has been successfully generated and used as a biological model in many experimental setups (Metwali et al., 2000; Hastings et al., 2003; Voice et al., 2003; Lee and Cox, 2006). However, there are no reports as with regard to the behavior, cognition and memory of these animals. However, there are some studies in which mice lacking the VPAC2 receptor, were used to study VPAC1 and VPAC2 receptors' role in rhythm generation in the suprachiasmatic nucleus (SCN) (Harmar, 2003; Hastings et al., 2003; Brown et al., 2005). In another study in the thalamus, using VPAC2 knockout mice, it has been shown that the excitatory actions on intrathalamic rhythmic activities, are predominantly mediated by VPAC(2) receptor subtype (Lee and Cox, 2006).

5.1.5 Aims:

In chapter three and chapter four, we demonstrated a variety of VIP effects on hippocampal progenitor cells that involve trophic effects self-renewal enhancement on the progenitor/ precursor cells. Trophic effects also extend to involve astrocytic and neuronal progeny. As suggested by the kinetic studies, these effects seem to be mediated by the high affinity VPAC receptors. In this chapter, we focused on VPAC1 and VPAC2 receptors and studied the expression of these proteins and their mRNAs by hippocampal cells *in vitro*. Using some of the available agonists and antagonist, we sought to study the contribution of each receptor subtype to VIP effects on nestin-expressing stem cells, GFAP cells and neuroblasts. We extended our study to involve evaluation of cell survival in adult mice lacking VPAC2 receptor, a model which has recently become available.

5.2 Methods

5.2.1 Introduction

In this section, I will point to some important points in cell culture generation, immunohistochemistry for VIP receptors, VPAC2 knockout mice breeding and generation, Immunocytochemistry for mice sections, BrdU counting and statistical analysis. I will also detail the design of the pharmacology experiments.

5.2.2 Generating primary hippocampal culture

In each experiment, primary hippocampal cultures were generated from 2-8 Wistar rats of P7-10 age. Viable cells were plated in 500μL NB/B27 and glutamine at a density of 100,000 cells directly onto poly-L-lysine coated 24-well plates or on glass cover slips for confocal images. At 2 hrs after plating cells were rinsed free of debris and replenished with fresh medium of NB/B27 and glutamine under control conditions and the addition of the indicated peptide at the right concentration for each experiment under other drug conditions as detailed in (**Section 5.2.3**). Again VIP and the other peptides from human, porcine and/or rat powder were dissolved in water. A working stock solution was prepared from 1ml aliquots of 1mM in sterile water and being stored as 10μL aliquots at -20C in the freezer.

5.2.3 Examining VPAC1 and VPAC2 mediating trophism and self-renewal

We have elucidated VIP survival-effects in hippocampal cell culture at physiological concentrations. We believe that these effects are mediated by the high affinity receptors VPAC1 and/or VPAC2. However, the possibility that VIP survival effects could be mediated by the low affinity receptor is much less likely, as it has been shown by many studies that VIP does not bind PAC1 receptor at concentrations of less than 500nM-1μM (Vaudry et al., 2000). To determine if the activity of VIP is mediated via VPAC1, VPAC2 or both, we used theVPAC1 receptor agonist [Lys15,Arg16, Leu27]-VIP (1-7) - GRF (8-27) (Phoenix Pharmaceutical, INC), the VPAC1 antagonist [Ac-His1, D-Phe2, Lys15, Arg16, Leu27]-VIP (3-7)-GRF (8-27) (Phoenix Pharmaceutical, INC) and the VPAC1/VPAC2 non selective antagonist [D-*p*-Cl-Phe^{6}, Leu^{17}]-Vasoactive Intestinal Peptide (Sigma).

5.2.4 Investigating the role of VPAC2 in cell survival *in vivo*

To confirm a VPAC2 involvement as indicated by our *in vitro* experiments (**Section 5.3.3**), we collaborated with Prof. T. Harmar, University of Edinburgh, Edinburgh, UK) who provided us with adult VPAC2 knockout ($VPAC2^{-/-}$) and wild type mice (C57BL/6J).

5.2.4.1 The VPAC2−/− mice generation

Professor Harmar's method of generation involved targeted disruption of the *VPAC2* gene that was generated in E14/4 embryonic stem cells by replacing a 132 bp sequence containing the translation start site of the *VPAC2* gene (**Figure 5.1A**) with a *LacZ-Neo*^{r} cassette (**Figure 5.1B**). *Vipr2*$^{-/-}$ mice have been found to exhibit no differences from wild type littermates in gross morphology or fertility. Gene targeting was confirmed by RT-PCR using mouse *Vipr2* and *Hprt* gene-specific primers and by radio-receptor autoradiography using the selective $VPAC_2$ receptor ligand Ro 25-1553 (**Figures 5.11 H–1M**). Specific binding of ^{125}I-Ro 25-1553 was detectable in tissues known to express the receptor, including the SCN, in wt mice but not in *VPAC2*$^{-/-}$ mice (**Figures 5.1H and 1K**). $VPAC_2$ receptors were detected in gastric mucosa of wt mice but not in *VPAC2*$^{-/-}$ mice, whereas $VPAC_1$ receptors were present in gastric mucosa from both wt and *VPAC2*$^{-/-}$ animals. Therefore, the gene targeting was considered to be specific for the $VPAC_2$ receptor and did not have any detectable effect on $VPAC_1$ receptor expression. There was no detectable expression of the *lacZ* transgene in heterozygous or homozygous *VPAC2* null mice at any stage of development, possibly as the result of transgenic silencing (Harmar et al., 2002).

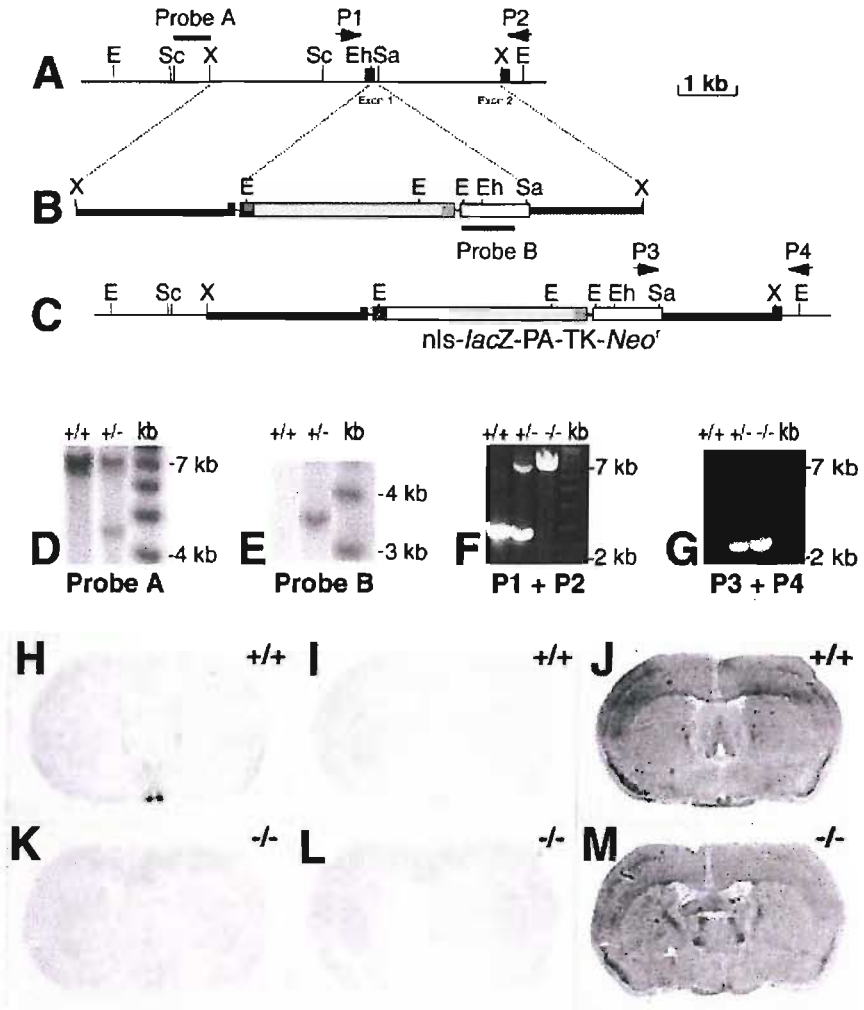


Figure 5.1: Generation of *VPAC2*$^{-/-}$ Mice and Binding of Ro25-1553 in the SCN .Schematic diagram of (A) wild-type allele, (B) targeting vector, and (C) targeted allele showing restriction sites for EcoRI (E), EheI (Eh) ScaI (Sc), SacII (Sa), and XbaI (X) and positions of probes A and B and primers P1, P2, P3, and P4. EcoRI digests of genomic DNA from ES cells hybridized with probe A (D) and probe B (E). Amplification of mouse genomic DNA by primer pair P1 and P2 (F) and P3 and P4 (G). Coronal sections of the brain from wt (H, I, and J) and *VPAC2*$^{-/-}$ (K, L, and M) mice were incubated with labeled ^{125}I-Ro25-1553. (H) and (K) are autoradiograms showing total binding of ^{125}I-Ro25-1553, while (I) and (L) show nonspecific binding (in the presence of 20 nM unlabeled Ro25-1553). (J) and (M) are the hematoxylin-eosin-stained sections equivalent to (H) and (K), respectively. Strong labeling of SCN is seen in wt mice only (H) (adopted from (Harmar et al., 2002)).

5.2.4.2 Injections, sacrifice and perfusion

All the experimental work related to BrdU injections and surgery was carried out in Edinburgh by Dr. W. J. Sheward, (The Queen's Medical Research Institute, Edinburgh) as detailed below. Eight-week-old mice received single daily dose of intra-peritoneal injections of 5-bromo-2-deoxyuridine (BrdU) at 50 μg/kg body weight (10 mg/mL stock, dissolved in normal saline (0.9% NaCl)) for 5 successive days, and were then allowed to live for four weeks before being sacrificed. For all mice, sacrifice was performed by administration of a terminal dose of phentobarbitone, followed by trans-cardiac perfusion initially with normal saline (0.9% NaCl) and followed by 4% PFA. Whole brains were then post-fixed in PFA and stored at 4°C and transported to our labs. This protocol allowed evaluation survival and characterization of newly born cells in the two groups of mice ($VPAC2^{-/-}$ and Wild type).

5.2.4.3 Sectioning and immunohistochemistry

Sections containing the hippocampal formation were obtained for immunochemistry on vibratome (Leica VT100M) cutting in the coronal plane at 40μm thickness. Approximately, 60 sections, comprising the entire hippocampal formation from each brain were collected and stored sequentially in a 96-well plate such that the position within the hippocampus of any individual section could be determined.

Pre-mounted sections were initially rinsed once in TBS before being denatured in 2MHCl at 37°C for 30min. After three times washing, sections were treated with 3%H2O2 in water for 30min to block endogenous peroxidase activity. Sections were then washed three times in TBS before being blocked in TBS.0.1% Triton (TBS.T) and 3% Goat blocking serum for 30 minutes at room temperature. After blocking the non-specific binding sites, sections were incubated in TBS.T Triton containing Rat anti-BrdU antibody (Biodesign #M20105S), diluted 1:500 in TBS.T. Following three TBS washes, primary antibodies were probed by biotinylated Goat anti-rat Secondary antibody in TBS.T for two hours at room temperature. To reduce background, slides were rinsed 3 times in TBS. Specifically bound biotin-conjugated secondary antibody was then probed using streptavidin ABC/ peroxidase complex (1;200; Dako, Glostrup, Denmark) and visualised by diaminobenzidine (DAB) (Vector Labs) staining. Mounted sections were rinsed free of salt in distilled water before being dehydrated

through a graded series of ethanol solutions (2x 70%, 2x 90% and 2x 100%) cleared in xylene and the cover-slipped in DPX mountant (Fisher). Staining was visualised and pictures were taken using inverted light microscope (Improvision, Lexington, MA, USA).

5.2.5 Cell counting, area measurement and data analysis

For the *in vitro* imaging was performed on an inverted DM IRB microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). The area of a 20× field was measured using a 255 μm grid graticule slide (Microbrightfield, Williston, USA). Cell counting was performed on 6 random 20× fields per well using the Open Lab image-capturing system version 2.1 (Improvision, Lexington, MA, USA). Raw data from the 20× field counts were averaged and plotted ± SEM and expressed as cells/mm² per well, based on a sample of four to eight wells per condition per experiment. All experiments were repeated at least three times. One experiment consisted of sixteen hippocampi from eight different animals, pooled and prepared as described above. Data points were plotted using GraphPad Prism data analysis software (GraphPad inc. USA). The statistical significances between the means was assessed by ANOVA followed by post hoc tests, with $P<0.05$ considered significant.

In vivo quantification analysis and sampling of BrdU-positive cells was performed throughout the DG in its rostrocaudal extension where the total $BrdU^+$ cells were determined in four sections per animal with the investigator blind to the identification of the animal. Every sixth section (40 μm) was used for counting, the numbers of BrdU cells from each animal were added and the total number was obtained by multiplying the value by 6. The cross-sectional area of the dentate gyrus was determined by use of Leitz Dialux22 microscope with 20x objective and the data were expressed as number of cells per dentate. Means were determined for these variables, and the data were subjected to two-tailed Student's *t* tests with $P<0.05$ considered significant. We also used the stereology Leitz Dialux22 microscope to measure the area of the GCL in both VPAC2-/-and wild type animals.

5.2.6 Investigating VPAC1 and VPAC2 mRNAs expression in hippocampal cell culture

To examine the expression of VPAC1 and VPAC2 in hippocampal cell cultures harvested from postnatal rats (P7-10), we carried out a semiquantitative- PCR. The aim was to determine if the mRNAs of these receptors are expressed by cultured hippocampal cells by the time we treat them with VIP or not.

5.2.6.1 RNA extraction and cDNA synthesis:

We used (as detailed in **Section 4.2.10**) the SuperScript™ III CellsDirect cDNA Synthesis Kit (Invitrogen™ life technologies) to directly synthesize the first-strand cDNA from our progenitor cell lysate without firstly isolating RNA. Primary hippocampal cultures were first generated as pointed in details in (**Sections 2.2.2-2.2.4**). At 2hrs after plating, we started the procedure of cDNA synthesis. cDNA concentration and purity were again determined using a spectrophotometere (NanoDrop® ND-1000 UV-Vis Spectrophotometer). Samples were accordingly either stored at -20°C in the freezer or directly diluted to the indicated concentration (5ng/ml) for PCR essay.

Using these single-stranded cDNA as templates, PCR was carried out via a one-step RT-PCR kit (Rat Custom real-time PCR assay for use with SYBRgreen chemistry) (PrimerDesign Ltd, Southampton) in a PCR machine (Rotor-Gene 6000, Corbett Robotics. Ltd). PCR reactions were performed according to the kit manufacturer instructions for 40 cycles (**see Sections 4.2.11 and 4.2.12 for details**). For VPAC1, VPAC2 and the housekeeping gene ß-actin mRNAs, primers were designed by PrimerDesign Ltd (Southampton) and provided in a lyophilised form. Each primer tube was first centrifuged to ensure that the primer mix is in the bottom and then reconstituted in RNase free water as indicated by the provider (i.e. 660µl). Forward and reverse primers, sequence, positions on the gene, melting temperatures and expected product lengths are as detailed (**Table 5.1**) below:

Primer	Sequence	Position	Tm	Product length
VPAC1	Forward:TAACTGAAGCGGGTGTGGAT	3,842	57	113
	Reverse:CCTCTCCTAGCCCTCAAACA	3,954	56.4	
VPAC2	Forward:CGGATTTCATAGATGCGTGTG	420	55.8	99
	Reverse:CACTGTAGCCCAAGGTATAAAATG	518	55.7	
ß-actin	PrimerDesign Ltd ß-actin primers			

Table 5.1: VPAC1, VPAC2, and ß-actin primers. This table shows the primers' sequences, position on the gene of origin, melting temperature and the amplicon length.

For this purpose the Rotor Gene machine was programmed according to instruction of the Kit provider. The PCR reaction amplification conditions were: enzyme activation for 10min at 95°C followed by 15 second for denaturation at 95°C and then the data was collected in 60 seconds at 60°C. Reaction amplifications for each product were then run on 2% agarose gel containing ethidium bromide and visualised using UV light.

5.6.6.2 Immunocytochemistry for VIP receptors:

At day 5, cells were fixed in 4% PFA for 30 minutes. Cells were then washed three times in PBS free of PFA. Non specific binding sites were blocked with 10% donkey blocking serum (DBS) in PBS for 20 minutes at room temperature. Then cells were incubated in goat anti-VPAC1 or anti-VPAC2 primary antibodies in PBS containing 1.5% DBS for 60 minutes at room temperature. After that, cells were washed and then incubated in Cy3-conjugated anti-goat secondary antibody in PBS containing 1.5% DBS for 45 minutes at room temperature in a dark chamber. Finally, cells were washed again three times with PBS and then counterstained in DAPI.

5.3 Results

5.3.1 The VPAC1 and VPAC2 receptors and their mRNA are expressed by cells in hippocampal cell culture

The expression of VPAC1 and VPAC2 receptors' mRNA was examined in hippocampal cultures at 2hrs after plating. We used two positive controls: cDNA extracted from whole Wistar rat brain and universal pooled cDNA extracted from many rat tissues. Our negative control included the addition of water instead of cDNA to the PCR reaction. ß-actin was used as our housekeeping gene. The amplification products of our PCR were run on 2% agarose gel stained with ethidium bromide and then visualised with UV light. As shown (**Figure 5.2**): VPAC1, VPAC2 and ß-actin are expressed in our hippocampal cell culture. Both of them were also expressed in the universal control and to a lesser extend in whole brain tissue. No bands were traced under negative control.

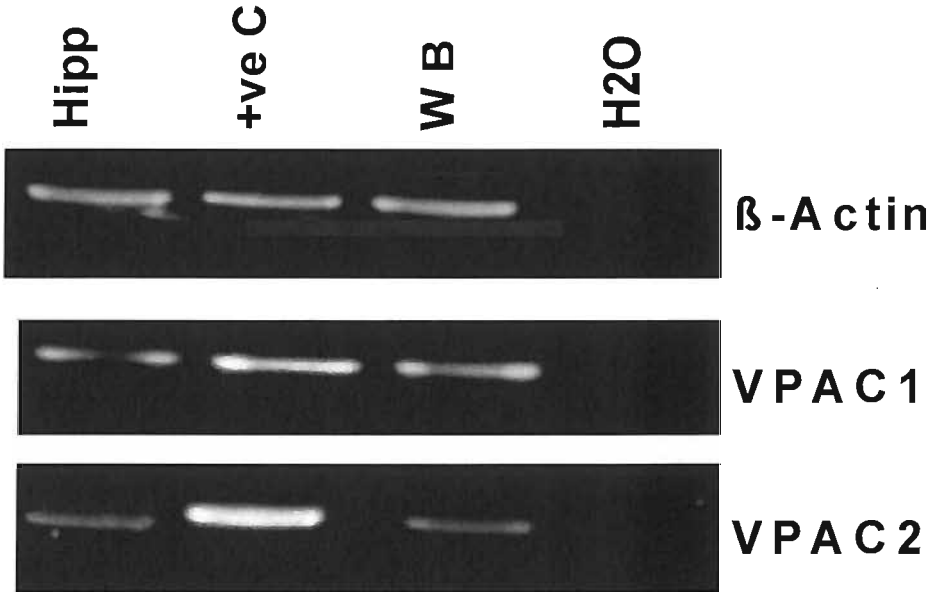


Figure 5.2: Expression of mRNAs for VPAC1 and VPAC2 in primary hippocampal neuronal progenitor cells derived from postnatal Wistar rats (P7-10), as shown by 2% agarose electrophoresis. Hipp: Hippocampal cell culture; +ve C: positive control (universal rat cDNA as a template); WB: whole brain. H2O; where water was added instead of cDNA as a negative control. ß-actin was used as a housekeeping gene.

Immuno-staining of 5 day-old hippocampal cultures grown in 30nM VIP revealed high levels of VPAC1 and VIPAC2 signals on the major cell specific phenotypes in culture.VPAC1 and VPAC2 immuno-reactivity was frequently observed to label flat

rounded cells with long and short processes. This was confirmed by double labelling visualized by sequential scanning of cyanin3 (red, VPAC1/VPAC2) and cyanin2 (green, nestin) channels (**Figure 5.3 & Figure 5.4**). In addition, both VPAC1 and VPAC2 showed immuno-reactivity in cells with neuronal morphology. These cells were again visualized using sequential scanning and found to be dual-labelled for VIP receptors and the neuronal marker class III ß-tubulin.

VPAC1 and VPAC2 receptors were also expressed by cells with astrocytic morphology that are co-expressing the astrocytic marker GFAP (**Figure 5.3 & Figure 5.4**). Generally, VPAC2 expression was more dominating than VPAC1 on the major cell phenotypes.

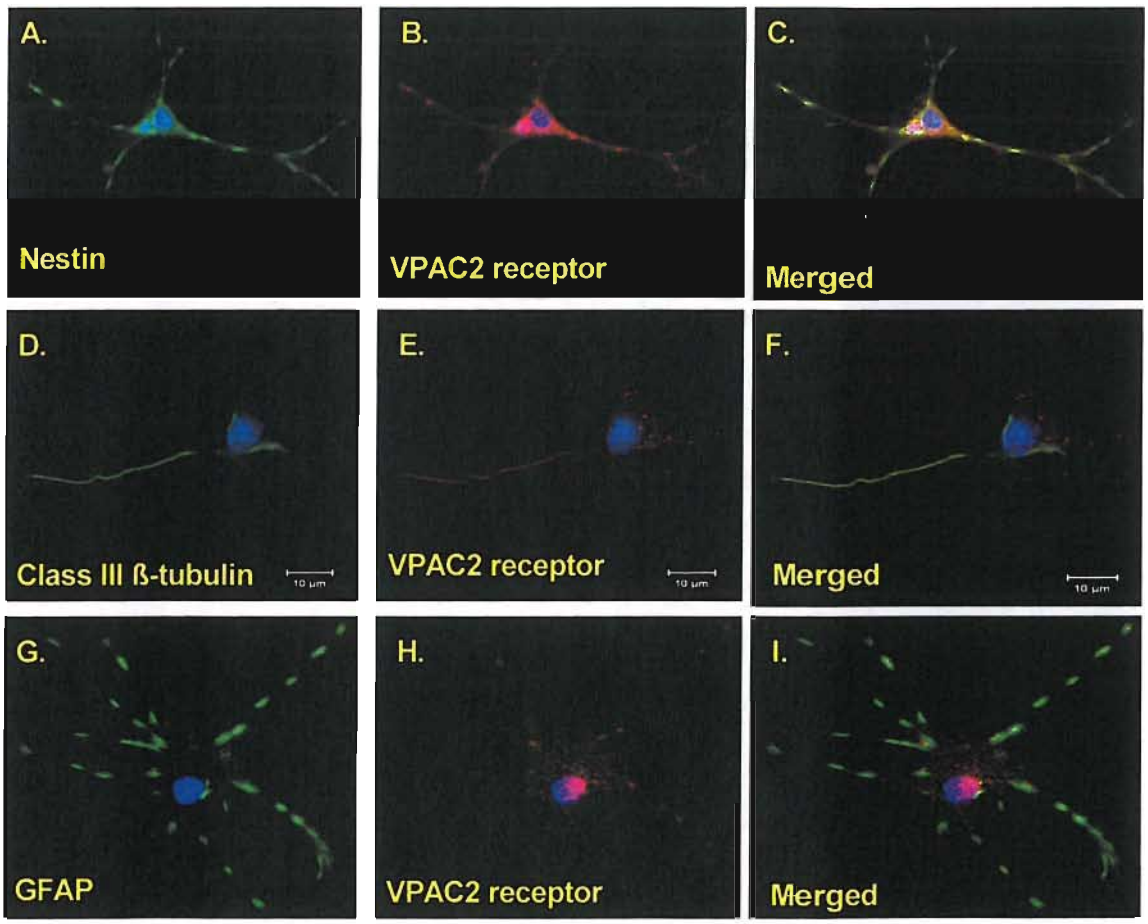


Figure 5.3: VPAC2 receptor is expressed by the major cell specific phenotypes in culture. Cells were grown for 5 days in culture then phenotyped for the expression of VPAC2 (B, E and H) co-labelled with nestin (A) , GFAP (H) and class III ß-tubulin (E) markers. D, F and I represent the merged images.

