UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Investigation into the Effects of Neuropeptides on Epileptiform Activity in the Hippocampus *in vitro*

by

Christopher Harper

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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Temporal Lobe Epilepsy (TLE) is one form of epilepsy where drug resistance presents a significant clinical problem. Due to this new drug treatments and targets could prove worthwhile. Neuropeptides have received increasing interest as endogenous anti-convulsants; because of this their receptors could be valuable targets for future drug intervention. One such compound is Nociceptin (Noc), which is the endogenous ligand for the OP4 receptor. The hippocampus is a useful model for the investigation of TLE as this is one of the regions frequently involved in the propagation of epileptiform activity associated with this condition.

I used Electrophysiological recording techniques to investigate the effects of Noc in various models of epileptiform activity, in acute hippocampal slices taken from adult rats. I subsequently looked at the effects of Noc in slices taken from an epileptic (EL) mouse model and in organotypic hippocampal slice cultures (OHSC's). In some models I compared these effects to the action of Neuropeptide Y (NPY) as this is a well studied anti-convulsant neuropeptide.

I found that Noc caused a blockade of the bursting activity seen in the 10μM Bicuculline (Bic) and 0 Mg^{++} models of epileptiform activity. I also found that Noc did not cause a change in the bursting activity seen in the High K^{+} (8.5mM) or 100μM 4-Aminopyridine (4-AP) models.
When I looked at the Noc effects on the Bic and 0 Mg++ models in EL mice I found that Noc showed a greater reduction in bursting activity when applied to slices taken from 'sensitised' mice as compared to 'non-sensitised'.
I found that Noc showed no effects on synaptic transmission or paired-pulse inhibition in OHSC's. The same result was seen with NPY. I showed through Western Blotting that the OP4, Y1, Y2 and Y5 receptors are present in OHSC's. I then showed that Noc caused a reduction in bursting activity in the Bic model in OHSC's and that no effect was seen when the experiment was repeated with NPY. I also experienced problems with the 4-AP model in OHSC's and subsequently showed that 4-AP causes neuronal death in the CA1 sub-field of OHSC's. Finally I showed that NPY caused a reduction in synaptic transmission and paired-pulse inhibition when administered to acute hippocampal slices.

This data shows that targets of the OP4 receptor pathway could prove a useful experimental tool, and potential future anticonvulsant treatment.

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Abbreviations

1^0	Primary
2^0	Secondary
4-AP	4-Aminopyridine
µl	Microlitre (10^{-6})
µM	MicroMolar (10^{-6})
°C	Degrees Celsius
AC	Adenyl Cyclase
aCSF	Artificial Cerebrospinal Fluid
AEDs	Anti Epileptic Drugs
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of Variance
Bic	Bicuculline
CA1	Cornu ammonis area 1
CA3	Cornu ammonis area 3
cAMP	Cyclic Adenosine MonoPhosphate
CNS	Central Nervous System
DG	Dentate Gyrus
EC	Entorhinal Cortex
EPSP	Excitatory Postsynaptic Potential
GABA	γ-Aminobutyric Acid
HBSS	Hank's balanced saline solution
HCN	Hyperpolarisation-activated cyclic-nucleotide gated
Hz	Hertz
I_H	Hyperpolarising cationic current
IPI	Inter Pulse Interval
IPSP	Inhibitory Postsynaptic Potential
KA	Kainic Acid (Kainate)
LM	Lacunosum Moleculare
MΩ	MegaOhms
mA	MilliAmp (10^{-3})
MEA	Multi Electrode Array
MEM	Minimum essential medium
min	Minute
ml	Millilitre (10^{-3})
mM	MilliMolar (10^{-3})
mRNA	Messenger RNA
ms	Millisecond (10^{-3})
mV	MilliVolt (10^{-3})
nM	NanoMolar (10^{-9})
NA	Noradrenaline
NMDA	N-Methyl D-Aspartate
Noc	Nociceptin
NPY	Neuropeptide Y
OHSC	Organotypic Hippocampal Slice Culture
PLC	Phospholipase C
PS	Population Spike
PTZ	Pentylenetetrazol

secs	Seconds
SOM	Somatostatin
StLac	Stratum Lacunosum
StOr	Stratum Oriens
StPyr	Stratum Pyramidale
StRad	Stratum Radiatum
S	Subiculum
TLE	Temporal Lobe Epilepsy

Chapter 1

Introduction

1.1 EPILEPSY

Epilepsy is defined by two or more unprovoked seizures, and is among one of the most prevalent neurological disorders seen in clinical medicine. The seizures result from dysfunction in the grey matter of the brain, and are not a disease in themselves. Seizures are caused by a number of factors (see Table 1.1), although the causes of most seizures are unknown. Seizures manifest as electrical discharges from one or more areas of the brain that result in anything from the briefest of lapses of attention to severe and prolonged convulsions.

Congenital	
	Maldevelopment
	Inborn metabolic errors
Perinatal	
	Immediate: hypoxemia, haemorrhage, trauma
	Latent: temporal lobe sclerosis
Metabolic	
	Hypocalcemia
	Hyponatraemia
	Hypoglycemia
Infections	
	Simple febrile convulsions
	Encephalitis
	Meningitis
Neoplastic	
	Primary
	Metastatic
Vascular	
	Arteriovenous malformation
	Postinfarction
	Posthaemorrhage
Trauma	
	Penetrating wounds
	Closed head injuries
Toxins	
	Drug abuse
	Withdrawal from alcohol and sedative drugs

Table 1.1 – Causes of Seizures. There are other unknown causes of seizures, but these are the main causes that have been identified. Taken from Neurobiology of Disease.

Epilepsy is one of the oldest conditions known to man, and a detailed account of various seizure types is found in records as old as 4000 years. Most of the early descriptions of epilepsy attribute it to the result of supernatural forces, although Hippocrates (around 500 BC) believed it was a disorder of the brain. However it

was not until the 18th and 19th Centuries that epilepsy was widely accepted as a brain disorder.

In 1873 Hughlings Jackson proposed that seizures were the result of electro-chemical discharges within the brain. Since then a large amount of work has been carried out to further the understanding of how and why epilepsy occurs. The incidence of Epilepsy worldwide has been estimated to affect approximately 8.2 people per 1000 of the general population (WHO, 2003). This is the number of people who have active epilepsy at any one time, although twice this number may have had epilepsy at some point in their lives.

1. Partial (focal, local) seizures

- **A. Simple partial seizures (consciousness is not impaired)**
- **B. Complex partial seizures (with impairment of consciousness)**
- **C. Partial seizures evolving to secondarily generalised seizures**

2. Generalised seizures (convulsive or non-convulsive)

- **A. Absence seizures**
- **B. Myoclonic seizures**
- **C. Clonic seizures**
- **D. Tonic seizures**
- **E. Tonic-clonic seizures**
- **F. Atonic seizures**

3. Unclassified epileptic seizures

These includes seizures that cannot be classified because of inadequate or incomplete data and some that defy classification in any of the above categories. This includes some neonatal seizures eg. rhythmic eye movements, chewing and swimming movements.

Table 1.2 – List of Seizure Types. This is a generalized list and most types can be expanded further, usually with reference to the parts of the brain affected. (Taken from the British Epilepsy Association website, and based on information from (Dreifuss, 1989))

The brain is responsible for a wide range of functions, and seizures usually present themselves as changes in the brain's normal activity. Therefore there could be a change in the level of consciousness of the patient, a change in their mood, or muscle spasms arising from a seizure, depending on where in the brain the epilepsy originates or occurs. There are two main categories of epilepsy (although there are over 30 different types of recognized seizure, See Table 1.2); Partial, where the seizure has focus in a particular area of the brain; and Generalized, where the seizure has no identifiable focal point. The Generalized type is evident from the general form of symptoms that occur. Epilepsies are also described as Simple or Complex, this refers to whether or not there is a change in the level of consciousness during the seizure. Seizures are described as Simple if the individual experiencing the seizure is conscious and aware of its occurrence. Complex conversely refers to a seizure whereby the individual loses consciousness and is not aware of its occurrence.

Within these four main groups: Simple Partial, Simple Generalized, Complex Partial and Complex Generalized; there are many different types and they are often named after how the symptoms present and also the location, if any, where the seizure arises.

The widely accepted cause of seizures is due to an imbalance between the inhibitory and excitatory systems (Moshe, 2000), within the Central Nervous System (CNS). Under physiological conditions a balance is maintained which allows the brain to function normally, but in the 'disease state' it is believed that the balance moves in favour of the excitatory system; and this in turn leads to an increase in excitation.

Of all the people that are affected with epilepsy the majority are able to have their symptoms treated with currently available Anti Epileptic Drugs (AEDs), although in about 20-30% of cases the epilepsy does not respond to drug treatment. In these cases, and as a last resort, surgical intervention may be necessary to improve the quality of life for the patient. At present most available treatments for epilepsy treat the symptoms of the disease but do not treat the underlying disease state.

Temporal Lobe Epilepsy is one of the more 'drug resistant' forms of epilepsy, this type of epilepsy having seizures that arise in the hippocampus and surrounding limbic area of the CNS.

As there are a large number of people who are unresponsive to currently available pharmacological treatments there is a necessity for new drug treatments that exploit alternative modes of action to that of the current treatments. The majority of the currently available treatments cause their effects by influencing neuronal transmission, i.e. suppressing the electrical activity of the brain either by inhibiting neuronal excitability or by enhancing the inhibitory circuitry. These effects are mainly on the GABA-ergic system or on the ionic channels of the neurons.

For the reasons stated above new treatments are required to attend the epilepsies that are currently unresponsive. As the majority of treatments use either GABA-mimetics or ionic channel blockers, there is good reason to believe that in order to treat the drug resistant forms of epilepsy new mechanisms need to be uncovered.

1.2 HIPPOCAMPUS

The Hippocampus is a specialized region of the limbic system. The hippocampal formation has been shown to be essential for the formation of long-term memories (Milner, 1972). The Hippocampus consists of two identical elongated C-shaped structures, one on either side of the brain located in the temporal lobe (See Figure 1.1). As the hippocampus is the region of the brain where Temporal Lobe Epilepsy (TLE) arises (see (Engel, Jr., 2001b) for review) it is an important tissue for the study of new mechanisms, and treatments, for this disease.

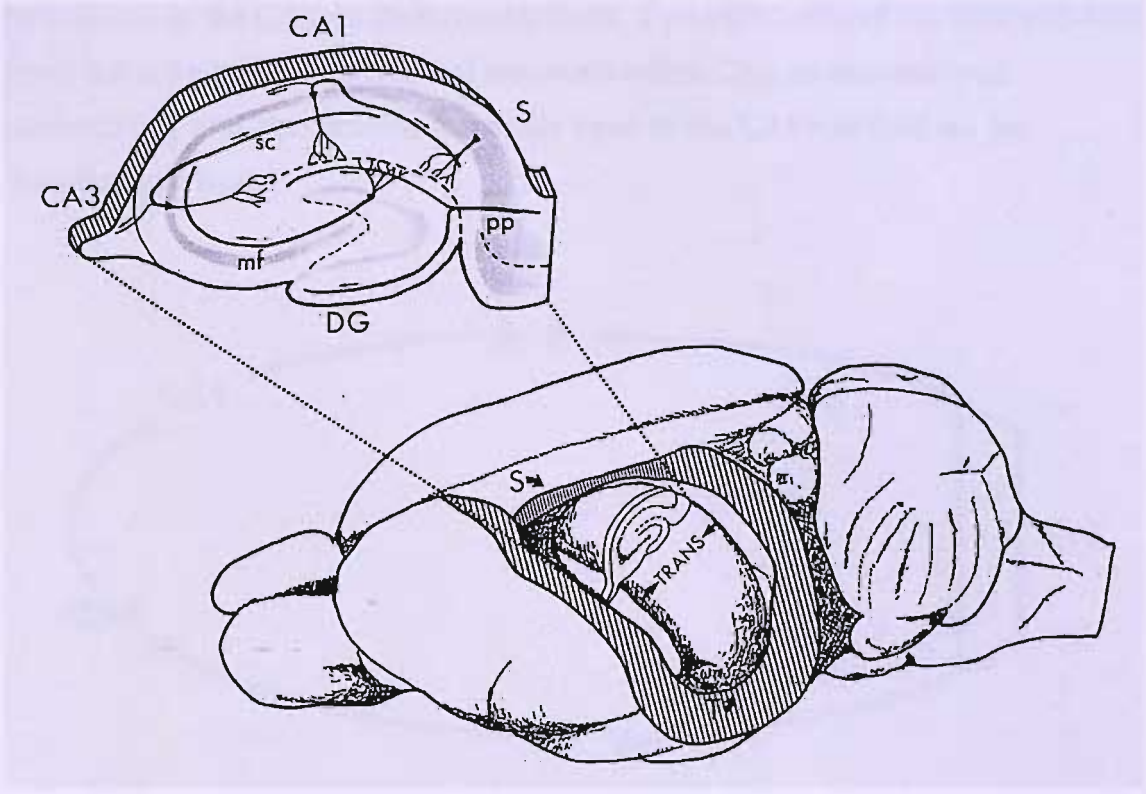


Figure 1.1 Picture of Rat brain from (Amaral and Witter, 1989).

1.2.1 Anatomy and Physiology of the Hippocampus

The Pyramidal cells of the hippocampus provide the major excitatory pathways of the hippocampus, and the interneurons (discussed further in section 1.2.3) provide the inhibitory connections that prevent the circuitry becoming hyper-excitable. The hippocampus receives signals via the entorhinal cortex, and then processes them via the unidirectional pathway illustrated in Figure 1.2.
The Hippocampus is composed of four cortical regions as illustrated in figure 1.2. These are the dentate gyrus, the hippocampus proper (which is sub-divided into the CA3, CA2 and CA1), the subicular complex (which is sub-divided into the subiculum, presubiculum and parasubiculum) and the entorhinal cortex (this is generally divided into medial and lateral subdivisions, particularly in the rat).
The fields of the hippocampus are linked by unique and largely unidirectional connections. The entorhinal cortex provides the major input for the dentate gyrus via the perforant pathway. The granule cells of the dentate gyrus send

projections to the CA3 via their mossy fibers. Pyramidal cells of the CA2 sub-field send out collateralized axons that terminate within CA3 as associational connections, and also provide the major input to the CA1 sub-field via the Schaffer collaterals.

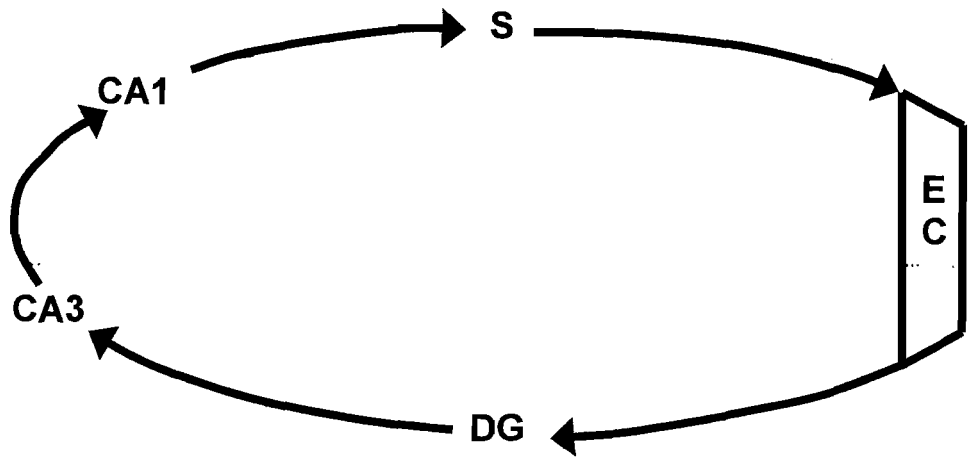


Figure 1.2 Diagram of Unidirectional Pathway from (Amaral and Witter, 1989). EC, Entorhinal Cortex; DG, Dentate Gyrus; S, Subiculum.

In the early 1970's the results of a number of physiological and anatomical studies were combined to provide a coherent hypothesis of the three-dimensional organization of the major hippocampal connections. This is referred to as the 'lamellar hypothesis' of hippocampal organization (Anderson et al., 1971). This hypothesis was arrived at because the four pathways of the hippocampus were shown to be successively activated when a stimulus is applied to the entorhinal area, i.e. the perforant path, the mossy fibres, the Schaffer collaterals, and finally the alvear fibres of CA1. The four pathways were also shown to all be orientated in the same direction, that is nearly transverse to the longitudinal axis. Therefore in a small slice, or lamella, of tissue a localized activation of the entorhinal cortex will lead to the four pathways being activated in succession. It was also described that due to the hippocampal cortex being organized into parallel lamellae, small slices of the hippocampus may operate as independent functional units, although excitatory and inhibitory transverse connections may modify the

behaviour of the neighbouring lamellae. This hypothesis is not the whole picture as it has been shown that information processing takes place in both the transverse and the longitudinal axes (Amaral and Witter, 1989).
Subsequently the information regarding this hypothesis have been revisited and it has been shown that although the Schaffer collaterals do not run simply from a CA3 cell to a CA1 cell in the same transverse plane, it has been shown that the majority of the signal strength acts within the transverse plane, and cells to the flanks of the plane are excited at a weaker level (Andersen et al., 2000). They also showed that Schaffer collateral branching is heaviest running along an oblique vector in relation to the longitudinal axis.
The organization described above is very useful when it comes to experimental models as it allows acute hippocampal slices to be taken and used for experiments with all the major connections of the hippocampus intact within a single slice. Although this does eliminate the longitudinal connections, this does still provide a very useful *in vitro* model of the hippocampus.

1.2.2 Major transmitter systems of the Hippocampus

Within the CNS there are a large number of neurotransmitters that play a role in regulating signalling behaviour either directly or by modulating the actions of other neurotransmitters. These neurotransmitters are split into two main types, broadly called either excitatory or inhibitory neurotransmitters. Some neurotransmitters can have both excitatory or inhibitory properties, this can depend on the receptor activated and also the region of the brain where the neurotransmitter is acting. Some of the major neurotransmitters of the brain are listed in table 1.3.
Along with the neurotransmitters listed in table 1.3 there are a large number of peptides that act as neurotransmitters, these are discussed further in section 1.4.
Although there are a large number of neurotransmitters in the CNS only a few have a ubiquitous role to play throughout the CNS, two of which are

Glutamate and GABA, described in more detail below; the others play a more limited role in certain brain regions and in different neuronal cell types.

Amino Acids	Biogenic Amines	Monoamines	Purines
Aspartate	Acetylcholine	Dopamine	Adenosine
Glutamate		Epinephrine	ATP
GABA		Norepinephrine	
Glycine		Serotonin	
		Melatonin	
		Histamine	

Table 1.3 List of the major small molecule neurotransmitters in the Central Nervous System.

1.2.2.1 Glutamate

Glutamate is the major excitatory neurotransmitter in the CNS (Fonnum, 1984) where it is expressed in about two thirds of synapses. It is a single amino acid transmitter and is found in many neuronal calls including pyramidal cells (Francis et al., 1993). Glutamate tends to act by depolarising the postsynaptic membrane by causing an excitatory postsynaptic potential (EPSP). This means that the membrane potential of the postsynaptic terminal is elevated towards threshold and therefore there is an increased probability that an action potential will result in the postsynaptic neuron.

The receptors for Glutamate are divided into two main groups: the ionotropic (ion channel receptors which when activated allow the influx of ions into the neuron), and the metatropic (G-protein coupled receptors that cause cellular changes in the neuron) receptors (see (Dingledine et al., 1999) for review). The ionotropic receptors are of primary interest in synaptic transmission as these are fast acting and cause propagation of the action potential. There are three classes and they are named for the agonists that most effectively activate them. These are N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-

isoxazole propionic acid (AMPA) and kainic acid (KA). The AMPA and KA receptors display similarities, while the NMDA receptors are distinct.

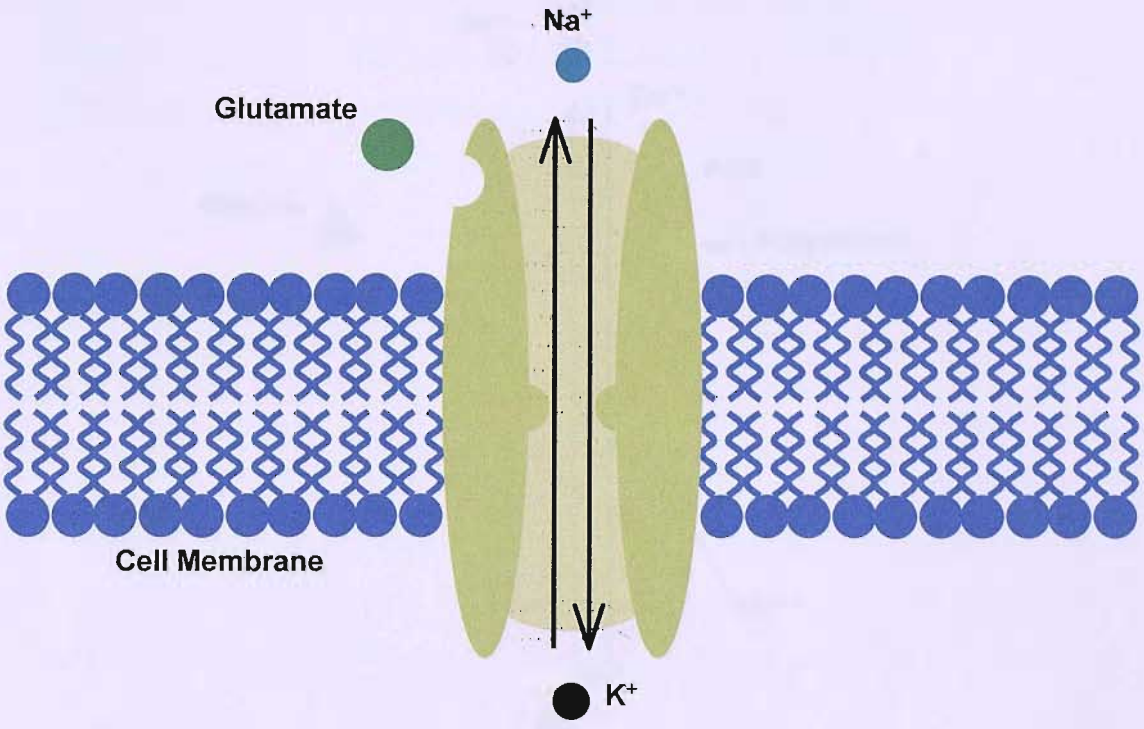
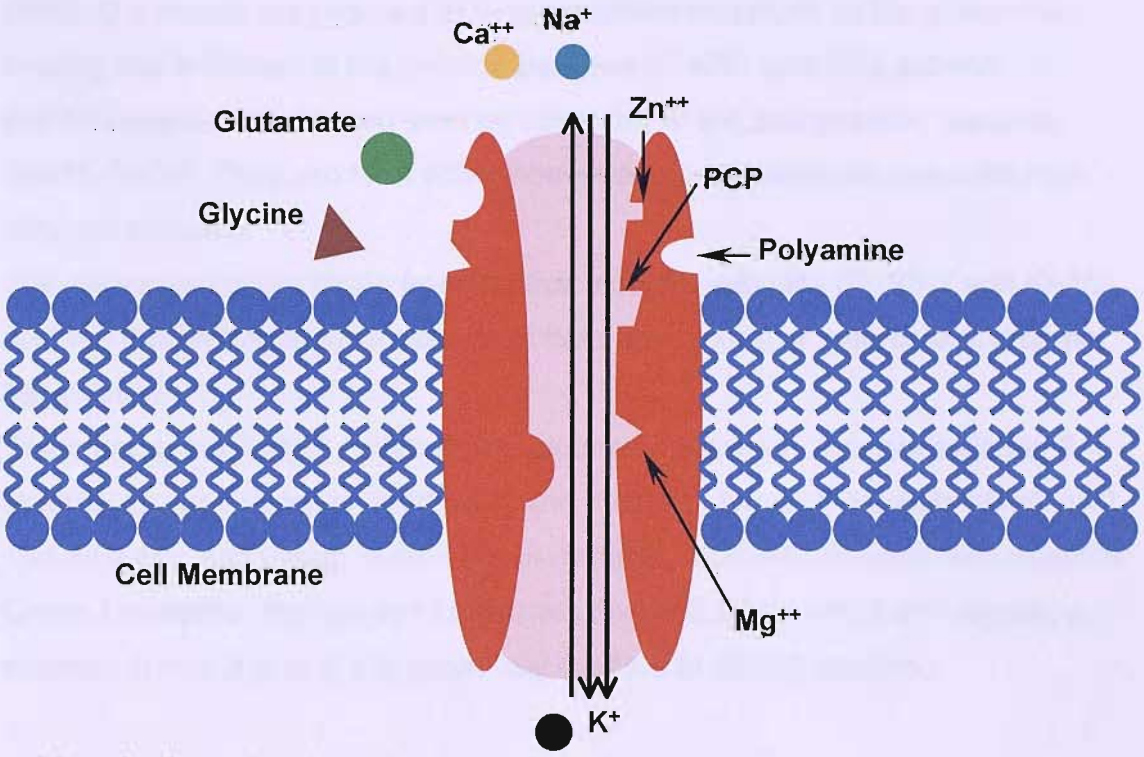


Figure 1.3 Schematic representation of the AMPA and KA receptors.