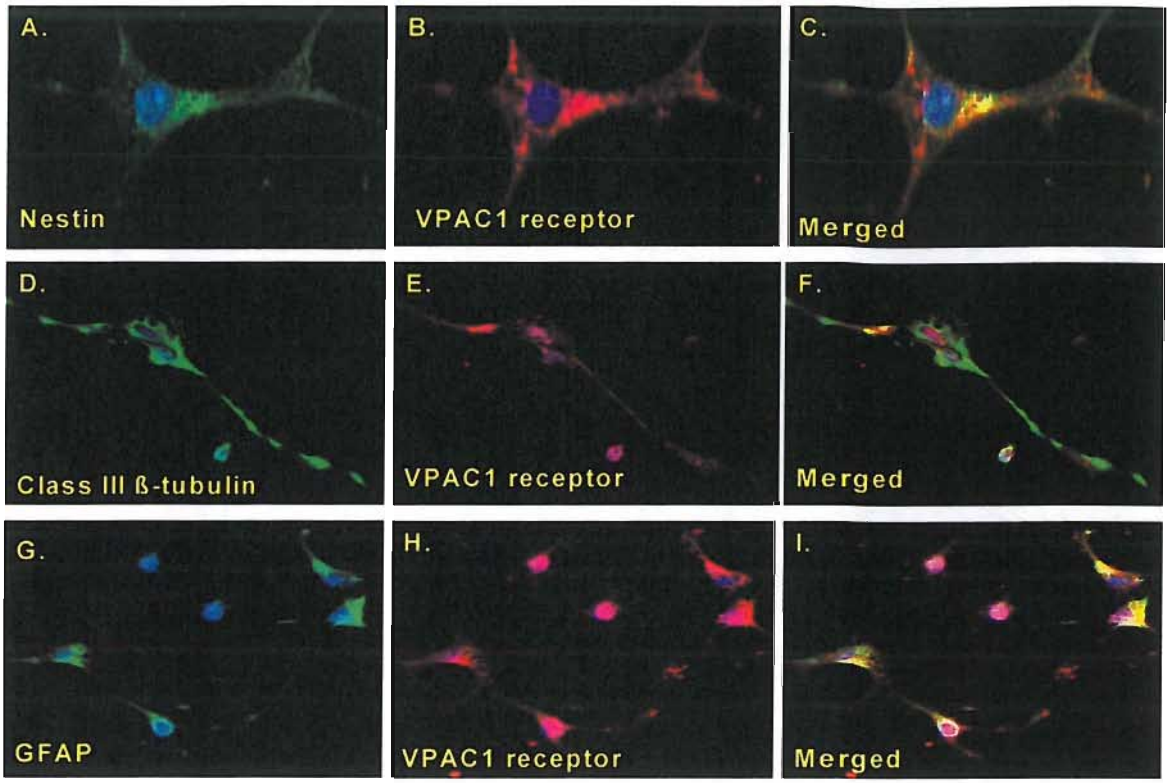


Figure 5.4: **VPAC1 receptor expression on the major cell-specific phenotypes**. Cells were grown for 5 days in culture then phenotyped for the expression of VPAC1 (B, E and H) co-labelled with nestin (A), GFAP (H) and class III ß-tubulin (E) markers. D, F and I represent the merged images.

5.3.2 VPAC2 mediates survival of hippocampal progenitor cells *in vitro*

Hippocampal progenitor cells were grown for 5 days under standard control conditions and control plus 30nM VIP, 1μM VPAC1 agonist, 1μM VPAC1 antagonist, 1μM VPAC1 antagonist and 30nM VIP, 10nM VPAC1/VPAC2 antagonist, or 10nM VPAC1/VPAC2 antagonist and 30nM VIP. As expected, the total number of cells as measured by DAPI cell counts increased significantly from 136.47 ± 1.76 cells / mm² under control conditions to 199.8447 ± 5.28 cells / mm² under VIP treatment (30nM) (**Figure 5.5**). This increase was statistically significant. The number of total cells slightly, but insignificantly increased under VPAC1 agonist reaching 146.75 ± 8.13 cells / mm². The addition of VPAC1 antagonist decreased the number of cells insignificantly to 133.93± 3.89 cells / mm² (**Figure 5.5**). Likewise, this change was statistically insignificant. Interestingly, the number of total cells in cell cultures under VIP combined with VPAC1 antagonist increased nearly the same as for VIP alone treatment compared to control conditions (217.45± 12.37 cells / mm²) (**Figure 5.5**). VPAC1/VPAC2 antagonist treatment had no effect on total cell counts (134.62± 8.13 cells / mm²) compared to control conditions. However, the

combined treatment of VIP plus VPAC1/VPAC2 antagonist has abolished VIP effects on total cell counts (135.06± 3.63 cells / mm²) as compared to control conditions (**Figure 5.5**).

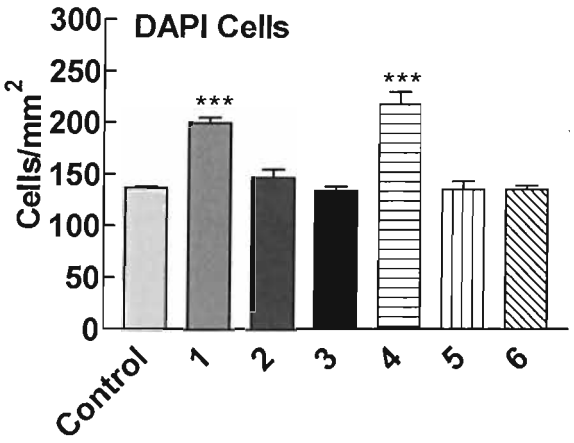


Figure 5.5: The VIP survival effect is mediated by VPAC2. Cell cultures were grown under control conditions or control plus **1**: 30nM VIP, **2**: 1μM VPAC1 agonist, **3**: 1μM VPAC1 antagonist, **4**: 1μM VPAC1 antagonist and 30nM VIP, **5**: 10nM VPAC1/VPAC2 antagonist, and **6**: 10nM VPAC1/VPAC2 antagonist and 30nM VIP. Data represent mean ± SE based on a sample that represent at least 12 wells per condition from three different experiments. Comparisons between different conditions are one way ANOVA with Dunnetts' multiple comparison test (***, $p < 0.001$).

5.3.3 VIP effects on putative hippocampal nestin-expressing cells are VPAC2-mediated

In this set of experiments, hippocampal cell cultures were generated from postnatal rats (P7-10). Cells in different wells were grown for 5 days under control conditions, 30nM VIP, 1μM VPAC1 agonist, 1μM VPAC1 antagonist, 1μM VPAC1 antagonist and 30nM VIP, 10nM VPAC1/VPAC2 antagonist, or 10nM VPAC1/VPAC2 antagonist and 30nM VIP. Cells grown under all conditions were fixed and then processed for the expression of the putative/stem cell marker nestin. Absolute numbers as well as proportions of cells expressing nestin, with respect to DAPI cells were then determined. As indicated in **(Figure 5.6 A)** , the absolute number of nestin cells, in parallel with DAPI counts, increased compared to control conditions under VIP treatment only and once combined with the VPAC1 antagonist, 150.55± 3.75 cells / mm² and 149.99± 9.93 cells / mm², respectively. Also VIP increased the proportions of nestin cells compared to control conditions (0.53± 0.04) by its own and once combined with the VPAC1 antagonist to 0.75± 0.2 and 0.69± 0.02, respectively **(Figure 5.6 A & B)**. However, VIP effect on the total numbers and proportions of

nestin expressing cells was abolished once VIP treatment was combined with 10nM VPAC1/VPAC2 antagonist, 82.34± 4.28 cells / mm² and 0.61 ± 0.03. VPAC1 agonist, VPAC1 antagonist or VPAC1/VPAC2 antagonist had slight but insignificant changes on the number and proportions of nestin cells in culture (**Figure 5.6 A & B**).

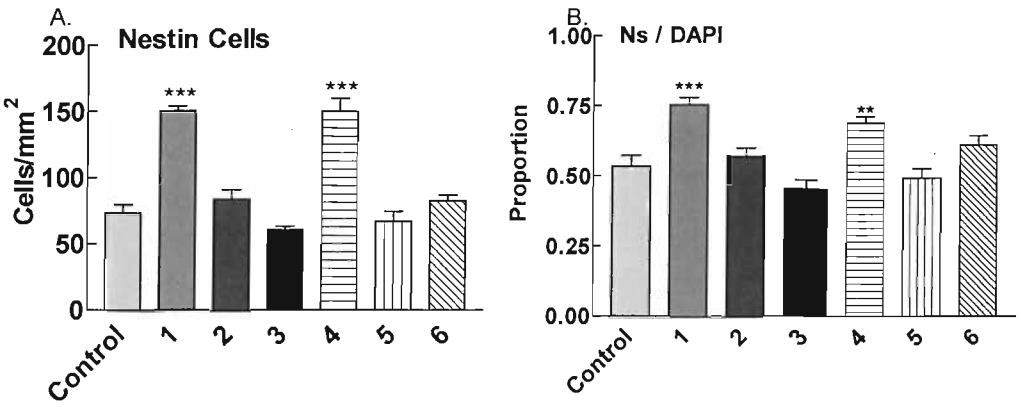


Figure5.6: VPAC2 mediates VIP effects on nestin cells' survival and self-renewal. Primary hippocampal cell culture were grown for 5 days under control conditions or control plus **1**: 30nM VIP, **2**: 1μM VPAC1 agonist, **3**: 1μM VPAC1 antagonist, **4**: 1μM VPAC1 antagonist and 30nM VIP, **5**: 10nM VPAC1/VPAC2 antagonist, and **6**: 10nM VPAC1/VPAC2 antagonist and 30nM VIP. Cells were then processed for the expression of the stem cell marker nestin. Absolute numbers and proportions of nestin-expressing cells were then worked out. Data represent mean ± SE based on a sample that represent at least 12 wells per condition from three different experiments. Comparisons between different conditions are one way ANOVA with Dunnetts' multiple comparison test (**, $p < 0.01$ and ***, $p < 0.001$).

5.3.4 While VPAC2 mediated VIP effects on the survival of neuroblasts, VPAC1 mediates neurogenesis

In a separate set of experiments, cells dissociated from postnatal hippocampi were grown for 5 days under control conditions or control plus 30nM VIP, 1μM VPAC1 agonist, 1μM VPAC1 antagonist, 1μM VPAC1 antagonist and 30nM VIP, 10nM VPAC1/VPAC2 antagonist, or 10nM VPAC1/VPAC2 antagonist and 30nM VIP. After 5 days, cells were fixed in 4% PFA and stained for the neuronal marker TuJ1. Counts of TuJ1-expressing cells and their proportions with respect to the total number of cells in culture were worked out. As shown in (**Figure 5.7 A**), VIP like before increased the number of TuJ1 cells from 30.00 ± 1.44 cells / mm² under control to 44.36 ± 2.14 cells / mm² under VIP tretment, with no proportional change in this phenotype of cells in culture (**Figure 5.7 A & B**). However, co-treatment of cultured hippocampal cells with VPAC1/VPAC2 antagonist and VIP abolished VIP effect

(21.97 ± 2.80 cells / mm²). This antagonist, by its own, had no significant effect on the number or proportion of TUJ1 cells. Interestingly, VPAC1 agonist increased the absolute number and the proportions of TuJ1 expressing cells to 43.52 ± 3.81 cells / mm² and 0.30 ± 0.01 compared to control conditions 30.00 ± 1.44 cells / mm² and 0.22 ± 0.01, respectively (**Figure 5.7 A & B**). VPAC1 antagonist by its own had significant effect on neither the absolute numbers nor the proportions of TuJ1 cells.

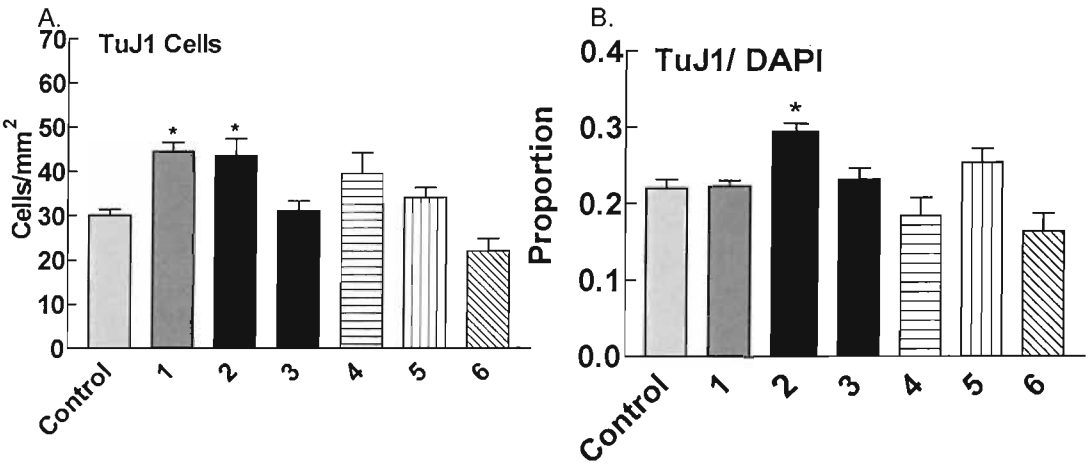


Figure 5.7: VPAC2 mediates VIP survival effects on TuJ1-expressing neuroblasts and VPAC1 mediates neuronal generation. Cultured hippocampal progenitor cells were grown for 5 days under control conditions or control plus **1**: 30nM VIP, **2**: 1μM VPAC1 agonist, **3**: 1μM VPAC1 antagonist, **4**: 1μM VPAC1 antagonist and 30nM VIP, **5**: 10nM VPAC1/VPAC2 antagonist, and **6**: 10nM VPAC1/VPAC2 antagonist and 30nM VIP. Cells were then processed for the expression TuJ1. Absolute numbers and proportions of neuronal cells expressing TuJ1 were then worked out. Data represent mean ± SE based on a sample that represent at least 12 wells per condition from two different experiments. Comparisons between different conditions are one way ANOVA with Dunnetts' multiple comparison test (*, $p < 0.05$).

5.3.5 VPAC1 and VPAC2 receptors mediated VIP-enhanced astrocytic cell survival

Cells from the hippocampus of postnatal rats were grown under either under standard control conditions, 30nM VIP, 1μM VPAC1 agonist, 1μM VPAC1 antagonist, 1μM VPAC1 antagonist and 30nM VIP, 10nM VPAC1/VPAC2 antagonist, or 10nM VPAC1/VPAC2 antagonist plus 30nM VIP. Cells in culture were then stained for the expression of the astrocytic cell marker GFAP. Total counts and proportions of astrocytic cells were then measured. As revealed by our results, VIP at 30nM peptide concentration increased the total number of GFAP cells to 146.47 ± 10.66 cells / mm²

compared to 93.80 ± 3.00 cells / mm² (**Figure 5.8 A**). As expected, the proportions of GFAP cells did not change under VIP treatment compared to controls. None of the peptides, including VPAC1 agonist, VPAC1 antagonist or VPAC1/VPAC2 antagonist had a significant effect on the total numbers of GFAP cells or their proportions (**Figure 5.8 A**). However, the VIP effect we have seen on the total number of astrocytic cells was abolished when VIP treatment was combined with either the VPAC1 antagonist or the VPAC1/VPAC2 antagonist were astrocytic cell counts dropped down to 101.11 ± 10.66 cells / mm² and 101.26± 10.66 cells / mm², respectively (**Figure 5.8 A**). There was no proportional change under VIP plus VPAC1/VPAC2 antagonist, but the proportion of GFAP cells significantly decreased to 0.41 ± 0.03 under VIP and VPAC1 antagonist treatment (Compared to controls; 0.63± 0.04) (**Figure 5.8 B**).

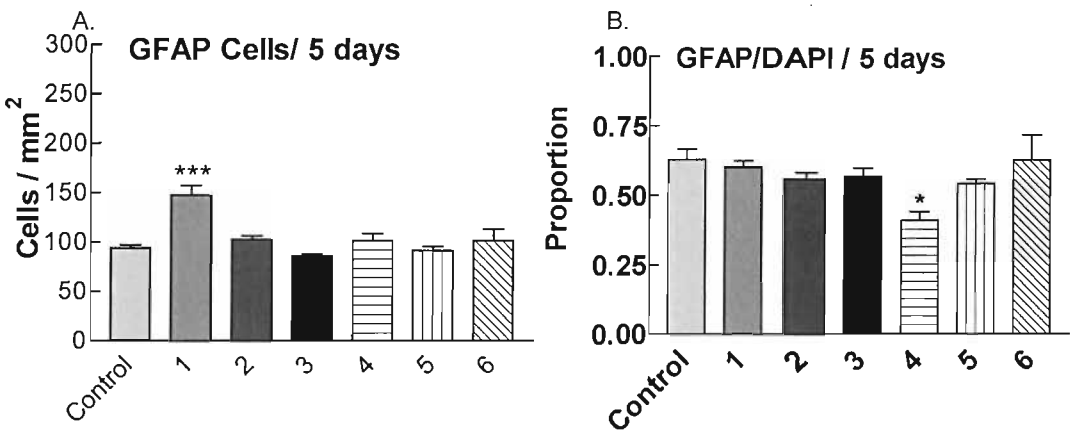


Figure 5.8: VIP enhances astrocytic cell survival via VPAC1 and VPAC2. Hippocampal progenitor cells harvested from postnatal Wistar rats (P7-10) were grown under control conditions or control plus **1**: 30nM VIP, **2**: 1μM VPAC1 agonist, **3**: 1μM VPAC1 antagonist, **4**: 1μM VPAC1 antagonist and 30nM VIP, **5**: 10nM VPAC1/VPAC2 antagonist, and **6**: 10nM VPAC1/VPAC2 antagonist and 30nM VIP. Cells were then processed for the expression TuJ1. Absolute numbers and proportions of neuronal cells expressing TuJ1 were then worked out. Data represent mean ± SE based on a sample that represent at least 6 wells per condition from only one experiment. Comparisons between different conditions are one way ANOVA with Dunnetts' multiple comparison test (*, $p < 0.05$ and ***, $p < 0.001$).

5.3.6 Cell survival is reduced in VPAC2 knockout mice

To evaluate if VIP and the VPAC2 receptor play a similar role *in vivo* to our reported findings of cell survival by VIP through VPAC2 receptor *in vitro*, we have compared the number of BrdU labelled cells within the dentate of the hippocampus of $VPAC2^{-/-}$ to wild type controls subjected to BrdU. Four adult wild type (control) and four adult $VPAC2^{-/-}$ mice from the same founder pair and housed under standard conditions were administered BrdU intraperitoneally for 5 consecutive days and allowed to survive for 4 weeks before being sacrificed and processed for BrdU immunoreactivity. Within the dentate gyrus, BrdU cells visualized with the chromagen DAB (**Figure 5.9 B & C**) were (although not many) scattered in the hilus, the subgranular cell layer, granule cell layer and even the outer region.

As expected, we had no staining in the negative controls (**Figure 5.9 D**). All counting was carried out blindly to the status of the animal. We have first counted the number of BrdU labelled cells in both dentates (left and right) in each section from the two groups of animals. We found no difference in the total number of BrdU cells in the left compared to the right dentate within each specific group of animal. Stereological estimation of dentate gyrus cells labelled with BrdU revealed a 40% decrease in the number of BrdU cells in the $VPAC2^{-/-}$ group in comparison to controls (174.0 ± 14.90 cell /dentate and 105.0 ± 12.61 cell / dentate for control and $VPAC2^{-/-}$ groups, respectively) (**Figure 5.9 A**) . Moreover, while the GCL looks thinner in the knockout mice compared to controls, the area of the GCL as measured by stereology microscope is signifacnty smaller in VPAC2-/- compared to wild type animals 0.16 ± 0.01 mm^2 vs. 0.22 ± 0.01 mm^2).

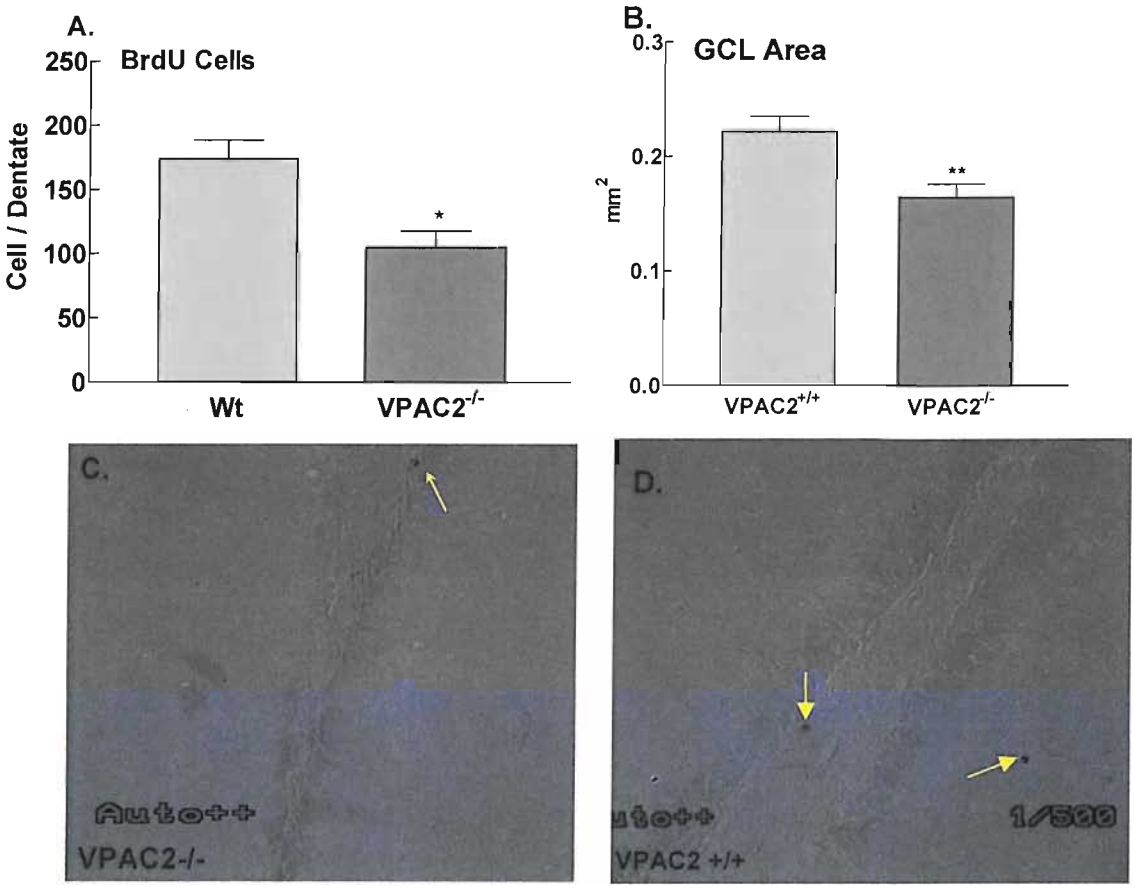


Figure 5.9: VPAC2$^{-/-}$ mice have reduces survival of BrdU cells in the dentate gyrus. Four wild type controls and four VPAC2$^{-/-}$ of 8 weeks old mice were given BrdU (50mg/Kg) intraperitoneally once per day for 5 days and sacrificed 4weeks after the final injection. (A) Total BrdU counts per dentate. Data represents BrdU counts from the whole dentate where in 4 sections per animal serially sampled along the rostro-caudal extent of the hippocampus from four animals per group, BrdU positive nuclei (arrowed) were scored at high magnification. Immuno-histochemical detection of BrdU is shown in representative sections of wild type animals (C), VPAC2 knockouts (D). (B) the area in mm^2 of the GCL as measured by the stereology microscopy based on sampling four sections per animal from four animals per group. Values (for BrdU counts and GCL areas are meanes ± SE, Comparisons between VPAC-/- and wild type animals are simple *t*-student test with *, $p < 0.05$ and **, $p < 0.01$ are considered significant.

5.4 Discussion

VIP mediates its effects via at least three ligand receptors: VPAC1, VPAC2 and PAC1. These receptors have been demonstrated to be widely expressed in the rodent hippocampus(Vaudry et al., 2000). However, there were few reports concerning their expression at the cellular level in the rodent brain(Cazillis et al., 2004; Hirose et al., 2005) and none of them demonstrated their cellular expression by the precursor cells and their progeny in the hippocampus.

5.4.1 VPAC1, VPAC2 and their mRNAs are expressed in hippocampal progenitor cell culture

VIP effects in the CNS are mediated by three receptors:VPAC1, VPAC2 and PAC1. The expression of these receptors has been studies extensively. The three receptors have been shown to be expressed by cortical astrocytes and embryonic stem cells (Masmoudi-Kouki et al., 2007). PAC1 has been shown to be also expressed by neuronal stem cells (Masmoudi-Kouki et al., 2007). However, we are not aware of any reports on the expression of VPAC1 and VPAC2 receptors by neural stem cells that are co-expressing nestin. Using specific antibodies for rat VPAC1 and VPAC2, we demonstrated the expression of VPAC1 and VPAC2 receptors by neuronal, astrocytic and most importantly putative/stem cells. Our findings with regard to the expression of VPAC1 by neuronal and astrocytic cells are in agreement with other studies (Joo et al., 2005; Masmoudi-Kouki et al., 2007), but the expression of these receptors by nestin expressing putative/stem cells is an original novel finding.

The mRNAs of VPAC1 and VPAC2 have been studied as well in more than one model in rodents. The distribution of mRNAs has been found to match quite well with the distribution of the proteins themselves (Joo et al., 2005). mRNAs encoding for VPAC1 and VPAC2 have been shown to be expressed by embryonic stem cells, but again we are not aware of any experimental work demonstrating their expression in hippocampal progenitor cell cultures. Using real-time-PCR, we have demonstrated clearly the expression of both VPAC1 and VPAC2 receptors in out hippocampal stem cell cultures.

Taken together, our findings indicate the expression of the high affinity receptors VPAC1 and VPAC2 and their related mRNAs. These findings strongly support our hypothesis that VIP trophic and self-renewal effect of hippocampal progenitor/precursor cells, at nanomolar concentrations, are mediated via the high affinity receptors. It is therefore, of great importance to elucidate the contribution of each one of these receptors to the process of hippocampal neurogenesis.