AMPA and KA receptors (figure 1.3) activate cation channels that allow Na^+ and K^+ ions to flow across the membrane. NMDA receptors (figure 1.4) activate cation channels that in addition to Na^+ and K^+ ions also allow Ca^{++} ions to pass through. Mg^{++} ions cause a voltage dependent block on the NMDA receptor at resting membrane potentials (Nowak et al., 1984;Mayer et al., 1984). This means that at resting potentials the NMDA receptor cannot be activated following the binding of glutamate. At this point the glutamate effect is mediated by AMPA and KA receptors. When the membrane depolarises by a sufficient amount, around 20mV, the Mg^{++} block is removed and the NMDA receptors become activated. When this happens then calcium ions, passing through the NMDA receptor channel, enter the cell in addition to the sodium ions passing through the AMPA

and KA channels. This causes a greater depolarisation in the membrane than is



achieved with sodium ions alone.

Figure 1.4 Schematic representation of an NMDA receptor and the major chemicals that affect its function

Along with the differences already mentioned the NMDA receptor is modulated by a number of different chemicals. Along with Mg^{++} that has already been mentioned Zn^{++}, polyamines and PCP's have been shown to interact with the receptor. Glycine has been shown to have a binding site on the receptor distinct from glutamate. It is thought that Glycine may in fact act as a coagonist with glutamate (Benveniste and Mayer, 1991;Clements and Westbrook, 1991), the precise role of this has not been determined but may act to fine tune the activity of NMDA receptors.

All three classes of receptor are made up of either 4 or 5 sub-units. These span the membrane and cluster together to form a pore that allows the ions to pass across the membrane.

The NMDA receptor is formed from an assembly of a mixture of four NR1 and NR2A-D subunits along with a NR3A subunit. Expression of both NR1 and NR2A-D subunits are required to form functional receptors as the glutamate binding site is formed at the junction between an NR1 and NR2 subunit.
AMPA receptors are formed from an assembly of the four possible subunits GluR1-GluR4. They can form either homomeric or heteromeric channels from different subunits.
The KA receptors are made from the five receptor subunits GluR5-7 and KA1/2, and like the AMPA receptors can form both homomeric or heteromeric channel assemblies.
There are at least eight metabotropic glutamate receptors that are split into 3 classes. Group I consists of mGluR1 and mGluR5, Group II consists of mGluR2 and mGluR3, and Group III consists of mGluR4, mGluR6, mGluR7 and mGluR8. Group I receptors are coupled to Phospholipase C (PLC) and Ca++ signalling, whereas Group II and III are negatively coupled to adenyl cyclase.

1.2.2.2 GABA (γ-aminobutyric acid)

GABA is the major inhibitory neurotransmitter of the CNS. It is found in GABA-ergic neurons and regulates the activity of the brain and assists in the prevention of hyper-excitability. There are 3 receptors for GABA: $GABA_A$, $GABA_B$ and $GABA_C$. $GABA_B$ is a G-protein coupled receptor, the remaining two receptors being ionotropic in nature. $GABA_C$ appears to be mainly located in the visual pathways. $GABA_A$ is therefore the main inhibitory receptor, of fast action and extensive distribution, within the CNS. These receptors are therefore of primary interest to inhibitory networks within synaptic transmission. The $GABA_A$ receptors, when activated, allow the passage of Cl^- ions across the cell membrane. The receptor is made of 5 membrane-spanning sub-units, similar to the ionotropic glutamate receptors, and the channel is similar in appearance to that of the AMPA and KA receptors (see figure 1.3), the major difference being that rather than allowing Na^+ and K^+ ions to pass it allows Cl^- ions to pass along

its concentration gradient. Under normal circumstances this results in chloride ions entering the neuron and causing a hyperpolarisation of the postsynaptic membrane thereby reducing the probability of an action potential propagating in the postsynaptic neuron. This process is known as an inhibitory postsynaptic potential (IPSP).

The $GABA_A$ receptor is formed from the assembly of 5 subunits from amongst the plethora of cloned subunits available. In excess of seven classes of subunit have been identified which each contain multiple isoforms. The most commonly expressed channel *in vivo* is made up of two α, two β and one γ subunit. The channel then contains three binding sites. A GABA binding site and a Benzodiazepine site located on the outside of the channel, and a Barbiturate site that is located within the channel.

The $GABA_B$ receptor is a metabotropic receptor that is a member of the mGlu superfamily. Activation of $GABA_B$ receptors causes a decrease in adenyl cyclase activity, and a decrease in Ca^{++} and increase in K^+ conductance within neuronal membranes.

1.2.3 Interneurons and Inhibition

Interneurons are found throughout the CNS including the hippocampus. Most of these interneurons are inhibitory in nature and the majority use GABA as their inhibitory neurotransmitter. There are currently at least 12 types of interneuron within the hippocampus that primarily innervate pyramidal neurons and an additional 4 types that innervate other interneurons. The primary role of interneurons in the hippocampus is to dampen down the excitatory activity and keep the whole system in check (see (Somogyi and Klausberger, 2005) and (Freund and Buzsaki, 1996) for reviews on interneurons).

Interneurons act to prevent hyper-excitability within the neuronal circuitry by innervating excitatory pyramidal cells reducing their probability of reaching threshold potential due to the induction of IPSP's in the postsynaptic neuron.

There is both feed-forward and feedback inhibition in the hippocampus which is illustrated in figure 1.5. These interneurons also allow an inhibitory surround to be formed in the brain of those experiencing a partial seizure. As the name suggest the interneurons cause an epileptic focus to become isolated within an area as the inhibitory circuitry prevents the excitation spreading to neighbouring neuronal tissue. After a time this inhibitory activity can weaken and it is at this point that the seizure can spread to other tissue within the brain and result in a generalized seizure.

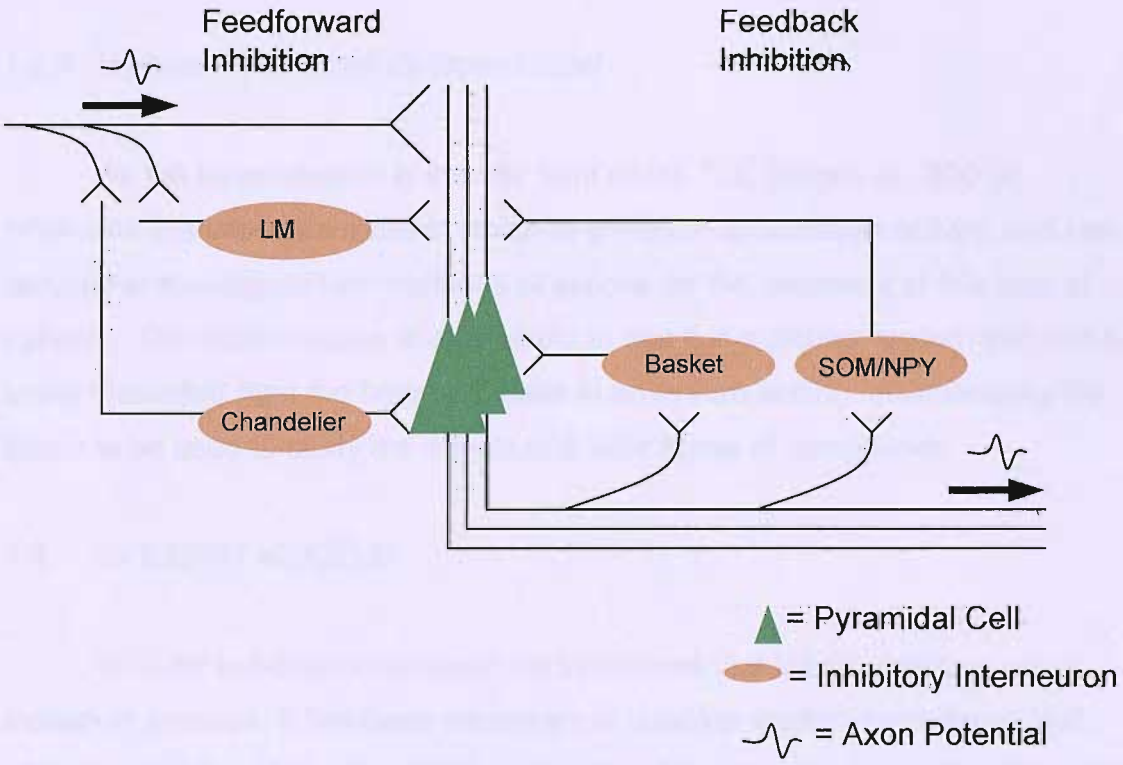


Figure 1.5 Schematic representation of the Inhibitory circuitry in the hippocampus. LM – Lacunosum moleculare.

Along with containing GABA as a neurotransmitter, interneurons can contain a range of co-transmitters such as parvalbumin, cholecystokinin, vasoactive intestinal peptide, somatostatin and neuropeptide Y to name but a few. Different classes of interneurons contain varying ranges of co-transmitters. When the

terminal of the interneuron is activated to release its vesicular contents into the synaptic cleft the neurotransmitters are released and act on numerous post- and presynaptic receptors. NPY has been identified in at least two sub-types of interneurons; Schaffer Collateral associated cells and Bistratified Cells. In these neurons NPY is co-localised with somatostatin and other peptide transmitters. Neuropeptide transmitters are often stored in large dense core vesicles within the synaptic terminal. These vesicles are not released under normal neuronal firing but are released when the terminal experiences high frequency bursting, such as that seen during epileptiform activity.

1.2.4 Hippocampus as an Epilepsy Model

As the hippocampus is the site from which TLE (Engel, Jr., 2001a) originates it is useful as a tool in which to generate epileptiform activity, and can be used to investigate new methods of actions for the treatment of this type of epilepsy. The hippocampus is also useful in that it is a distinct system that can be easily dissected from the brain and used in an *in vitro* setting, thus allowing the tissue to be used to study the effects of a wide range of compounds.

1.3 EPILEPSY MODELS

In order to better understand the processes that lead to epilepsy and to individual seizures, it has been necessary to develop models for epilepsy that allow the condition to be investigated outside of the human patient. For this both *in vitro* and *in vivo* animal models have been developed, and their variety allows various epileptic phenomenon to be investigated. Broadly speaking models are split into three categories. These are acute, chronic and genetic.
The Acute models are generally achieved by the addition of pharmacological agents to the neurons or the removal of specific ions. The Acute models can in the majority of cases be used both *in vitro* and *in vivo*, and usually cease when the system is returned to normal conditions.

The Chronic models are mainly *in vivo* models, although *in vitro* experiments may be carried out on tissue obtained from these models, and are typically caused by injected toxins, local lesions, repeated stimulation, or the implantation of heavy metal salts.
The Genetic models involve the identifying of epilepsy prone animals; or the manipulation of the genetic code of the animal to produce a genetically distinct animal. This may involve removing or inserting a gene, or up-regulating one that is already present.

1.3.1 Description of Models

1.3.1.1 Acute Models

All the Acute models of epilepsy are in fact models of bursting, and as such are not as close to the disease states as the chronic models. However they do provide a valuable way of investigating the effects of drugs on the increased activity that occurs during a seizure, and can be used in the evaluation of anti-convulsants.

1.3.1.1.1 Penicillin

Penicillin is traditionally accepted as an antibiotic, but it is also effective as a blocker of the $GABA_A$ receptor. This leads to a decrease in synaptic inhibition, which results in spontaneous epileptiform bursting. Penicillin can be applied topically to regions of the brain, and to brain slice preparations, in which case it produces acute recurring interictal spikes within a few minutes.
Penicillin has also been widely studied following systemic administration. When injected systemically or applied to the brain *in vivo* at low doses, penicillin generates a model of epilepsy in cats that is similar to primary generalized epilepsy (Gloor and Fariello, 1988). The penicillin model has been known for a

number of years and has played an important role in the discoveries that have been made into the neuronal basis of epilepsy.

1.3.1.1.2 Bicuculline and Picrotoxin

Similar to the inhibitory effect of Penicillin, both Bicuculline and Picrotoxin are antagonists for the $GABA_A$ receptor, and cause a reduction in the level of synaptic inhibition. This leads to an increase in synaptic excitation and generation of spontaneous epileptic activity. Bicuculline blocks the $GABA_A$ receptor binding point, whereas Picrotoxin blocks the Cl^- channel of the $GABA_A$ receptor. These models and also the penicillin model further support the hypothesis that epilepsy results from disinhibition within the neuronal cells affected.

1.3.1.1.3 4-Aminopyridine

The 4-Aminopyridine (4-AP) model of epileptiform activity is one that does not rely on GABA-mediated inhibition to bring about its effects (Chesnut and Swann, 1988). 4-AP appears to cause spontaneous epileptiform activity due to its action as a blocker of K^+ currents. These effects can lower the threshold and reduce the latency of action potential generation (Rudy, 1988). This model is discussed further in chapter 2.

1.3.1.1.4 Pentylenetetrazol

Pentylenetetrazol can be administered topically or systemically, and appears to cause its convulsive action by interfering with GABA-mediated inhibition. This may occur through an interaction with the GABA receptor complex (Olsen, 1981), although the mechanism is not fully understood.

1.3.1.1.5 Non Synaptic model

The Non-Synaptic model exploits the action of Gap junctions within the tissue. Its effects are caused by the removal of Calcium ions from the aCSF inhibiting synaptic transmission. The concentration of Potassium ions is increased causing the tissue to generate epileptiform activity. Since there is no possibility of synaptic transmission the only manner by which the activity can propagate throughout the tissue is via the gap junctions which link the neuronal cells together.

1.3.1.1.6 Zero Magnesium

The Zero Magnesium model works by removing the Mg++ ions from the artificial cerebrospinal fluid (aCSF). This removes the Magnesium block from the N-methyl D-aspartate (NMDA) receptor, and leads to an increase in the action of glutamate. This in turn causes spontaneous epileptiform activity to propagate throughout the tissue. This model is discussed further in chapter 2.

1.3.1.2 Chronic Models

The Chronic models of epilepsy rely on the generation of an underlying disease state, but how close this is to the processes involved in the human patient are not fully understood. The models do however have a closer resemblance to the disease that exists in the human patient, and so they are useful for evaluating the efficacy of new treatments and modes of action.

1.3.1.2.1 Kainic Acid

Kainic acid (Kainate, KA) is an endogenous agonist for the Kainate receptor, which is one of the ionotrophic glutamate receptors. When KA is administered to the hippocampus *in vivo* it causes lesions to develop in the CA3 region. This results in cell loss within the CA3 region and mossy fibre sprouting

(Cronin and Dudek, 1988). KA at low doses selectively targets the hippocampus, even when administered systemically. Therefore it is useful as a model of Temporal Lobe Epilepsy. It has been shown that the KA model causes plasticity of the NMDA and AMPA receptors following intracerebroventricular injection (Bernard and Wheal, 1995).

1.3.1.2.2 Tetanus Toxin

Administration of Tetanus Toxin intracerebrally, causes reduction of the IPSPs (Inhibitory Post Synaptic Potentials), due to impaired release of GABA (Jefferys et al., 1991). This reduction continues after the toxin is no longer present within the tissue, and so the toxin disrupts inhibitory transmission. It does not however destroy inhibitory neurons (Najlerahim et al., 1992).

1.3.1.2.3 Metal Salts

This group of models relies on the implantation of metals into the brain. These lead to recurrent seizures that arise spontaneously one or two months after the administration of the metal salts. Although the mechanism of action is not completely known these metals seem to develop a focus which has been shown to display a loss of GABA-ergic neurons (Ribak et al., 1986;Ribak et al., 1989) and axon terminals (Ribak et al., 1979;Ribak et al., 1982). This would lead to a reduction in the inhibitory input and then lead to an increase in excitatory activity. The most commonly used metal is alumina but cobalt, zinc and iron can also be used.

1.3.1.2.4 Kindling

Kindling is achieved by the repeated stimulation of an area of the brain by either an electrical or a chemical stimulus (Goddard et al., 1969). The stimulus could be from an electrode implanted within the brain or an agent such as pentylenetetrazol. Repeated application of the stimulus, at least once a day,

leads to an afterdischarge that over time increases in magnitude. The feature of this model is that progressively the response to the stimuli increases, both electrically and behaviourally. This eventually leads to a situation where an initially non-convulsive response can trigger a generalized motor seizure.

1.3.1.3 Genetic Models

There are many genetic animal models that have been developed. These have been generated either by selective breeding or the molecular manipulation of genes to provide animals with a desired phenotype. Two of the many models are mentioned below.

1.3.1.3.1 EL mice

This model is a result of a naturally occurring mutant that spontaneously generates seizure activity when the mouse reaches maturity and following a conditioning programme. This model was first described in 1959 (Imaizumi et al., 1959). Conditioning requires the mouse to be 'tossed' into the air on repeated occasions. Following several weeks an epileptic condition occurs. It is believed that this model contains a vestibular alteration that predisposes the mice to seizure activity.

1.3.1.3.2 $GABA_B$ Receptor Knockout mice

This knockout model is the result of molecular manipulation of the genetic code of the mice. It has been found that the removal of the gene for the $GABA_B$ receptor results in an epileptic phenotype. This leads to a decrease in inhibitory activity within the brain, and causes seizure activity to manifest. Unfortunately the knockout mice also have a reduced life expectancy, and therefore have to be utilised while young.

As epilepsy, in humans, has not been attributed to a single identifiable cause then it is difficult to say which of the models most closely resembles the clinical state. Most of the models that have been identified to cause an epileptic state in animals show characteristics that do not completely resemble the disease in humans. As a result of this it is useful, and in fact necessary, to utilise a number of different models in order to maximise the information that is garnered from any hypothesises regarding the pathophysiological state and potential anticonvulsant treatments.

1.4 NEUROPEPTIDES

A number of Neuropeptides have been identified within the CNS, table 1.4 contains a partial list of these. These have been shown to play a role in various neuronal functions, such as feeding, anxiety, etc. (Hughes and Woodruff, 1992) for review. In recent years it has been reported that some neuropeptides may play a role in the modulation of synaptic activity and could act as endogenous anti-epileptic agents (Tallent et al., 2001d;Tallent and Siggins, 1999;Vezzani et al., 1999;Smialowska et al., 2003;Patrylo et al., 1999;Klemp and Woldbye, 2001a;Rubaj et al., 2002c;Gutierrez et al., 2001). If this is the case then the receptors for these substances may prove a novel and potentially very exciting target for new AEDs.

Numerous studies have shown that in models of epilepsy, an increase in several neuropeptides and their receptors is observed following seizures (Vezzani et al., 1996;Takahashi et al., 2000;Takahashi et al., 1997;Sundstrom et al., 2001;Sperk et al., 1996). Several studies since this time report that the exogenous application of these neuropeptides can cause a reduction in the level of synaptic activity and a decrease in the severity of seizures that are witnessed (Woldbye et al., 1997;Tallent et al., 2001c;Rubaj et al., 2002b;Gutierrez et al., 2001;Woldbye et al., 2002;Klemp and Woldbye, 2001b;Klapstein and Colmers, 1997).

Although many neuropeptides within the CNS have attracted interest two have attracted attention as potential anti-convulsant agents. These are Neuropeptide Y

and Nociceptin. Both are G protein coupled receptors, and are found in high concentrations within areas of the brain that are involved with the propagation and transmission of epileptic activity. Neuropeptide Y has been, by far, the most extensively studied neuropeptide with regard to possible anti-convulsant actions.

Bombesin	Opioids
Gastrin releasing peptide	Corticotropin (adrenocorticotropic hormone, ACTH)
	Beta-lipotropin
Gastrins	Dynorphin
Gastrin	Endorphin
Cholecystokinin	Enkephalin
	Leumorphin
Neurohypophyseals	
	Secretins
Vasopressin	
Oxytocin	Secretin
Neurophysin I	Motilin
Neurophysin II	Glucagon
	Vasoactive intestinal peptide (VIP)
Neuropeptide Y	Growth hormone releasing factor (GRF)
Neuropeptide Y	Tachykinins
Pancreatic polypeptide	
Peptide YY	Neurokinin A
	Neurokinin B
Somatostatins	Neuropeptide A
	Neuropeptide gamma
Somatostatin	Substance P

Table 1.4 List of some of the neuropeptides identified within the CNS.

1.4.1 Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide that was first identified in the enteric nervous system. NPY is the most abundant neuropeptide within the brain, and has been shown to affect a number of physiological and pathological processes with the CNS. These include actions on food intake, learning and memory, and cardio-respiratory effects.

1.4.1.1 Distribution of Peptide

NPY is the most abundant neuropeptide in the CNS. It can be found in a number of areas of the brain.
Under normal physiological conditions the hippocampus contains a comparatively large amount of NPY compared to other neuropeptides (Iritani et al., 2000). NPY is found both in cells and in fibres. Cells where NPY is present include the hilus of the dentate gyrus (DG), stratum oriens (StOr) of CA2 and CA3 and near or in the stratum pyramidale (StPyr) of CA1. NPY positive fibres are seen in the molecular layer of the DG, and also running along the stratum radiatum (StRad) and stratum lacunosum (StLac) molecular layers. These later fibres appear to be projecting to the subiculum and entorhinal cortex.

1.4.1.2 Distribution of Receptors

There are 4 receptors identified for NPY (Y1, Y2, Y4 and Y5). There is also a y6 receptor, but this has not been identified in humans or rats. Recent evidence has also indicated the presence of a y7 receptor, although this has only been identified in lower organisms. All the NPY receptors are 7 transmembrane spanning G-protein coupled receptors (see figure 1.6).
The distribution of the four main receptors has been carried out to show the location in rat hippocampus. The Y1 receptor is present in moderate levels in the molecular layer of the DG, and in low, or moderate to high, levels in the StOr, StRad and StPyr depending on whether the measurements are carried out dorsally or ventrally respectively. The Y2 receptor is shown to be present in high levels in the StOr and StPyr, and in lower levels in the StPyr. Y2 receptors have also been identified in very low levels in the molecular layer of the DG, but in very high levels in the Fimbria and Stria terminalis. The Y4 receptor is almost undetectable in the hippocampus. The Y5 receptors have been located in very low or moderate levels in the StOr, StRad and StPyr depending on whether it is measured dorsally or ventrally respectively. Low levels of the Y5 receptor have also been identified in the molecular layer of the DG, although at much lower levels than the Y1 receptor.