5.4.2 VPAC2 mediates cell survival and self-renewal of hippocampal progenitor cells

VPAC1, VPAC2 and PAC1 subtypes of the VIP receptor family have been demonstrated to be expressed in the adult rat hippocampus and dentate gyrus (Joo et al., 2004). We have not accounted for any possible PAC1 receptor-subtype mediated effects on our culture system as VIP at concentrations of less that 500-1000nM does not bind to this low affinity receptor (Vaudry et al., 2000; Moody et al., 2003). We believe that VIP trophic effects (as shown in **Sections 3.4.2 and 3.4.3**) on cultured hippocampal cells are mediated by VPAC1 and/or VPAC2. To further address this issue, we used the synthetic peptide [Lys15, Arg16, Leu27]-VIP (1-7) - GRF (8-27) and [Ac-His1, D-Phe2, Lys15, Arg16, Leu27]-VIP (3-7)-GRF (8-27) as a VPAC1 receptor agonist and antagonist, respectively. We also used the VPAC1/VPAC2 non selective antagonist [D-*p*-Cl-Phe^{6}, Leu^{17}]-Vasoactive Intestinal Peptide in an attempt to abolish VIP effects in culture. Although a VPAC2 selective agonist has been reported for VPAC2 (Gourlet et al., 1997c), there is a considerable debate whether this peptide is a true selective agonist for VPAC2 receptor or not (Laburthe et al., 2007) and it is commercially unavailable.

As our results indicate, the addition of VPAC1 selective agonist did not mimic VIP effect on total cell counts, on the absolute number of nestin cells or their proportions. In addition, the co-treatment of cells with VIP and the VPAC1 selective antagonist had no effect at all on VIP effects on total cell counts or the counts and proportions of nestin-expressing cells. It was not until we co-treated the VIP treated cells with the VPAC1/VPAC2 non-selective antagonist when we completely abolished VIP effects on nestin cells. These findings clearly implicate VPAC2 receptor in the survival of hippocampal cells, and the self-renewal of putative/stem cells expressing nestin.

The development of VPAC2 receptor deficient mice provided us with a useful tool to further evaluate the role of VPAC2 receptor subtype in mediating trophic effects of VIP. Using a protocol as detailed in **section 5.2.4** and as mentioned else where (van Praag et al., 2005), we have been able to quantify the survival of newly born cells in the dentate gyrus in VPAC2 knockout mice compared to controls (wild type). Consistent with our finding *in vitro*, we found that the number of BrdU cells that have survived over a periods of 4 weeks were 40% less than the VPAC2 knockout mice compared to their counteract wild type group. This decrease was statistically significant (p <0.01). in addition and as might be expected the area of the GCL is reduced in the VPAC2 knockout. These findings taken together with the fact that the GCL is largely formed postnatally (Namba et al., 2007), support our hypothsis that VIP is an important mediator of hippocampal neurogenesis.

5.4.3 VPAC1 is an important mediator for hippocampal neurogenesis *in vitro*

VIP, in agreement with our previous findings (**Section 3.4.5**), increased the number but not the proportions of neuronal precursor cells expressing TuJ1. Interestingly, the addition of VPAC1 agonist mimicked VIP action on the absolute number of cells. Furthermore, this VPAC1 selective agonist enhanced a significant proportional increase in this phenotype of cells. Because VPAC1 agonist effects were devoid from any affect on nestin cells, this proportional increase in TuJ1 may indicate a pure differentiation effect toward neuronal lineage of cells. Also because the proportions of nestin cells did not drop under VPAC1 agonist treatment, this population of TuJ1 cells is going to be nestin positive as well. Our findings are in agreement with previous results in which VPAC1 has been reported to play a crucial role in the regulation of neurogenesis in the rat brain (Basille et al., 2000). Both the trophic effect of VIP on this cell population and the differentiation effect of VPAC1 agonist were abolished as we added the VPAC1/VPAC2 antagonist. These results are in agreement with the findings of Hirose et al and others in which he has shown that VPAC1 along with VPAC2 are expressed in embryonic stem cell cultures as they go through neuronal differentiation (Cazillis et al., 2004; Masmoudi-Kouki et al., 2007). This may partially contribute to the role diversity of the two receptors in the modulation of hippocampal neurogenesis. The fact that hippocampal neurogenesis is decreasing with age (Sohur et al., 2006), taken collectively with findings of Joo et. al. in which he showed a marked reduction in the expression of VPAC1 in the hippocampus of aged rats, is a

possible evidence for the involvement of this receptor subtype in the VIP modulation of hippocampal neurogenesis (Joo et al., 2005).

5.4.4 VPAC1 and VPAC2 receptors are necessary for VIP trophic effect on astrocytic cells.

Our results presented in **(section 5.3.5)** indicate that VIP effects on astrocytic cells expressing GFAP are abolished by the addition of VPAC1 selective antagonist. However, the VPAC1 agonist failed to mimic the effects of VIP on this subpopulation of cells on its own. Two possible explanations can be derived from these findings. Firstly, both VPAC1 and VPAC2 are required for VIP to enhance survival of astrocytic cells. Secondly, VIP enhanced the survival of these cells through another mechanism that involves neither. However, the fact that VPAC1/VPAC2 antagonist by itself abolished VIP effects supports the first hypothesis. Yet, this needs to be further investigated through the use of other VPAC2-selective peptide agonist and antagonist as they become available.

5.5 Summary

VPAC1 and VPAC2 receptors are important for VIP effects on culture hippocampal cells. VIP is likely to enhance the survival and self-renewal of putative/stem cells expressing nestin through binding the VPAC2 receptor. Similarly, via VPAC2 VIP also enhanced the survival of TuJ1-expressing neuronal precursor cells. *In vivo*, $VPAC2^{-/-}$ mice displayed a 40% reduction in the newly-born cells compared to their counteract controls. While VPAC1 on its own mediated hippocampal neurogenesis, appeared to interact with VPAC2 in the modulation of the survival of astrocytic cells expressing GFAP. These findings support our hypothesis that VIP effects, at physiological concentrations, on cultured hippocampal cells are mediated by the high affinity receptors.

Chapter 6

VIP interactions with other neuropeptides and growth factors in the modulation of hippocampal neurogenesis

6.1 Introduction

There is increasing evidence that the regulation of progenitor cell states involves interactions between different intracellular mechanisms and environmental signals, including growth factors (Abrous et al., 2005). We believe that the regulation of the survival and renewal is one of the most important aspects of progenitor cell development that needs to be further investigated. In this regard, we will be addressing growth factor interactions in the modulation of hippocampal neurogenesis, particularly VIP interactions with the neuropeptide NPY or the cytokine FGF-2. We will also examine the effects of the neuropeptide galanin on postnatal hippocampal stem cell survival and proliferation.

6.1.1 NPY is a proliferative neuropeptide that mediates hippocampal neurogenesis

Neuropeptide Y (NPY) has been reported to be involved in many physiological and pathological sates including depression, cognitive function and seizure like hyper-excitability (Scharfman and Gray, 2006; Laskowski et al., 2007). Events such as mood disorders, seizures, memory and learning involve the hippocampus and have been reported to influence hippocampal neurogenesis (Kuhn et al., 1996; Kempermann, 2002; Encinas et al., 2006; Scharfman and Gray, 2006). In this regard, NPY has been investigated thoroughly in our lab. The findings emerged from our group demonstrated NPY as proliferative peptide for nestin-expressing hippocampal progenitor cells and TuJ1-expressing neuroblasts with no effect on glial cells expressing GFAP (Howell et al., 2003). These effects have been shown to be mediated via Y1 receptor subtype (Howell et al., 2003). Also Y1 knockout mice displayed a reduction in cell proliferation and neurogenesis in the granular cell layer of the dentate gyrus of the hippocampus (Howell et al., 2005). These findings demonstrated NPY as an important modulator of hippocampal neurogenesis. Furthermore, Howell demonstrated a significant reduction not only in basal but also seizure-induced proliferation in the DG of NPY(-/-) mice (Howell et al., 2007).

6.1.2 VIP and NPY in the CNS / hippocampus

In the hippocampus, anatomical and physiological studies have revealed a wide spectrum of different interneurons with considerable differences in physiological parameters (Gulyas et al., 1996). Interneurons can be classified on the basis of their neurochemical marker content, which is considered a reliable marker of its place, and probably of its function, in the hippocampal neuronal networks (Gulyas et al., 1996). GABAergic neurons have been reported to play key roles in synaptic plasticity, learning and memory (Chen et al., 2007; Mohler, 2007). The proportions of GABAergic neurons containing the neuropeptides VIP and NPY among the total GABAergic neurons have been studied in mice hippocampus. In the whole area of the hippocampus, NPY and VIP-positive neurons accounted for about 31% and 8% of GABAergic neurons, respectively (Jinno and Kosaka, 2003).

In relation to the postnatal development of the hippocampus, the level of mRNA of both VIP and NPY has been demonstrated to steadily increase during the early postnatal life hitting the peak at postnatal day 10 (P10). This increase has been observed to accompany the gradient maturation of the different subfields of the hippocampus including the dentate gyrus (Lopez-Tellez et al., 2004). As pointed above, the hippocampus and hippocampal neurogenesis are implicated in pathological states like, epilepsy and mood disorders (Jacobs et al., 2000; Scharfman and Gray, 2006). In this regard, VIP and NPY expression has been shown to be augmented after acute seizure induced by kainate injection (Marksteiner et al., 1989). Similarly, studies on the effects of electroconvulsive treatments, which is used in the treatment of depressive disorders, on hippocampal distribution of NPY and VIP, have shown a significant increase in the expression of the two peptides in rats (Stenfors et al., 1989). Electroconvulsive treatment is an effective treatment for major depression in humans that has been shown to induce hippocampal neurogenesis in nonhuman primates (Perera et al., 2007).

Taking together, the present data may establish potential roles of VIP and NPY in the hippocampal network activity and neurogenesis. Therefore, we will be investigating any possible interactions between VIP and NPY in the modulation of hippocampal cell proliferation and differentiation *in vitro*.

6.1.3 FGF-2: interactions in the modulation of neurogenesis

Fibroblast growth factor-2 (FGF-2, also called basic FGF), a member of the FGF superfamily, is considered a key regulator of hippocampal neurogenesis (Gage et al., 1995; Palmer et al., 1997; Yoshimura et al., 2001; Bull and Bartlett, 2005). Injection of FGF-2 into the brain increases neurogenesis in the dentate gyrus of the hippocampus *in vivo* (Jin et al., 2003). FGF-2 has been shown to modulate adult hippocampal and postnatal cortical neurogenesis (Gage et al., 1995; Tao et al., 1996; Laskowski et al., 2005). Fibroblast growth factor-2 knockout mice have been shown to display significant impairments in cortical neurogenesis (Yoshimura et al., 2001). In fact, in the mice lacking FGF-2, about half of the progenitor cells at the beginning of neurogenesis are missing (Vaccarino et al., 1999).

While FGF-2 by its own is an important regulator, it has been shown to interact variably with other growth and peptide factors in the modulation of neurogenesis. In cultured cells derived from the embryonic hippocampus or cortex, FGF-2 by its own enhanced proliferation of progenitor cells, but when it was combined with neurotrophin-3 treatment, they enhanced neuronal differentiation of these precursor cells with no change in cell proliferation rates (Ghosh and Greenberg, 1995; Temple and Qian, 1995; Jin et al., 2005). In cultured neural precursors cells isolated from embryonic mice hindbrain, cell proliferation has been shown to be enhanced and inhibited when the cells were treated with PACAP alone and PACAP plus FGF-2, respectively (Lelievre et al., 2002). In hippocampal slice cultures, FGF-2 has been shown to enhance proliferation and neurogenesis at the same time while EGF has been shown to enhance proliferation with no effect on neuronal differentiation (Laskowski et al., 2005). In the same model, the combined treatment of the two factors has been demonstrated to enhance cell proliferation with no effect on hippocampal neurogenesis (Laskowski et al., 2005).

We are in this chapter interested in investigating FGF-2 interactions with VIP in the modulation of the survival and proliferation of hippocampal progenitor cells *in vitro*.

6.1.4 VIP and ADNP

In many CNS culture systems, this neuroprotective/ trophic role of VIP has been described to be secondarily modulated by the cytokine interleukin-1 alpha, the serine protease inhibitor (protease nexin I), insulin growth factor I (IGF-I), activity-dependent neurotrophic factor (ADNF) and / or activity-dependent neuroprotective protein (ADNP) (Brenneman et al., 1995; Brenneman et al., 1997; Servoss et al., 2001; Brenneman et al., 2003).

ADNP has been completely sequenced in mouse neuroglial cells and found to contain 828 amino acids (Bassan et al., 1999). Moreover, it has been revealed that ADNP contains the neuroprotective small peptide NAP, which has immunological and structural homology with ADNF (Leker et al., 2002). The gene expression of ADNP has been demonstrated in the mouse hippocampus and cerebellum and augmented in the presence of VIP (Bassan et al., 1999). Embryonic ADNP mRNA has been shown to display a temporal pattern with greater amounts present from gestational days 9 to 16 (Poggi et al., 2002). ADNP as a VIP responsive gene has been shown to play a role in embryogenesis and neurodevelopment (Pinhasov et al., 2003). ADNP-knockout embryos die by embryonic day 8.5-9, primarily because of cranial neural tube closure failure (Pinhasov et al., 2003). In a more recent study, an ADNP +/- mice strain has been generated and found to exhibit cognitive deficits, neurodegeneration, and significant increases in phosphorylated tau, tangle-like structures which are known to cause memory impairments in neurodegenerative diseases, such as Alzheimer's disease (Vulih-Shultzman et al., 2007).

Variants of ADNF, as another peptide factor that mediates VIP trophic effects, have been described; a fourteen-amino-acid fragment of ADNF (ADNF-14), and the more potent, nine-amino-acid derivative (ADNF-9). NAP on the other hand is an eight-amino-acid peptide derived from ADNP (sharing structural and functional similarities with ADNF-9), which has been described as the most potent neuro-protectant to-date (Gozes and Brenneman, 2000; Zemlyak et al., 2000). NAP as an eight amino acid peptide that has been demonstrated to be neuroprotective against toxicity associated with oxidative stress, glucose deprivation, β-amyloid and NMDA (Bassan et al., 1999; Offen et al., 2000; Leker et al., 2002). At the time that ADNP deficiency has been reported to be associated with a down regulation in gene clusters associated

neurogenesis, such as Ngfr, neurogenin1 and neurod1 (Vulih-Shultzman et al., 2007), little is known about the expression and any possible role of ADNP in the postnatal and adulthood neurogenesis, particularly in the hippocampus.

6.1.5 Galanin: biological activity and role in neuroprotection and neurogenesis

Galanin is a 29/30 amino acids long neuropeptide which does not belong to any known peptide family (Hokfelt, 2005). Galanin was first isolated by Tatemoto et al. in 1983 from porcine upper intestine (Floren et al., 2000). It has been demonstrated to be widely distributed both in the central and peripheral nervous systems where it plays variety of physiological effects, acting mostly as an inhibitory neuromodulator (Floren et al., 2000; Katsetos et al., 2001). Galanin has been reported to play multiple biological effects including, food intake, memory and pain modulation (Branchek et al., 2000). It has been suggested recently that galanin acts as a trophic factor during neurogenesis and neural injury and repair (Hokfelt, 2005; Jungnickel et al., 2005). Galanin (GAL) and GAL receptors (GALR) are over expressed in limbic brain regions associated with cognition in Alzheimer disease (AD) (Counts et al., 2003). Thus, GAL may also be neuroprotective for AD (Counts et al., 2003).

Galanin coexists with NPY in several regions of the brain, including the hippocampus (Hokfelt, 2005). It has been indicated that galanin has anti-seizure effect in the model of febrile convulsions in children--hyperthermia induced seizures in neonatal rats in the age from 5 to 13 days (Chepurnov et al., 1997; Mazarati et al., 2000). Also Status epilepticus-induced galanin depletion in the hippocampus may contribute to the maintenance of seizure activity, whereas the increase in galanin concentration and the appearance of galanin-immunoreactive neurons may favor the cessation of self-sustaining SE (SSSE) (Mazarati et al., 1998). The seizure-protecting action of galanin at the onset of SSSE opens new perspectives in the treatment of SE (Mazarati et al., 1998). In another study, it has been shown that in the dentate gyrus, galanin, acting through GalR2, inhibited seizures, promoted viability of hilar interneurons and stimulated seizure-induced neurogenesis (Mazarati et al., 2004b). Other studies have indicated that, GalR1 mediate galanin protection from seizures and seizure-induced hippocampal injury in Li-pilocarpine and perforant path stimulation models of limbic SE, but not under conditions of KA-induced seizures (Mazarati et al., 2004a). It has been demonstrated that galanin acts as an endogenous neuroprotective factor to the

hippocampus against Kainate, glutamate or staurosporine -induced hippocampal cell death (Elliott-Hunt et al., 2004). In addition, it has been indicated that galanin may act at the molecular level to influence learning and memory in vertebrates (Coumis and Davies, 2002).

6.1.6 Molecular biology and CNS distribution of Galanin

The sequence of galanin has been described in six species including the human. The N-trminal 15 amino acids are conserved among these species suggesting that this portion is important for galanin binding to its receptors (Chepurnov et al., 1997). The 16-29/30 amino acid portion of the peptide is variable among these six species.

Galanin as a neuropeptide is widely distributed in the CNS (Shen et al., 2003). In the rat brain, the highest densities of galanin immuno-reactive (producing) neurons have been demonstrated in the amygdaloid complex, the hypothalamus and the brainstem (Hokfelt, 2005). In the hippocampus very few galanin-immuno-reactive neurons were seen. However, an abundance of galanin-positive nerve terminals projecting from the septum and locus coeruleus are dominating in the area of the hippocampus (Shen et al., 2003). In addition, galanin-mRNA has been detected in the SVZ as one of the neurogenic areas in the postnatal brain (Shen et al., 2003).

In the CNS, three galanin receptor subtypes have been described: GalR1, GalR2 and GalR3 (Hokfelt, 2005). They all belong to the superfamily of the G-protein-coupled receptors (Hua et al., 2004). GalR1 is the first described galanin receptor and has been isolated from the human Bowes melanoma cell line (Branchek et al., 2000). Human GalR1 contains 349 amino acids with a 42% and 38% homology with GalR2 and GalR3, respectively. The rat GalR1 contains 346 amino acids and shares the same consensus for the N-linked glycosalation with the human GAlR1 (Branchek et al., 2000). In rat and human brains, GalR1 mRNA has been detected by northern blot analysis in many areas in the brain, particularly the ventral hippocampus and the subventricular zone of lateral ventricles (Branchek et al., 2000).

Rat and human GalR2 consists of 372 and 387 amino acids, respectively. Rat GalR2 share a 40% and 55% amino acid similarity with rat GalR1 and GalR3, respectively (Branchek et al., 2000). GalR2 mRNA is widely expressed in the rat brain with the

hippocampus among the areas of the highest expression (Branchek et al., 2000). In fact, GalR2 is highly expressed in the granule layer of the dentate gyrus where about 57% of the galanin receptors are GalR2 and the remained are GalR1. GalR3 is a 370 amino acid protein with 36% and 55% similarities to GalR1 and GalR2, respectively. GalR3 has been reported to be expressed in many areas of the brain but not in the hippocampus (Branchek et al., 2000).

Investigations of the contribution of each galanin receptor to the effects of galanin are a bit difficult due to the lack of highly GalR1 and GalR2 selective agonists and antagonists. However, there are some galanin ligands that have been reported to show a higher affinity for one galanin receptor over the others. Galanin (2-11) is a high affinity GalR2 agonist that prefers GalR2 over GalR1. In deed, galanin (2-11) can be used at high micro-molar concentrations (>10μM) without affecting GalR1 (Branchek et al., 2000). Galanin (2-29) is another galanin agonist that has been shown to have GalR1/GalR2 mixed affinity. Another useful method that will help to characterise galanin activity on these receptors is the generation of a transgenic mice strain carrying a null mutation of GalR1 which has been recently introduced by Jacoby and co workers (Mazarati et al., 2004a). Moreover; there has been preliminary data presented on the generation of transgenic mice carrying a null mutation of GalR2 (Hokfelt, 2005).

6.1.7 Aims

The main aim of this chapter is to elucidate and unveil possible interactions between VIP and either NPY or FGF-2, as mitogenic factors of hippocampal cells, in the modulation of postnatal hippocampal neurogenesis *in vitro*. In addition we will be investigating the expression of ADNP and its mRNA in the postnatal hippocampal progenitor cell cultures and its regulation by VIP. We will also address the effects of the neuropeptide galanin on hippocampal neurogenesis. Therefore, we will again be using the primary hippocampal cultures to investigate possible roles of galanin in the modulation of the survival and proliferation of hippocampal progenitor cells.

6.2 Methods

6.2.1 Introduction

In this section, I used the same protocols for cell culture generation, cell proliferation measurement, immunocytochemistry and cell counting as detailed in **Chapter 2**.

6.2.2 Generating primary hippocampal culture

In each experiment, primary hippocampal cultures were generated from 2-8 Wistar rats of P7-10 age. Viable cells were plated in 500μL NB/B27 and glutamine at a density of 100.000 cells directly onto poly-L-lysine coated 24-well plates or on glass cover slips for confocal images. At 2 hrs after plating cells were rinsed free of debris and replenished with fresh medium of NB/B27 and glutamine under control condition or the addition of the indicated concentration for each experiment under peptide treatments. In interactions experiments, VIP, at 30nM peptide concentration, was used alone or combined with NPY or FGF-2 treatments whereas NPY and FGF-2 were added at 1μM and 20ng/ml, respectively.

In galanin investigation experiments, Galanin (1-29) was used as GAlR1/ GalR2 non-selective agonist and galanin (2-11) as GalR2 selective agonist. The two peptides were obtained from human, porcine and rat powder and supplied by Sigma-Aldrich. A working stock solution was prepared from 1ml aliquots of 1mM in sterile water and being stored as 10μL aliquots at -20C in the freezer.

6.2.3 Examining VIP and NPY interactions to affect hippocampal cells in culture

In three different sets of experiments, cells were grown for 5 days under control conditions, 30n MVIP, 1μM NPY, or 30nM VIP plus 1μM NPY. To assess cell proliferation, cells from the first set of experiments were pulsed with BrdU for the last 4 hrs before fixation. Cells were then immuno-stained for BrdU incorporation and counterstained with DAPI. Total cell counts and proportions of BrdU immuno-positive cells were calculated. The second set of experiments was used to quantify cell death as detailed in **Section 2.6 for details**. Total cell counts and proportions of dead cells were determined. The numbers of cells expressing nestin, class-III ß-tubulin and GFAP were also determined

6.2.4 Determining VIP effects on cell cycle exit index

Cells were grown for 5 days under control conditions and under 30nM VIP. However, in this experimental set up, cells were exposed to BrdU early on at 2 hrs after initial plating. Another dose of BrdU was loaded again at 3 DIV. At 5 DIV cells were fixed and immuno-stained for BrdU incorporation and Ki-67 expression. Cells were then counterstained with DAPI. Proportions of BrdU positive but Ki-67 negative cells were calculated with respect to the total number of cells under different conditions. Confirmation that BrdU incorporation by cells undergoing DNA repair was not a confounding factor comes from BrdU/Ki-67 co-labelling of cells exposed to short BrdU pulse (4,6 and 8 hrs at) at three days and five DIV. All the BrdU positive cells in these different experiments were dual labelled with Ki-67, indicating that all the BrdU cells had been at some stages in the cell cycle.

6.2.5 Defining VIP and bFGF interactions in the modulation of precursor cell survival and proliferation *in vitro*

To investigate the interactions between VIP and bFGF, cells were grown for 5DIV under control, 30nMVIP, 20ng/ml bFGF or a combination of 30nM VIP plus 20ng/ml bFGF conditions. Then cells were pulsed for BrdU for the last 4 hrs before fixation to quantify cell proliferation. In addition cells were stained for nestin and counterstained with DAPI. The total number of cells, the number of BrdU, and the number of nestin labelled cells were determined under different conditions.

6.2.6 Examining VIP effects on the expression of FGF-receptor 1 mRNA (FGFR1) subtype.

In order to investigate VIP effects on the expression of the FGFR1 mRNA, hippocampal cells were harvested from postnatal rat hippocampi and grown for three days under control conditions. At day three, cells in half of the wells were pulsed with 30nM VIP for 3 hrs, before being processed for RNA extraction and cDNA synthesis. cDNA synthesised from control or VIP treated cells was then evaluated for purity and used as a template to amplify FGFR1 mRNA on real time thermocycler (Rotor-Gene 6000, Corbett Robotics. Ltd)(as detailed in the method **Section 4.2.10**). The forward and reverse primers, sequence, positions on the gene, melting temperatures and expected product lengths are as detailed (**Table 6.1**) below:

Primer	Sequence	Position	Tm	Product length
FGFR1	Forward:CGGGTAACTCTATCGGACT CT	1,028	56.4	79
	Reverse:ATCACGGCTGGTCTCTCTTC	1,106	57.2	
ß-actin	As designed by "PrimerDesign Ltd (Southampton)			
	As designed by "PrimerDesign Ltd (Southampton)			

Table 6.1: FGFR1 and ß-actin primers. This table shows the primers' sequences, position on the gene of origin, melting temperature and the amplicon length.

As detailed in **Section 4.2.11**, fluorescent data was collected at least once during each cycle of amplification during the real time PCR experiment, and the Ct-values were obtained then processed and analysed using the 2[-Delta Delta C(t)] method (2[-ΔΔCt]).

6.2.7 Examining the expression of ADNP and its mRNA by cultured hippocampal cells

For staining for ADNP expression, hippocampal cells were grown under standard control conditions for three days and then fixed and processed for the expression of ADNP (see **Section 6.2.14**) and nestin. The proportions of cells expressing ADNP were then calculated.