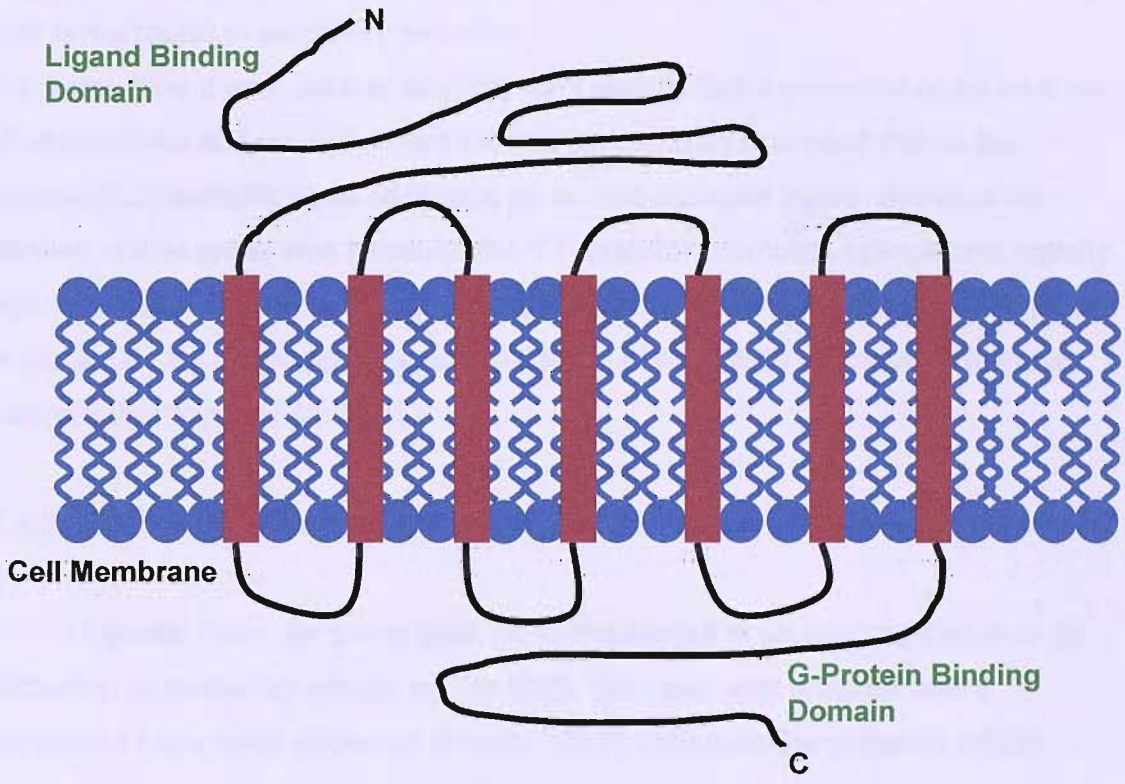


Figure 1.6 Diagram of a typical seven trans-membrane spanning G-protein coupled receptor. Examples of which are NPY and OP4.

1.4.1.3 NPY expression in Epilepsy

The previous paragraphs show the location of NPY and its receptors in non-epileptic animals. However in epilepsy models and in human epilepsy patients an alteration in the location and abundance of both NPY and its receptors is observed.

It has been demonstrated that NPY protein expression in rats is increased in mossy fibres in KA and PTZ models (Marksteiner et al., 1990). The same study

also showed that after KA treatment NPY expression is increased in the hilus and the Stratum Lucidum of CA3, and later within the Dentate Gyrus cells. This study showed no change in either the protein expression or mRNA of Somatostatin in granule cells or mossy fibres. This is in contrast to normal conditions where NPY is only constitutively expressed in GABAergic interneurons (Kohler et al., 1986) and is not found in excitatory neurons.

For some time it was unclear whether NPY was in fact a promoter or an inhibitor of epileptiform activity, but recently it has been widely accepted that in the pathological epileptic state NPY acts as an anti-epileptic agent. Some of the confusion that arose was because the Y1 receptor promotes epileptiform activity (Gariboldi et al., 1998), although this receptor has been shown to down-regulate in the tissue from epileptic animals (Redrobe et al., 1999) and human epilepsy patients (Furtinger et al., 2001).

1.4.2 Opioids and Nociceptin

Opioids have, for some time, been implicated in causing an elevation or reduction of excitatory effects on the CNS. Both pro- and anticonvulsant properties have been observed (Frenk, 1983). Whereas the classical opioid receptors δ, κ and μ (now called OP1, OP2 and OP3 respectively (Dhawan et al., 1996)) have caused some confusion over the role of opioids, in that they seem to both elevate and reduce convulsant activity, the recent discovery of a fourth member of the opioid family, OP4, seems to show a role in anticonvulsant behaviour (Tallent et al., 2001b). OP4 (sometimes called ORL-1 and Orphanin FQ) was cloned in the early 1990's (Mollereau et al., 1994;Bunzow et al., 1994;Fukuda et al., 1994;Nishi et al., 1994;Wang et al., 1994) and for some time the endogenous ligand for it was unidentified.

Nociceptin is a 17 amino acid peptide of the opiate family. It has been identified as the endogenous ligand for the Orphan Opioid receptor-like receptor (ORL1/OP4) (Meunier et al., 1995;Reinscheid et al., 1995). This neuropeptide has been shown to have varying effects on nociception (Calo et al., 1998) (Rossi

et al., 1998). It also has numerous other effects including anxiolytic-like activity (Jenck et al., 1997), reduction in heart rate and blood pressure (Kapusta et al., 1997), stimulating food intake (Pomonis et al., 1996) and impairing spatial learning (Sandin et al., 1997).

1.4.2.1 Distribution of Peptide

Nociceptin (also called Orphanin FQ) is distributed throughout the CNS of both rats and humans. Light to moderate staining of the peptide is seen throughout the CNS of rats (Neal, Jr. et al., 2001;Neal, Jr. et al., 1999b) and high levels are seen in some areas including the hippocampus, with the strongest expression seen in CA1, CA3 and the granule cell layer. Expression of the peptide is found in mouse hippocampus where the characteristic localisation and morphological characteristics suggest that it is most likely that it is contained in interneurons (Ikeda et al., 1998).

1.4.2.2 Distribution of Receptors

The receptor for nociceptin is found throughout the brain of the wild-type mouse (Clarke et al., 2001). Similarly to the distribution of the peptide, the OP4 receptor is found throughout the CNS of humans and rats (Anton et al., 1996;Peluso et al., 1998). Strong expression of receptor mRNA is seen in the hippocampus and others structures (Neal, Jr. et al., 2001a). The OP4 receptor shows strong expression throughout all the hippocampal sublayers (Anton et al., 1996).

Figure 1.6 shows a graphical representation of what the OP4 receptor looks like. At present there are limited ligands that bind to the OP4 receptor and unlike all the other opioid receptor it does largely not interact with the opioid receptor antagonist Naloxone.

1.4.2.3 Nociceptin expression in Epilepsy

Whereas Neuropeptide Y has been investigated by many laboratories to discover its role in epileptiform activity at present Nociceptin is a fairly new drug in this field and so very few studies have been carried out. Most of these studies have been undertaken in the last couple of years and show that Nociceptin has an anticonvulsive effect in several models of epilepsy including Zero Magnesium, High Potassium (Tallent et al., 2001), Pentylenetetrazole, Bicuculline (Rubaj et al., 2002), Kindling (Gutierrez et al., 2001) and Penicillin (Feng et al., 2004). Whereas some early studies indicated that Nociceptin may cause both pro and anti convulsant actions, these studies now firmly establish that Nociceptin is a peptide with anticonvulsive actions within the CNS.

1.5 Purpose of Project

The aims of my work were to investigate the effects of neuropeptides in various in vitro models of convulsant activity. In order to achieve this I used acute hippocampal slices and organotypic hippocampal slice cultures. These were then exposed to convulsant models and the effects of Nociceptin and also, to a limited extent, Neuropeptide Y were investigated by applying these neuropeptides to the perfusion medium.

I choose to look at Nociceptin because, when I started my studies, it had only recently been identified as a possible anticonvulsant and because of this the precise mechanism of action wasn't, and still isn't, yet known. It would therefore be useful to identify a possible mechanism as this could be a useful receptor target for future drug treatments.

I used NPY as a reference neuropeptide at certain stages of my work to compare my observations with Nociceptin back to the large quantity of published data in reference to the effects of NPY on convulsant models.

Chapter 2

Effects of Nociceptin on Epileptiform Activity in Acute Slices

2.1 Introduction.

Recent studies have indicated that Nociceptin may act as an endogenous anticonvulsant (Feng et al., 2004;Gutierrez et al., 2001;Rubaj et al., 2002;Tallent et al., 2001). Nociceptin was identified in 1995 (Meunier et al., 1995;Reinscheid et al., 1995) to be the endogenous ligand for the previously discovered orphan opioid receptor known as either ORL-1 or OFQR (Bunzow et al., 1994;Mollereau et al., 1994;Fukuda et al., 1994;Nishi et al., 1994;Wang et al., 1994), but now known as the OP4 receptor (Dhawan et al., 1996).

The OP4 receptor has been identified in numerous structures within the mouse, rat and human brain (Florin et al., 1997;Neal, Jr. et al., 1999b;Peluso et al., 1998;Anton et al., 1996;Ikeda et al., 1998).

Most of the investigations carried out with Nociceptin have focussed on its actions in relation to nociceptive pathways (Meunier, 2003;Harrison and Grandy, 2000). I was interested in finding out its role in mediating anti-convulsant activity.

Although the actions of Nociceptin in convulsant models in slices has been investigated (Tallent et al., 2001) so far there have not been a large number of studies looking at the mechanism by which Nociceptin exerts its anti-convulsant action and so this requires further investigation.

I utilised a number of epileptiform models to investigate the effects of Nociceptin on acute hippocampal slices.

2.2 Materials and Methods

2.2.1 Acute Hippocampal Slice Preparation.

Hippocampal slices were prepared from 6-8 week old male rats (Biomedical Research Facility, Southampton UK: and LAS, GlaxoSmithKline, Harlow, UK). Following cervical dislocation and decapitation, in accordance with the Animal Scientific Procedures Act 1996, the brain was quickly removed and immersed in cold (4°C) cutting solution, containing in mM: 189 Sucrose, 10 Glucose, 26 $NaHCO_3$, 2.5 KCl, 5 $MgCl_2$,

0.1 $CaCl_2$ and 1.2 NaH_2PO_4, gassed with 95% O_2 and 5% CO_2. The mean osmolarity of the cutting solution was 275 mosmoles/litre and pH 7.2.

Following removal of the brain, it was mounted on a Vibratome VT1000S (Leica, UK), and immersed in cutting solution and horizontal slices, 400μm thick, were prepared. Slices were kept at room temperature in a holding chamber (Figure 2.1) in artificial cerebrospinal fluid (ACSF) containing in mM: 124 NaCl, 3 KCl, 1 NaH_2PO_4, 1 $MgSO_4$, 25 $NaHCO_3$ 10 Glucose, 2 $CaCl_2$, pH 7.2, gassed with 95% O_2 and 5% CO_2. The mean osmolarity of the ACSF was 310 mosmoles/litre. Slices were allowed to recover for an hour before transferral to the recording chamber.

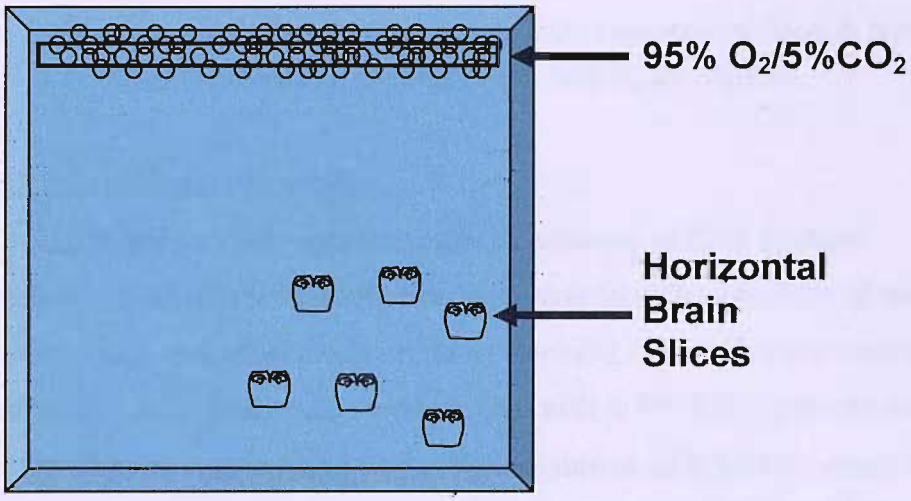


Figure 2.1 Diagram of Holding Chamber containing aCSF, to a depth of around 5mm, gassed with 95% O_2 and 5% CO_2. The holding chamber is viewed from above and the brain slices stay in the chamber in order to receive O_2 and nutrients from the aCSF.

2.2.2 Electrophysiological Recording System.

2.2.2.1Interface Chamber.

Electrophysiological recordings were carried out in an interface chamber (Figure 2.2). Slices were transferred from the holding chamber to the recording chamber and allowed to equilibrate for a period of 30 minutes prior to recording. The slice was maintained on a thin nylon mesh at the interface between the aCSF and warmed (31±0.5°C), humidified gas (95%

O_2 and 5% CO_2). The lower surface of the slice was perfused with warmed (31±0.5°C) aCSF at a flow rate of 2ml min^{-1}. Warmed and humidified gas was passed over the top of the slice.

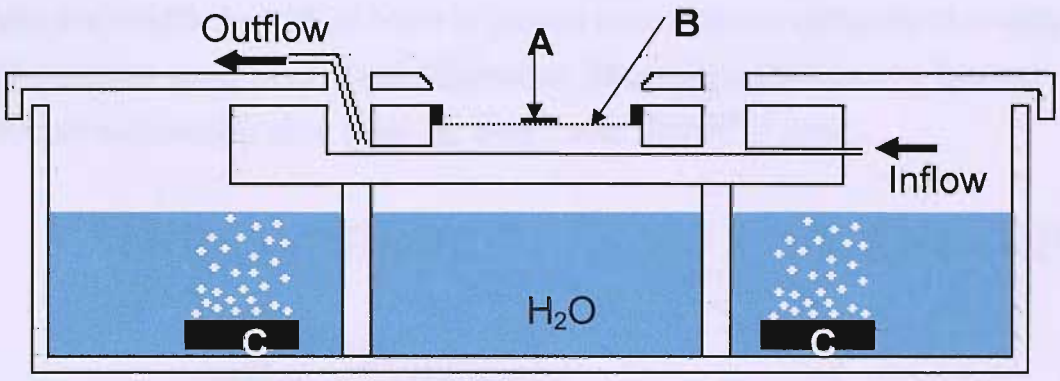


Figure 2.2 Diagram of the Interface Chamber. A: Acute Hippocampal Slice, B: Nylon Mesh, C: Air stone to bubble water with 95% O_2 and 5% CO_2.

2.2.2.2 Extracellular recordings.

Extracellular field potentials were recorded in CA1 stratum pyrimidale, using glass microelectrodes made from borosilicate glass (1.5mm outside diameter) with an inner filament (Clark Electromedical Instruments, UK). Electrodes were pulled with a PP-830 microelectrode puller (Narishige, Japan) and had a tip resistance of 5-10 MΩ when filled with 2M sodium chloride. The microelectrode was connected to the recording circuit by means of a chlorided silver wire with a Ag/AgCl reference electrode placed in the bath. Manipulation of the microelectrode was achieved by using a Leitz micromanipulator that allowed the microelectrode to be lowered gently onto the slice.

Data was collected using an Axopatch 220B linked by a Digidata 1322A to a computer running pClamp 8 (all hardware and software, except computer, from Axon Instruments, Inc. U.S.A.). The data was recorded at a sampling frequency of 10 KHz. The software used was Clampex 8.0, run in gap-free mode to continually record the spontaneous activity from the slices.

2.2.3 Epilepsy Model Protocols.

The viability of the slices was confirmed by stimulating in the dentate gyrus, if a population spike was observed in the CA3 stratum pyrimidale then the slices were used for the experiments. The slices were perfused with the modified aCSF in order to induce spontaneous epileptiform activity. The models used were 10µM Bicuculline (Bicuculline Methiodide, Sigma), 100µM 4-Aminopyridine (Sigma), $0Mg^{++}$ and High K^{+}(8.5mM).

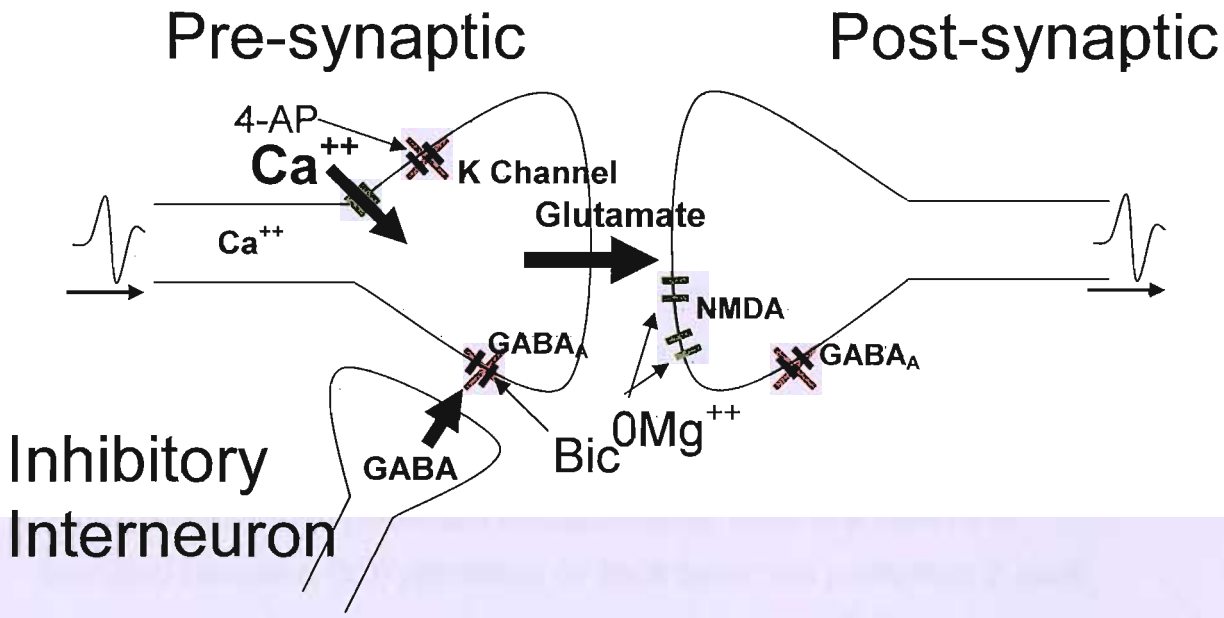


Fig 2.3 Diagram illustrating the sites of action of the various models used to induce spontaneous epileptiform bursting. (K, Potassium Channel; Bic, Bicuculline)

Figure 2.1 illustrates the mechanism of action by which the various models achieve their convulsant activity.

Following induction of epileptiform activity the neuropeptide under investigation was washed on for periods of 30 minutes in increasing concentrations (see Figure 2.4). After all the drug concentrations had been washed on to the slice then normal aCSF was re-perfused and a wash-off value was recorded.

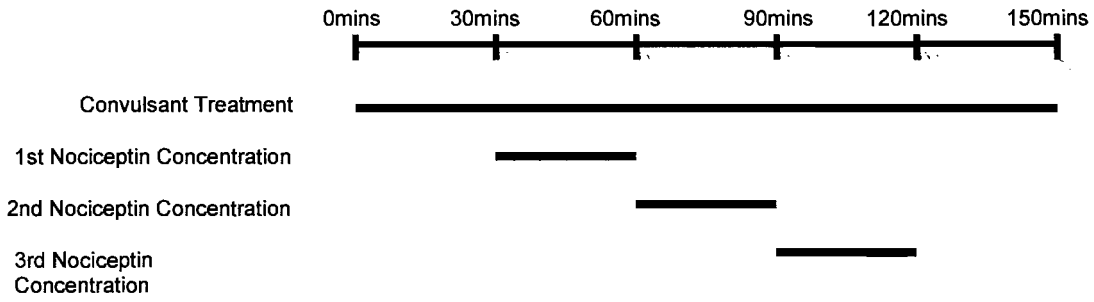


Figure 2.4 Diagram illustrating the time course used for the epilepsy model experiments. Increasing concentrations of Nociceptin were washed onto the slice depending on the number of differing concentrations under investigation.

2.2.4 Data Analysis.

Data was analysed using Clampfit 8.0 (Axon Instruments).

During analysis of the data, the level of spontaneous activity was measured during a ten minute time interval prior to the change of concentration. A frequency of bursting per minute was then calculated for each treatment carried out.

In the tables that are presented for each model, there is a mean and Standard Deviation (SD) presented for each treatment performed in each individual experiment. An average value for all the experiments was then calculated and this is presented in the figures.

There are two types of graph presented for each model. The first contains data that has been normalised, i.e. each treatment has been calculated as a percentage of the control group. This allows a comparison between experiments where the baseline burst frequencies may differ. The second graph represents the mean of the raw values (in Hertz) obtained for that group of experiments.

The data was then subjected to one-way analysis of variance, to show any statistical significance. If the data showed a statistical significance then a Dunnett's post-hoc test was carried out to find out which of the treatment groups showed any significant difference from the control group.

2.3 Bicuculline Model

Figure 2.5 shows sample traces of the experiments carried out using the 10μM Bicuculline convulsant model. This is a typical experiment and the first trace shows the recordings obtained before 10μM Bicuculline is added to the perfusion medium. The trace on the left shows a time period of 10 mins and the right hand trace shows a period of about two seconds. The other traces show the effect of adding 10μM Bicuculline to the perfusion medium and then supplementing this with 100nM, 300nM and 1μM Nociceptin and then removing the Nociceptin altogether to leave 10μM Bicuculline in the perfusion medium. The left and right hand traces for all the different drug solutions have the same time periods as that of the pre Bicuculline traces. The traces show that when 10μM Bicuculline is washed onto the slices then the neuronal tissue begins to display spontaneous bursting behaviour. This behaviour builds up over a 10-15 minute period (data not shown) and then begins to settle into a regular pattern.

When Nociceptin is added to the perfusion medium a reduction in bursting frequency is evident.

The right hand traces show that the Nociceptin does not change individual bursting events. This is demonstrated by the events maintaining the same shape as the concentration of Nociceptin is increased.

When Nociceptin is washed off the slice by removing it from the perfusion medium, and bathing the slice in aCSF + 10μM Bicuculline alone, the amount of bursting looks to increase again, although is does not appear to be as frequent as in the 10μM Bicuculline baseline.

Figure 2.6 shows the mean effect of increasing doses of Nociceptin on 10μM Bicuculline induced bursting activity. The data in this graph is normalised to the baseline, which is the frequencies for each of the drug treatments are calculated as a percentage compared to their individual baseline value and then these results are compared to corresponding treatments in the other experiments. Therefore the Baseline value is fixed at 100% and has no error value. All the treatments are then compared to this and have error bars displaying the standard deviation of the data sets.