For PCR studies on the ADNP mRNA expression, hippocampal progenitor cells were grown for 2 days under standard control conditions (Neurobasal A/B27 and glutamine). On day 2 cells in half of the wells were pulsed with 30nM VIP and rest wells with culture medium. After three hrs cells were taken and processed for cDNA synthesis using the SuperScript™ III CellsDirect cDNA Synthesis Kit (Invitrogen™ life technologies) as detailed in **Section 4.2.9** (**Chapter 4**).

For ADNP mRNA quantitation, PCR was carried out on our single-stranded cDNA using a one-step real time-PCR kit (Rat Custom real-time PCR assay for use with SYBRgreen chemistry) (PrimerDesign Ltd, Southampton) in a PCR thermocycler

(Rotor-Gene 6000, Corbett Robotics. Ltd). PCR reactions were performed according to the kit manufacturer instructions for 40 cycles. Both ADNP and ß-actin mRNAs' primers were designed by PrimerDesign Ltd (Southampton) and provided in a lyophilised form. Each primer tube was first centrifuged to ensure that the primer mix is in the bottom and then reconstituted in RNase free water as indicated by the provider (i.e. 660µl). Forward and reverse primers, sequence, positions on the gene, melting temperatures and expected product lengths are as detailed (**Table 6.2**) below:

Primer	Sequence	Position	Tm	Product length
ADNP	Forward:CCACCATCGTTTATACCATCTAATAT	4,390	56.1	91
	Reverse:TGCTTAAAATGAATACAAAGGGAAAT	4,480	55.7	
ß-actin	As designed by "PrimerDesign Ltd (Southampton)			
	As designed by "PrimerDesign Ltd (Southampton)			

Table 6.2: ADNP and ß-actin primers. This table shows the primers' sequences, position on the gene of origin, melting temperature and the amplicon length.

In each PCR reaction tube, the mix contains 1µl of the reconstituted primer mix, 10µl of PrimerDesign 2X Precision^{TM} MasterMix with SYBRgreen and 4µl PCR-Grade water. PrimerDesign 2X Precision^{TM} MasterMix is basically made of 2X reaction buffer, 0.025 U/ µl Taq polymerase, 5 mM MgCl2 and DNTP mix (200µM each dNTP). To this 15µl mix of each tube, 25ng/µl of cDNA was added to be transverse transcribed at a final volume of 20µl. For this purpose the Rotor Gene machine was programmed according to instruction of the Kit provider. The PCR reaction amplification conditions were: enzyme activation for 10min at 95°C followed by 15 second for denaturation at 95°C and then the data was collected in 60 seconds at 60°C.

6.2.8 Data collection and Analysis:

Data with regard to ADNP mRNA expression was analysed initially by semiquantitative PCR and then by quantitative real time-PCR.

For semiquantitation, the PCR products corresponding to each primer were separated on a 2% agarose gel. The signals from each particular band were quantified and background-corrected using ImageQuant TL software (Amersham Biosciences Corp). Using this software, the density of each band for ADNP and ß-actin transcripts was digitized and defined in terms of pixels (which is the total pixel minus the background). Finally, the levels of rat ADNP mRNA products were related to the amount of rat ß-actin mRNA values and normalized as percentages of the vehicle treated controls as 100%.

For quantitative real time PCR analysis, (as detailed in **Section 4.2.11**) fluorescent data was collected at least once during each cycle of amplification and the Ct-values were obtained. Raw data was then processed and analysed using the 2[-Delta Delta C(t)] method (2[-ΔΔCt]).

6.2.9 Defining the effects of galanin (1-29) and galanin (2-11) on the cell density of cultured hippocampal cells

Hippocampal cells were plated as mentioned in **Chapter two**. At 2hrs, cells were replenished with either control medium or medium containing 1μM of either galanin (1-29) or galanin (2-11). In this initial set of experiments the cells were fixed and only stained with the nuclear stain DAPI to assess total cell counts under different conditions.

6.2.10 Examining galanin (1-29) effects compared to NPY on cultured hippocampal cells

Cells were dissociated from postnatal rat hippocampi and plated under control conditions plus 1μM galanin (1-29) or 1μM NPY as a positive control. Cells were pulsed with the S-phase marker BrdU for the last 24hrs before fixation. At 3 DIV, cells were fixed and processed for BrdU incorporation. Cells were then counterstained with DAPI to assess total cell counts.

6.2.11 Characterising the dose response curve for galanin (1-29) effects on hippocampal cells in culture

Hippocampal cells were cultured from postnatal rat hippocampi aged 7-10 days. Cells in culture were grown either under standard control conditions (Neurobasal/ B27 plus glutamine) or control plus varying concentrations of galanin (1-29) (0.1-100nM). At 5 DIV, cells were pulsed with BrdU for the last 4 hrs before being fixed. Immunocytochemistry was used to label the cells that have incorporated BrdU. Cells were eventually counterstained with the nuclear stain DAPI to assess total cell counts.

6.2.12 Defining Galanin (1-29) time course effects on hippocampal cells

To study galanin (1-29) effects on total cell number and cell proliferation at different time points in culture, hippocampal cells generated from the same cell stock were plated and grown for 3, 5 and 7 days. To measure cell proliferation, cells were again pulsed with 20μM BrdU 4hrs before fixation at the indicated time point. Cells were then processed for BrdU incorporation and counterstained with DAPI to assess total cell counts.

6.2.13 Determining Galanin (2-11) dose response effects on cultured hippocampal cells

Hippocampal cells were grown for 5 days in culture under standard control conditions or varying concentrations of galanin (2-11) (1nM-1μM). Cells in some of the wells were pulsed with 1μMNPY as a positive control. In this initial set of experiments, cells were fixed and only stained with the nuclear stain DAPI to assess total number of cells in culture under different conditions.

6.2.14 Immunocytochemistry protocol

At the indicated times for each experiment, cells were rinsed free of medium with phosphate buffer solution (PBS) and immediately fixed in 4% Para formaldehyde (PFA) for a minimum of 30 mins at 4°C. Fixed cells were then washed 3 times in PBS to remove the PFA. For BrdU detection, cells were incubated for 30 mins with 2M HCl in a water path at 37°C to unfold the double stranded DNA. This was followed by rinsing in PBS 3 times to remove any remained HCl (as it is associated with horrible DAPI staining). After rinsing, non-specific binding sites were blocked with 5% donkey blocking serum in 0.1%Triton-X in Tris-buffered saline (TBS). Primary

antibodies in TBS-0.1% Triton-X including rat anti-BrdU diluted 1:200, mouse anti-rat nestin diluted 1:500, rabbit anti-ADNP diluted 1:1000, mouse anti–GFAP diluted 1:400, rabbit anti-GFAP diluted 1:500 and/or mouse anti-Class III ß-tubulin diluted 1:200 were incubated with cells overnight at 4°C.

Cells were then rinsed once with PBS and incubated with TBS-0.1% Triton-X containing Cy2 or Cy3-conjugated anti-rat diluted 1:500, anti-mouse diluted 1:500 and/or anti-rabbit diluted 1:200 secondary antibodies for 2 hrs at room temperature. After that cells were washed once with PBS and counterstained with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI;5 µg/mL) (Sigma) for 6 min. Optimum antibody concentrations were determined by testing each antibody on cultures, and these ratios mentioned above for each antibody are the concentrations of antibody that provided optimal signal. For each experiment, negative controls (the addition of secondary in the absence of primary antibodies) were obtained to rule out any non-specific secondary antibody binding.

6.2.15 Cell Counting and statistical analysis:

Imaging was performed on an inverted DM IRB microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). The area of a 20× field was measured using a 255 µm grid graticule slide (Microbrightfield, Williston, USA). Cell counting was performed on 5-6 random 20× fields per well using the Open Lab image-capturing system version 2.1 (Improvision, Lexington, MA, USA). Raw data from the 20× field counts were averaged and plotted ± SEM and expressed as cells/mm² per well, based on a sample of four to eight wells per condition per experiment. All experiments were repeated at least two to three times. One experiment consisted of four hippocampi from two animals, pooled and prepared as described above. Data points were plotted using GraphPad Prism data analysis software (GraphPad inc. USA). The statistical significances between the means was assessed by either Student's test for single comparisons and by ANOVA followed by post hoc tests for multiple comparisons, with $P<0.05$ considered significant.

6.3 Results

6.3.1 The effects of VIP combined with NPY on the total number of cells, proliferation, death, and specific phenotypes of cells in culture

Once cells were exposed to 30nM VIP in conjunction with 1μM NPY, the total number of cells did not change in comparison to either (**Figure 6.1 A**). However, the total number of cells increased significantly under the combined treatment in comparison with control cell counts. In addition, there was no significant change in cell death in comparison with either VIP alone or NPY alone conditions (**Figure 6.1 B**). However, there was a significant drop in the proportion of nonviable cells under VIP combined with NPY conditions compare to control conditions. Interestingly enough, the addition of VIP plus NPY has abolished the proliferative effect of NPY. The number of BrdU positive cells under NPY conditions was 25.4 ± 2.5 cells/mm^2 declined significantly to 6.0 ± 3.6 cells/ mm^2 under combined NPY and VIP treatment (**Figure 6.1 C**).

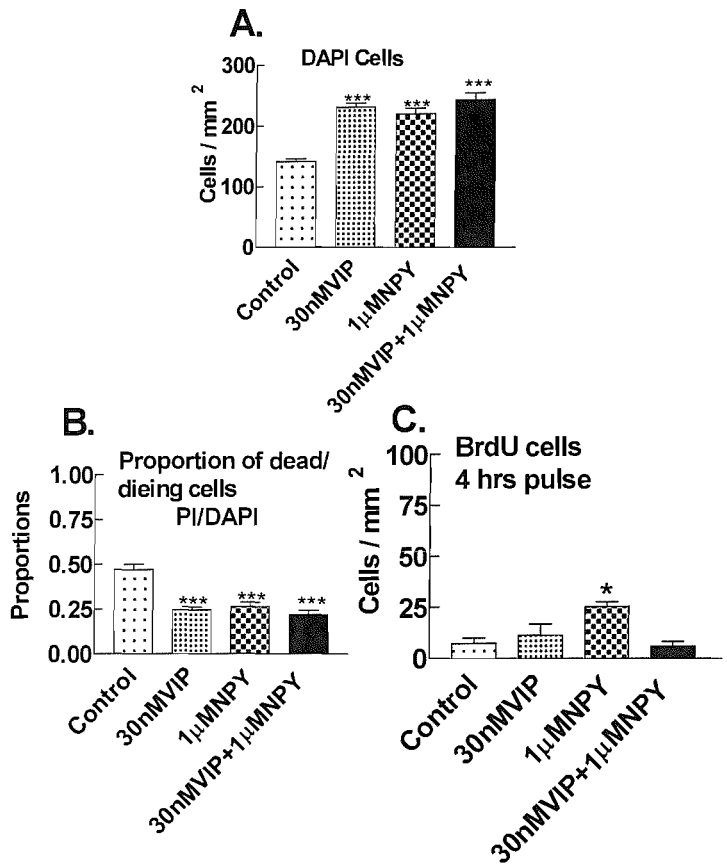


Figure 6.1: VIP combined with NPY has no additive effect on cell density and cell death but abolished proliferative activity of NPY. Hippocampal cultures were grown for 5 days either in standard control conditions, 30nM VIP, 1μM NPY or a combination of 30nM VIP and 1μM NPY. PI was added to the medium for 40 minutes. Cells were then exposed to DAPI while still alive for another 40minute. BrdU was added for the last 4 hrs. (A) Significant increases in the total number of cells under different conditions with respect to control. However, no additive increases in the total number of cells under the combination of NPY and VIP over each individual one. (B) A drop in cell death under the different conditions with compared to control. VIP plus NPY decreased cell death more than each one did alone. This difference was insignificant. (C) NPY by its own increased the number of BrdU incorporating cells but VIP did not. Once NPY combined with VIP, increase in the number of BrdU incorporating cells was not observed. Values represent mean ± SE based on a sample that represent at least 4 wells per condition from two experiments. Comparisons between different conditions, one way ANOVA with Bonferronis multiple comparison test (*, $p < 0.05$ and ***, $p < 0.001$).

6.3.2 VIP in conjunction with NPY enhanced neurogenesis

To quantify the numbers and proportion of the cells expressing the major cell-specific phenotypes under the combined treatment of VIP and NPY, we grew hippocampal cells in culture and stain them for, nestin, GFAP and class III ß-tubulin. The numbers and proportions of nestin positive cells and GFAP positive cells did not change under the effect of VIP in conjunction with NPY compared to each one alone. The proportions of nestin positive cells increased under the mixture of the two peptides compared to the control conditions (**Figure 6.2 A**). This effect was mirrored by VIP alone conditions. However the proportions of GFAP positive cell did not change under VIP combined with NPY conditions compared to control conditions (**Figure 6.2 B**). It was only the proportions of cells that are immuno-positive for the neuronal marker class III ß-tubulin that increased significantly under the combined VIP and NPY treatment with respect to control conditions (**Figure 6.2 C**). Neither of the two neuropeptides could replicate this effect when applied by its own to cultured cells.

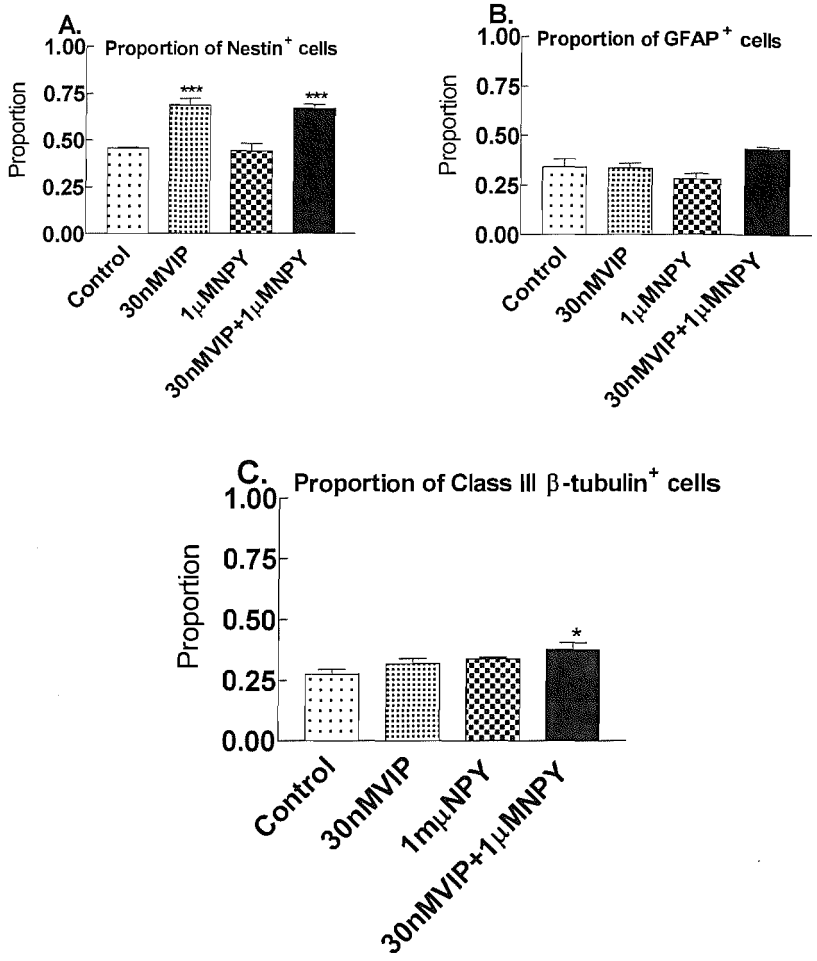


Figure 6.2: Hippocampal cells were grown for 5 days under either control, 3nM VIP, 1μM NPY or the combination of both. Cells were then processed for the stem/ precursor cell marker nestin, the neuronal marker class III ß-tubulin and the astrocytic marker GFAP. Proportions of each cell specific phenotype with respect to the total cell density were determined. (A)VIP enhanced the proportion of nestin positive cells but NPY did not. The combination has no significant effect over the effect of VIP alone. (B) The proportion of the cells expressing the astrocytic marker GFAP has not changed under any of the conditions compared. (C) The combination of 30nM and 1μM NPY increased the proportion of the neuronal cells expressing the marker class III ß-tubulin. Data represent mean ± SE based on a sample that represent at least 8 wells per condition from three different experiments. Comparisons between different conditions are one way ANOVA with Dunnetts' multiple comparison test (*, $p < 0.05$ and ***, $p < 0.001$).

6.3.3 VIP enhanced cell cycle exit

To assess the cell cycle exit index, hippocampal cells were grown for 5 days under control, 30nM VIP, 1μM NPY and 30nM VIP plus1μM NPY and pulsed with 20μM BrdU for the whole time in culture. To obtain an index of cell cycle exit, cultured cells were double-immuno-labelled for BrdU and Ki-67 (**See Section 2.5**). The proportion of cells that exited the cell cycle was calculated by dividing the number of cells immunoreactive for BrdU but not Ki-67 (BrdU+/Ki-67- = postmitotic/ non-mitotic) by the total number of cells. The number of non-mitotic/ postmitotic cells under 30nM VIP and 30nM plus 1μM NPY was 53.9 ± 1.7 cells/mm^2 and 61.8 ± 9.5 cells/mm^2, respectively, compared to control conditions (24.3 ± 4.3 cells/mm^2) (**Figure 6.3 A**). This increase was statistically significant. In parallel, the index of the cell cycle exit increased from 13.0 ± 1 % under control conditions to 22.3 ± 3 % and 26.6 ± 1 % under 30nM VIP and 30nM plus 1μM NPY conditions, respectively (**Figure 6.3 B**). However, the number of non-mitotic/ postmitotic cells and the index of the cell cycle exit under 1μM NPY alone were not different from control conditions.

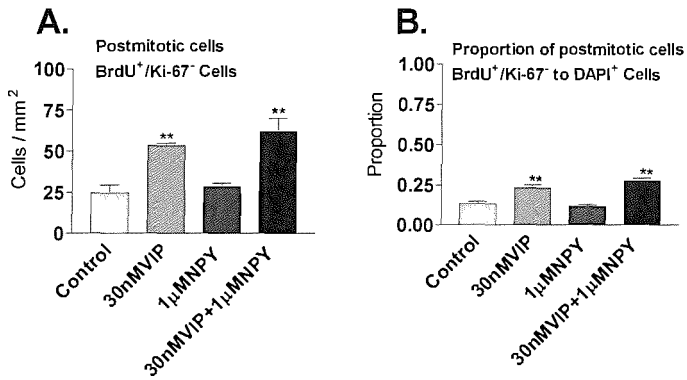


Figure 6.3: **VIP enhanced cell cycle exit.** Hippocampal cultures were grown for 5 days either in standard control conditions, 30nM VIP, 1μM NPY or a combination of 30nM VIP and 1μM NPY. Cells in culture were exposed to BrdU from the start at 2 hrs after plating. Another pulse of BrdU was given on the third day. Cells were stained for Ki-67 expression and BrdU incorporation. (A) Significant increase in the numbers of BrdU+/Ki67- cells under 30nM VIP and the combination of VIP and NPY but not under NPY alone condition compared to control condition. (B) the proportion of BrdU+/Ki67- to the total number of cells in culture also increased significantly under 30nM VIP and the combination of VIP and NPY conditions but not under NPY alone condition compared to control conditions. Data represent mean ± SE based on a sample that represent at least 8 wells per condition from two different experiments. Comparisons between different conditions, one way ANOVA with Dunnetts' multiple comparison test (**, $p < 0.01$).

6.3.4 VIP in conjunction with bFGF additively increased the numbers of precursor cells in culture

To assess the effects of combined VIP and bFGF treatment on cells in culture, hippocampal cells dissociated from postnatal rat hippocampi were cultured under control, 30nM VIP, 20ng/ml bFGF or VIP in conjunction with 20ng/ml bFGF. Two thirds of the medium were changed at 3DIV. Cells were pulsed with BrdU 4 hrs before fixation; then cells were processed for BrdU incorporation and nestin expression. Total cell counts were determined by counterstaining cells with DAPI. In control cultures, the total number of cells was only 138 ±7.3 cells/mm^2. Treatment with VIP or bFGF resulted in an increase in total cell counts to 232.7±16.3 cells/mm^2 and 420.8±17.1 cells/mm^2, respectively. A further increase in total cell counts (593.3± 15.7cells/mm^2) was also observed once cultured hippocampal cells were treated with VIP and bFGF together (**Figure 6.4 A**). This represents a 30% and 60% increase over VIP and bFGF treatment alone, respectively. The same results were mirrored on the number of cells expressing the stem/progenitor cell marker nestin. Treatment with VIP alone, bFGF alone or VIP combined with bFGF resulted in about 4, 7 and 11-fold increases in the number of nestin expressing cells over control conditions (**Figure 6.4 C**). The number of proliferating cells in the S-phase of the cell cycle has not significantly changed under 30nM VIP conditions. In contrast, counts of BrdU positive cells showed a significant increase under bFGF conditions (13.7±0.23 cells/mm^2 vs. 49.0±6.1 cells/mm^2). However, once cells were treated with bFGF combined with VIP, the number of BrdU cells (31.5 ± 2.5 cells/mm^2) dropped significantly compared to bFGF alone although the number of BrdU positive cells are still significantly higher under the combined factor treatment compared to control conditions (**Figure 6.4 B**).

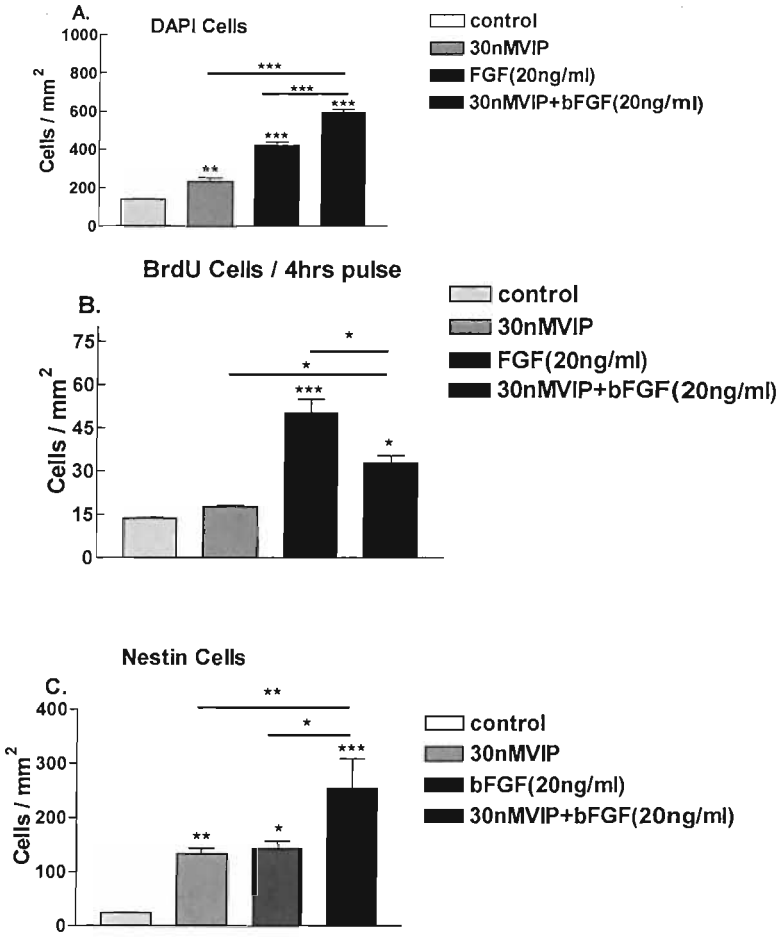


Figure 6.4: Effects of VIP and bFGF combined treatment on culture hippocampal precursor cells. Cells were grown for 5 days under control, VIP, bFGF or VIP plus bFGF conditions. Cells were pulsed with BrdU for the last 4hrs before fixation. Cells were immuno-stained for BrdU and nestin. (A) Total cell counts, (B) Counts of BrdU positive cells, and (C) numbers of nestin expressing cells. . Data represent mean ± SE based on a sample that represent at least 8 wells per condition from two different experiments. Comparisons between different conditions, one way ANOVA with Bonferroni's Multiple Comparison Test (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

6.3.5 VIP downregulates FGFR1 mRNA expression in hippocampal cell culture

Three day hippocampal cell cultures were grown under standard control conditions. Cells in half of the wells were treated with 30nM VIP for 3 hrs while the rest of them were maintained under control conditions. Cells were then processed for cDNA synthesis, which was then used as a template to amplify FGFR1 and β-actin mRNAs using FGFR1 and β-actin primers; respectively (table 6.1). Using the delta-delta Ct-method of analysis, the folds of change in the expression of FGFR1 under each condition were determined (**Figure 6.5 A & B**). 3hrs VIP treatment downregulated the expression of FGFR1 significantly by more than six folds compared to control expression (0.36 ± 0.03 vs. 1.03 ± 0.16).

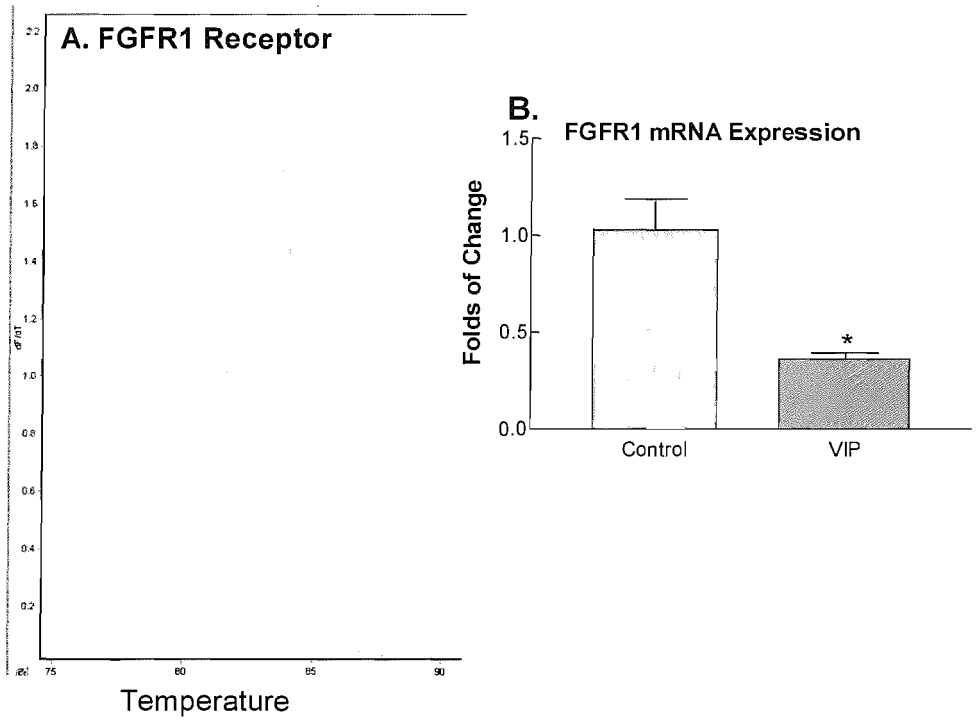


Figure 6.5: VIP regulates the expression of FGFR1 mRNA in hippocampal cell cultures. (A) The melt curve for FGFR1, as the uniqueness of the amplicons synthesized using the FGFR1 primer and amplification conditions were assessed by SYBR Green I dye melting analyses. Ramping of the temperature from 65°C to 95°C produced a single unique DNA dissociation curve in each case. **(B)** FGFR1 mRNA expression as measured by real time-PCR. Data represent mean ± SE for folds of expression as calculated by the delta-delta Ct method. Comparisons between control and VIP conditions are simple *t*-student test with *, $p < 0.05$ is considered significant.

6.3.6 Hippocampal progenitor cells including nestin-expressing cells express ADNP

Hippocampal cells harvested from postnatal rat were processed for the expression of ADNP. Our results demonstrate the expression of ADNP by the majority of (97%) of cultured postnatal hippocampal precursor cells at 3 DIV (**Figure 6.6**). The intensity of expression is strong and the distribution is exclusively in the cytoplasm with no detected staining in the nuclei of our cells. Co-labeling these cells with nestin demonstrated the expression of ADNP by all the nestin positive (**Figure 6.7**).

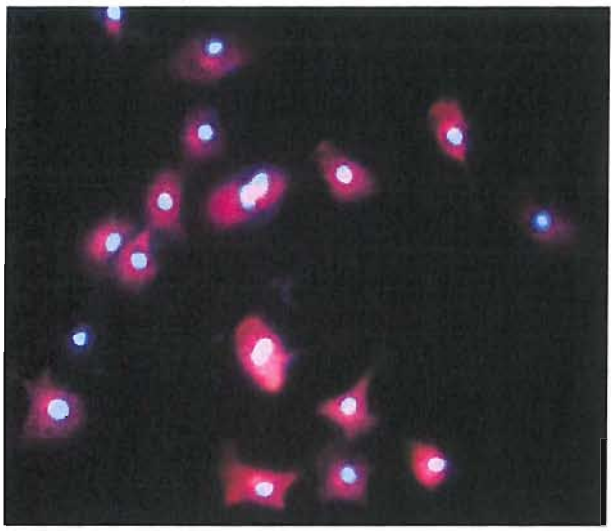


Figure 6.6: hippocampal cells strongly express ADNP. Hippocampal cells grown for three days were processed for the expression of ADNP. The majority of the cells are expressing ADNP.

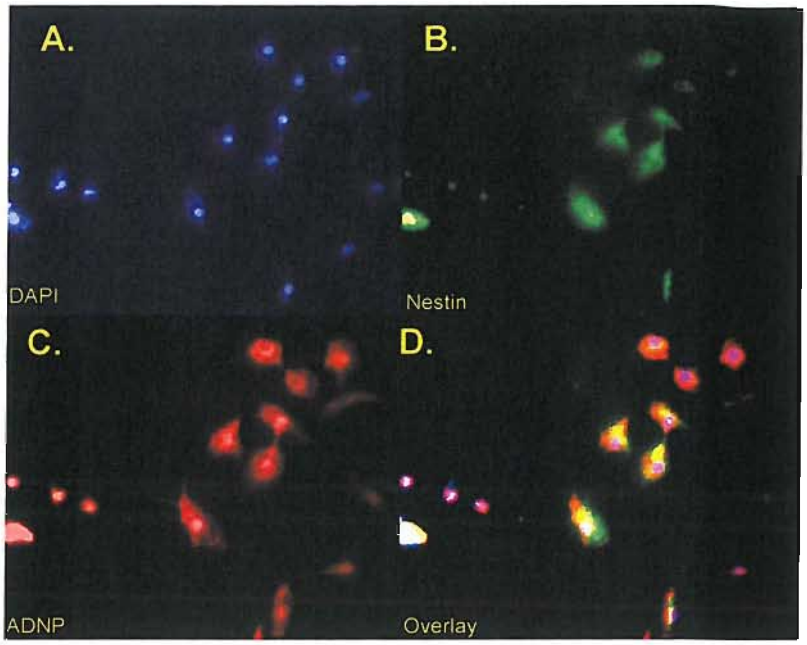


Figure 6.7: hippocampal nestin positive cells strongly express ADNP. Hippocampal cells grown for three days were processed for the expression of ADNP and nestin. All nestin-expressing cells are expressing ADNP.

6.3.7 VIP regulates ADNP mRNA expression in postnatal hippocampal cell culture

In hippocampus progenitor cell culture grown under control conditions or 30nM VIP, the expression of ADNP mRNA is regulated by VIP. Under control conditions ADNP is expressed in our cell cultures. According to our semiquantitative data, ADNP mRNA was increased three folds after a short exposure (3hrs) to 30nM VIP; this increase is statistically significant (**Figure 6.8 B**). In contrast, our quantitative real time-PCR data (**Figure 6.9 A & B**) showed a slight but insignificant drop in ADNP mRNA expression after a 4 hr VIP exposure (1.02 ± 0.12 vs. 0.80 ± 0.02). Furthermore, 30nM VIP significantly down-regulated the expression of ADNP at 1 and 8hrs of exposure times (1.05 ± 0.23 vs. 0.11 ± 0.01 and 1.00 ± 0.04 vs. 0.06 ± 0.01).

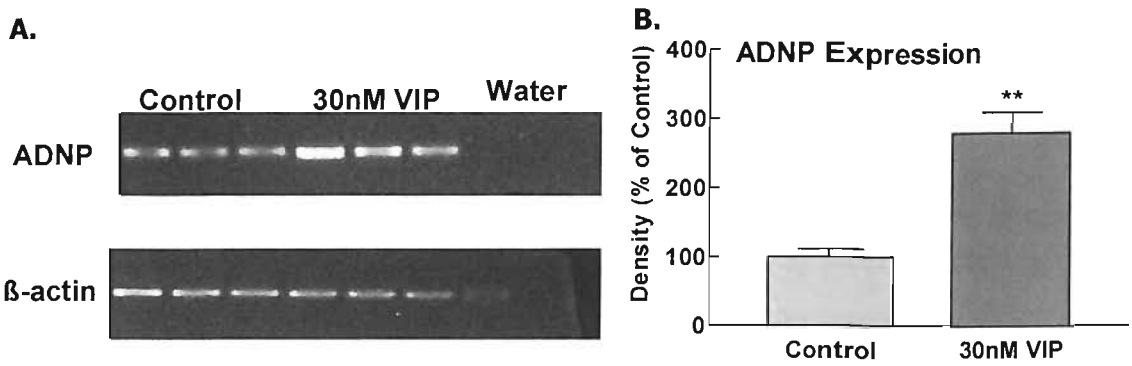


Figure 6.8: ADNP mRNA expression is regulated by VIP. Hippocampal cell cultures were harvested from postnatal hippocampal rats' tissue. cDNA was synthesised from the cell lysate of our culture and amplified by RT-PCR. The amplicons for ADNP and ß-actin mRNA were run on a 2% agarose gel (A). (B) the density of each band was digitized and defined in terms of pixels then the level of ADNP mRNA products were related to the amount of rat ß-actin mRNA values and normalized as percentages of the vehicle treated controls as 100%. Comparisons between control and VIP conditions are simple *t*-student test with *, $p < 0.05$ is considered significant.

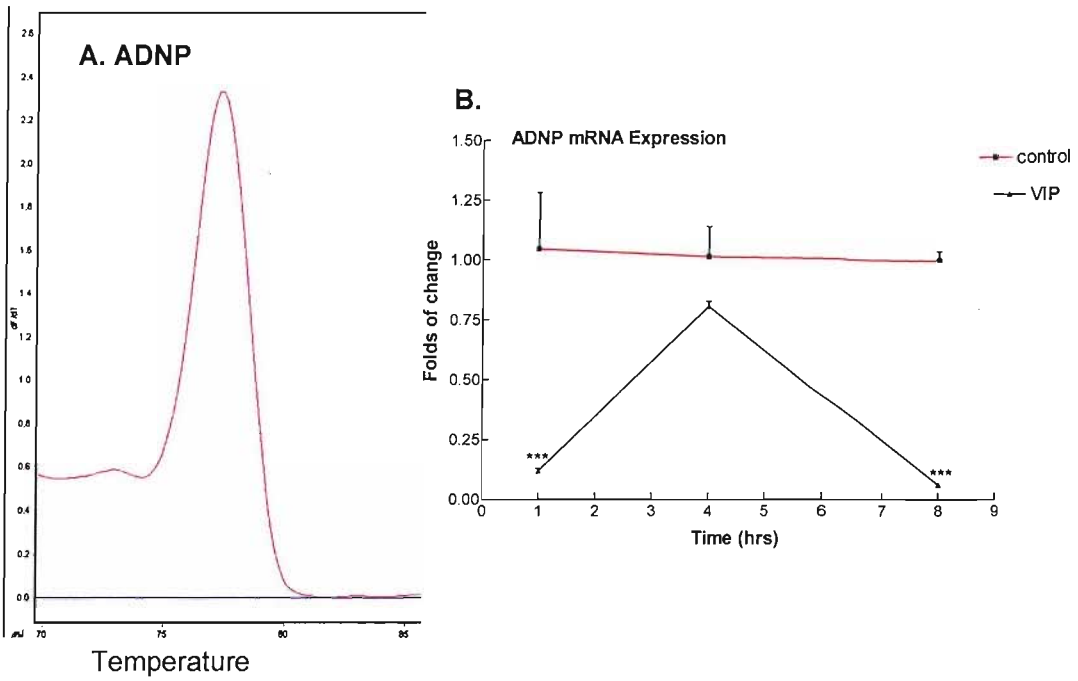


Figure 6.9: VIP regulates the expression of ADNP mRNA in hippocampal cell cultures. (A) The melt curve for ADNP, as the uniqueness of the amplicons synthesized using the ADNP primer and amplification conditions were assessed by SYBR Green I dye melting analyses. Ramping of the temperature from 65°C to 95°C produced a single unique DNA dissociation curve in each case. **(B)** ADNP mRNA expression as measured by delta-delta Ct method under control and VIP conditions at 1, 4, and 8 hrs. Data represent mean ± SE for folds of expression as calculated by the delta-delta Ct method. Comparisons between different conditions with respect to time is two way ANOVA with Bonferroni's Multiple Comparison Test (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

6.3.8 Galanin1-29 and galanin 2-11 increase the total number of cells in culture

Hippocampal cells were grown under control, 1μM galanin(1-29) or galanin(2-11) for three days. Cells were then washed and stained with the nuclear stain DAPI to assess total cell counts under different conditions. Total cell counts under control conditions were 84.7±4.0 cells/mm², grew up to 144.7±4.3 cells/mm² and 140.4±18.3 cells/mm² under 1μM galanin(1-29) and 1μM galanin(2-11) conditions, respectively (**Figure 6.10**).

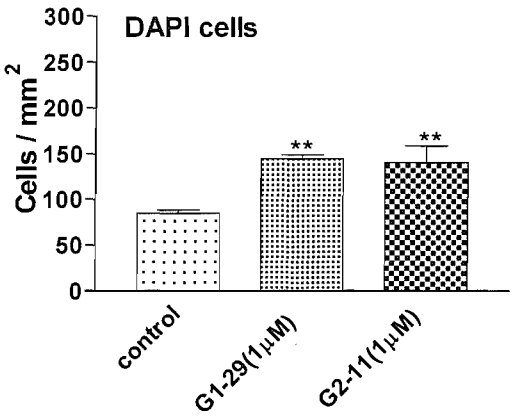


Figure 6.10: **Galanin (1-29) and galanin (2-11) increased cell density in culture.** Hippocampal cells were grown under control, 1μM Galanin (1-29) or 1μM galanin (2-11). At 3 DIV, cells were fixed and just stained with DAPI to assess total cell counts under different conditions. Data represent mean ± SE based on a sample that represent at least 8 wells per condition from one experiment. Comparisons between different conditions, one way ANOVA with Dunnetts' multiple comparison test (**, $p < 0.01$).

6.3.9 Galanin (1-29) increased the survival of BrdU cells in cultured hippocampal cells

Cultures grown for 3 DIV under 1μM galanin (1-29) or NPY displayed a significant increase in total cell counts (131.7 ± 6.5 cells/ mm², 131.1 ± 5.3 cells/ mm² for galanin (1-29 and NPY, respectively vs. 86.5 ± 3.2 cells/ mm² for control conditions). Similarly, the numbers of BrdU-incorporated cells were 79.2 ± 5.7 cells/ mm² and 76.2 ± 7.4 cells/ mm² under galanin (1-29) and NPY treatments, respectively, compared to 33.9 ± 5.6 cells/ mm² under control conditions (**Figure 6.11 A & B**). These differences between either peptide treatments in comparison to controls are statistically significant. However, there were no significant differences between NPY and galanin effects neither on the total number of cells no on the number of BrdU cells.

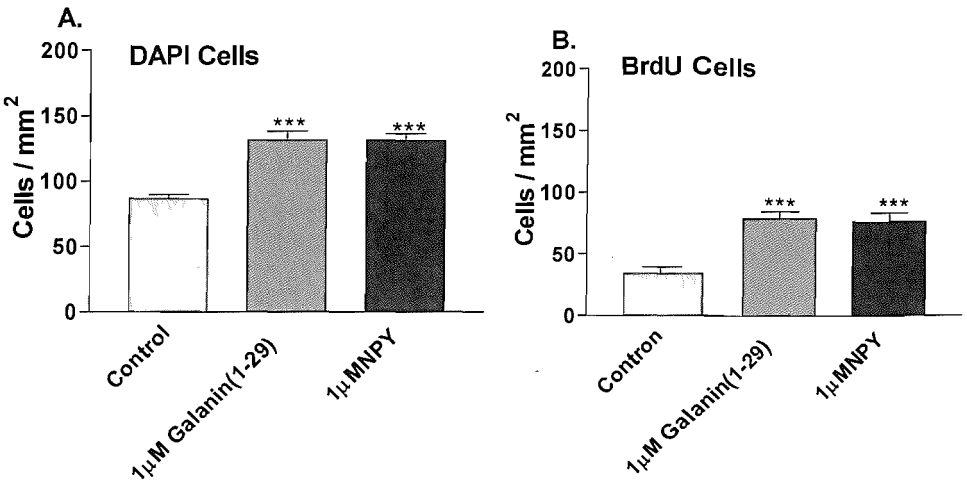


Figure 6.11: NPY effects were mirrored by galanin (1-29) effects in cell culture. Hippocampal cells were grown for 3 days under control, 1μM galanin (1-29) or 1μM NPY. Cells were given a 24 hr pulse of BrdU before fixation. (A) Total cell counts, (B) BrdU incorporating cells counts. Data represent mean ± SE based on a sample that represent at least 6 wells per condition from one experiment. Comparisons between different conditions, one way ANOVA with Dunnetts' multiple comparison test (***, $p < 0.001$).

6.3.10 Galanin1-29 supports the survival of proliferating cells at concentrations as low as 1nM

Hippocampal cultures generated from postnatal rat hippocampi displayed a significant increase in the total number of cells under 0.1-100 nM galanin (1-29) concentrations (279.7 ± 6.5 – 361.5 ± 18.2 cells/ mm² vs. 198.6 ± 11.6 cells/ mm²). However, the relative increase in BrdU incorporating cells was a significant event at concentrations between 1-100nM galanin (1-29) (**Figure 6.12 A & B**) (67.7 ± 3.4 – 91.2 ± 10.6 cells/ mm² vs. 37.9 ± 3.9 cells/ mm²). There were no significant differences neither in the total number of cells or in counts of BrdU cells between different galanin (1-29) concentrations used. There were also no significant differences in the proportion of BrdU cells with respect to DAPI cells between any galanin (1-29) and control conditions (**Figure 6.12 C**).

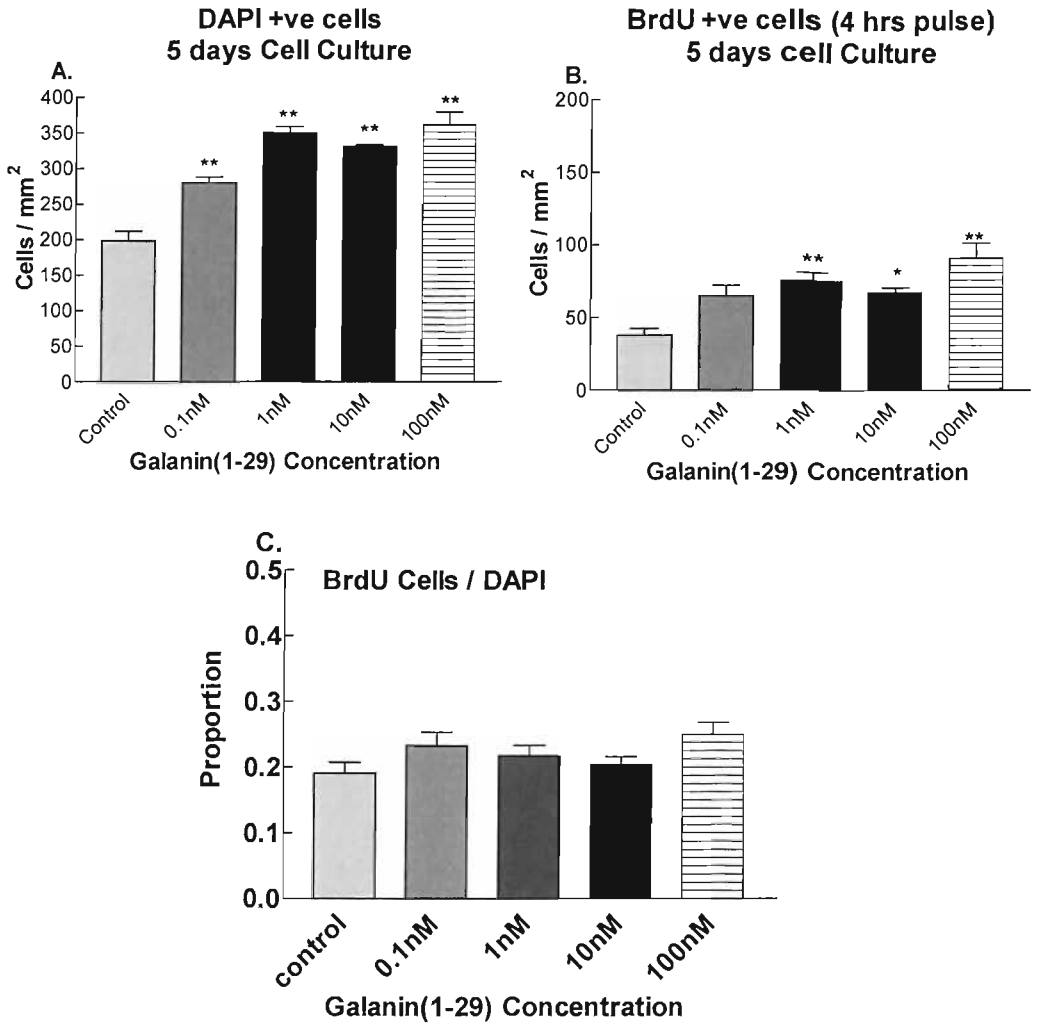


Figure 6.12: **Nanomolar concentrations of galanin (1-29) enhanced the survival of hippocampal cells**. Hippocampal cells were culture under control conditions or in the presence of 0.1-100nM galanin (1-29).cells were pulsed with BrdU for the last 4 hrs before fixation. (A) total cell counts in culture, (B) numbers of BrdU incorporating cells and (C)the proportion of BrdU cells with respect to DAPI. Data represent mean ± SE based on a sample that represent at least 6 wells per condition from one experiment. Comparisons between different conditions, one way ANOVA with Dunnetts' multiple comparison test (*, $p < 0.05$ and **, $p < 0.01$).

6.3.11 Cultured hippocampal cells responded to galanin (1-29) over time

Primary hippocampal cell cultures were established from post-natal 7-10 day-old rats and cultured under standard control conditions (Neurobasal A/B27/glutamine) or control plus 100nM galanin 1-29. Cells were grown under the two conditions for 3, 5 and 7 days. Cells were given a BrdU pulse just 4hrs before fixation to assess the number of proliferating cells in the S-phase of the cell cycle at each time point.

Cultured hippocampal cells in the presence of 100nM galanin (1-29) displayed an increase in total number of cells as indicated by counts of DAPI cells at 3, 5 and 7 days compared to control conditions (**Figure 6.13 A**). Interestingly, the increase in the total number of cells was also significant with galanin treatment with respect to time (**Figure 6.13 A**). The same trend of increase was seen in the numbers of BrdU incorporating cells under 100nM galanin (1-29) compared to control conditions with no significant changes under either condition with respect to time (**Figure 6.13 B**).

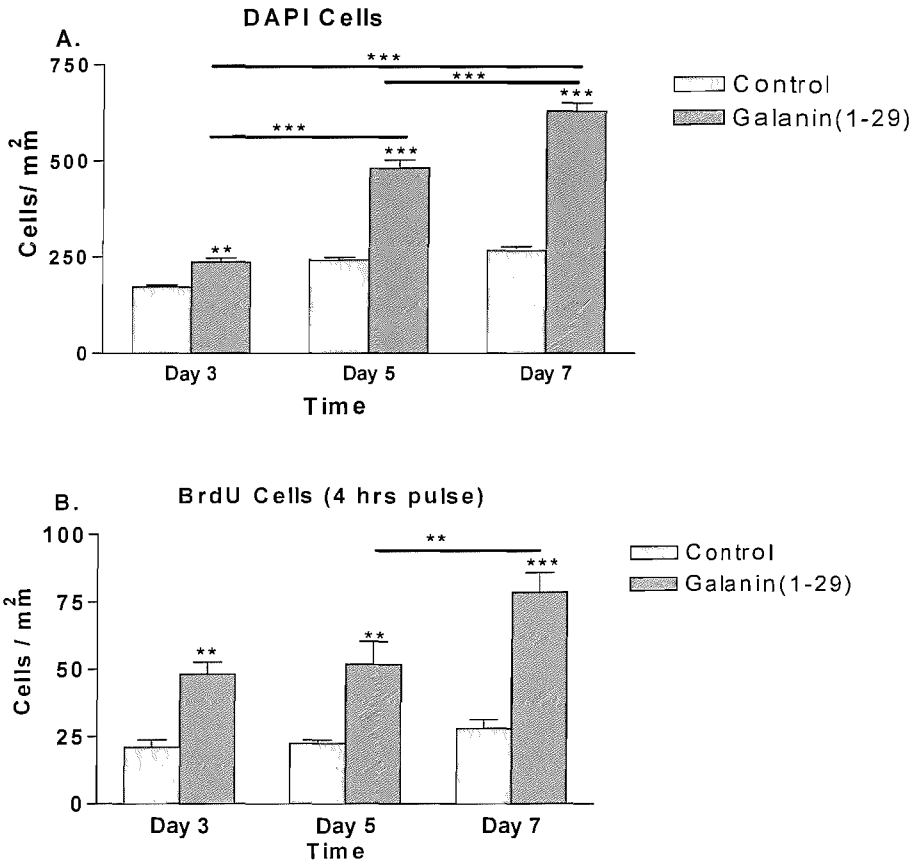


Figure 6.13: **Galanin (1-29) supports cell proliferation of cultured hippocampal cells over time.** Cells were grown under control or 100nM galanin (1-29) for 3, 5 and 7 DIV. (A) Total number of cells under the tow conditions with time, (B) the number of BrdU incorporating cells under the two conditions after a 4 hr pulse at each time point. . Data represent mean ± SE based on a sample that represent at least 8 well per condition from one experiment. Comparisons between different conditions with respect to time, two way ANOVA with Bonferroni's Multiple Comparison Test (**, $p < 0.01$ and ***, $p < 0.001$).