The data can be seen to show that increasing concentrations of Nociceptin

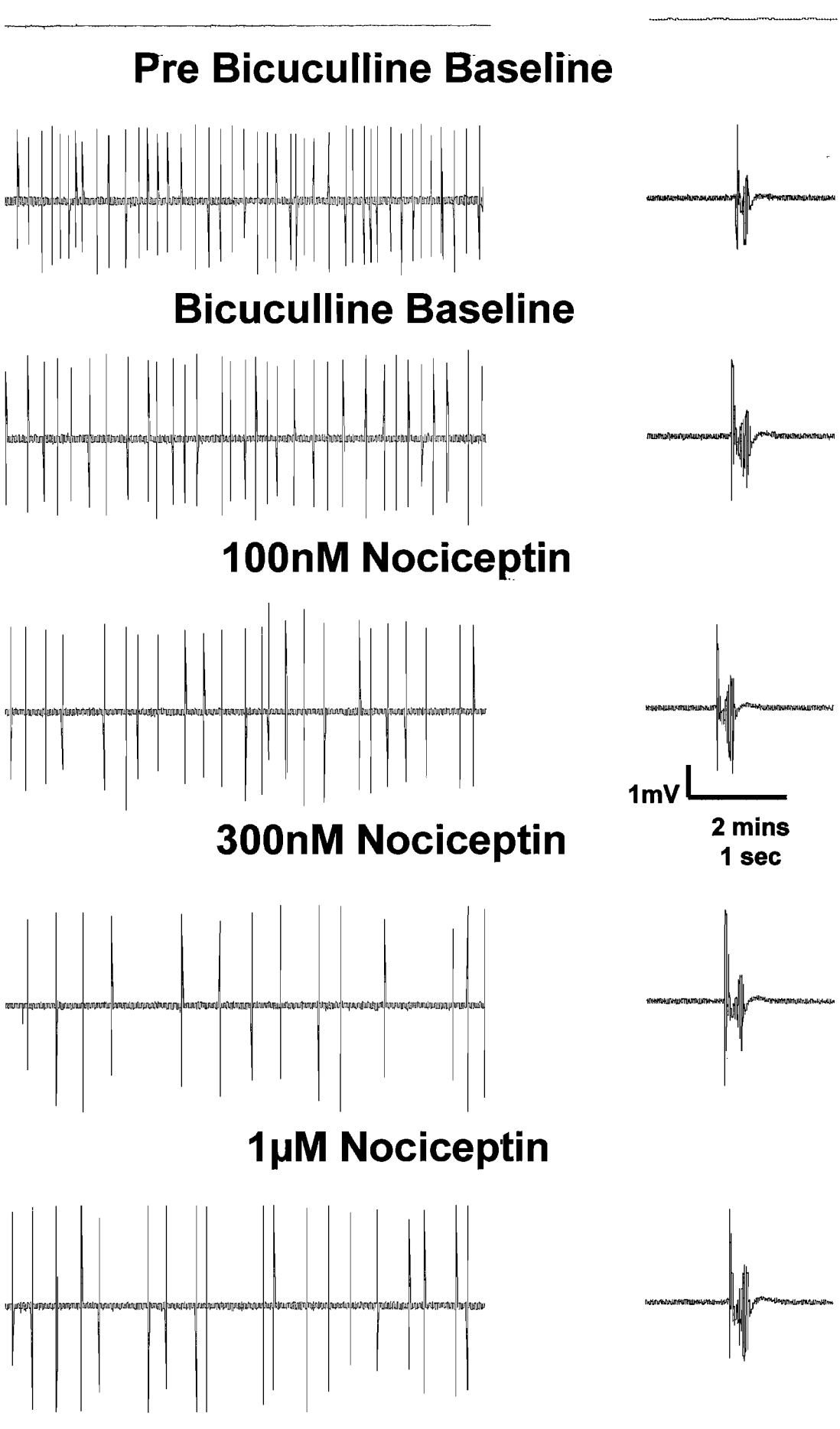

Pre Bicuculline Baseline
Bicuculline Baseline
100nM Nociceptin
300nM Nociceptin
1mV
2 mins
1 sec
1μM Nociceptin
Bicuculline Baseline (Wash-Off)

Fig 2.5 (previous page) Traces showing the effect of Nociceptin treatments on 10μM Bicuculline generated spontaneous epileptiform activity in Acute Hippocampal Slices. The traces on the right side represent expanded views of the traces that are shown on the left side of the page. The scale bar shows 2 values for the x-axis; the upper one refers to the left trace, and the lower to the right trace.

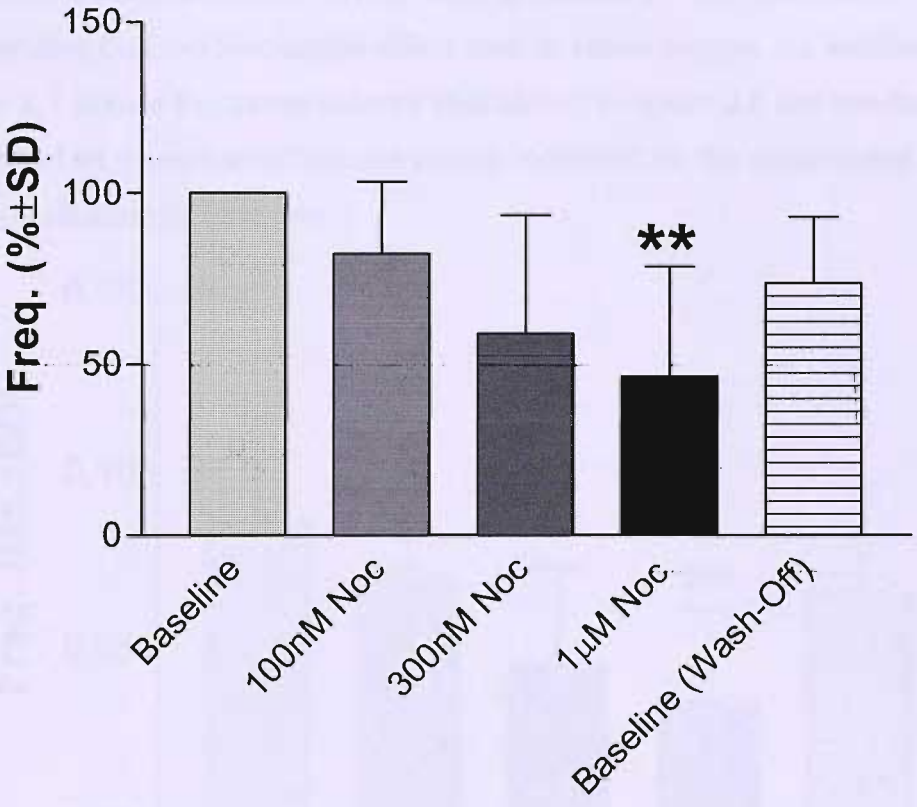


Fig 2.6 Graph showing the effect of varying doses of Nociceptin on Acute Hippocampal Slices pre-treated with 10μM Bicuculline. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. **=p<0.01 (n=5).

result in a decrease in the mean frequency of bursting. This effect of Nociceptin also increases as the concentration of Nociceptin increases and so can be said to be acting in a concentration dependent manner. The 100nM concentration of Nociceptin causes a reduction in bursting behaviour to a mean value of 82%, of that of the baseline, 300nm causes reduction to 59% of baseline and 1μM results in a decrease to 46% of baseline.

When the concentration of Nociceptin added to the perfusion medium is at 1μM then the data shows a statistically significant effect $p<0.01$, a reduction

to 46% of the amount of bursting seen in the baseline experiments. The final column in this figure shoes that when Nociceptin is removed from the slice then bursting activity begins to return. Although this activity does not return to the same value at that seen before Nociceptin is added, bursting at 74% of that seen in the baseline experiments is recorded, it is at a higher frequency than that seen when the slice is bathed in 1μM Nociceptin, and so indicates that the Nociceptin effect can, to some degree, be washed off. Figure 2.7 shows the same data as that shown in figure 2.6 but the data is presented as the mean of the raw values recorded for the experiment with no normalisation to baseline.

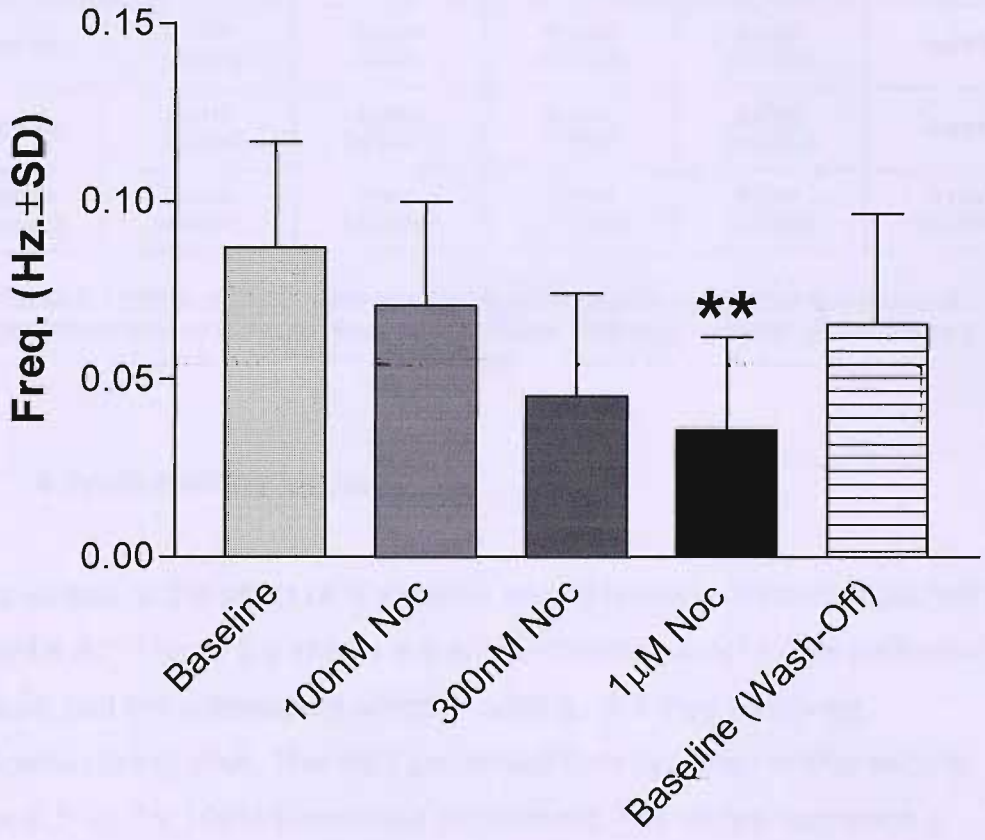


Fig 2.7 (previous page) Graph showing the effect of varying doses of Nociceptin on Acute Hippocampal Slices pre-treated with 10μM Bicuculline. All frequencies are calculated using the raw data values originally recorded, and there is no normalising to baseline. **=p<0.01 (n=5).

Table 2.1 shows the individual data sets for the experiment carried out. This data shows that over the course of the experiment carried out, except for

experiment B, the bursting activity decreases as the concentration of Nociceptin increases and that the activity then increases when Nociceptin is removed from the perfusion medium. Experiment B shows a slight increase in activity following addition of 100nM Nociceptin, but after this the recorded pattern is similar to that seen in all the other experiments.

Exp. ID	A	B	C	D	E
Baseline	0.0633 (±0.0153)	0.1083 (±0.0059)	0.0717 (±0.0158)	0.0633 (±0.0217)	0.1283 (±0.0112)
100nM Noc	0.0550 (±0.0112)	0.1217 (±0.0075)	0.0550 (±0.0081)	0.0517 (±0.0091)	0.0683 (±0.0123)
300nM Noc	0.0467 (±0.0105)	0.0750 0.0000	0.0650 (±0.0123)	0.0383 (±0.0046)	0.0000
1μM Noc	0.0417 (±0.0088)	0.0600 (±0.0037)	0.0583 (±0.0088)	0.0183 (±0.0037)	0.0000
Baseline (Washoff)	0.0450 (±0.0112)	0.0667 (±0.0059)	0.0700 (±0.0105)	0.0317 (±0.0070)	0.1133 (±0.0383)

Table 2.1 Effect of Drug treatments on 10μM Bicuculline generated spontaneous epileptiform activity in Acute Hippocampal Slices. Values are Means (of frequency in Hertz).

2.4 4-Aminopyridine Model

I also looked at the effect of Nociceptin on the bursting behaviour elicited by 100μM 4-AP. Figure 2.8 shows the effect of adding 4-AP to the perfusion medium and the subsequent effect of adding, and then removing, Nociceptin to the slice. The data presented here is similar to that seen in figure 2.5 for the 10μM Bicuculline experiment. The traces represent a typical experiment that I carried out.

It can be seen that when 100μM 4-AP is added to the slice then bursting activity occurs. The bursting activity seen with this model is of a higher frequency than that seen in the Bicuculline model described previously, the baseline frequency in this experiment is around 0.5Hz. When increasing concentrations of Nociceptin, 100nM, 300nM and 1μM are added to the perfusion medium then no apparent reduction in bursting activity appears evident. The activity appears to remain unchanged when the Nociceptin is

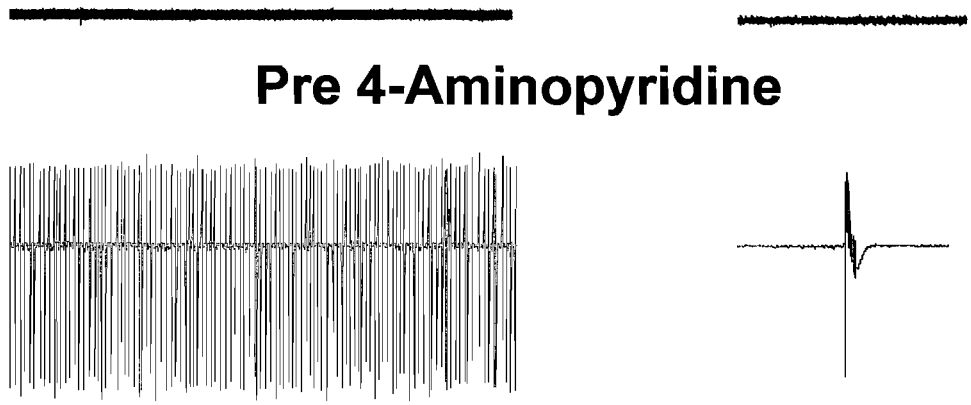

Pre 4-Aminopyridine
4-Aminopyridine Baseline
100nM Nociceptin
2mV
1min
0.5 sec
300nM Nociceptin
1µM Nociceptin
Nociceptin Wash-Off

Fig 2.8 (previous page) Traces showing the effect of Nociceptin treatments on 100μM 4-Aminopyridine generated spontaneous epileptiform activity in Acute Hippocampal Slices. The traces on the right side represent expanded views of the traces that are shown on the left side of the page. The scale bar shows 2 values for the x-axis; the upper one refers to the left trace, and the lower to the right trace.

removed from the slice. This is shown by the final trace, Nociceptin wash-off. The expanded view of the individual bursts show that throughout the experiment these do not change in amplitude or envelope.

If we then look at the cumulative data for a series of experiments examining the effect of varying concentrations of Nociceptin on the 4-AP model of bursting.

Figure 2.9 shows this data, and is laid out in the same way as described earlier (for figure 2.6). The figure shows that when Nocieptin is added to the perfusion medium then no significant changes in bursting activity are observed.

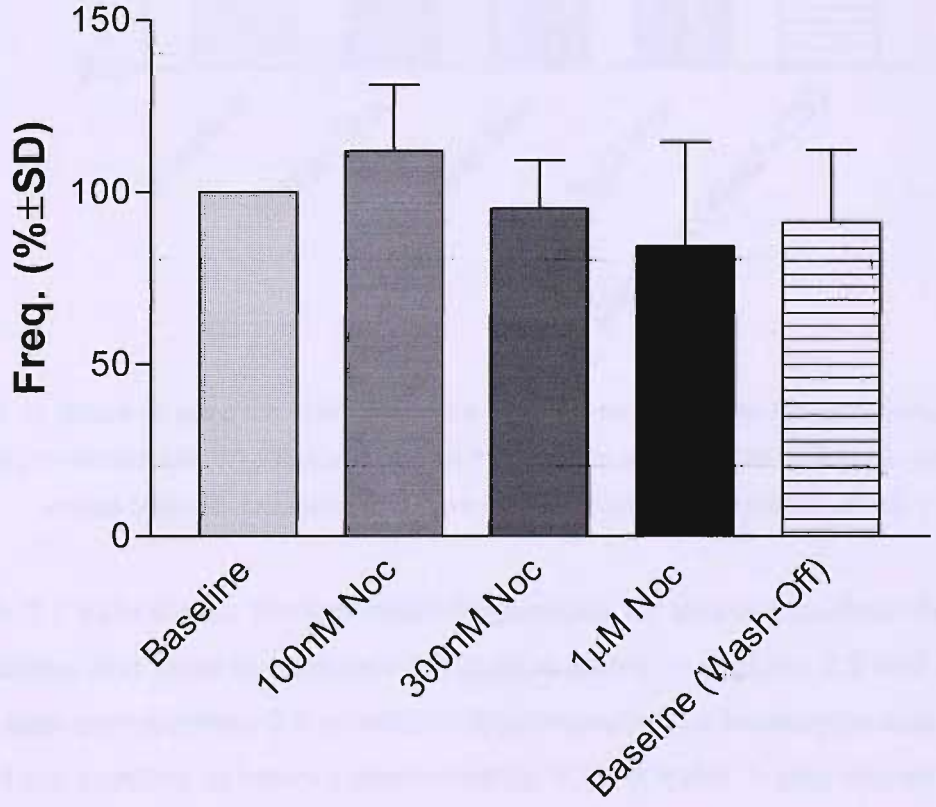


Fig 2.9 Graph showing the effect of varying doses of Nociceptin on Acute Hippocampal Slices pre-treated with 100μM 4-AP. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=6).

Figure 2.10 shows the same data as that displayed in figure 3.9, but gives the mean frequencies recorded and does not set a 100% baseline to which all other drug treatments can be compared.
The graph supports the observation reached from the previous graph that Nociceptin does not show an effect on the bursting behaviour generated by the 100µM 4-AP model of epileptiform activity.

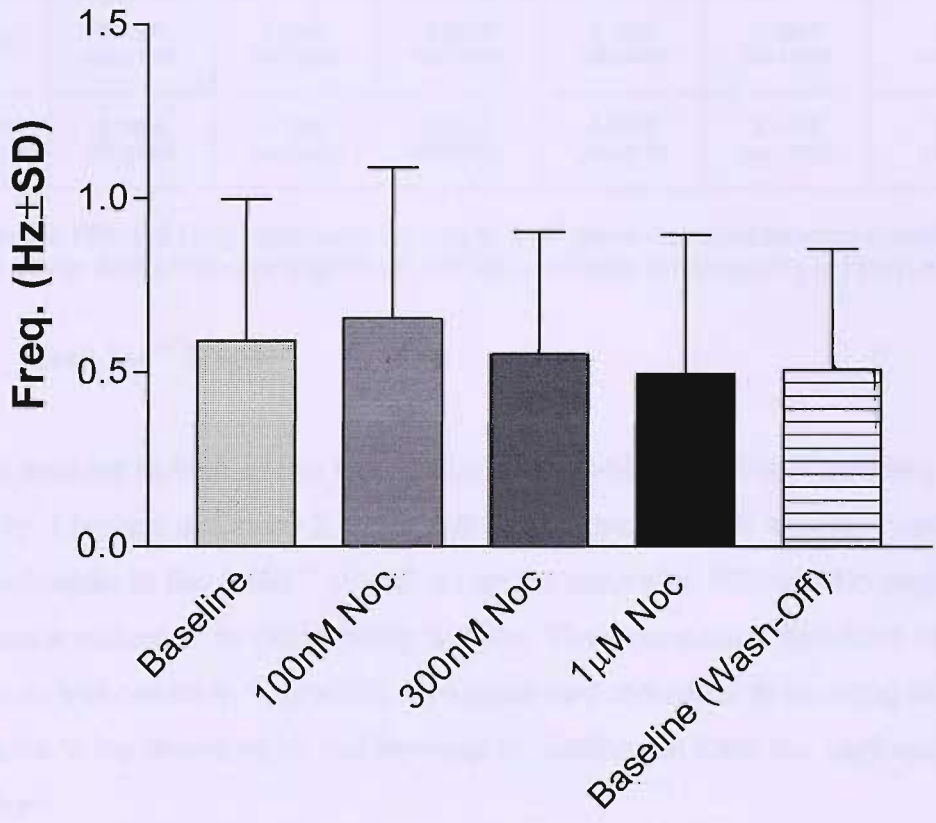


Fig 2.10 Graph showing the effect of varying doses of Nociceptin on Acute Hippocampal Slices pre-treated with 10µM Bicuculline. All frequencies are calculated using the raw data values originally recorded, and there is no normalising to baseline. (n=6).

Table 2.2 then shows the individual frequencies for all the experiments performed and used to calculate the graphs shown in Figures 2.9 and 2.10. This data corroborates the previously seen results that Nociceptin does not affect the bursting behaviour generated by 100µM 4-AP. It also shows that no one experiment was distinctly different from the mean result shown in the previous figures.

Exp. ID	A	B	C	D	E	F
Baseline	0.1685 (±0.0100)	1.3033 (±0.0757)	0.6217 (±0.0354)	0.7517 (±0.1304)	0.3167 (±0.0778)	0.3783 (±0.0112)
100nM Noc	0.1667 (±0.0111)	1.3400 (±0.0654)	0.5850 (±0.0426)	1.0000 (±0.0556)	0.4417 (±0.1137)	0.3900 (±0.0306)
300nM Noc	0.1483 (±0.0095)	1.0733 (±0.2841)	0.5117 (±0.0343)	0.8717 (±0.0393)	0.3433 (±0.1601)	0.3567 (±0.0117)
1uM Noc	0.1200 (±0.0105)	1.2000 (±0.0583)	0.2017 (±0.0500)	0.7083 (±0.0579)	0.3867 (±0.1561)	0.3550 (±0.0209)
Baseline (Wash-Off)	0.1800 (±0.0105)	1.1283 (±0.0445)	0.3217 (±0.0502)	0.6983 (±0.0532)	0.3467 (±0.1503)	0.3767 (±0.0141)

Table 2.2 Effect of Drug treatments on 100µM 4-AP generated spontaneous epileptiform activity in Acute Hippocampal Slices. Values are Means (of frequency in Hertz) ±SD.

2.5 Zero Mg^{++} Model

I next wanted to look at the Nociceptin in the 0 Mg^{++} model of bursting activity. Looking at Figure 2.11, which shows the effect of one concentration of Nociceptin in the 0 Mg^{++} model, it can be seen that 500nM Nociceptin causes a reduction in the bursting activity. The information provided in this figure is laid out as in figure 2.5. The observed reduction in bursting activity appears to be reversed by the removal of Nociceptin form the perfusion medium.

The expanded traces, on the right hand side of the figure, show that when Nociceptin is administered to the slice then the envelope of the bursting event changes. It appears that the event breaks down into a few spikes within the bursting as opposed to the mass of spikes seen in the Baseline and Wash-off traces. The amplitude of the event also seems to increase slightly. Therefore as well as reducing the frequency of the bursting events, nociceptin also reduces the number of bursts within each individual event. Figure 2.12 shows the mean data obtained for the series experiments performed on the 0Mg^{++} model. This data shows that when 500nM is added to the perfusion medium then a reduction in bursting activity is seen. The mean value is 70% that of the baseline. This effect is not however significant by the statistical tests described in the Materials and Methods.

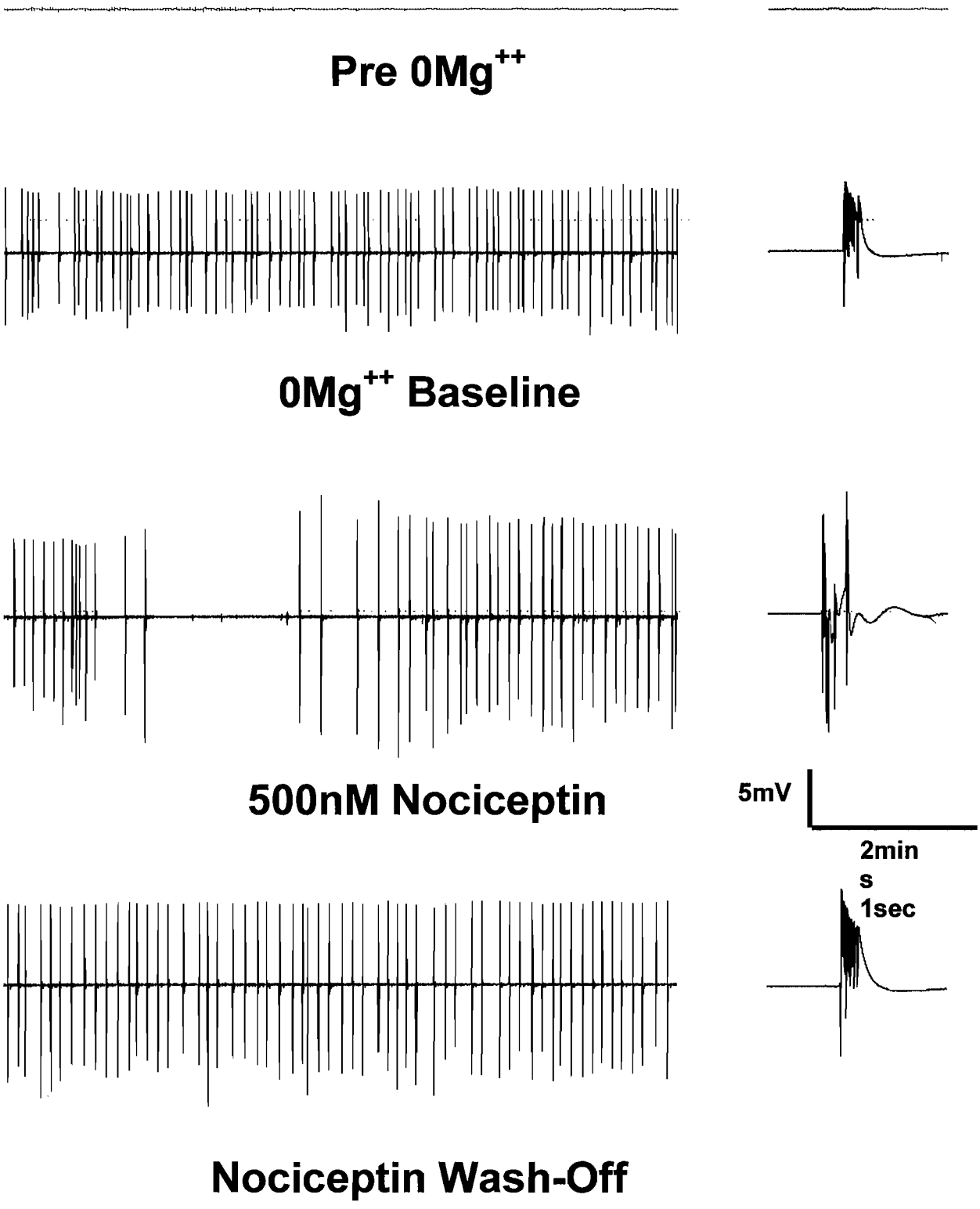

Pre 0Mg++
0Mg++ Baseline
500nM Nociceptin
Nociceptin Wash-Off
5mV
2min
s
1sec

Fig 2.11 (Previous page) Traces showing the effect of Nociceptin treatment on $0Mg^{++}$ generated spontaneous epileptiform activity in Acute Hippocampal Slices. The traces on the right side represent expanded views of the traces that are shown on the left side of the page. The scale bar shows 2 values for the x-axis; the upper one refers to the left trace, and the lower to the right trace.

Although there is not a statistically significant reduction following Nociceptin administration there does appear to be an apparent decrease in bursting frequency following addition of 500nM Nociceptin.

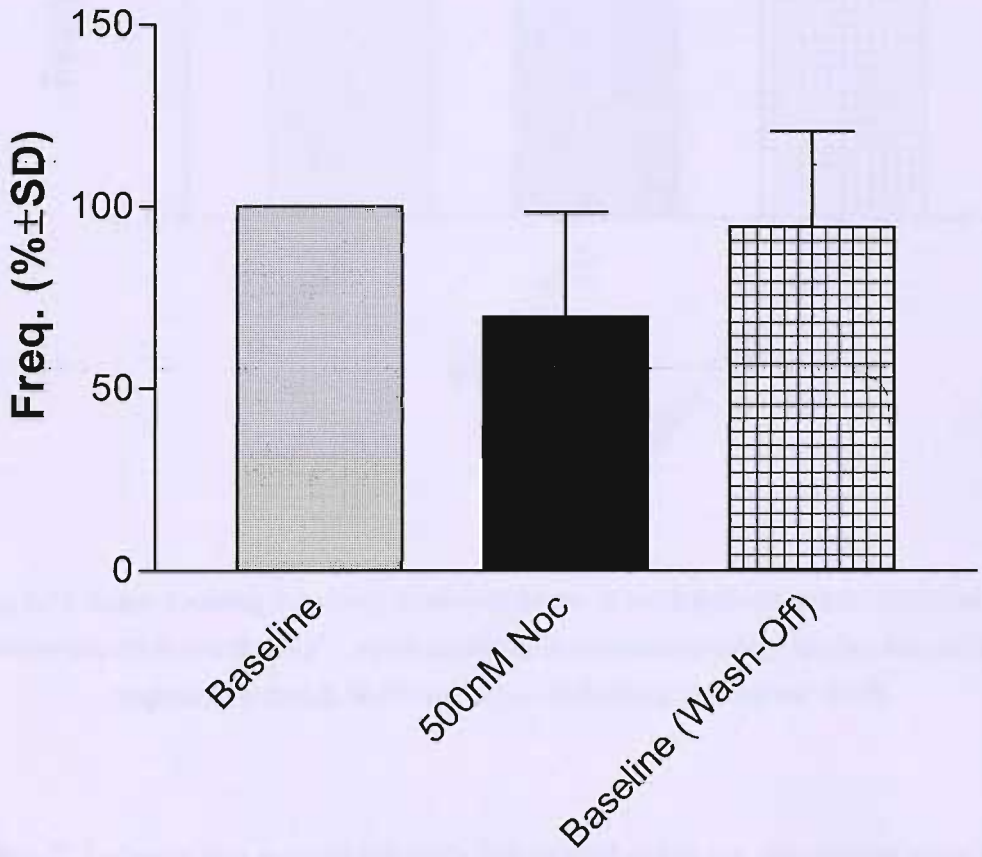


Fig 2.12 Graph showing the effect of varying doses of Nociceptin on Acute Hippocampal Slices pre-treated with $0Mg^{++}$. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=6).

The Wash-off data set shows that the bursting frequency increases towards that of baseline following the removal of Nociceptin from the perfusion medium.

Figure 2.13 confirms that the mean value of the bursting in the presence of 500nM Nociceptin is lower that that of Baseline and Wash-Off. It does not

however prove conclusive, as the error bars (standard deviations) are too large to draw any definitive results.

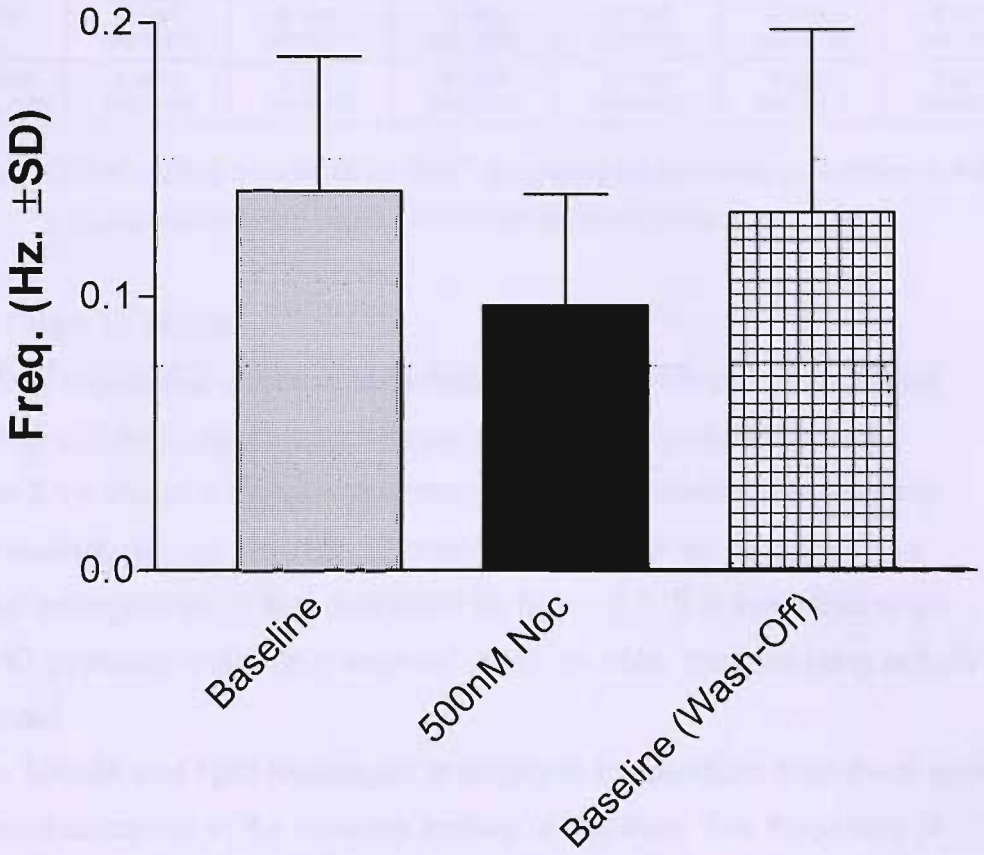


Fig 2.13 Graph showing the effect of varying doses of Nociceptin on Acute Hippocampal Slices pre-treated with $0Mg^{++}$. All frequencies are calculated using the raw data values originally recorded, and there is no normalising to baseline. (n=6).

Table 2.3 shows the individual data obtained for the six experiments carried out in the $0Mg^{++}$ model. It shows that in general the 500nM Nociceptin caused a reduction in bursting activity, and that this effect was reversed when Nociceptin was washed off the slice. Five out of six of the experiments performed showed a decrease in bursting frequency following the addition of the nociceptin. The only experiment where the wash off frequency was lower than that seen when Nociceptin was present, was experiment C. These two observations are not outside what would be considered acceptable from expected variation.

Exp. ID	A	B	C	D	E	F
Baseline	0.0683 (±0.0095)	0.1517 (±0.0166)	0.1650 (±0.0254)	0.0933 (±0.0117)	0.1517 (±0.0183)	0.2017 (±0.0053)
500nM Noc	0.0217 (±0.0137)	0.1450 (±0.0177)	0.0981 (±0.0386)	0.1033 (±0.0070)	0.1000 (±0.0572)	0.1117 (±0.0137)
Baseline (Wash-Off)	0.0617 (±0.0209)	0.1517 (±0.0095)	0.0867 (±0.0131)	0.1100 (±0.0086)	0.1233 (±0.0141)	0.2517 (±0.0123)

Table 2.3 Effect of Drug treatments on $0Mg^{++}$ generated spontaneous epileptiform activity in Acute Hippocampal Slices. Values are Means (of frequency in Hertz).

2.6 High K^+ Model

The final model that I utilised to investigate the effects of nociceptin on bursting activity in acute hippocampal slices was the High K^+ Model. Figure 2.14 shows a typical experiment where the effects of Nociceptin were investigated on the High K^+ model. The traces are arranged in a similar arrangement to that described for figure 2.5. It is seen that when High K^+ perfusion medium is washed on to the slice, then bursting activity is observed.

When 500nM and 1μM Nociceptin is added to the medium then there is no apparent decrease in the bursting activity of the slice. The frequency of bursting appears to remain constant when the slice is exposed to different drug treatments, and the wash off frequency is similar to that seen during baseline and Nociceptin treatments. The expanded view traces, on the right, show that throughout the experiment the size and shape of the bursting events remain consistent.

The graph in figure 2.15 shows the mean values for the various drug treatments performed on the High K^+ model for the six different experiments carried out.

The data shows that there is no significant change in the bursting activity when Nociceptin is added to the perfusion medium. The changes seen for the Nociceptin treatments are 95% that of baseline for 500nM Nociceptin and 96% for 1μM Nociceptin.

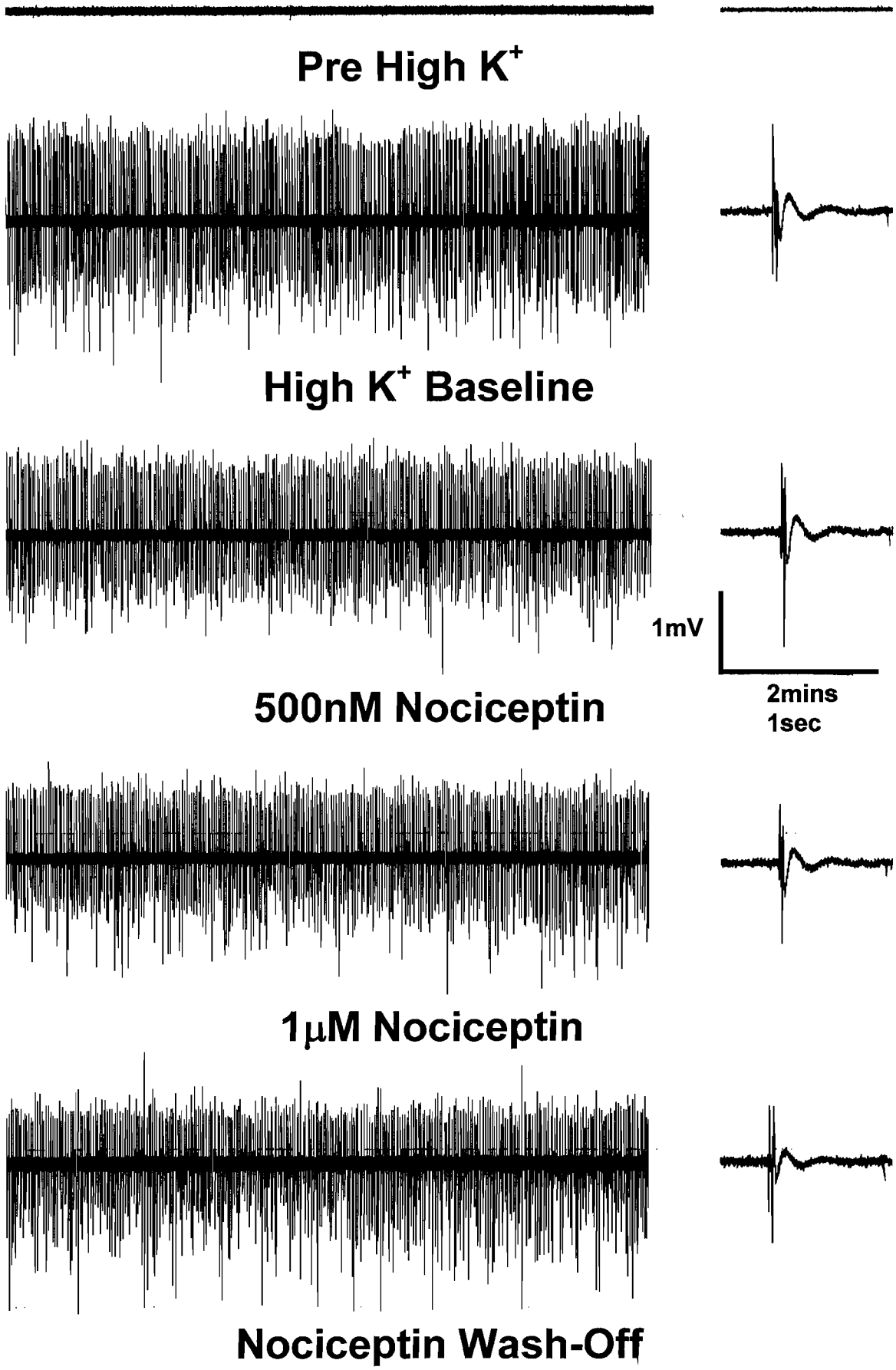

Pre High K+
High K+ Baseline
500nM Nociceptin
1mV
2mins
1sec
1μM Nociceptin
Nociceptin Wash-Off

Fig 2.14 (Previous page) Traces showing the effect of Nociceptin treatments on High K^+ generated spontaneous epileptiform activity in Acute Hippocampal Slices. The traces on the right side represent expanded views of the traces that are shown on the left side of the page. The scale bar shows 2 values for the x-axis; the upper one refers to the left trace, and the lower to the right trace.

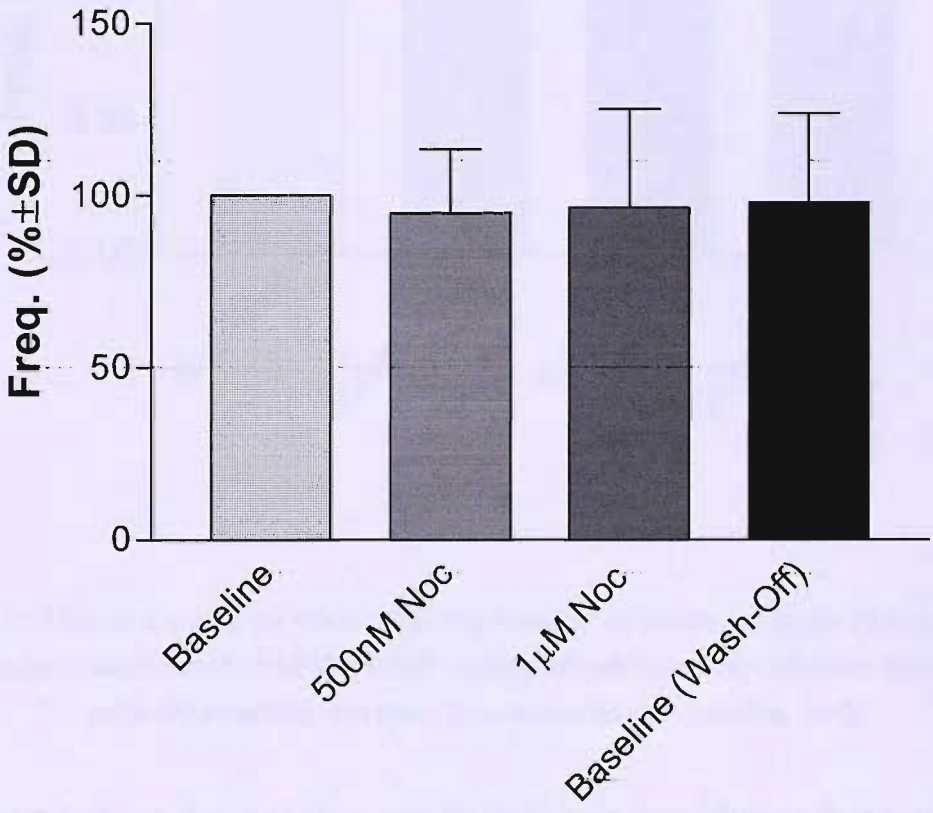


Fig 2.15 Graph showing the effect of varying doses of Nociceptin on Acute Hippocampal Slices pre-treated with 8.5mM K^+. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=6).

Figure 2.16 confirms the findings found in figure 2.15 that 500nM and 1μM Nociceptin do not show any effect on the activity generated by the High K^+ model of spontaneous bursting.

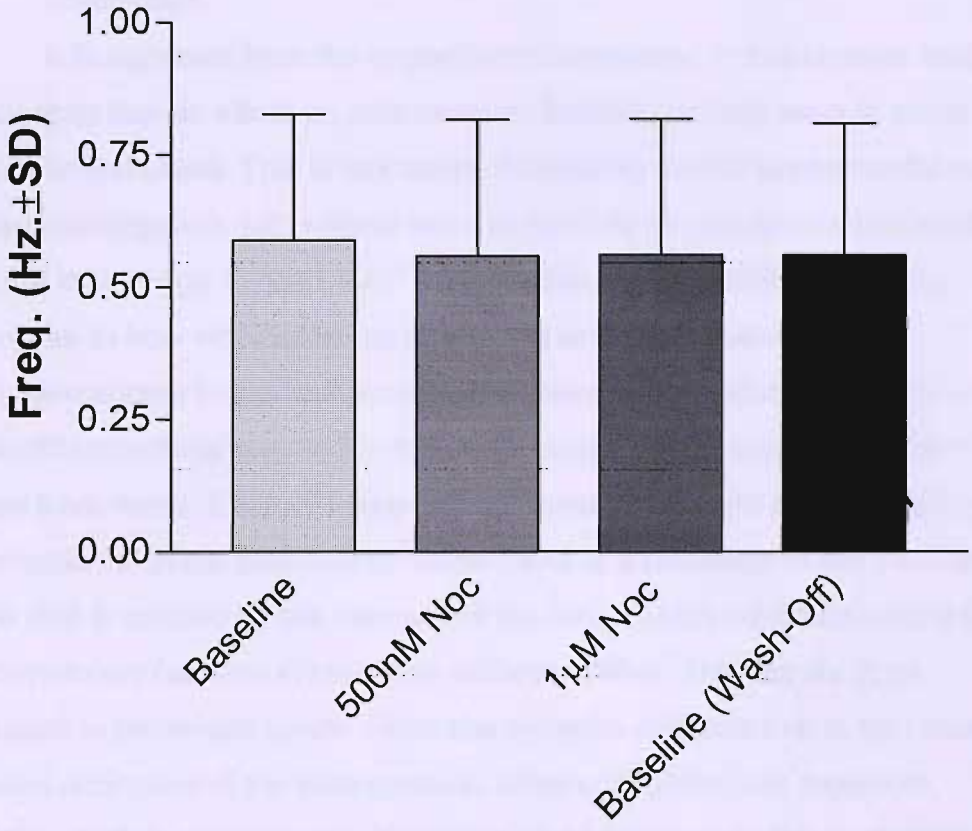


Fig 2.16 Graph showing the effect of varying doses of Nociceptin on Acute Hippocampal Slices pre-treated with 8.5mM K^+. All frequencies are calculated using the raw data values originally recorded, and there is no normalising to baseline. (n=6).

Table 2.4 shows the individual results for the six experiments that were carried out. It can be seen that, with the possible exception of experiments A and B, that all the frequencies stay the same, or increase, throughout the time course of the experiment .

Exp. ID	A	B	C	D	E	F
Baseline	0.2317 (±0.0328)	0.8550 (±0.1034)	0.6400 (±0.0473)	0.4100 (±0.2457)	0.5833 (±0.0603)	0.8133 (±0.0258)
500nM Noc	0.1933 (±0.0211)	0.6133 (±0.3942)	0.6200 (±0.0463)	0.4600 (±0.2391)	0.4900 (±0.0086)	0.9783 (±0.0209)
1µM Noc	0.1683 (±0.0166)	0.4817 (±0.4236)	0.6167 (±0.0283)	0.5500 (±0.1887)	0.5783 (±0.0249)	0.9700 (±0.0172)
Baseline (Wash-Off)	0.2183 (±0.0146)	0.4933 (±0.3490)	0.6567 (±0.0141)	0.5317 (±0.2061)	0.4917 (±0.0297)	0.9767 (±0.0417)

Table 2.4 Effect of Drug treatments on 8.5mM K^+ generated spontaneous epileptiform activity in Acute Hippocampal Slices. n=10 points for each treatment. Values are Means (of frequency in Hertz) ±Standard Deviation.