6.3.12 Galanin (2-11) enhanced total cell counts in culture

In an initial set of experiments, cultured hippocampal cells were grown under control conditions or control plus 1nM-1μM galanin (2-11) or 1μM NPY for 5 days. After cells were fixed they were washed and stained with the nuclear stain DAPI (20μ/ml); cells were then imaged. Total cell counts under each condition were determined. Results presented in (**Figure 6.14)** revealed that galanin (2-11) at concentrations of 100nM and 1μM significantly increased total cell counts to 212.4 ± 5.6 cells/ mm^2 and 226.7 ± 8.7 cells/ mm^2, respectively, compared to 151.4 ± 13.3 cells/ mm^2 under control conditions. NPY at 1μM as a positive control significantly increased total cell counts to 215.3 ± 8.9 cells/ mm^2

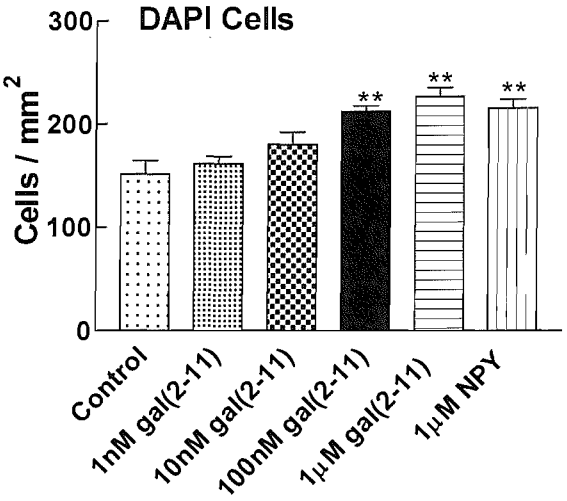


Figure 6.14: Galanin (2-11) increased cell density in culture. Hippocampal cells were grown under control, or 1nM-1μM galanin (2-11). 1μM NPY was used as a positive control. At 5 DIV, cells were fixed, washed and just stained with DAPI to assess total cell counts under different conditions. Data represent mean ± SE based on a sample that represent at least 6 wells per condition from one experiment. Comparisons between different conditions, one way ANOVA with Dunnetts' multiple comparison test (**, $p < 0.01$).

6.4 Discussion

6.4.1 VIP combined with NPY enhanced neurogenesis *in vitro*

The neuropeptides VIP and NPY and their receptors are highly expressed in the rat hippocampus (Gulyas et al., 1996; Scharfman and Gray, 2006). VIP and NPY levels peak in the rat brain at postnatal day 10 (Lopez-Tellez et al., 2004); the time when more than half of the GCL is formed (Namba et al., 2007) and severely declined with age (Cha et al., 1997) as hippocampal neurogenesis does (Jin et al., 2003). As anticonvulsants, both VIP and NPY have been implicated in the modulation of cell survival and proliferation in the hippocampus after seizure activity in epileptic patients (de Lanerolle et al., 1995; Howell et al., 2007). In addition, the levels of both peptides have been shown to increase acutely in a model of status epilepticus in rats (Marksteiner et al., 1989). NPY has been demonstrated in our lab as a proliferative factor that plays key roles in hippocampal neurogenesis (Howell et al., 2003). In fact, in an elegant study conducted by Howell and Gray, NPY, via Y1 receptor subtpe, has been shown to enhance cell proliferation in the DG of the hippocampus both under basal conditions and in status epilepticus ; where the Y(1) receptor(-/-) mice displayed a significant reduction in basal and seizure-induced proliferation in the DG (Howell et al., 2007).

Herein, we have demonstrated VIP as a trophic (at physiological concentrations) and proliferative factor (at elevated levels) that also enhanced the self-renewal of the transient amplifying cell population during postnatal hippocampal neurogenesis. These facts have driven us to search for any possible interactions between the two neuropeptides in the modulation of neurogenesis in cultures generated from the postnatal rat hippocampus. Our observations suggest that hippocampal cells in culture responded well to NPY (1μM) and VIP (30nM) as indicated by the increase in the total cell counts and BrdU incorporating cell numbers. In fact, the response of hippocampal cells in postnatal cultures to NPY (as an internal control) is in accordance with previous work done in our lab (Howell et al., 2003).

When VIP and NPY were added together to cultured hippocampal cells, the response as indicated by total cell counts was not additive. This lack of an additive effect shows that the same population of cells responds to VIP and NPY. In addition, our analysis

of cell proliferation assessed by the proliferation marker BrdU revealed that the number of BrdU labelled cells decreased under combined treatment of both NPY and VIP compared to NPY conditions. These observations contrast with the proliferative effects of NPY on cultured precursor cells generated from the postnatal rat hippocampus. The interpretation of this data is that, primary hippocampal cultures grown for 5 days contain different populations of cells, including proliferating and differentiating ones. Combined treatment of these cells with VIP and NPY may have yielded in a shift of these cells from the proliferating population to exit the cell cycle and become terminally differentiated. Consistent and in agreement with these results is our data retrieved from the experiments in which we studied the cell cycle exit index. Making use of BrdU and Ki-67, this index has been reported to effectively measure the proportion of cells that exit the cell cycle in hippocampal organotypic slice cultures (Siegenthaler and Miller, 2005). In our experiments, cells were grown in monolayer cell cultures under standard control conditions, 30nM VIP, 1μM NPY or combined VIP and NPY. Similar to what Siegenthaler and Miller did, cells were pulsed with BrdU at the time of peptides application (2 hrs after initial plating). However, because BrdU gets incorporated to the daughter cells as cells proliferate, it gets diluted after 4 cell-cycles (Dayer et al., 2003), cells were loaded with another pulse of BrdU on the third day. In this setup, all proliferating cells are going to incorporate BrdU. After 5 days in culture, cells were fixed and processed for BrdU incorporation and the co-expression of the proliferative marker Ki-67. A cell exiting the cell cycle to become post-mitotic/ non-mitotic was defined as the cell that has incorporated BrdU some time early on and has become Ki-67 negative. The cell cycle exit index was calculated by dividing the number of BrdU positive but Ki-67 negative cells by the total number of cells.

Our results show that the combined VIP and NPY treatment of cells in culture increased the number and the proportions of cells leaving the cell cycle compared to control conditions. These cells either become non-mitotic in a resting state or postmitotic terminally differentiated cells. Regardless of the state of this population of cells, it apparently less responsive to NPY proliferative effects thus explains the drop in BrdU positive cell numbers.

This conclusion is supported by the phenotyping data, which indicate that NPY and VIP combined treatment, yielded a proportional increase in cells expressing the neuronal marker class III ß-tubulin; an effect that none of the two neuropeptides duplicates when added to cells alone. In fact, we noted that under the combined treatment the increase in the percentage of post-mitotic cells (13%) over control is slightly higher than the increase in the percentage of class III ß-tubulin cells (10%), suggesting that a large portion of the cells that prematurely exited the cell cycle under VIP and NPY combined treatment terminally differentiated and adopted a neuronal fate.

Howell as well as our pharmacological studies (**Chapter 5**) indicate that while VPAC2 and VPAC1 receptors mediate VIP trophic self-renewal and neuronal differentiation effects; respectively, Y1 receptor mediates the NPY proliferative effects in postnatal hippocampal cell cultures (Howell et al., 2003). This ligand/receptor interrelationship may provide a model that explains our findings. For instance, while VIP may have downregulated Y1 receptor expression, hence the decreased cell proliferation, NPY on the other hand may have upregulated VPAC1 with no effect on VPAC2; thus the neuronal differentiation and the maintained trophic and self-renewal effects of VIP, respectively, under the combined treatment. Yet, this needs to be further studied in future work.

In addition, in our literature review with regard to the intracellular pathways that may be involved in the interactions between VIP and NPY, we found that cAMP is a common pathway. Whilst NPY is proliferative to a wide range of cell types, it has been shown to inhibit cAMP production in many of these systems, including bovine chromaffin cells (Zheng et al., 2000) melanotrope cells (Scheenen et al., 1995), prostate cancer cells (Ruscica et al., 2006) and rat left ventricular cells (Raimondi et al., 2002). In contrast, while VIP survival effects are often cAMP-independent (Muller et al., 1995), it has been shown at nanomolar concentration to enhance the production of cAMP in cultured neurons (Pincus et al., 1990a; Nowak et al., 2007). In this regard, in many cell culture systems including, neocortical precursor cells, vascular smooth muscle cells and keratinocytes, elevated cAMP has been shown to decrease cell proliferation (Indolfi et al., 2000; Takahashi et al., 2004; Meyer, 2006).

This could have relevance to the fact that the addition of VIP abolished NPY proliferative effects.

In conclusion, these observations all together suggest that the combined treatment of cultured hippocampal cells with the neuroproliferative (NPY) and neurotrophic (VIP) peptides resulted in enhanced survival and differentiation of precursor cells toward the production of neuronal restricted progeny cells. Indeed, these results are in agreement with other reports that have shown that the treatment of embryonic hippocampal precursor cells with a mitogenic factor like bFGF combined with a trophic factor like neurotrophin-3 yielded an enhanced survival and differentiation of neuronal cells (Ito et al., 2003). Therefore, we suggest that these neuropeptides and probably others may act as a novel control system that influences hippocampal neurogenesis depending on their differential release by local interneurons in response to different stimuli.

6.4.2 Combined VIP and bFGF treatment enhanced the survival of precursor cell population in culture

Consistent with our findings in **Chapter 3**, 30nM concentration of VIP increased nestin positive cells' counts as well as the total numbers of cultured hippocampal cells. Moreover, in agreement with observations of other studies (Babu et al., 2007), bFGF increased the total cell numbers, BrdU and nestin-expressing hippocampal cells. Interestingly, while the treatment with VIP and bFGF increased the survival of nestin expressing stem/ progenitor cells, treatment with either factor alone failed to duplicate the massive increase of combined treatment. In fact, the increase in counts of nestin positive cells under combined treatment was an additive effect. This may suggest that multiple factors are required in specific combinations for the survival of progenitor cells at this particular stage of development. In agreement with our observations, in primary dentate cultures generated from neonatal rats, it has been shown that the growth properties of cultured cells are influenced by exogenously applied BDNF or bFGF in a time- and age-dependent manner (Lowenstein and Arsenault, 1996). To interpret this data we need to recall that bFGF is a trophic factor that has been shown to have a mitogenic activity on cultured hippocampal precursor cells at 20ng/ml (Gage et al., 1995; Gage et al., 1998). Also our data demonstrated VIP as a trophic factor for dividing cells with no direct proliferative effects. This may

explain the additive effect we observed on total cell counts and on the number of nestin positive cells over each factor alone. VIP and bFGF combined treatment may have yielded a population of cells that are less responsive to bFGF and/or more dependent on VIP or a combination of both.

The other important finding that our results revealed is that the number and proportion of proliferating cells in the S-phase of the cell cycle dropped significantly under combined VIP and bFGF treatment compared to bFGF conditions; indicating decreased rates of cell proliferation. Yet, these counts are still significantly higher than those under control conditions. The drop in the number of actively dividing cells at that particular time (5DIV) under combined treatment compared to bFGF alone may be due to VIP-enhanced cell cycle exit at 5 days, like in the NPY experiment. Therefore, the cells may have become less responsive to the proliferative activity of VIP. In agreement with this finding it has been shown that neural stem cells derived from the mice telencephalon respond distinctly to the proliferative effect of FGF and EGF in sphere cultures. Indeed, neural stem cells in the anterior neural plate respond well to FGF but not EGF by E8.5 whereas they respond to EGF but not FGF by E15.5 (Tropepe et al., 1999).

More importantly, this decrease in cell proliferation under the combined treatment could be attributed to the ligand/ receptor interactions. Because FGFR1 receptor is known to mediate the proliferative effect of bFGF (Maric et al., 2007), we have investigated the effect of VIP on the regulation of FGFR1 mRNA expression. As expected VIP downregulated the expression of FGFR1 mRNA by more than 50% after 4 hrs exposure. This finding may explain the decrease in cell proliferation under combined treatment, although a western blot study may be needed to investigate the expression of the FGFR1 protein as well. In addition, and as I mentioned above, VIP may have resulted in elevated level of cAMP, which may have influenced the proliferative activity of bFGF as it does so in other systems; another thing that may be needed to consider in future work.

6.4.3 ADNP: regulation by VIP in postnatal hippocampal cell cultures

Activity dependent neuroprotective protein (ADNP) is a gene that has been reported to be essential for the brain formation (Pinhasov et al., 2003). This gene has been shown to be developmentally regulated by VIP in rat astrocytes cultures through VPAC2 receptor (Zusev and Gozes, 2004). Within the developing brain, ADNP has been co-localised at the cellular level both in astrocytes and in cells expressing tubulins (Furman et al., 2004). Herein, we present the expression of ADNP and its mRNA in postnatal hippocampal progenitor cultures. Indeed, we found that ADNP is expressed by merely all the cells in cultures, which may include nestin, astrocytic, neuronal and NG2 cells. Our double immunohistochemistry clearly demonstrates the expression of ADNP by all nestin-expressing putative/ stem cells. Using primers for ADNP and cDNA extracted from hippocampal progenitor cell-lysate, we managed to amplify the expression of the mRNA of this gene under standard control conditions. Semiquantitative analysis of the RT-PCR products separated on 2% agarose gel, indicates that VIP treatment for three hrs upregulated the expression of ADNP mRNA by three times. In contrast, our quantitative real time PCR data revealed that 30nM VIP significantly downregulated ADNP mRNA expression after 1 and 8 hrs by nearly 10-fold. However, although VIP resulted in a 2-fold drop in ADNP mRNA expression after 4hrs exposure, this decrease is statistically insignificant.

In the literature, while VIP has been shown to be an important regulator of the expression of ADNP mRNA, the net effect varied depending on the system and the period of development in and during which it has been investigated, respectively. For instance, when whole post-implantation embryos (E8.5) were incubated for 4hrs with 100nM VIP, the ADNP mRNA expression doubled (Pinhasov et al., 2003). Interestingly, in cortical astrocyte cultures generated from neonatal rats, the effect of VIP on ADNP expression appears to be age dependent; a 3hr incubation resulted in an increase by 29% in 4 days old astrocyte and 19% decrease in 8 days astrocytes (Zusev and Gozes, 2004), an effect which is VPAC2 mediated (Zusev and Gozes, 2004). This could have relevance as, in our hippocampal progenitor cell cultures, which closely age-matched with the 8-day-astrocyte cultures, 30nM VIP resulted in ADNP mRNA downregulation, significantly at 1 and 8 hrs incubation times. These data suggest the regulation of ADNP mRNA by VIP, and taken together with the extensive expression of ADNP by cultured hippocampal cells, further suggest that ADNP may

be involved in the mediation of VIP self-renewal effects in hippocampal cell cultures. Indeed, while ADNP has been shown to mediate the VIP-differentiation effect of embryonic hippocampal precursor cells toward a neuronal lineage (Blondel et al., 2000), its downregulation by VIP (probably via VPAC2 receptor) in our postnatal hippocampal culture system may fits well with the VIP self-renewal affect rather than differentiation.

In adult life, the impact of VIP *in vivo* on ADNP expression has recently been examined in adult wild-type (VIP +/+) and VIP null (VIP -/-) offspring of VIP deficient mothers (VIP+/-) comparing them to wild-type offspring of wild-type mothers (Giladi et al., 2007). Using quantitative real time PCR, Giladi has shown reductions in ADNP mRNA expression in the brains of VIP null mice compared with the brains of wild-type offspring in the cortex and hypothalamus, but not in the hippocampus or thalamus.

Taken together, our data with others' observations suggest that ADNP gene's responsiveness to VIP is developmentally regulated. Whether ADNP mediates VIP survival and self-renewal effect on hippocampal stem cells during the postnatal period is yet to be further investigated. This inconsistency between our semiquantitative and quantitative real time results is yet to be further examined. However, real time PCR has been suggested as a more accurate method of quantifying changes in gene expression (Heid et al., 1996).

6.4.4 Galanin is an important neuropeptide for the survival of hippocampal progenitor cells

Galanin receptors are widely distributed in the brain, particularly the hippocampus (Branchek et al., 2000; Mazarati et al., 2004b). Galanin is expressed by some neurons in the hippocampus in addition to a lot of nerve terminals supplying the area (Shen et al., 2003). GalR1 and GalR2 are highly expressed in the dentate gyrus of the hippocampus and the subventricular zones of the lateral ventricle; two important neurogenic areas in the postnatal and adult brain (Mazarati et al., 2004b; Hokfelt, 2005). This expression of galanin and its receptors in the areas of neurogenesis suggests that galanin may play a role in the survival and proliferation of stem cells and the survival of their progeny in the hippocampus. In addition, galanin has been

reported to be involved in many conditions such as, exercise (Legakis et al., 2000), stress (Kehr et al., 2001), epilepsy (Mazarati et al., 2004a), learning (Kinney et al., 2003), cognition and affect (Wrenn and Crawley, 2001). All these conditions have been shown to modulate hippocampal neurogenesis. Therefore, this part of my PhD project was designed to preliminarily investigate galanin roles in hippocampal neurogenesis *in vitro*. Primary hippocampal neuronal cultures generated from postnatal rat hippocampus have been our methodology to investigate this hypothesis. We have shown that cells in culture grow, proliferate and respond well to growth factors, like bFGF and neuropeptides like NPY; in accordance with others observations (Howell et al., 2003; Howell et al., 2005).

To test galanin effects on hippocampal neurogenesis *in vitro*, we exposed cultured hippocampal cells to galanin (1-29) and galanin (2-11) which are GalR1/GalR2 mixed and GalR2 high affinity agonists, respectively (Floren et al., 2000). Our preliminary data indicate that both galanin (1-29) and galanin (2-11) enhanced the survival of hippocampal cells in culture as indicated by the increase in the total number of cells under galanin compared to control conditions. This data was not surprising because galanin has been reported to have trophic and developmental roles in the peripheral and the CNS (Hokfelt, 2005) and neuroprotective effects against excitotoxicity in the hippocampus; which is accompanied by altered hippocampal neurogenesis (Elliott-Hunt et al., 2004).

Our further investigations have shown that one of the galanin analogues, galanin (1-29), has potent trophic effects at as low as 1nM peptide concentration. In deed, galanin (1-29) increased the number of BrdU cells after 24hr as equal as NPY in cultured hippocampal cells. These results are in agreement with the observations of Mazarati and his co-workers, which have shown that galanin acting on GalR2 to induce seizure accompanied neurogenesis and increase the number of proliferating cells in the dentate gyrus (Mazarati et al., 2004b). However, our results are in disagreement with other reports in which low concentrations of galanin has been shown to attenuate cell proliferation in cultured neurospheres (Hokfelt, 2005). It is important to note that these neurosphere cultures are usually generated with support of mitogenic factors, like bFGF and EGF. Therefore, galanin may have interactions with these factors which may affect the net effect on cells growing in these sphere cultures.

The fact that Galanin (2-11) (a GalR2 agonist) increased total cell counts strongly suggests mediation of GALR2 in galanin-modulation of cell survival and proliferation *in vitro*; yet this needs to be further examined.

Our preliminary data with regard to galanin indicate that galanin is involved in the modulation of hippocampal neural stem cell survival during the postnatal period. It would be interesting to further study any possible proliferative activity of galanin on these cells. Moreover; future work should focus on the pharmacology to study the contribution of each receptor subtype in its effects. This is in addition to phenotyping the cells to identify the galanin-responsive cell population and then any possible interactions with NPY and or VIP.

6.5 Summary

Our results which are listed in this chapter can be divided into three main groups. Firstly, results dealt with VIP interactions with NPY or FGF-2. Secondly, VIP regulation of ADNP expression and lastly, results obtained from galanin investigations. For the interaction results, we have demonstrated that the combined treatment of VIP and NPY reduced rates of proliferation but enhanced neuronal differentiation (neurogenesis). On the other hand, bFGF-2 combined with VIP enhanced the survival of nestin-expressing cells in an additive manner, despite a reduction in the rate of cell proliferation, which may be due to FGFR1 downregulation. Our findings further indicate that ADNP is highly expressed by hippocampal progenitor cells *in vitro*. In addition, the expression of ADNP in these cultures is regulated by VIP.

For the galanin data, we herein present for the first time preliminary work which strongly suggests proliferative and trophic activity of galanin on cultured hippocampal cells. Galanin (1-29) increased total cell counts of hippocampal cells *in vitro* at concentrations between 0.1nM and 1μM. Cells displayed enhanced cell proliferation at concentrations of 1nM and above. In addition, the GalR2 agonist enhanced the survival of cultured hippocampal cells at concentrations of 100-1000nM.

Taking together, this data demonstrate that VIP is an important trophic factor for hippocampal neurogenesis and interacts with NPY and FGF-2 to modulate the proliferation of stem cells and the survival of their progeny. Galanin is also another putative neuropeptide that influences the proliferation and the survival of these progenitor cells. These neuropeptides and others (smatostatin; another neuropeptide that is expressed in the DG (Sloviter and Nilaver, 1987)) may provide a novel control system for hippocampal neurogenesis depending on their differential release from hippocampal interneurons. Characterizing this control system will provide a better understanding of control mechanisms of hippocampal neurogenesis.

Chapter 7

General Discussion

7.1 Overview and summary of the major findings

In mammals, including humans, new neurons are continuously added to neuronal circuits in the hippocampus (Doetsch, 2003). Newly-born neurons in the hippocampus are generated in the subgranular cell layer of the dentate gyrus, (Doetsch, 2003; Seri, 2004) in a process which may represent a mechanism for learning and memory formation and which is influenced by other physiological and pathological activities such as exercise, stress, mood disorders and seizures (Kempermann et al., 1997; Parent et al., 1997; van Praag et al., 1999; Jacobs et al., 2000; McCabe et al., 2001; Kempermann, 2002; Parent et al., 2002; Eriksson and Wallin, 2004). Much of the control of neurogenesis centres on altering the survival of proliferating precursors and their progeny rather than in the proliferation rate of cycling precursors (Dayer et al., 2003). Despite the dominance of trophism in adult dentate neurogenesis, our knowledge of its mechanisms, with the exception of BDNF, are poorly understood. In particular, how neural activity exerts trophic effects on stem cells and their progeny is unclear.

Vasoactive intestinal peptide (VIP) is a 28 amino acid neuropeptide that is highly expressed in GABAergic interneurons in the adult hippocampus and dentate gyrus, and in common with other neuropeptides is released under specific firing conditions (Acsady et al., 1996; Hokfelt, 2005). It is also widely expressed along with its receptors in the developing rodent and human CNS (Besson et al., 1986; Hill et al., 1994a; Usdin et al., 1994), where it has prominent trophic and proliferative effects. In whole embryo cultures, VIP stimulated growth as assessed by the increase in embryonic volume, DNA and protein contents, and the number of cells in S-phase of the cell cycle (enhanced cell proliferation) (Gressens et al., 1997; Cazillis et al., 2004). In addition, in embryonic stem cell culture, VIP potently enhanced differentiation specifically towards a neuronal lineage without altering glial cell numbers (Cazillis et al., 2004). With respect to the dentate gyrus, VIP levels are markedly increased in the rat hippocampus postnatally hitting a peak at P10 (Lopez-

Tellez et al., 2004) ,which is exactly the period of time during which the granule cell layer of the dentate gyrus is largely generated (Namba et al., 2005). Interestingly, in adulthood, VIP has been implicated in hippocampal dependent learning and memory as the intracerebral administration of a VIP receptor antagonist results in learning impairments in rats (Glowa et al., 1992), and the transgenic mice harboring a chimeric VIP gene displayed learning impairment and prolonged retardation in memory acquisition (Gozes et al., 1993). While these studies strongly suggest VIPs involvement in the modulation of stem cell development during embryonic life, and in adulthood learning and memory which also modulate hippocampal neurogenesis, to our knowledge, there are no studies examining VIPs possible involvement in postnatal hippocampal neurogenesis. Therefore, we have designed this project to investigate VIP effects in the modulation of hippocampal neural progenitor/ stem cells during postnatal and adult life.

In this study, I have demonstrated a trophic effect of VIP, at physiological concentrations (30nM), on nestin-expressing neural stem cells, GFAP-expressing astrocytic cells and TuJ1-expressing neuronal cells, but not oligodendrocyte-NG2-expressing precursor cells. This trophic effect is mainly on actively proliferating/ cycling hippocampal precursor cells. At these nanomolar concentrations, VIP also increased the proportion of nestin-expressing cells, particularly the amplifying actively dividing cell population which expresses nestin, but not GFAP nor TuJ1 (primitive progenitors). At elevated concentrations (1μM), we demonstrated a proliferative effect of VIP on both nestin-expressing cells as well as neuroblasts and a trophic (survival) effect on NG2 positive oligodendrocyte precursor cells. Using postnatal micro-dissected cell cultures, we have shown that VIP, in both the dentate gyrus and hippocampal subventricular zone cultures, is trophic to nestin-expressing progenitors and to neuronal progeny cells. However, VIP enhances the self-renewal of nestin positive but TuJ1 negative cells (primitive amplifying cell population) derived from the dentate gyrus, but not the hippocampal subventricular zone.

I have also looked at the expression of the VIP high affinity receptors; and have demonstrated the expression VPAC1 and VPAC2 in the granule cell layer of the dentate gurus in postnatal rat brain sections and in hippocampal cell cultures. At the cellular level, we have further demonstrated the expression of the two receptors by the

major cell populations *in vitro*, including nestin-expressing progenitor cells. Using PCR techniques, we have also demonstrated the expression of the mRNA of VPAC1 and VPAC2 by cultured hippocampal cells. Pharmacologically, our *in vitro* results implied that VPAC2 mediates VIP self-renewal and trophic effects of nestin-expressing cells whereas VPAC1 mediates differentiation of hippocampal stem cells towards a neuronal lineage. To build on our *in vitro* data, we further studies VPAC2 mediation of cell survival *in vivo* using VPAC2 Knockout mice strain. In these young adult VPAC2 knockout mice, we have shown a reduction in cell survival of newly-born cells in the dentate gyrus and in the overall area of the SGZ in comparison to respective littermate controls.