2.7 Discussion

It is apparent from the experiments presented in this chapter that Nociceptin has an effect on spontaneous bursting activity seen in acute hippocampal slices. This effect varies depending on the experimental model under investigation, with effects seen in the 0Mg++ and Bicuculline models but not in the High K+ and 4-AP models, this allows conclusions to be drawn as to how Nociceptin may cause its anti-convulsant actions.

As I have shown in the four models, that were investigated, spontaneous epileptiform activity resulted in the acute hippocampal slices exposed to these treatments. Each of these causes bursting brought about by differing mechanisms. In the Bicuculline model there is a reduction in the inhibitory drive that is caused by the removal of the tonic GABA inhibition onto the glutamatergic neurons (Traub and Jefferys, 1994). This results in an increase in glutamate levels within the synaptic cleft and this in turn causes greater activation of the post-synaptic ionotropic glutamate receptors (NMDA, AMPA and Kainate). This causes an increase in the excitability of the post-synaptic neuron and means that there is an increased probability of the neuron firing and thus causing spontaneous bursting activity, as seen in figure 2.5.

In the zero magnesium model, bursting activity results from the lack of magnesium ions in the extracellular space that results in the removal of a voltage dependent magnesium block from the NMDA receptor.

Under normal physiological conditions the magnesium block prevents ions from passing through the NMDA receptor while the membrane potential is at resting level, around –70mV. When glutamate is released into the synapse it then activates AMPA and Kainate receptors that allow sodium and potassium ions to pass through the receptor associated ion channels. When the neuron is near to its resting potential then the electrochemical gradient is such that the drive is towards sodium entering the cell, as opposed to potassium leaving the cell. The influx of sodium results in a depolarisation of the membrane. When this depolarisation begins it causes the magnesium ions to dissociate from the NMDA channel and so allows the passage of sodium, potassium and calcium ions to occur. This change happens at around –50mV and added to the continued passage of ions

through the AMPA and Kainate channels, results in an increase in the rate of depolarisation. When the membrane potential depolarises sufficiently to reach the threshold potential then the voltage dependent sodium channels open and this leads to the neuron firing an action potential.

The removal of the magnesium block in this model means that the NMDA receptor will allow the passage of ions in response to glutamate stimulation at more negative membrane potentials and this results in an increase in the amount of sodium and calcium ions that can pass into the cell for a given amount of glutamate and so will result in an increased probability of the neuron reaching its threshold potential and thus resulting in an increase in excitability of the tissue, which is seen in figure 2.11.

The 4-Aminopyridine model results from a blockade of K^+ channels by 4-AP (Traub et al., 1995). This means that potassium ions are unable to leave the neurons during repolarisation. This leads to a depolarisation of the neuron that means that the neuron is nearer to threshold and so more likely to result in an action potential for a given signal received at the post-synaptic terminal on the neuron. This results in spontaneous bursting activity as seen in figure 2.8

The high potassium model results from a similar mechanism to that which causes the epileptiform activity in the 4-Aminopyridine model. In the high potassium model the elevation of the extracellular potassium concentration from 3mM to 8.5mM results in a change in the resting potential of the neuron, due to a change in the ionic balance of the potassium. This again results in the resting potential of the membrane being nearer to that of the threshold potential than would be observed when the normal aCSF concentration of potassium is used, this causes spontaneous epileptiform activity as seen in figure 2.14.

The modes of action described above explain how the models used bring about an increase in neuronal excitability that presents as an increase in burst frequency. How do the results seen in this chapter for Nociceptin fit with these?

It has been shown that in three of the models investigated; Bicuculline, 0Mg++ and 4-AP; a NMDA receptor antagonist (APV) blocked the frequency of bursting seen in these models (Gulyas-Kovacs et al., 2002).

This would seem to suggest that the NMDA receptor would not be the direct target of Nociceptin because two of these models were blocked by Nociceptin and the other one was not.

Nociceptin is the endogenous ligand for the OP4 receptor. This receptor has been shown to be present in the CA1-CA4 fields of Ammon's Horn and in the dentate gyrus of the hippocampus within rodents (Anton et al., 1996;Florin et al., 1997;Foddi and Mennini, 1997;Neal, Jr. et al., 1999a) and OP4 receptor transcripts have also been found in the hippocampus of human brain tissue (Peluso et al., 1998).

The physiological presence of OP4 has been demonstrated in rat cerebrocortical slices where Nociceptin inhibits the potassium-evoked release of glutamate (Nicol et al., 1996).

The transduction mechanism by which the OP4 receptor brings about its actions has been investigated. It has been found that OP4 is linked to the G_i/G_0 class of G-proteins. Nociceptin causes an inhibition in forskolin-stimulated cAMP production (Okawa et al., 1998) and it has also been shown that Nociceptin is able to inhibit the elevation in cAMP levels caused by other receptor driven systems (e.g. D1 dopamine receptor) (Chan et al., 1998).

Nociceptin has been demonstrated to increase potassium conductance in rat brain (Connor et al., 1996a;Vaughan and Christie, 1996;Vaughan et al., 1997) and to stimulate a Pertussis Toxin sensitive inhibition of Ca^{2+} channel currents in an OP4 expressing neuroblastoma cell line (Connor et al., 1996b) and in the hippocampus (Knoflach et al., 1996).

There is some evidence that opioid receptor activation can lead to regulation of a membrane potassium current (I_h), via changes in the level of cAMP (Ingram and Williams, 1994;Ludwig et al., 1998). The potassium channel is involved in the repolarisation of the membrane following an action potential and so could play a role in any anti-convulsant activity of Nociceptin, cAMP has been shown to enhance the potassium conductance of this channel (Ingram and Williams, 1996).

The channels that cause the I_h potassium current have been identified as a family of ion channel subunits that have been identified as the hyperpolarisation-activated cyclic-nucleotide gated (HCN) channels

(Santoro et al., 2000). The HCN channels allow K^+ ions to depolarise hippocampal neurons when hyperpolarisation occurs (Simeone et al., 2005) with a half-activation voltage of –81.3mV.

There is also some evidence that OP4 receptor activation may cause stimulation of Phospholipase C via G_q (Lou et al., 1997).

Drugs that effect cAMP levels have been shown to affect the duration of after discharges in rat hippocampal slices exposed to tetanic stimulation. Forskolin, which increases cAMP levels, causes an increase in the number of after discharges observed (Higashima et al., 2002).

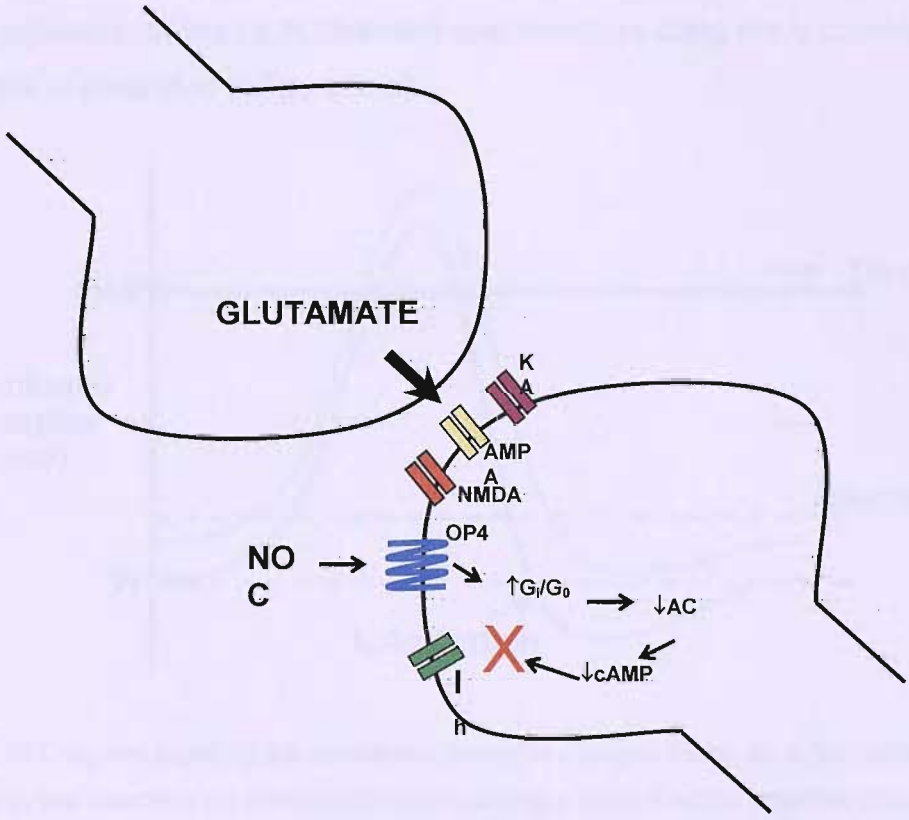


Fig 2.17 Diagram of the possible coupling of the OP4 receptor to the I_h potassium channels. All three types of Ionotropic glutamate receptors are represented on the postsynaptic membrane.

As I have mentioned above, activation of OP4 receptors can lead to a decrease in cAMP levels. This in turn leads to a decrease in the conductance of the I_H potassium current, which would slow down the rate of

membrane repolarisation following an action potential (see figure 2.17 for illustration).

This could cause the effects seen in the Bicuculline and Zero Mg^{++} model where 1μM Nociceptin caused a reduction in the frequency of bursting. As the High K^+ and 4-Aminopyridine models rely on changes in potassium ion concentrations to bring about their epileptiform activity then this may explain why Nociceptin does not have any effect in these systems. As these two models rely on causing an increase in the resting potential of the neuron, then it follows that the K^+ I_H current will have less of a role to play as there is less chance that the membrane will reach the potential required to bring about activation of the HCN channels and therefore bring the I_h current into play, this is illustrated in figure 2.18.

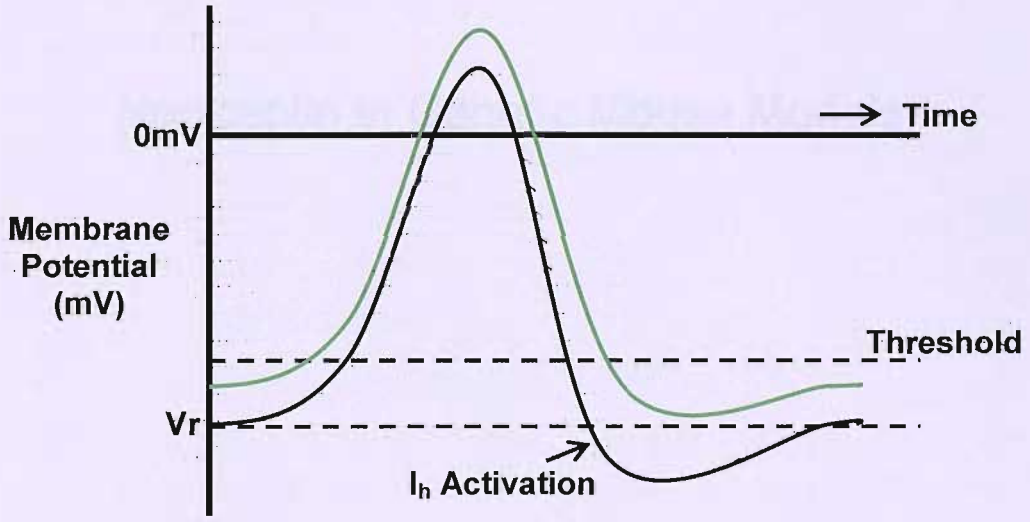


Fig 2.18 Diagram depicting the membrane potential changes during an action potential. The black line indicates the observed changes during a normal action potential that applies to the Bicuculline and 0Mg++ models. The green line represents a possible trace seen from the High K+ and 4-AP models. V_r = resting membrane potential in normal neuronal tissue.

Chapter 3

Nociceptin in Genetic Mouse Models

3.1 Introduction.

As described in the introduction there are numerous genetic models where the animals show epileptic type behaviours. This can be the result of either transgenic manipulation or by selectively breeding animals to emphasise particular traits.

One such example of this selective breeding is the EL mouse. The El mouse is a model of hereditary sensory precipitated temporal lobe epilepsy (see (King, Jr. and LaMotte, 1989) for review) which is bred from a ddY mouse strain. The El mouse was first described in 1959 (Imaizumi et al., 1959) following discovery in 1954. Since this time many studies have been carried out on this model to investigate the cause and function of the epileptiform activity exhibited by these animals.

EL mice display spontaneous epileptiform activity following a conditioning period. This conditioning period requires that the animals receive weekly vestibular stimulation (for example 30 vertical, 10-15cm, 'tosses'). These mice have been selectively inbred, from a ddY 'mother strain', to maximise the tonic-clonic seizures induced by vestibular stimulation. When the animals undergo the conditioning treatment they display characteristic prodromal, ictal and postictal phases. The prodromal phase consists of squeaking and transient immobility, which is followed by 'running fits'. The ictal phase presents as convulsions, which start with the hind limbs before quickly progressing to generalized tonic-clonic; salivation, defacation, urination and ventroflexion. The final postictal phase comprises lethargy and adoption of the 'kangaroo posture' (sitting on hind legs with paws drawn up to torso). The average duration of the seizure in one group of animals was 16±3s (Naruse et al., 1960) and the animals displayed a refractory period following seizures where no further seizures could be induced. Although this refractory period varied between animals, it was always a minimum of 30 minutes. Along with the 'sensitised' animals that were given the pre-conditioning treatment there was a group of age-matched 'non-sensitised' animals.

As the conditioning treatment, carried out on the sensitised animals, causes a seizure then the animals used in this series of experiments will have experienced a prolonged period of seizures by the time that their hippocampi are used for the electrophysiology experiments. This will mean that the animals will have undergone a treatment that may be similar in effect to prolonged kindling.

Although the stimulation of normal tissue, as has been shown in the previous chapter, to create a convulsant state is a very useful model of epileptiform activity and can help to investigate the mechanism of action, I was interested in seeing if there would be any difference in the action of Nociceptin, as an anticonvulsant, if it is administered to tissue from a mutant mouse with a lower seizure threshold.

In order to achieve this I used slices from EL mice and exposed them to the different convulsants' to induce epileptiform activity.

3.2 Materials and Methods

The experiments presented in this chapter were carried out in a similar way to those performed in chapter 2, the only major difference is that the tissue is prepared from EL mice that were between 18-22 weeks old, and had undergone conditioning for about 15-20 weeks. During the conditioning period the animals were thrown up and down vertically (10-15cm) 30 times on one day each week.

Age matched controls (non-sensitised) were also used and their tissue was prepared in a similar manner.

I choose to use a concentration of 1μM Nociceptin in this series of experiments as the experiments carried out in the previous chapter showed that this is the concentration that causes the most reliable and significant effect. I also choose to use the 10μM Bicuculline and $0Mg^{++}$ aCSF convulsant models as in the previous chapter Nociceptin had reduced the bursting activity in these two models.

As in the previous chapter each treatment was carried out for a period of 30 minutes. This meant that once spontaneous activity was seen in the slice then the bursting would be recorded for 30 minutes, following

this 1μM Nociceptin was added to the perfusion medium for a further 30 minutes, and once this period was up then the Nociceptin was removed from the medium and the slice recorded for a final 30 minutes.

3.2.1 Data Analysis

Data analysis is similar to that carried out in chapter 2. The difference being that the bursting frequency is measured for a period of 5 minutes between 25-30 following the introduction of a treatment. A frequency for this time period is then calculated and the values are then either worked out, as a percentage of the baseline and averaged or the means are worked out directly from the raw frequencies.

3.3 Nociceptin Results

3.3.1 Bicuculline Model (Non-Sensitised Animals)

The next experiments were performed on tissue taken from the 'non-sensitised' age matched controls. These animals had not undergone the conditioning period and so were not prone to spontaneous seizures.

Figure 3.1 shows the traces for a typical experiment from this group. The baseline before Bicuculline addition to the slice shows that there is no spontaneous activity in the slice at the start of the experiment.
When 10μM Bicuculline is added to the slice then bursting behaviour results and settles into a regular pattern. When 1μM Nociceptin is added to the perfusion medium no noticeable change in the burst frequency is observed and no changes are seen when the Nociceptin is subsequently removed from the slice. The right hand traces show that throughout the experiment there are no changes in the burst events that take place.

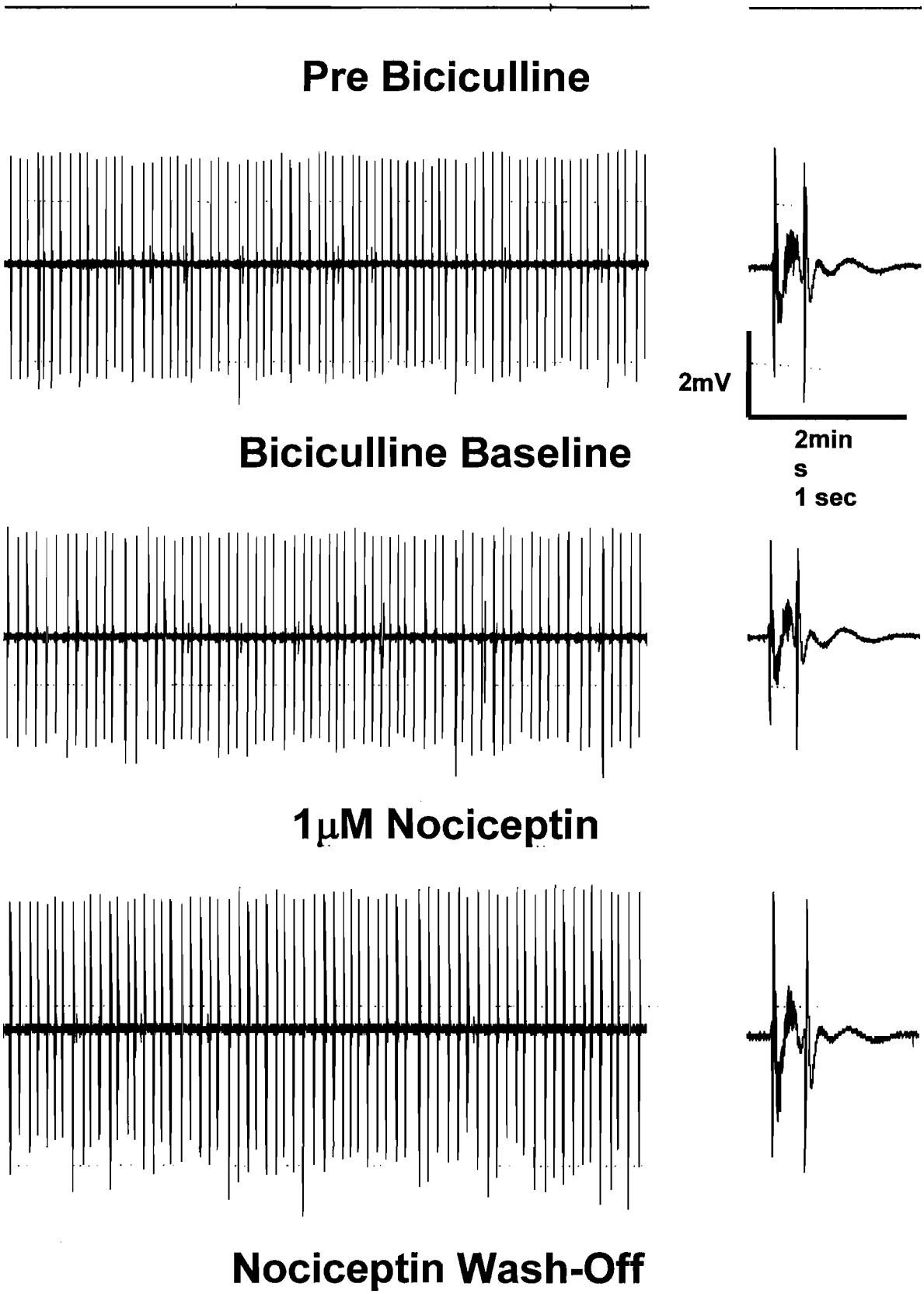

Pre Biciculline
2mV
2min
s
1 sec
Biciculline Baseline
1μM Nociceptin
Nociceptin Wash-Off

Fig 3.1 (previous page) Traces showing the effect of 1µM Nociceptin treatment on 10µM Bicuculline generated spontaneous epileptiform activity in Acute Hippocampal Slices from 'non-sensitised' EL mice. The traces on the right side represent expanded views of the traces that are shown on the left side of the page. The scale bar shows 2 values for the x-axis; the upper one refers to the left trace, and the lower to the right trace.

Figure 3.2 shows the mean data for this group of experiments, and confirms the results seen in the individual traces. That is that there is no significant effect of the Nociceptin on the bursting behaviour. There does appear to be a slight reduction in the activity following addition of 1µM Nociceptin, 77% as compared to baseline, but this effect is not statistically significant. When the Nociceptin is washed off the mean burst frequency increases very slightly to 83% of that seen in the baseline responses.

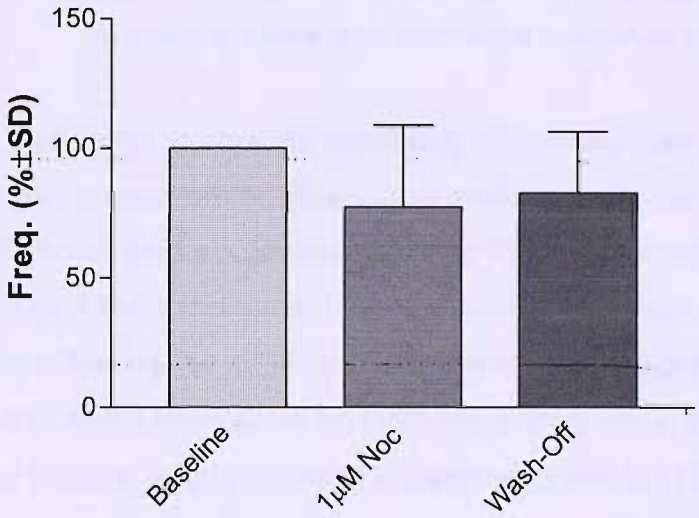


Fig 3.2 Graph showing the effect of 1µM Nociceptin on EL mice (non-sensitised) acute Hippocampal Slices pre-treated with 10µM Bicuculline. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=8).

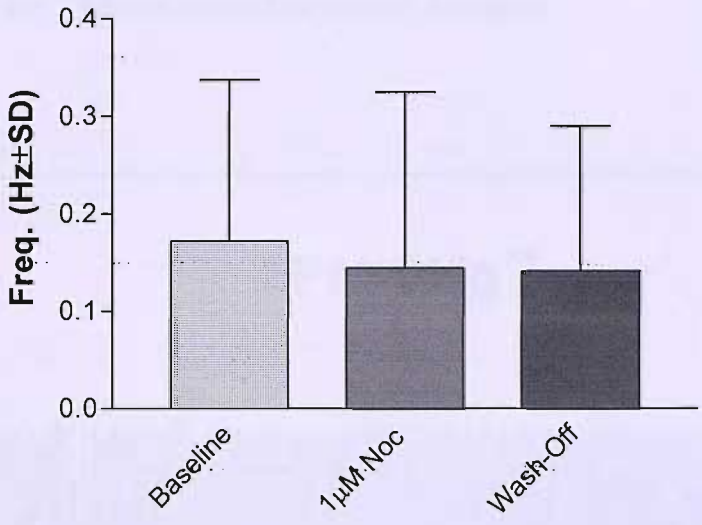


Fig 3.3 Graph showing the effect of 1µM Nociceptin on EL mice (non-sensitised) Acute Hippocampal Slices pre-treated with 10µM Bicuculline. All values are those originally recorded, and there is no normalising to baseline. (n=8).

Figure 3.3 confirms the results previously shown in figure 3.2, that no effect of Nociceptin is seen on the Bicuculline model in 'non-sensitised' EL mice. Table 3.1 shows the frequencies recorded from the individual experiments in this group. Although a couple of the experiments show a large reduction in bursting following the addition of Nociceptin, the majority show little if any change, and some even show an increase in frequency. The baseline frequency induced in this series of experiments was 0.172±0.165Hz.

Exp. ID	02708	02709	02710	02711a	02712	02621	02730a	02620
Baseline	0.103 100%	0.067 100%	0.183 100%	0.160 100%	0.147 100%	0.063 100%	0.567 100%	0.087 100%
1µM Noc	0.027 25.806%	0.037 55%	0.077 41.818%	0.130 81.250%	0.140 95.455%	0.070 110.526%	0.580 102.353%	0.090 103.846%
Wash-Off	0.040 38.710%	0.060 90%	0.103 56.364%	0.127 79.167%	0.137 93.182%	0.070 110.526%	0.500 88.235%	0.090 103.846%

Table 3.1 Effect of 1µM Nociceptin treatment on 10µM Bicuculline generated spontaneous epileptiform activity in Acute Hippocampal Slices from 'non-sensitised' EL mice. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

3.3.2 0 Mg^{++} Model (Non-Sensitised Animals)

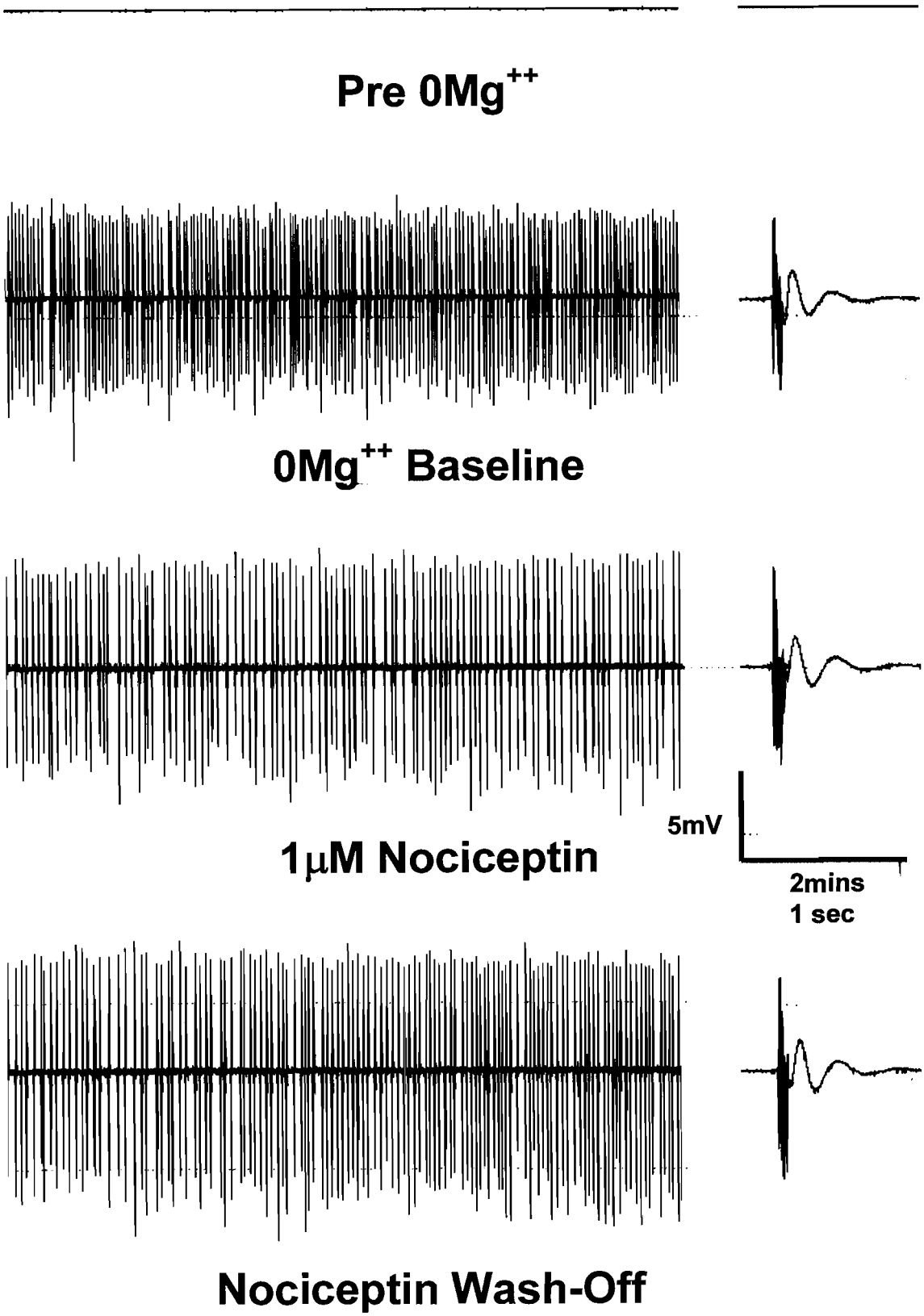


Fig 3.4 (previous page) Traces showing the effect of 1μM Nociceptin treatment on 0 Mg^{++} generated spontaneous epileptiform activity in Acute Hippocampal Slices from 'non-sensitised' EL mice. The traces on the right side represent expanded views of the traces that are shown on the left side of the page. The scale bar shows 2 values for the x-axis; the upper one refers to the left trace, and the lower to the right trace.

Figure 3.4 shows the typical traces seen in the series of experiments carried out in the zero magnesium model in 'non-sensitised' EL mice. The traces show that at the start of the experiment no spontaneous bursting is evident in the slice. Following the removal of magnesium from the perfusion medium the slice begins to display marked bursting activity. This activity is reduced by the addition of 1μM Nociceptin to the slice, although the bursting is still very high. When the Nociceptin is removed from the slice then the frequency of bursting appears to increase. The right hand traces show that the bursting events recorded during the experiment appear to stay the same throughout the duration of the experiment.

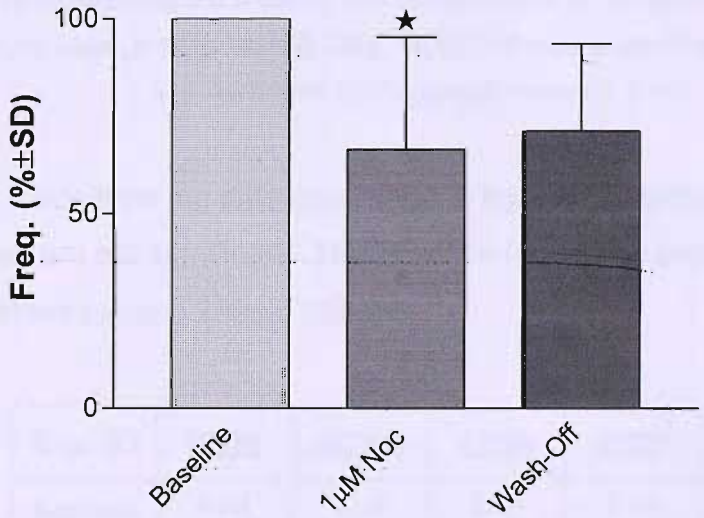


Fig 3.5 Graph showing the effect of 1μM Nociceptin on EL mice (non-sensitised) acute Hippocampal Slices pre-treated with 0Mg^{++} aCSF. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. *=$p<0.05$ (n=5).

Figure 3.5 shows the mean data for this series of experiments. The graph shows that when 1μM Nociceptin is added to the perfusion medium then the frequency of bursting reduces, 66% that of baseline, and this reduction is

significant at $p<0.05$. When the Nociceptin is removed from the slice the bursting increases slightly, 71% that of baseline, and the mean data is now not significant as compared to the baseline response.

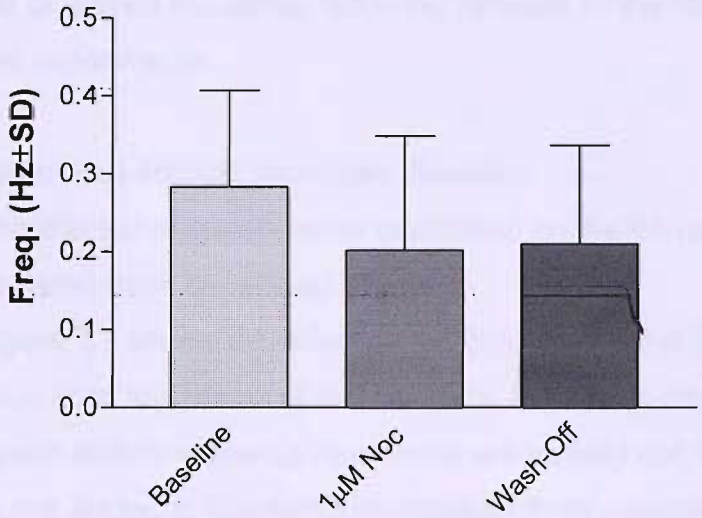


Fig 3.6 Graph showing the effect of 1µM Nociceptin on EL mice (non-sensitised) Acute Hippocampal Slices pre-treated with $0Mg^{++}$ aCSF. All values are those originally recorded, and there is no normalising to baseline. (n=5).

Figure 3.6 confirms the data presented in figure 3.15 although the responses are not significant. The baseline frequency induced in this series of experiments was 0.283±0.123Hz.

Exp. ID	02709	02711b	02726	02729	02731
Baseline	0.357 100%	0.197 100%	0.203 100%	0.193 100%	0.467 100%
1µM Noc	0.230 64.486%	0.067 33.898%	0.087 42.623%	0.190 98.276%	0.433 92.857%
Wash-Off	0.273 76.636%	0.087 44.068%	0.107 52.459%	0.190 98.276%	0.393 84.286%

Table 3.2 Effect of 1µM Nociceptin treatment on 0 Mg^{++} generated spontaneous epileptiform activity in Acute Hippocampal Slices from 'non-sensitised' EL mice. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

Table 3.2 shows the individual results for all the experiments in this group and makes it clear that 1μM Nociceptin caused a reduction in bursting, to a greater or lesser extent, in all the slices. The frequency of bursting then increased or stayed the same, following removal of the Nociceptin, in all but one of the experiments.

3.3.3 Bicuculline Model (Sensitised Animals)

The first set of experiments performed on the EL mice was done in slices prepared from 'sensitised' animals.

Figure 3.7 shows the effect of the Bicuculline and Nociceptin on a typical slice from this series of experiments. Firstly the slice shows no spontaneous activity when no treatments are carried out. When 10μM Bicuculline is added to the perfusion medium then spontaneous activity is observed and this activity reduces when 1μM Nociceptin is added to the perfusion medium along with the Bicuculline. Following removal of the Nociceptin from the slice then the activity increases, although it does not appear to be as great as that observed in the Bicuculline baseline trace. The right hand traces show that throughout the experimental period the size and shape of the bursting activity remains constant.

Figure 3.8 and 3.9 show the mean data for all nine experiments that were carried out. Fig 3.8 shows the normalised data where each data set is calculated as a percentage of its baseline value before a mean value for all the experiments is calculated. This graph shows that 1μM Nociceptin causes a reduction in the frequency of bursting to 50% of that seen in the baseline response. This effect is significant at a value of $p<0.01$. When the Nociceptin is washed off the slice the bursting comes back slightly, 60% as compared to baseline, but this effect is still significantly lower than seen in the baseline treatment.

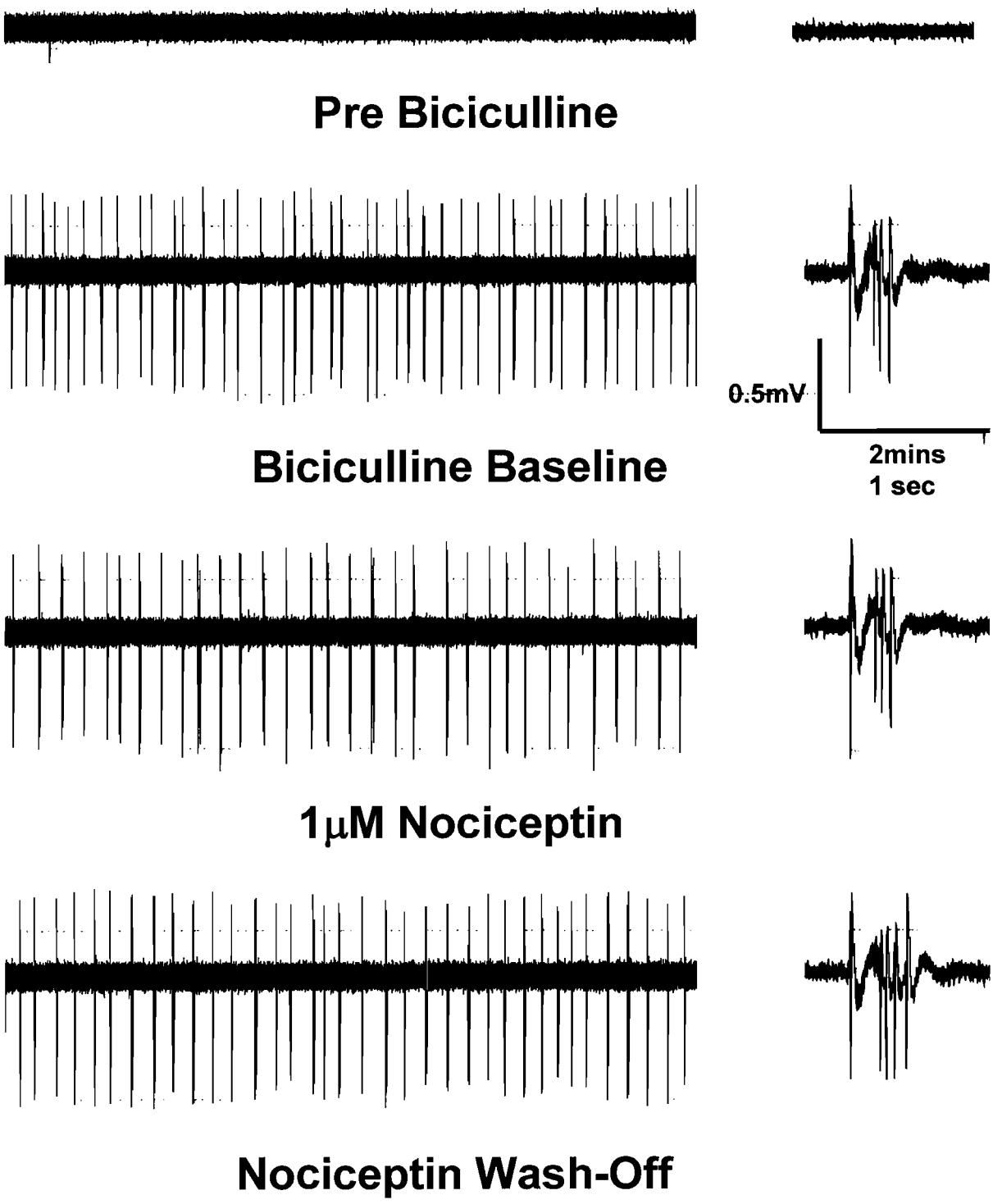


Fig 3.7 Traces showing the effect of 1μM Nociceptin treatment on 10μM Bicuculline generated spontaneous epileptiform activity in Acute Hippocampal Slices from 'sensitised' EL mice. The traces on the right side represent expanded views of the traces that are shown on the left side of the page. The scale bar shows 2 values for the x-axis; the upper one refers to the left trace, and the lower to the right trace.

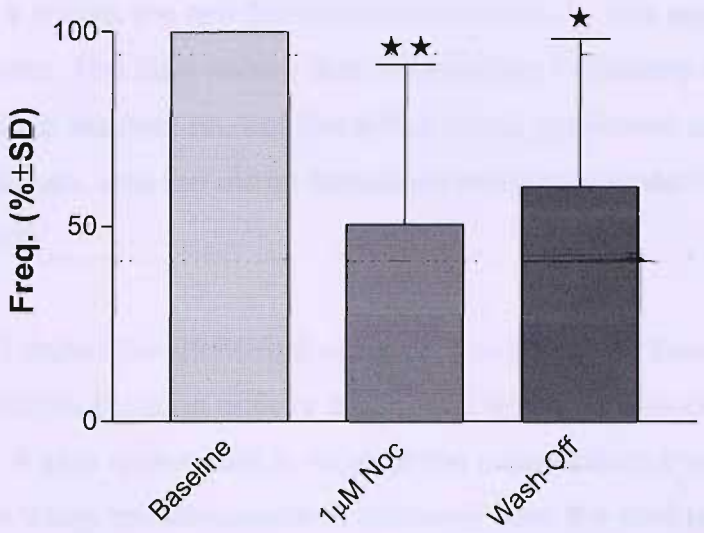


Fig 3.8 Graph showing the effect of 1μM Nociceptin on EL mice ('sensitised') Acute Hippocampal Slices pre-treated with 10μM Bicuculline. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. *=p<0.05 **=p<0.01 (n=9).

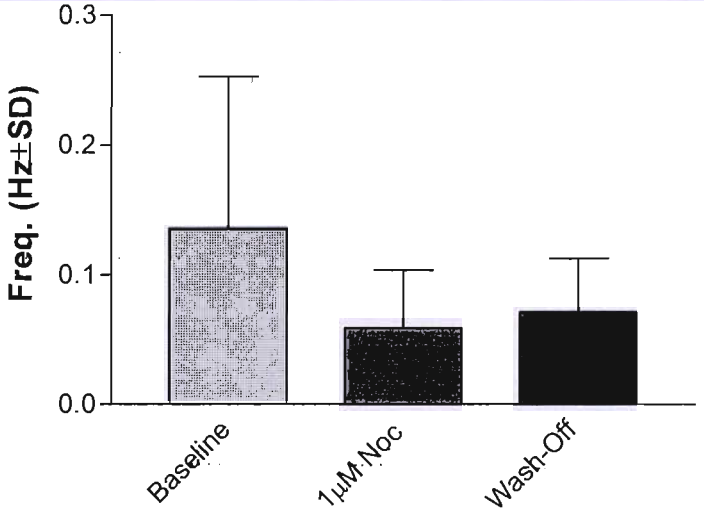


Fig 3.9 Graph showing the effect of 1μM Nociceptin on EL mice ('sensitised') Acute Hippocampal Slices pre-treated with 10μM Bicuculline. All values are those originally recorded, and there is no normalising to baseline. (n=9).

Figure 3.9 shows the raw frequencies recorded for this series of experiments. The data shows that the bursting frequency reduces when Nociceptin is washed on, but this effect is not significant under these circumstances, and the mean frequency increases when Nociceptin is washed off.

Table 3.3 shows the individual values recorded for all these experiments. It shows that the bursting activity decreases when 1μM Nociceptin is added to the slice. It also shows that in most of the experiments the bursting activity increases when the Nociceptin is removed from the perfusion medium. The baseline frequency induced in this series of experiments was 0.135±0.118Hz.

Exp. ID	**02723**	**02724**	**02725**	**02618**	**02703**	**02704**	**02705**	**02701**	**02702**
Baseline	0.073 100%	0.443 100%	0.113 100%	0.083 100%	0.073 100%	0.133 100%	0.070 100%	0.117 100%	0.107 100%
1μM Noc	0.003 4.545%	0.103 23.308%	0.093 82.353%	0.063 76.000%	0.067 90.909%	0.000 0.000%	0.003 4.762%	0.110 94.286%	0.083 78.125%
Wash-Off	0.010 13.636%	0.120 27.068%	0.103 91.176%	0.083 100%	0.063 86.364%	0.057 42.500%	0.003 4.762%	0.113 97.143%	0.083 78.125%

Table 3.3 Effect of 1μM Nociceptin treatment on 10μM Bicuculline generated spontaneous epileptiform activity in Acute Hippocampal Slices from 'sensitised' EL mice. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

3.3.4 0 Mg^{++} Model (Sensitised Animals)

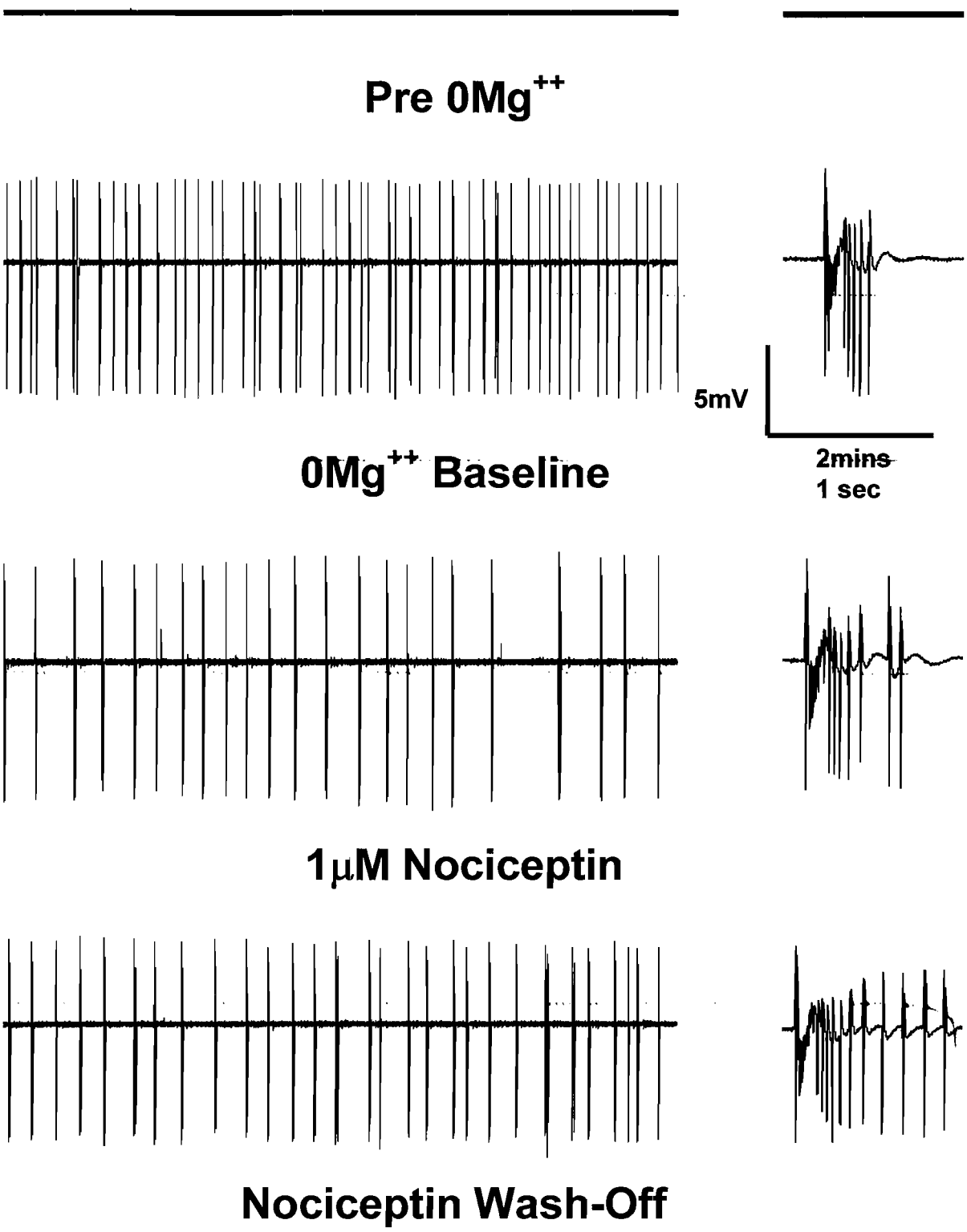


Fig 3.10 (previous page) Traces showing the effect of 1μM Nociceptin treatment on 0 Mg^{++} generated spontaneous epileptiform activity in Acute Hippocampal Slices from 'sensitised' EL mice. The traces on the right side represent expanded views of the traces that are shown on the left side of the page. The scale bar shows 2 values for the x-axis; the upper one refers to the left trace, and the lower to the right trace.