We have also extended our study to look at VIP interactions with the proliferative neuropeptide NPY and the mitogen FGF-2 which play key roles in hippocampal neurogenesis (Yoshimura et al., 2001; Howell et al., 2003; Howell et al., 2007). Our results in this regard showed that when cells were exposed to VIP in conjunction with NPY or FGF-2, VIP abolished or decreased their proliferative effects, respectively. Interestingly, the proportion of neuroblasts increased (enhanced neurogenesis) under VIP plus NPY conditions while the number of progenitor cells increased under VIP plus FGF-2 conditions.

As the neuroprotective effects of VIP are mediated in part through the action of glial-derived substances, such as ADNP (Brenneman et al., 2000), we have looked at VIP interactions with ADNP. In this regard, we have demonstrated that the majority of hippocampal precursor cells express ADNP *in vitro*. We further looked at VIP regulation of ADNP mRNA; and our initial data implies that VIP down-regulated ADNP mRNA in cultured postnatal hippocampal cells.

We have also looked preliminarily at the neuropeptide Galanin which is likewise expressed along with its receptors in the hippocampus (Floren et al., 2000). Our preliminary data implies that galanin is a trophic factor that enhances the survival of hippocampal progenitor cells *in vitro*.

In summary, VIP enhances the survival and self-renewal of hippocampal nestin putative/stem cells and their progeny specifically via VPAC2 receptor subtype *in vitro*. VIP via VPAC2 also enhances the basal cell survival in the dentate gyrus of the adult mice hippocampus *in vivo*. This strongly suggests a similar modulatory role for VIP in the control of hippocampal neurogenesis through development and into adulthood. Our results also indicate that VIP interacts with other regulators of the stem cell niche in the hippocampus, including FGF-2, NPY and ADNP.

7.2 VIP is trophic for neural stem cells and their progeny

VIP effects on cells in cultures varied depending on the type of cells and stage of development. (Brenneman et al., 1985; Brenneman et al., 1998). In some systems VIP has been shown to enhance survival, and/ or induce cell differentiation, while in others it induces cell proliferation (Waschek, 1996). For example, VIP regulated cell survival in sympathetic ganglion (Brenneman et al., 1985), cerebellar cortex (DiCicco-Bloom, 1996), and the early embryo (Hill et al., 1996a). In cultured sympathetic ganglion neuroblasts, micromolar concentrations of VIP have been shown to enhance the survival of proliferating neuronal precursor cells (Pincus et al., 1990b; DiCicco-Bloom, 1996). Furthermore, in dissociated spinal cord cultures generated form foetal rats (E12-14), 100nM VIP has been shown to enhance neuronal survival with no significant effect on thymidine incorporation (Waschek, 1996). In addition, VIP increased the survival of both dividing and non-dividing neuroblasts in cultures generated from E15.5 rat superior cervical ganglia (Pincus et al., 1994). Moreover, in embryonic rat hippocampal culture, 100nM of VIP enhanced neuronal differentiation (Blondel et al., 2000). In whole embryonic cultures, nanomolar concentrations of VIP enhanced growth of post-implanted embryo as indicated by the number of somites and proliferating cells (Hill et al., 1996a).

As far as hippocampal stem cells are concerned, only one study attempted to elucidate VIP effects in hippocampal stem cells generated from adult female rats supplemented by FGF-2 (Antonawich and Said, 2002). In this study, VIP, only at 5μM and above, inhibited apoptotic cell death resulting from FGF-2 withdrawal, as indicated by cytochrome C translocation. These effects were suggested to be mediated by the high affinity receptor; PAC1. This study could be criticized for two pitfalls. Firstly; the supplementation with FGF-2, may have led to interactions with VIP, and thus, never

purely reflects VIP effects. Secondly, this study failed to elucidate VIP effects on basal cell survival. In our study, we designed our cell culture paradigm to study VIP effects on the modulation of hippocampal stem cell basal survival. Other growth or peptide factors were not added, except in the interactions experiments.

In primary hippocampal cell cultures generated from postnatal rats (P7-10), nanomolar concentrations of VIP, dramatically decreased cell death with no effects on the rates of cell proliferation or the recruitment of quiescent cells. It was not until 1μM that VIP enhanced the proliferation of the nestin-expressing stem cells and their neuronal progeny without affecting the recruitment of quiescent cells. Consistent with these findings, the Ara-C experiments and time-lapse live imaging experiments confirmed that the trophic effect of VIP, at 30nM peptide concentration, was specific for proliferating progenitor cells and their newly-born daughter cells.

The trophic effect of VIP was mediated by the VPAC2 receptor *in vitro*. This was confirmed *in vivo* with a significant reduction in the survival of newly-born cells in adult germline VPAC2 -/- mice. The importance of VPAC2 mediated trophism was underlined by the significant reduction in the size of the granule cell layer in adult VPAC2-/- mice.

By analyzing cell phenotypes at 3 and 5 DIV, we noted that VIP at physiological concentrations enhanced the survival of nestin-expressing stem cells, TuJ1-expressing neuronal cells, GFAP-expressing glial cells, but not NG2-expressing oligodendrocyte precursor cells. VIP displayed a trophic effect on the NG2 cells only at 1μM peptide concentration. This finding is consistent with the work of DiCicco Bloom et al who found atrophic effect of VIP at 5μM as there are many NG2 expressing cells in hippocampal cultures from the postnatal hippocampus (Lee et al., 2001).

These observations suggest that VIP, at physiological concentrations, modulates the survival of postnatal hippocampal progenitor cells and their progeny. These observations, in agreement with the high levels of VIP during postnatal life, strongly imply a role for VIP in the survival of newly-born progeny during the postnatal and adult development of the GCL of the DG. Although the majority of studies on hippocampal neurogenesis have focused on proliferative factors, the accumulating

evidence indicates that cell survival is the key parameter that regulates net hippocampal neurogenesis *in vivo* and perhaps certain forms of hippocampal dependant learning and memory (Prickaerts et al., 2004). In support of this hypothesis, VIP has been shown to play a role in hippocampal dependant learning and memory. Indeed, while transgenic mice harboring chemric VIP genes (20% VIP reduction) has been shown to have learning and memory impairments, the chronic administration of Met-Hybird, a potent inhibitor of VIP function *in vivo*, results in impairment of learning abilities (Gressens et al., 1997). Therefore, VIPs role as a trophic factor for precursor cells may represent an important mechanism for its role in learning and memory. Clearly this will be an interesting avenue for future research.

Furthermore, VIPs trophic and proliferative effects on NG2 cells and progenitor/ precursor cells respectively at elevated pharmacological levels (1μM), are not of less importance. These elevated levels of VIP may represent the peptide levels in pathological states, like in epilepsy. VIP has been found to be elevated acutely (3 days) after status epilepticus, before returning to basal levels (Marksteiner et al., 1989). This may, at least in part, explain the enhanced cell proliferation in the DG acutely after seizures. In addition, during pathological states including epilepsy, NG2 cells are needed for remyelination as part of the repair mechanisms (Dawson et al., 2000; Dawson et al., 2003).

The exact mechanisms through which VIP mediates the survival of neuronal precursor cells are yet to be determined. However, the neurotrophic and neuroprotective properties of VIP are mediated in part through the action of glial-derived substances, including cytokines, like interleukin-1 alpha (IL-1 alpha), protease nexin I, insulin growth factor-I (IGF-I) and ADNF and ADNP. Indeed, while VIP has been shown to enhance the release of IL-1 alpha in astroglial cultures generated from the cerebral cortex in a dose-dependent manner; consistently, neutralizing antiserum to IL-1 alpha or its receptor decreased neuronal survival (Brenneman et al., 1995). In addition, protease nexin I is another trophic factor that has been shown to be upregulated by VIP in primary schwan cell cultures generated from postnatal rats (P4-5) (Bleuel and Monard, 1995). IGF-I is a growth factor that has been shown to mediate VIP trophic effects in whole cultured mouse embryos (E9.5) (Servoss et al., 2001). Indeed, Servoss showed that while the IGF-I induced a

significant, dose-dependent increase in growth as measured by somite number, as well as DNA and protein contents, VIP stimulated growth was blocked by anti-IGF-I. Furthermore, he demonstrated by PCR techniques that 100 nM VIP resulted in a 2-fold increase in IGF mRNA. Similarly, VIP has been shown to upregulate ADNP mRNA in whole embryo cultures (Pinhasov et al., 2003) and downregulate it in P8 day astrocytic cultures (Zusev and Gozes, 2004). In agreement, anti-ADNP serum has been shown to decrease neuronal survival in hippocampal cell cultures generated from foetal mice (Gozes et al., 1997).

7.3 VIP enhances the self-renewal of nestin-expressing stem cells

Consistent with other studies (Seri, 2004; Conover and Notti, 2007; Riquelme et al., 2007), using immunochemistry to detect the expression of nestin, GFAP and TuJ1, we have shown that our hippocampal cell cultures contain distinct but overlapping cell populations. Nestin is a marker of true stem cells, which is also expressed in more restricted progenitor cell types (Howell et al., 2007). In early stages of neural stem cell development within the dentate gyrus, nestin cells can be classified into two categories (Encinas et al., 2006). Firstly, a slowly divining primitive stem cell population which co-expresses nestin and the glial cell marker GFAP. This cell population functions to maintain the pool of the stem cells within the SGZ (Seri, 2004). Secondly, a more actively dividing cell population which represents the amplifying cell population. This subpopulation of cells in contrast expresses nestin, but not GFAP (**Figure 7.1**). In agreement with these findings, we observed the two populations of nestin cells in our postnatal hippocampal cultures (**Section 3.4.8**). Indeed, about 25% and 22% of cultured hippocampal cells were nestin+ GFAP+ (primitive stem cells) and nestin+ GFAP- (amplifying cell population) under standard control conditions, respectively. In addition, nestin has been found to be expressed by a small subpopulation of restricted neuronal precursor cells expressing TuJ1 (**Figure 7.1**) (Howell et al., 2007). Making use of double immunochemistry, we identified a subpopulation (~3% of total hippocampal cells) of nestin cells that coexpresses TuJ1 in hippocampal progenitor cell culture whilst the majority of nestin cells were TuJ1 negative (~33% of total cells on culture).

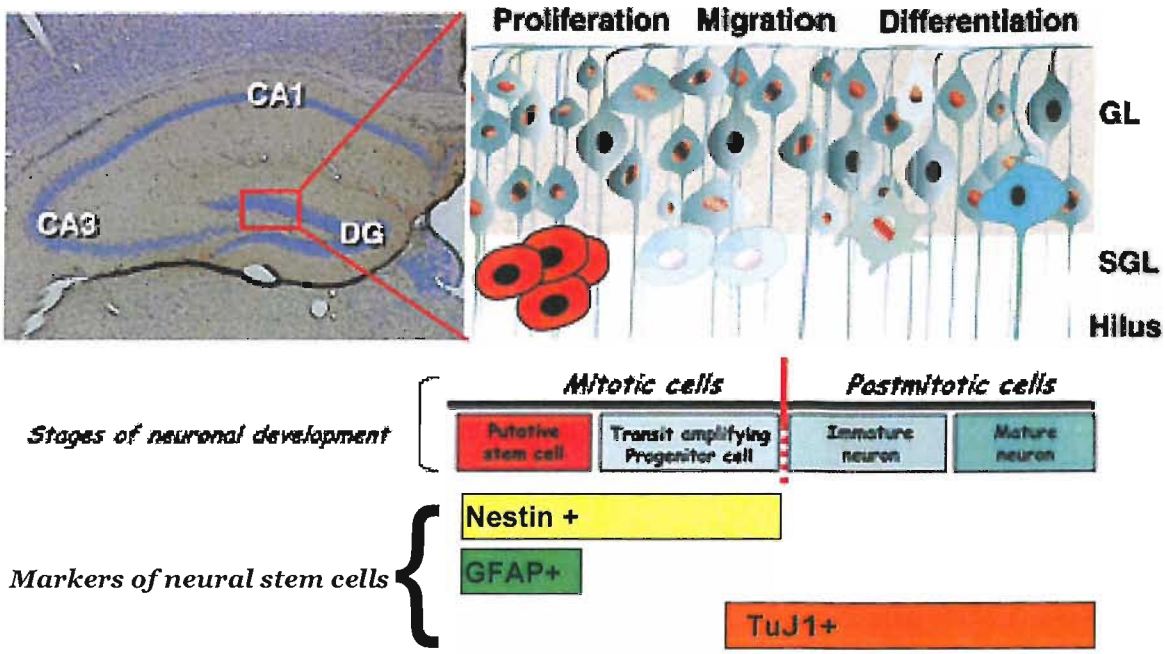


Figure 7.1: Neurogenesis in the dentate gyrus of the hippocampus. *Top panel*: a frontal view of a rodent brain showing the sites of neurogenesis in the dentate gyrus (DG) of the hippocampal formation (HF) is given. Cells proliferate in the subgranular layer (SGL) located at the interface between the hilus and the granular layer (GL), where they migrate and differentiate into mature neurons. *Bottom panel*: a sequence of cell types involved in neuronal development, along with specific markers allowing cell identification, is proposed (see text for further details). Adopted and developed from (Abrous et al., 2005).

We observed that VIP increased the overall proportion of nestin-expressing hippocampal progenitor cells in culture with no effect on the proportion of TuJ1 neuronal or GFAP astrocytic precursors. Within the stem cell pool, VIP increased the proportion of the amplifying population of cells which express nestin but not GFAP by nearly 33% with no effect on the more primitive (nestin+ GFAP+) cells. Looking at the subpopulation of nestin precursor cells that have committed to a neuronal lineage, VIP appeared not to drive the cells towards a neuronal lineage. In fact, VIP increased the proportion of nestin positive cells that did not express TuJ1 (amplifying cell population) both in whole hippocampal cell culture as well as in dentate micro-dissected cultures. VIP had no effect on this subpopulation of cells generated from the HSVZ.

Stu the mechanisms through which VIP enhanced self-renewal of nestin progenitor cells hippocampal cell culture; we found that VIP enhances nestin positive cells to divide symmetrically giving two similar progenitor daughter cells (nestin +). These observations were confirmed in two different experimental designs; firstly, we found that VIP enhanced nestin expression in the cycling / proliferating cell population. Secondly we found that VIP reduced the number of nestin positive that were NUMB negative (as a measure of asymmetric cell division (Shen et al., 2002)) significantly compared to control. These results are in agreement with other observations in which they have shown trophic factors to mediate self-renewal of precursor cells. For instance, FGF-2 has been shown to enhance the survival and self-renewal of telencephalon precursor cells in rats via the activation of FGFR1 and/or FGFR3 (Maric et al., 2007). Similarly, a novel secretory molecule; stem cell-derived neural stem/progenitor cell supporting factor (SDNSF) (secreted by hippocampal neural stem cells), has been shown recently to enhance the survival of the adult hippocampal progenitor cells and potentiates their self-renewal (Toda et al., 2003). These observations with other trophic factors while it mirrored VIP effects on postnatal hippocampal progenitors cells, the exact mechanism through which trophic factor enhance cell self-renewal without altering cell proliferation is yet to be elucidated.

Therefore, VIP is a trophic factor that enhances the self-renewal of the transient amplifying cell population. Taking this finding together with our *in vivo* findings in VPAC2 -/- mice and elevated levels of VIP in the postnatal hippocampus strongly suggests its implication in the maturation and development of the GCL postnatally. The trophic activity of VIP on hippocampal stem cells via the VPAC2 receptor, in parallel with its neuronal fate-determining effect via the VPAC1 receptor, makes VIP an important modulator of postnatal hippocampal neurogenesis.

7.4 VIP effects are specifically mediated via VPAC2

VIP exerts its biological effects at nanomolar concentrations via the high affinity receptors: VPAC1 and VPAC2 (Moody et al., 2003). We have shown the expression of the VPAC1 and VPAC2 mRNAs in hippocampal progenitor cell culture. We also demonstrated the expression of these two receptors by the nestin putative/ stem cells *in vitro*. Our findings clearly show reduced cell survival of hippocampal newly born cells and a smaller area of GCL within the dentate gyrus of VPAC2 receptor knockout

mice compared to controls. This finding seems quite relevant if taken together with the fact that more than half of GCL is formed postnatally (Namba et al., 2005). Indeed, VIP through VPAC2 appears to be an important regulator of cell survival in the rat postnatal and the adult mice hippocampus. In primary cell cultures of postnatal hippocampus we have demonstrated that a specific VPAC1 receptor agonist is unable to mimic the trophic effects of VIP. However, both the trophic and self-renewal effects of VIP are abolished in the presence of a VPAC1/VPAC2 antagonist. Furthermore, the addition of VPAC1 receptor specific antagonist to VIP treated cells has not influenced VIP effects on cell survival or fate-determination. Consistently, our *in vitro* and *in vivo* data suggests that VPAC2 is an important modulator of VIP effects on postnatal and adult hippocampal stem/precursor cells.

These findings are supported by many *in vivo* and *in vitro* studies: VPAC2 is expressed in neuronal tissue, including olfactory bulb and hippocampus, from the embryonic to the postnatal stages and through to adulthood, suggesting a key role in neurodevelopment (Loren et al., 1979; Vaudry et al., 2000; Joo et al., 2004). In the adult mice, VPAC2 is expressed in all the subfields of the hippocampus, including the dentate gyrus (Loren et al., 1979; Joo et al., 2004). VPAC2 mRNA expression has been found to be markedly elevated in embryonic stem cell culture at the time these cultures were enriched for nestin cells (Hirose et al., 2005). This finding may suggest important roles of VPAC2 in the modulation of nestin stem cells during development. These observations suggest that VPAC2 is an important modulator of VIP effects on neuronal development during embryonic and adulthood periods. Our finding that VPAC2 is expressed in the postnatal GCL of the DG in parallel with our *in vitro* observations, indicate that VPAC2 is a mediator of VIP trophic effects on postnatal hippocampal neurogenesis as well.

This is in agreement with other studies on postnatal rats (P5), in which VIP, selectively through VPAC2 receptor subtype, has been shown to induce neuroprotection against the excitotoxic drug; ibotenate, a glutamate agonist that is known to alter hippocampal neurogenesis, and cause white matter lesions mimicking human periventricular leukomalacia (Rangon et al., 2005). In addition, the VPAC2 receptor subtype has been shown to mediate VIP-induced changes in ADNP expression in astrocytes (Zusev and Gozes, 2004). ADNP knockout mice (which die

at E8.5) have been shown to have no expression of transcriptional factors that are associated neuronal differentiation, such as Pax6 (Pinhasov et al., 2003). This is in keeping with our preliminary finding (Chapter 6) that VIP downregulates ADNP by 10-fold in postnatal hippocampal cultures, since a shift towards self renewal of TUJ1 negative nestin cells should be accompanied by a down regulation in neuronal differentiation and hence ADNP expression. The role of ADNP in the VIP mediated effects on stem/precursor cells is however likely to be complex and worthy of further study.

While VPAC1 receptor has no role in mediating VIP trophic effects or nestin cell self-renewal, it appeared to modulate neuronal differentiation. This finding may be relevant to experimental works showing that a decline in the VPAC1 receptor expression in the rat hippocampus with age is associated with deficits in learning and memory (Joo et al., 2005). The fact that VIP did not enhance neuronal cell differentiation whilst VPAC1 selective agonist did, may indicate that VPAC2 activation interacts with VPAC1 to down regulate its differentiation effects. This may also be related to the different intracellular pathways which may have been triggered by the activation of the two receptors by VIP.

In summary, VIP itself is expressed by the different subtypes of hippocampal interneurons (Jinno and Kosaka, 2003). Therefore, the VPAC1, VPAC2 receptor subtypes and their ligand appear to be well placed to mediate the survival and determine the fate of hippocampal nestin stem cells, both in the postnatal as well as in the adult hippocampus. While GABA has recently emerged as another candidate for excitation neurogenesis coupling (Ge et al., 2007b), nearly 40% of the VIPergic interneurons are GABAergic (Kosaka et al., 1985; Wang et al., 1997). Interestingly, VIP has been shown to enhance both pre- and postsynaptic GABAergic transmission to hippocampal interneurons leading to increased excitatory synaptic transmission (Cunha-Reis et al., 2004). This effect has been shown to be dependent on VPAC1 as well as VPAC2 receptors (Cunha-Reis et al., 2005). While these observations were suggested to implicate VIP and its high affinity receptors in the mediation of GABA enhanced potentiation and learning they may indicate important role for VPAC1 and VPAC2 in the neuronal modulation of hippocampal neurogenesis.

7.5 VIP affects dentate gyrus nestin-expressing cells

The subgranular zone is a well defined neurogenic area within the hippocampal formation. However, our whole hippocampal cell culture may have involved stem cells harvested from adjacent stem cells niches, such as the subventricular zone of the lateral ventricles and the subcallosal region. Therefore, we sought to generate region specific cultures from the dentate gyrus only, and the rest of the hippocampus which we have referred to as HSVZ cultures. Using real time-PCR, we demonstrated the expression of VPAC1 and VPAC2 mRNAs in both cell cultures. We observed that dentate cell cultures contain a lower proportion of nestin stem cell population and a higher proportion of neuronal precursor cells compared to HSVZ cultures in agreement with previous studies from our lab (Howell et al, 2007). Although nestin cells from the two distinct areas responded to VIP, a prominent proportional increase was only observed in dentate-specific cell cultures. Furthermore, a proportional increase in the subpopulation of nestin positive but TuJ1 negative cells was confined only to dentate cell cultures. VIP was equally trophic to the neuronal cell population in both cell culture systems with no effects on neuronal differentiation.

One explanation of these observations is that the VPAC2 is more abundant in the dentate than in the HSVZ. In fact, the distribution of VPAC2 receptor, as shown by situ hybridization, during the embryonic development of the rat brain, has been demonstrated to be higher in the nongerminal zones compared to the neuroepithelium (Basille et al., 2000). In contrast, the VPAC2 receptor subtype has been found to be extensively expressed in the dentate gyrus of the adult mice (Joo et al., 2004). Our real time-PCR results indicate that VPAC2 mRNA is equally expressed in the postnatal dentate and HSVZ cell cultures. At the cellular level, VPAC2 mRNA is expressed by the neuronal and glial cells in the adult mice dentate gyrus (Joo et al., 2004); our immunohistochemistry results indicate that VPAC2 is exclusively expressed by all the nestin-expressing cells. Therefore, the receptor distribution does not appear to be conclusive.

The other explanation is related to the responsiveness of nestin positive cells to VIP. In fact, while the nestin-positive-neural stem cells in the SVZ comprise both the quiescent and the actively dividing transient amplifying cell populations, the proportion of nestin cells has been found to decrease with time in sphere cultures; an

effect that has been attributed to the emergence of a slower cycling (amplifying) cell population (Ma et al., 2006a). Moreover, the nestin-GFP-expressing cells that are isolated from the CA1, unlike those of the dentate gyrus, have been shown to lack the self-renewal capacity (Kronenberg et al., 2007). Taken together, these observations may explain why the dentate nestin positive amplifying cell population, but not the HSVZ cells (which largely comprise CA1 and the adjacent SVZ precursor cells) responded to VIP. Likewise, specific responses of neural precursor cells have been reported in literature. For instance, in the mice telencephalic germinal zone, FGF-responsive (but not EGF) neural stem cells are present as early as E8.5 in the anterior neural plate, whereas, the EGF-responsive neural stem cells emerged by E15.5 (Tropepe et al., 1999). Therefore, this specific response of dentate amplifying neural stem cell population to VIP may strongly implicate this neuropeptide in the development of the GCL postnatally.

7.6 VIP interacts with NPY and FGF-2 to modulate hippocampal neurogenesis

Neuropeptide Y (NPY) is a neuropeptide that is expressed in the hippocampus throughout development and adulthood (Hokfelt et al., 1999). The levels of both VIP and NPY in the rat hippocampus are high during the first two postnatal weeks (Lopez-Tellez et al., 2004). Indeed, there levels continue to increase during the first postnatal week hitting a peak at P10; these levels stay high till P20 as they drop to adulthood levels (Lopez-Tellez et al., 2004). Interestingly, these first two postnatal weeks are the key period when the GCL of the dentate is largely formed (Namba et al., 2007) with marked progenitor cell proliferation, differentiation and migration (Namba et al., 2005). At the other end of the age spectrum, neurogenesis in the aging brain is dramatically reduced (Kuhn et al., 1996). Moreover, both VIP and NPY containing interneurons markedly decrease in the aging dentate gyrus and hippocampus (Cha et al., 1997). It is interesting to speculate whether this drop in their level is causally related to the decline in hippocampal neurogenesis.

In the mice hippocampus, while 31% and 8% of GABAergic neurons contain NPY-, and VIP-positive neurons, the two peptides were never found co-localized within the same neuron (Jinno and Kosaka, 2003). This neurochemical segregation may suggest differential secretion under different stimuli. Therefore, it is relevant to study how these two peptides interact in the modulation of hippocampal neurogenesis.

In our lab, NPY has been shown to enhance the proliferation of nestin-expressing cells and neuroblasts, but not GFAP cells (Howell et al., 2003). Herein, we have shown that VIP enhances the survival and generation of nestin-expressing cell with a pure trophic effect on the neuronal progeny cells. Using hippocampal progenitor cell cultures, we have shown that the addition of VIP to NPY-treated cells abolishes the proliferative effect of NPY with no effect on the survival or self-renewal effect of VIP on nestin-expressing cells. However, as pointed in chapter one, progenitor/ precursor cells in the dentate gyrus stem cell niche exist in at least three overlapping cell subpopulations (i.e nestin+GFAP+, nestin+GFAP-, and nestin+TuJ1+). The fact that VIP trophic and self-renewal effect was not affected by NPY co-treatment may indicate that, within the cascade of neural stem cell development, the VIP-response is dominant over that of NPY.

Whilst NPY is proliferative to a wide range of cell types, it has been shown to inhibit cAMP production in many of these systems, including bovine chromaffin cells (Zheng et al., 2000) melanotrope cells (Scheenen et al., 1995), prostate cancer cells (Ruscica et al., 2006) and rat left ventricular cells(Raimondi et al., 2002). In contrast, while VIP survival effects are cAMP-independent (Muller et al., 1995), it has been shown at nanomolar concentration to enhance the production of cAMP in cultured neurons (Pincus et al., 1990a; Nowak et al., 2007). In this regard, in many cell culture systems including, neocortical precursor cells, vascular smooth muscle cells and keratinocytes, elevated cAMP has been shown to decrease cell proliferation (Indolfi et al., 2000; Takahashi et al., 2004; Meyer, 2006). This could have relevance to the fact that the addition of VIP abolished NPY proliferative effects.

Interestingly, while VIP on its own is trophic and fate-determining (**Chapter 3**) the combined treatment of VIP and the neuroproliferative peptide; NPY, resulted in enhanced neurogenesis *in vitro*. These findings are in accordance with observations of Colbert, showing that VIP stimulates neuropeptide Y gene expression and causes neuronal maturation manifested by neurite extension PC12 cell lines (Colbert et al., 1994). These observations are also relevant to studies on the expression of NPY and VIP in the sympathetic ganglion, suggesting that VIP and NPY expression regulates neuronal development (Jinno and Kosaka, 2003). Indeed, VIP in the embryonic

sympathetic ganglia peaked at the time of neuronal precursor proliferation, then declined exactly when NPY expression started to increase; this was accompanied by neuronal survival rather than proliferation (Jinno and Kosaka, 2003). It is worth noticing that in contrast to the postnatal hippocampus, VIP and NPY have proliferative and trophic effects; respectively, in the embryonic sympathetic ganglion. This may reflect the diversity of the effects of these neuropeptides through out development and in the different parts of the CNS.

The other possibility could be that VIP may downregulate the expression of the Y1 receptor subtype, which mediates the proliferative effect of NPY in postnatal hippocampal cultures (Howell et al., 2003). We have shown that VPAC1 stimulation increases neuronal differentiation and so another possibility is that NPY increases VPAC1 receptor signaling. Neither of these possibilities is exclusive and it may well be that such a bidirectional interaction underlies the observed effects. Clearly this will be another area for future research.

FGF-2 and its receptors belong to a family of ligand and receptors that includes at least 23 different FGFs and FGF receptors (FGFR) (Maric et al., 2007). FGF-2 is able to interact with at least FGFR1, FGFR2 and FGFR3 (Maric et al., 2007). It has been shown that, exogenous bFGF stimulates neuronal proliferation in the hippocampus and neuronal generation (Tao et al., 1996; Cheng et al., 2002).While FGF-2 is a mitogen, it has also been demonstrated to have trophic effects (Ohgoh et al., 1998) and to interact with other trophic factors, like ADNF, to interrupt excitotoxic neurodegenerative cascades in the hippocampus (Guo et al., 1999). These findings suggest that FGF-2 is an important factor in the regulation of hippocampal cell proliferation and neurogenesis. In our project we investigated the interactions of VIP and NPY in postnatal hippocampal cell cultures, in an attempt to understand the interrelationship between proliferation and survival of progenitor cells in maintaining basal hippocampal neurogenesis. In terms of cell proliferation, VIP and FGF-2 combined treatment resulted in reduced cell proliferation, but enhanced the generation of nestin-expressing cells. In an elegant study, it has been shown that FGF-2 mitotic activity and self-renewal effect are mediated by FGFR1 receptor subtype activation and the FGFR1 and FGFR3 coactivation; respectively (Maric et al., 2007). Therefore, we studied the expression of FGFR1 mRNA under VIP treatment, using real-time

PCR. Our results indicate that VIP down regulates the expression of FGFR1 mRNA by 50%. This may explain the mechanism of decrease in cell proliferation. Interestingly, the combination of VIP and FGF2 further enhanced the generation of nestin positive cells compared to either factor alone. This suggests that the combination of these factors further augments the self renewal of nestin positive cells; may be through FGFR3, as suggested by (Maric et al., 2007). Whether VIP affects FGFR3 signaling remains to be investigated as do the intracellular signaling pathways subserving symmetrical division by each factor. These results may further suggest that VIP and FGF-2 support the survival and probably the self-renewal of the same subpopulation of nestin positive cells.

Similar interactions between FGF-2 and other factors have been reported. For instance, FGF-2 combined with PACAP has been shown to convert the proliferative effects of PACAP on hindbrain precursor cells to inhibition (Lelievre et al., 2002). This effect was demonstrated to be mediated by FGF-2 switching off the PACAP signals to induce cell proliferation through phosphate kinase-A (PK-A) pathway (Lelievre et al., 2002). Neurotrophin-3 (NT-3) is another trophic factor that has been shown to inhibit the proliferative effect of FGF-2 on embryonic cortical precursor cells (Jin et al., 2005). This inhibition effect has been found to be mediated by NT-3 inhibiting FGF-2-induced cell proliferation through the phosphorylation of Akt and glycogen synthase kinas 3ß, a downstream kinase of Akt (which is involved in cell proliferation) (Jin et al., 2005). Collectively, our results from the interactions experiments, in agreement with literature, show how such ligand/ receptor systems might change the action of each others on different subpopulations of cells in the postnatal hippocampus.

7.7 VIP: a regulator postnatal hippocampal neurogenesis through adulthood

VIP is a major factor in neuroendocrine functions, cardiac activity, respiration, digestion, sexual potency and importantly brain activity (Gozes and Brenneman, 1989). VIP effects are universal and involve neuronal development during the embryonic period and through adulthood.

During embryonic development, VIP by influencing the development of the neural tube and the precursors of the cerebral cortex (DiCicco-Bloom, 1996), represents a

new member of broadly active neurogenesis-regulator. Indeed, defects in the VIP-ADNP regulation system in mice has been shown to cause failure of the neural tube closure and death by E8.5-9 (Pinhasov et al., 2003). In another study VIP has been shown to upregulate NGF expression in the neural tube, which is an important factor for neural tube development (Hill et al., 2002). In these methods, the effect of VIP is mainly on cell survival rather than cell proliferation. However, it has also been shown that VIP has proliferative activity on neural precursor cells during mice embryonic development as it increases the rates of neural cell proliferation by shortening the G1 and S phases of the cell cycle (Gressens et al., 1998).

While these observations, taken together, suggest that VIP modulates the proliferation and the survival of neuronal precursor cells during CNS embryonic development, it has been shown that blockade of VIP during neonatal development induces neuronal damage in the form of severe microcephaly (Hill et al., 1994b). In addition, VIP levels peak in the rat hippocampus on postnatal day 10 (Lopez-Tellez et al., 2004). During this period of time, more that 50% of the GCL of the DG is formed (Namba et al., 2005). Despite of this considerable amount of literature, which suggests possible effects of VIP in the modulation of neurogenesis, there were no studies on VIP roles in the modulation of hippocampal neurogenesis, particularly, during the postnatal period.

We have shown that both VPAC1 and VPAC2 receptors are expressed in the granule cells layer of the postnatal rat DG. *In vitro,* we have also demonstrated the expression of these receptors by the putative/ precursor cells generated from the postnatal hippocampus. This dense expression of VIP receptors along with, the findings of increased levels of VIP during the postnatal period strongly supports our hypothesis that VIP is a modulator of postnatal hippocampal neurogenesis. This hypothesis is further supported by our findings, which demonstrate that VIP enhances the survival of putative/ precursor cells, as well as neuronal and astrocytic progeny cells and the self-renewal of the amplifying cell population. This particular cell population comprises the actively dividing cells that give rise to neuronal progeny cells (Encinas et al., 2006), which are then added to the granule cell layer at this particular period of time (Namba et al., 2005). In fact, these findings strongly support the role of VIP under physiological conditions in the modulation of postnatal hippocampal

neurogenesis and GCL development. This period of development is very important for the understanding of how neurogenesis continues in the hippocampus from embryonic life throughout adulthood (Namba et al., 2007). Therefore, trophic support for neuronal precursor cells is of major importance during this period as death of new cells in the granule cell layer occurred at a steady rate between 6 and 28 postnatally, resulting in loss of 50% of cells over this 22-day period (Dayer et al., 2003). Importantly, new granule cells that survive this first month live for at least 5 additional months (Dayer et al., 2003). These observations reinforce the importance of survival factors, like VIP, during the postnatal period as this will affect the net neurogenesis in adulthood. These findings are supported by our observation in the adult VPAC2 knockout mice. In these young adult mice, we have shown a significant decrease in the survival of newly born cells in the DG and a reduction in the area of the GCL. These observations can be explained at least, in part, by our *in vitro* findings; which have demonstrated that VPAC2 mediates the survival and self-renewal of hippocampal progenitor cells in the postnatal hippocampus. We conclude that VIP is an important factor for the survival and self-renewal of hippocampal precursor cells during the postnatal period, which may modulate alterations in hippocampal neurogenesis accompanied by physiological states, including learning and memory.

Experimental treatments of adult rats have shown that blockade of VIP causes impairment of performance and retardation of spatial discrimination, and reduction in performance on behavioral tests related to learning and memory, suggesting impairment of memory and cognition (Glowa et al., 1992; Wu et al., 1997; Shors et al., 2001). Consistently, transgenic mice harboring a chimeric VIP gene (leading to 20% VIP reduction) displayed learning impairment and prolonged retardation in memory acquisition (Gozes et al., 1993). These observations are relevant to hippocampal neurogenesis as it is essential for certain forms of hippocampal dependant learning and memory providing a plausable mechanism for at least some aspects of VIPs involvement in hippocampal memory function (Kempermann et al., 1997; Shors et al., 2001). In addition, learning has been shown to increase the number of new neurons in the hippocampus by altering cell survival or cell proliferation (Gould and Gross, 2002). In fact, it has recently been emerged that neuronal survival with or without proliferation is the key regulator of learning-dependent neurogenesis

(Prickaerts et al., 2004). Furthermore, newly generated neurons in the adult have been shown not only to be affected by the formation of a hippocampal-dependent memory, but also to participate in it (Shors et al., 2001). Therefore, while the exact mechanisms controlling learning and memory-associated hippocampal neurogenesis are not clear, our observations that demonstrated VIP as a trophic factor that modulate the survival of neuronal progenitor cells and their progeny under physiological levels provides a better understanding of these control mechanisms. To further elucidate these mechanisms, we believe that future work should focus on stu changes in cognition and memory in the VPAC2 knockout adult mice.

The other physiological / environmental factor that is been shown to interfere with hippocampal neurogenesis is the enriched environment, of which; the most important component is voluntary exercise. Voluntary exercise including running enhances the survival and differentiation of newly born neuronal cells in the adult mouse dentate gyrus (van Praag et al., 1999). While enriching environments with physical, social and sensory stimuli are now established to be beneficial to brain development and ageing, neurotrophic factors, which are essential for neural development and survival, are likely to be involved in the consequences modified by enriched experiences (Pham et al., 2002). Indeed, voluntary exercise induces the expression of many neurotrophic factor in the hippocampus, including, BDNF, NT-3, NGF and VIP (Eilam et al., 1999; Olson et al., 2006). In addition, BDNF as a neurotrophic factor has been shown to enhance VIP gene expression in rat cerebrocortical cultures(Villuendas et al., 2001). VIP has also been to interact with NGF and NT-3 to enhance neuroblast proliferation and differentiation, respectively in embryonic hippocampal cell cultures (Blondel et al., 2000). To sum up, while neuronal survival is the hall-mark of exercise-induced hippocampal neurogenesis, neurotrophic factors are the key regulators of the process. Importantly, VIP is not only upregulated shortly after exercise (Eilam et al., 1999), but it also appears to be a central trophic factor that interacts with other trophic factors to modulate hippocampal neurogenesis. Taken together, these observations, with our findings, support our hypothesis that VIP is a trophic factor that modulates hippocampal neurogenesis under physiological conditions.

7.8 VIP and pathological neurogenesis:

VIP appears to be involved not only with physiological states modulating hippocampal neurogenesis, but also under disease states like epilepsy and depression. Indeed, patients with seizure disorders, have elevated levels of VIP both in the serum and the CSF (Ko et al., 1991). In autopsy on hippocampi from patients with chronic temporal lobe epilepsy, elevated levels of VIP high affinity receptors have been found in the hippocampus, especially in the dentate gyrus molecular cell layer (de Lanerolle et al., 1995). Interestingly, the SGZ in hippocampi resected from patients with chronic temporal lobe epilepsy have normal levels of proliferation, but reduced levels of neurogenesis (Shetty and Hattiangady, 2007). Given that VIP promotes the maintenance of precursor cells in a more primitive nestin positive state; could the increased levels of VIP receptors and signaling in chronic epilepsy be relevant to this chronically altered neurogenesis? In addition to reduced levels of neurogenesis there are also chronic alterations in the quality of neurogenesis with several lines of evidence implicate newly generated neurons in structural and functional network abnormalities in the epileptic hippocampal formation of adult rodents (Parent et al., 2002). These abnormalities include aberrant mossy fiber reorganization, persistence of immature DG structure (e.g. basal dendrites), and the abnormal migration of newborn neurons to ectopic sites in the dentate gyrus (Parent et al., 1998; Scharfman and Gray, 2006). Given VIPs role in neural survival and maturation it may well be involved in these processes in chronic epilepsy as well.

In the more acute situation, studies in the adult rodent dentate gyrus, indicate that single brief or prolonged seizures, as well as repeated kindled seizures, transiently increase dentate granule cell (DGC) neurogenesis (Parent, 2002). Given that there are increased levels of VIP expression in the hippocampus acutely after Kainate induced seizures (Marksteiner et al., 1989), could VIP play a role in the modulation of seizure-associated hippocampal cell proliferation, since we have found that VIP is proliferative to hippocampal progenitor cells and their neuronal progeny at higher levels (1μM)?

Chronic treatment with antidepressants increases adult hippocampal neurogenesis which may be required for some behavioral effects of antidepressants (Encinas et al., 2006). In this regard, the selective serotonin reuptake inhibitor antidepressant fluoxetine has been shown not to affect division of stem-like cells in the dentate gyrus

but increases symmetric divisions of an amplifying cell population (nestin+GFAP- cells) (Encinas et al., 2006). It is exactly this cell population that we demonstrate VIP to enhance self-renewal through symmetric cell division. This finding may implicate VIP in the mediation of antidepressant-associated enhanced hippocampal neurogenesis and therefore in the modulation of the behavioral changes that antidepressants modulate as well. These finding are also supported by the observations of other studies which have shown that while VIP increases the number of specific high affinity receptors for 5-HT in the rate hippocampus (Rostene et al., 1983); an effect which has been shown in other studies to enhance cell proliferation in the dentate gyrus (Brezun and Daszuta, 2000). VIP receptors may therefore be an attractive target for antidepressant drug design.

7.9 Do interneuron-secreted neuropeptides act as a novel control system of hippocampal neurogenesis?

In mammalians CNS, the hippocampus is classically characterized as the gateway to information flow. In this gateway structure, information must pass through pathways from the entorhinal cortex and other regions projecting to granule cells of the dentate gyrus. In fact, the hippocampal formation forms a trisynaptic network composed of: multimodal inputs which are transferred from neocortical regions to the DG via the entorhinal cortex; information is then transferred from the DG to CA3 through the mossy fibers, onward through the Schaffer collaterals to CA1 (and the subiculum) and then via the entorhinal cortex back out to the cortical associative areas (Kempermann, 2002). Therefore, the DG acts as a high pass pathway filter. Adult hippocampal neurogenesis occurs at exactly the narrowest spot within the three-synaptic circuits (Kempermann, 2002). New granule cells are generated, and mossy fibers are the axons of the granule cells. VIP neurons (VIPergic) in the hippocampal formation are exclusively interneurons, sparsely scattered throughout both Ammon's horn and the dentate gyrus, sending their axons, to the nearby pyramidal or granule cells. In fact, these VIPergic neuron axonal terminals course through all the layers of the hippocampus (Leranth et al., 1984). The morphology of VIPergic neurons is diverse and they receive inputs from the GABAergic hippocampal neurons (Acsady et al., 1996). One-third to one-half of the VIPergic neurons also contains the neurotransmitter γ-aminobutyric acid (GABA). In fact, 40% 0f VIPergic neurons in

the hippocampus contain the GABA-synthesizing enzyme glutamate decarboxylase (GAD), and therefore GABAergic (Kosaka et al., 1985). Not surprisingly, the activation of pre-synaptic VIP high affinity receptors enhances the GABAergic synaptic transmission in cultured hippocampal neurons as shown by patch-clamp techniques (Wang et al., 1997). More interestingly, this study revealed that VIP increased GABA release by directly stimulating the vesicular release apparatus. In addition, VIP has been shown to enhance pre-synaptic as well as postsynaptic release of GABA in the hippocampus (Cunha-Reis et al., 2004). GABA-ergic neurons on the other hand have been suggested as a candidate for excitation-neurogenesis coupling (Ge et al., 2007b), as progenitors cells of the dentate gurus have been shown to receive GABA-ergic inputs and to respond by differentiating into neuronal cells under non-synaptic GABA stimulation (Liu et al., 2005).

Interneurons and fibers participating in these complex links express / secrete a wide range of neuropeptides, including VIP, NPY and galanin (Skofitsch and Jacobowitz, 1986; Jinno and Kosaka, 2003). The levels of these peptides alter in response to different activities in the CNS (Marksteiner et al., 1989; Stenfors et al., 1989). Furthermore, the receptors for these neuropeptides are expressed in the different subfields of the hippocampus at variable levels throughout development (Kohler et al., 1986a; Kohler et al., 1986b; Hill et al., 1994a; Mazarati et al., 1998). The presence of VIP along with NPY and galanin appear to be ideal to the demand of information throughout, and to regulate the process of hippocampal neurogenesis accordingly. Indeed, VIP-ergic interneurons listen to the input from entorhinal cortex projections and the output from granule cells and respond by the release their neuropeptides in the SGZ. Different patterns of activity result in different patterns of neuropeptide release. More NPY increases proliferation, more VIP increases the number of transiently amplifying cells and therefore net neurogenesis as well as maintaining the progenitor pool is dependent on the balance between the levels of these neuropeptides. In fact, these neuropeptides may act as a novel control system of postnatal hippocampal neurogenesis.

7.10 VIP release from immune cells: neuroimmunomodulation of the stem cell niche

While VIP is released by interneurons in the hippocampus, they have also been shown to be secreted by immune cells, including CD4 and CD8 T-lymphocytes, B-lymphocytes and mast cells (Delgado et al., 2004a). In fact, the controlled activity of T-lymphocytes has been shown to be needed for the postinjury neuronal survival (Moalem et al., 1999). Furthermore, such T-cells, once in the site of injury and locally activated, they shape the behavior of microglia in a way that makes them supportive for neuronal survival and renewal (Shaked et al., 2005; Butovsky et al., 2006). Not only that, but T-cells, as part of the innate immunity in the healthy individual, have also been shown, in association with microglial activation, to mediate hippocampal neurogenesis induced by an enriched environment (Ziv et al., 2006). In addition, the immune deficient mice have been shown to have markedly impaired hippocampal neurogenesis that could also not be induced by enriched environment (Ziv et al., 2006). While these observations implicate immune cells, particularly T-cells and microglia in hippocampal neurogenesis, VIP is not only secreted by these immune cells, but also appears to modulate their function and response and therefore may be their involvement in hippocampal neurogenesis. Furthermore, T-cells and microglia have been shown to express variably the VIP receptors; T-cells express VPAC1 and VPAC2 receptors, but not PAC1 whereas microglial cells express VPAC1 and PAC1 receptors but not VPAC2 receptor subtype (Delgado et al., 2004a). With reference to our pharmacological results which implicate VPAC2 in the VIP effects on hippocampal neurogenesis; therefore VIP modulation of hippocampal neurogenesis may be through T-cells rather than microglia.

While T-cells and microglia appear to support hippocampal neurogenesis in the healthy individual, microglial cells have been shown to be detrimental to the newly born neuronal cells in neuro-inflammatory diseases (Ekdahl et al., 2003). In fact, most of these effects were attributed to the microglia secreting proinflammatory cytokines such as, interleukin-6 (IL-6), tissue necrosis factor and interleukin-10 (IL-10) (Ekdahl et al., 2003; Butovsky et al., 2006). In this regard, VIP has been shown to play anti-inflammatory effect by down regulating the release of Il-6, Il-10 and TNF (Bik et al., 2004). Therefore, VIP may also be important in the immunomodulation of

hippocampal neurogenesis under pathological states, but this time via VPAC1 or PAC1 which we have shown to modulate VIP effects at elevated levels.

7.11 ADNP: a glial mediator of VIP effects

Recent studies have demonstrated that astrocytes express variety of neurotransmitter receptors and can modulate the activity of neurons. In fact, the existence of bidirectional interactions between neurons and perineuronal astrocytes has been clearly demonstrated (Yamazaki et al., 2005). Since a single astrocyte makes tight contacts with many neighboring neuronal cells, they can provide efficient and wide modulation of neuronal networks. This makes VIP particularly important as we have demonstrated in agreement with other studies the expression of VIP receptors by astrocytes (Masmoudi-Kouki et al., 2007). Indeed, astrocytes in many biological systems have been suggested to modulate the VIP survival effect during embryonic neurogenesis (Brenneman et al., 1987; Masmoudi-Kouki et al., 2007). More precisely, these VIP-astrocyte- mediated effects have been, in the majority of studies, attributed to activity dependent neuroprotective protein (ADNP); as a VIP responsive gene (See (Gozes and Brenneman, 2000) for a review). Activity-dependent neuroprotective protein (ADNP) was first isolated from a cDNA library from P19, a neuroglial differentiated carcinoma cell line and then from a mouse fetal brain cDNA library (Zamostiano et al., 2001). ADNP is an 828-residues protein that has been shown to mediate the neuroprotective effects of VIP in many culture systems (Brenneman et al., 2000). For instance, ADNP has been shown to be a VIP-responsive gene in astrocytes derived from the cerebral cortex of newborn rats (Zusev and Gozes, 2004). In hippocampal cell cultures, we have shown that ADNP is abundantly expressed by the majority / all of cells *in vitro*. While ADNP at the cellular level was demonstrated to be expressed by astrocytic and neuronal like cells in the rat brain, there were no reports on its expression by nestin progenitor cells. Herein, we have shown that ADNP is expressed by all nestin positive cells in the postnatal hippocampal cell culture.

Using real-time PCR, we have shown that VIP at physiological concentrations regulates the ADNP mRNA *in vitro*. This regulation appears to be somewhat complex as our preliminary data shows both an upregulation of ADNP using semi quantitative PCR techniques and a down regulation at 1 and 8 hours but no change at 4 hours

using quantative PCR. These findings are in agreement with findings of others work which has shown that the changes induced by VIP on ADNP expression were developmentally dependent. Indeed, VIP upregulates ADNP expression of P4 astrocytic cultures, but downregulated its expression in P8 cultures. These observations are in accordance with our findings as our cultures are P7-P10 in age.

This suggests that VIP effect on ADNP is mediated via the high affinity receptors; VPAC1 and VPAC2. In agreement with these findings, using VIP agonists selectively-specific for the VPAC1 and the VPAC2 receptors, it has been demonstrated that VIP induced changes in ADNP expression in astrocytes via the VPAC2 receptor (Zusev and Gozes, 2004). Our real-time PCR data further indicate that VIP downregulated the expression of ADNP in postnatal hippocampal cell culture by 10 times at 1 and 8 hrs. While VIP has been shown upregulate ADNP expression in whole mice cultures, the knocking down of ADNP has been shown to b associated with down regulation of transcriptional factor that are associated with neuronal differentiation (Pinhasov et al., 2003). This is in keeping with our preliminary finding (**Chapter 6**) that VIP downregulates ADNP by 10-fold in postnatal hippocampal cultures, since a shift towards self renewal of TUJ1 negative nestin cells should be accompanied by a down regulation in neuronal differentiation and hence ADNP expression. The role of ADNP in the VIP mediated effects on stem/precursor cells is however likely to be complex and worthy of further study.

7.12 Conclusions

VIP is a trophic factor for nestin-expressing stem/ progenitor cells and their progeny in the subgranular zone of the dentate gyrus, specifically via the VPAC2 receptor. It also instructs neuronal differentiation via the VPAC1 receptor. VIP survival effects are both in postnatal hippocampal cultures in vitro and in young adult tissue *in vivo*. Additionally, VIP potentiates the self-renewal of the nesting positive amplifying stem cell population in the dentate. Our work identifies VIP as a key regulator of stem cell renewal, and survival and uniquely identifies a neurotransmitter that couples neuronal activity with the control of stem cell fate and trophism. We also show that VIP interacts with other growth factors to modulate hippocampal neurogenesis. We believe that these neuropeptides form a novel control system for the proliferation of

hippocampal stem cells and the survival of their progeny depending on their differential release from their corresponding interneurons.

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