Figure 3.10 shows the traces taken from a typical experiment is this group. The tissue from the 'sensitised' EL mice shows no activity in the first trace, but when the magnesium is removed from the perfusion medium bursting activity results.

When 1μM Nociceptin is added to the slice then the frequency of bursting reduces and this effect is reversed when the Nociceptin is washed off.

The right hand traces show that over the course of the experiment the number of after discharges associated with the burst events increases.

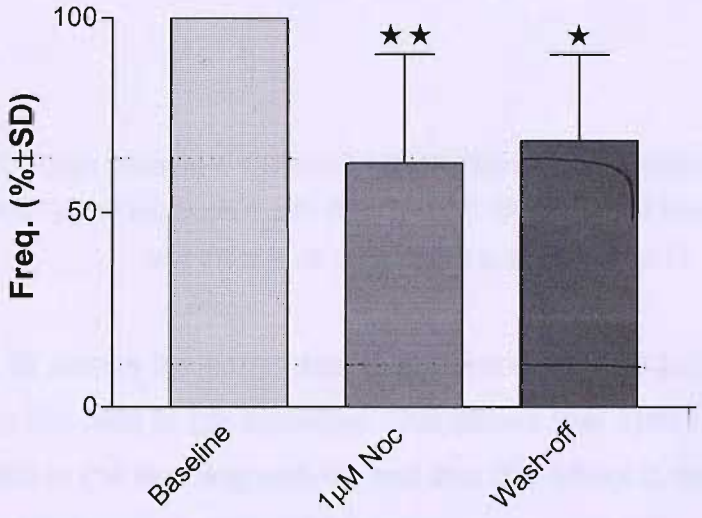


Fig 3.11 Graph showing the effect of 1μM Nociceptin on EL mice ('sensitised') Acute Hippocampal Slices pre-treated with 0Mg^{++} aCSF. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. *=p<0.05 **=p<0.01 (n=7).

Figure 3.11 confirms the results seen in the individual experiment displayed in fig 3.10. When 1μM Nociceptin is washed on to the slices then a mean reduction in activity is seen, 63% as compared to baseline, and this effect is significant at $p<0.01$. In a similar result to that seen in the Bicuculline model the effect is not totally washed off when the Nociceptin is removed from the slice, 68% as compared to baseline.

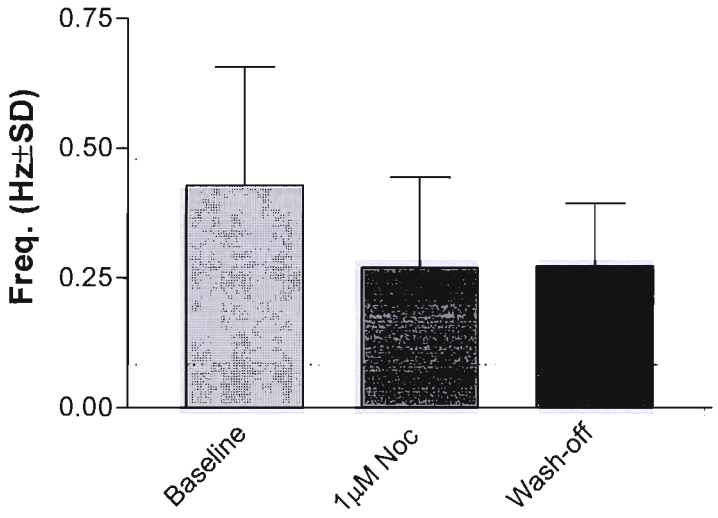


Fig 3.12 Graph showing the effect of 1μM Nociceptin on EL mice ('sensitised') Acute Hippocampal Slices pre-treated with 0Mg^{++} aCSF. All values are those originally recorded, and there is no normalising to baseline. (n=7).

Figure 3.12 shows the same data displayed in fig 3.11 but does not normalise the data to the baseline. This shows that 1μM Nociceptin causes a reduction in the bursting activity and that this effect is not washed off when the Nociceptin is removed from the slice.

Table 3.4 shows the individual data for all seven experiments carried out on the 0 Mg^{++} model in 'sensitised' EL mice.

This shows that the 1μM Nociceptin causes a reduction in bursting activity in all the experiments carried out. However, the removal of the Nociceptin did not result in an increase in burst frequency and in fact half the experiments showed a further reduction in frequency following Nociceptin

removal. The baseline frequency induced in this series of experiments was 0.429±0.228Hz.

Exp. ID	02722	02723	02724	02725	02704	02614	02617
Baseline	0.097 100%	0.563 100%	0.753 100%	0.287 100%	0.377 100%	0.623 100%	0.300 100%
1μM Noc	0.043 44.828%	0.303 53.846%	0.430 57.080%	0.270 94.186%	0.060 15.929%	0.513 82.353%	0.270 90%
Wash-Off	0.060 62.069%	0.283 50.296%	0.327 43.363%	0.313 109.302%	0.223 59.292%	0.460 73.797%	0.243 81.111%

Table 3.4 Effect of 1μM Nociceptin treatment on 0 Mg^{++} generated spontaneous epileptiform activity in Acute Hippocampal Slices from 'sensitised' EL mice. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

3.4 Discussion.

I have seen in this chapter that when the convulsant model, either bicuculline or zero Mg^{++}, is performed then convulsant activity results in both the slices from sensitised and non-sensitised mice. The frequency of the bursting activity seen is close to the same whether sensitised or non-sensitised tissue is used. This shows that even though the non-sensitised animals do not undergo the conditioning treatment, they still show a similar frequency of bursting to that seen in the sensitised mice.

It has been shown that in EL mice there is a disinhibition in the dentate gyrus (Ono et al., 1997) and paired pulse facilitation in the CA3 area (Fueta et al., 1998) of hippocampal slices, as compared to ddY wild-type mice. The results presented in this chapter demonstrate that Nociceptin has effects on the zero magnesium and Bicuculline models of epileptiform activity when carried out in hippocampal slices taken from EL mice.

When the Nociceptin is administered to slices exposed to the Bicuculline bursting model there was a difference between the slices taken from 'sensitised' (fig 3.8) and 'non-sensitised' (fig 3.2) EL mice. In the slices prepared from 'sensitised' animals the Nociceptin was able to reduce the bursting by a significant amount when it was administered at a

concentration of 1μM, whereas in the slices from 'non-sensitised' mice although there was a reduction in the bursting activity it was not by a significant amount.

In the Zero Magnesium model, slices from both the 'sensitised' (fig 3.11) and the 'non-sensitised' (fig 3.5) mice showed a reduction in bursting activity following administration of 1μM Nociceptin. However, the effect on the slices from 'sensitised' animals was more pronounced and this effect was significant to a greater level than for the slices from 'non-sensitised' animals.

These results seem to indicate that the slices from 'sensitised' EL mice were more susceptible to the anti-convulsant action of Nociceptin than the 'non-sensitised' EL mice.

The results also show that the baseline frequency of bursting induced by both the Bicuculline and the Zero Mg++ model did not differ markedly between the slices from 'sensitised' and 'non-sensitised' animals.

It is interesting to note that although these animals are more prone to seizures throughout their lives the baseline frequency induced by the epilepsy models does no markedly differ between the 'sensitised' and 'non-sensitised' animals and despite this the Nociceptin still exhibits a tendency towards causing a greater reduction in bursting behaviour within the 'sensitised' animals.

One possibility for this effect could be that due to the seizures that the 'sensitised' mice have been exposed to over a number of months, Nociceptin may play a role as an endogenous anti-convulsant to mediate this condition.

If this is so then the ORL-1 receptors may be up-regulated and so the tissue will have an increased susceptibility to the addition of exogenous Nociceptin.

The slices taken from 'sensitised' animals will have been subjected to repeated seizures throughout their lives in a similar way to animals exposed to the kindling model of epileptiform activity and the EL mice model may have similarities to the kindling model.

Chapter 4

Nociceptin and Neuropeptide Y in Organotypic Hippocampal Slice Cultures

4.1 Introduction.

Slice cultures of brain slices provide a useful method for the *in vitro* investigation of physiological, pathological and pharmacological situations. Since their initial introduction over 20 years ago (Gahwiler, 1981) they have gone on to become invaluable tools for the study of brain function. In the intervening years, modifications to the culturing techniques have been carried out that allow more reliable and robust cultures to be produced (Gahwiler et al., 1997;Gahwiler, 1988).

Cultures of hippocampal slices have been around for a large part of this time and provide a useful model for the investigation of conditions relevant to this structure. During the culture period hippocampal slices have been proven to show normal neuronal organisation (Beach et al., 1982;Gahwiler, 1984b;Gahwiler, 1984a;Frotscher and Gahwiler, 1988;Caeser and Aertsen, 1991;Stoppini et al., 1991;Bahr et al., 1994;Kunkel et al., 1994) and also demonstrate the formation of synaptic connections (Gahwiler, 1984a;Buchs et al., 1993;Muller et al., 1993;Robain et al., 1994;Debanne et al., 1995;Frotscher et al., 1995). It has also been shown that in long term hippocampal slices, up to 4 weeks in culture, NMDA and AMPA receptors and also several synapse-related proteins are maintained at levels comparable to that seen in equivalent adult rats (Bahr et al., 1995). Cultures prepared according to the interface method (Stoppini et al., 1991;Bahr, 1995) have also been shown to express normal synaptic waveforms and develop long term potentiation in an apparently identical manner to that seen in acute slice or in vivo preparations (Bahr et al., 1994;Muller et al., 1993;Vanderklish et al., 1995;Vanderklish et al., 1992;Stoppini et al., 1991).

The use of organotypic cultures to investigate various underlying brain functions, and potential treatments, is becoming increasing popular due to their ease of use and the ability to carry out non-invasive manipulation of the environment over a prolonged period of time (Sundstrom et al., 2005;Noraberg et al., 2005). This would not be possible with acute slices as you would need to manipulate the tissue in the *in vivo* situation in order to produce slices with different pre-conditioning treatments. This approach,

potentially, allows for a reduction in the amount of animals used and a refinement in the amount of *in vivo* work that needs to be performed. This is in line with the general approach of the three R's (reduce, refine, replace). Hippocampal cultures have been used to a limited degree to examine electrochemical processes involved in epileptic behaviour (Chen et al., 2004;Lahtinen et al., 2001;Pomper et al., 2001;Bausch and McNamara, 2000;Egert et al., 1998;Stoppini et al., 1997;McBain et al., 1989;Routbort et al., 1999), but they have only been used on a small number of occasions to elicit bursting behaviour caused by *in vitro* epilepsy models (Kovacs et al., 1999;Gutierrez et al., 1999;Duport et al., 1997;Bingmann et al., 1988;Fowler and Crain, 1984). I have been unable to find any published articles where organotypic hippocampal slices have been used to investigate the effects of exogenously applied neuropeptides on epilepsy models.

The data presented in chapter 2 demonstrates that Nociceptin causes an anti-convulsant effect in acute slices. I wanted to investigate these effects further in organotypic cultures to see if this would provide a suitable model where it would be possible to carry out various pre-conditioning treatments to further investigate the mechanisms of action of neuropeptides in convulsant activity.
I used Neuropeptide Y in this series of experiments as a large amount of work has been carried out looking at the effects of this neuropeptide, on convulsant models, in acute slices (Klapstein and Colmers, 1997;el Bahh et al., 2002;Smialowska et al., 1996); and felt that it would provide a useful comparison between the organotypic and acute slice models.

4.2 Materials and Methods

4.2.1 Organotypic Hippocampal Slice Cultures

Experiments presented in this chapter were carried out on Organotypic Hippocampal Slice Cultures (OHSC's) prepared according to (Stoppini et al., 1991) and (Pringle et al., 1997). These were prepared from

8-10 day-old wistar rat pups that were euthanised by decapitation in accordance with the Animal Scientific Procedures Act (1996). The brains were then removed and the two hippocampi were dissected out and placed on a McIlwain tissue chopper and cut into 400µm sections. The sections were then put into Gey's balanced salt solution (Gibco Life Technologies, UK) supplemented with 4.5 mg/ml glucose, ready for plating. Slices were seperated out and placed onto Millicell CM Tissue culture inserts (four per well) and maintained at 37°C and 5% CO2 for at least 14 days. Each well was placed in a 6 well plate which each had 1ml of culture medum in. The medium consisted of 50% minimum essential medium with added Earle's salts (MEM, ICN Biochemicals,UK), 25% heat –inactivated horse serum and 25% Hank's balanced salt solution (HBSS) supplemented with 1mM glutamine and 4.5 mg/ml glucose. The medium was changed every 3-4 days.

4.2.2 Multi-Electrode Array (MEA).

Electrophysiology experiments within this chapter were carried out on a multi electrode array. This consisted of 64 electrodes etched onto the base of a well, in an 8x8 arrangement. This allows the multiple recordings to be observed and recorded simultaneously. It also allows the stimulation of the tissue without having to move electrodes around the slice, therefore reducing any trauma that the slice may suffer.

The Multi-channel system used was supplied by MED systems (www.med64.com). The Electrophysiology rig (see Fig 4.1) consisted of a microscope with the MEA mounted on its stage. This is then attached to a 64-channel Integrated Amplifier that is in turn attached to a computer. The MEA is perfused with Normal aCSF, or aCSF and Drug, by means of a perfusion pump. The perfusion medium passes through an inline heater that maintains the bath temperature at around 36-37°C.

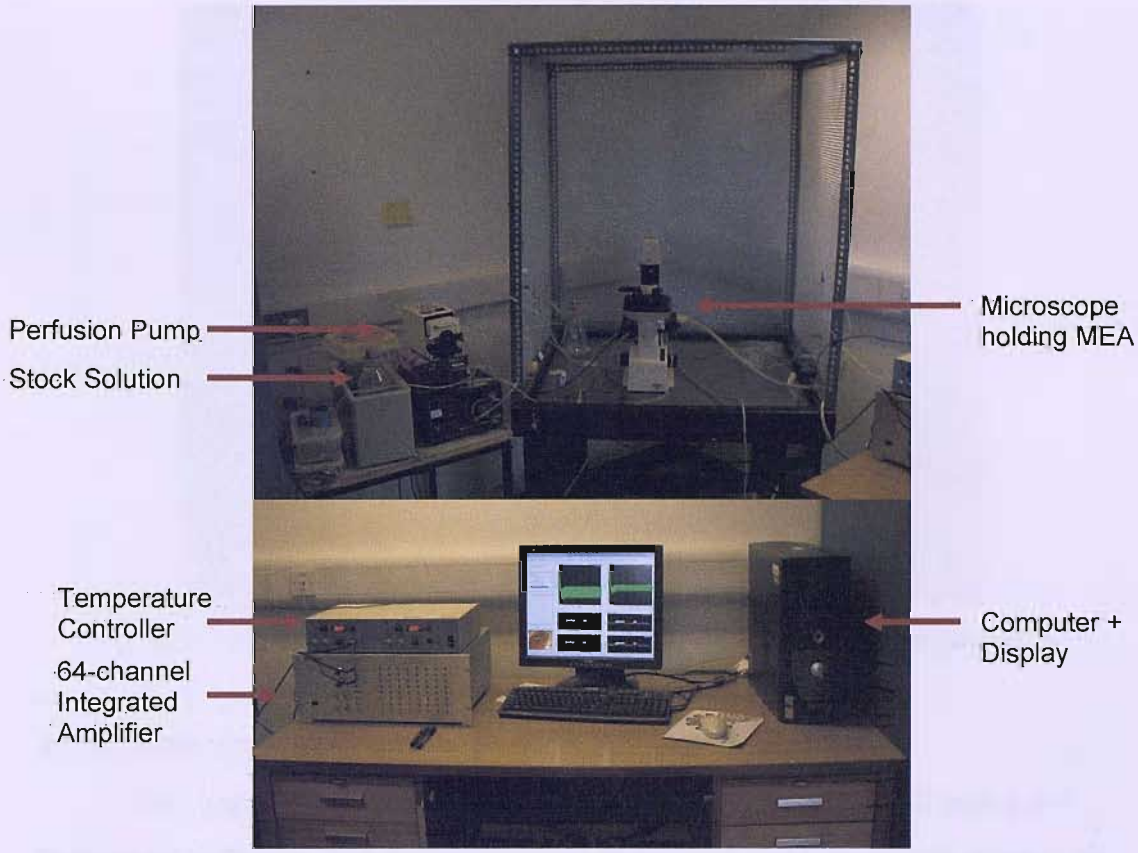


Fig 4.1 Photographs of MEA rig set-up. Top photograph shows the microscope that holds the well containing the 64 electodes, and the attached perfusion system. Bottom photograph show the Integrated Amplifier and attached Computer, also shows the temperature controller used to regulate the bath temperature.

Experiments where performed by mounting an OHSC onto the MEA and then performing stimulus response or spontaneous bursting experiments. The Stimulus response experiments where performed by applying a 100mA stimulus to an electrode in the Schaffer Collateral area of the slice and then recording the resultant stimulus from the CA1 area. Figure 4.2 shows the arrangement of the 64 electrodes in relation to the culture.

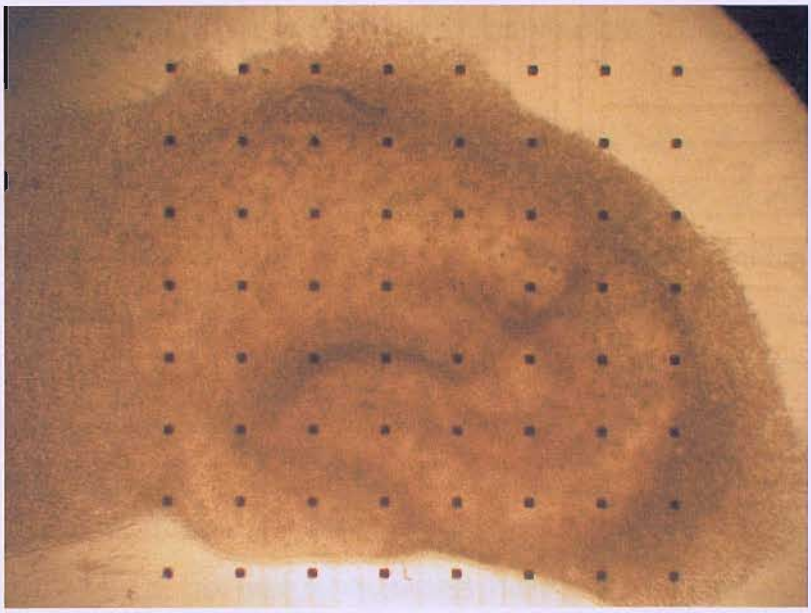


Fig 4.2 Photograph of the MEA with an OHSC placed on to the electrodes.

4.2.3 MEA Analysis

The experiments carried out on the MEA were analysed using the Performer software and measuring the amplitude (for population spikes) or frequency of the elicited responses. Further details of this analysis is contained in the relevant results chapters. The data was then subject to the same statistical tests used in chapters 2 and 3. That is One-Way ANOVA followed by Dunnett's post-hoc test.

4.2.4 Western Blotting

In order to investigate the presence of receptor protein in the slices it was decided to carry out Western Blotting. Firstly the protein was isolated from a number of sample slices and then gels were run to look for the presence of receptor protein.

4.2.4.1 Protein purification

Hippocampal slices from 8 day-old rat pups, 60 day old rats, and Organotypic Hippocampal Slice Cultures incubated for 2 weeks, were each dissolved in lysis buffer. Approximately 9 slices/cultures were dissolved in 800µl of lysis buffer with protease inhibitors (see Appendix 1 for details), and were put in a glass mortar/pestle to aid breakdown of cell membranes. Following this process the samples were left for 30 minutes to lyse and then

they were centrifuged to remove any solid debris. The supernatant was frozen and kept for protein assay.

In order to determine the level of protein in the samples a Bio-rad Protein Assay was used. This involved diluting the samples along a concentration gradient in order to determine the protein concentrations in the samples. Following this the samples were diluted with lysis buffer to give a concentration of 1mg of protein for each 1ml. These samples were then frozen and stored pending the running of the gel.

4.2.4.2 SDS-PAGE Gel and Western Blotting

An SDS-PAGE gel was run with the three protein samples under investigation, and a rainbow ladder to determine the molecular weight of the resulting bands.

The gels were run using the ATTO apparatus. The gels were made by layering a 4% acrylamide stacking gel on top of a 12.5% acrylamide separating gel, between two glass plates that were in a sandwich configuration. The glass sandwich is initially held together with clamps and a rubber gasket runs around three sides of the plates. 15ml of 12.5% acrylamide solution (see Appendix 1) was added to a beaker and to this was added 100µl of a 10% ammonium persulphate solution and 10µl of TEMED. This solution was then mixed thoroughly and poured into the glass sandwich prepared earlier. Isobutanol was poured on top and the gel was allowed to set at room temperature, approximately 30mins. When the gel had set the isobutanol was removed and the gel surface washed with water. Next 10ml of 4% acrylamide solution was made up in the same way as the 12.5% solution i.e. 10µl of TEMED and 100µl of 10% ammonium persulphate was added to the solution. This was then mixed and a small amount of it is used to wash the surface of the separating gel, 2-3ml was then poured on top of the 12.5% gel to fill it. A comb that forms 12 wells was then placed into the top of the gel. Once the gel had set (approximately 10mins) the comb, clamps and gasket was removed and the gel was then loaded into the electrophoresis tank. The lower and upper reservoirs of the tank are then filled with 1x electrode buffer (diluted with water, see appendix 1).

The samples were then loaded into the wells and the gel connected up to the power pack. The power pack was then set to maximum voltage and 20mA constant current and switched on at maximum power for 90 mins. After electrophoresis the apparatus was disassembled and the gel put into blotting buffer (see appendix 1) to await western blotting.

The proteins were transferred to nitrocellulose membrane blotting paper using a BioRad semi-dry blotter. Once the proteins had been transferred to the blotting paper then immunoassays could be performed to determine the presence of any receptor.

The blots where left overnight in 1° antibody to either the OP4, Y1, Y2 or Y5 receptors in a TBS blocking solution containing Tween-20. Following this the relevant secondary was then added and left for 2 hours to allow binding to occur. The blots were then exposed on photographic film to visualise the protein bands.

4.2.5 Toxicity of aCSF solutions on cultures

I carried out some further experiments in order to investigate whether the aCSF and drug solutions used for the Electrophysiology experiments were causing CA1 neurotoxicity, within the cultures. I exposed 2 week old cultures to either 10µM Bicuculline aCSF, 100µM 4-AP aCSF or normal aCSF. 2 wells (8 cultures) were incubated for 2 hours with each of the three different solutions. 1ml of the solution under investigation, along with 5µl of the fluorescent exclusion dye propidium iodide (PI) (Vitale et al., 1993), was added to each well. The cultures were then photographed under transmission microscopy to have a record of the CA1 layer. The cultures were then returned to the incubator for a 2 hour incubation period. After two hours the aCSF solution was removed from each well and replaced with a serum free medium (SFM, 75% MEM, 25% HBSS supplemented with 1mM glutamine and 4.5 mg/ml glucose) containing 5µg/ml PI. The cultures were then returned to the incubator overnight. At t=24 hours the cultures were removed from the incubator and PI fluorescence images of all the cultures were made.

In order to calculate the amount of CA1 damage in the cultures the images were analysed using OpenLab 2.1 (Improvision, UK). This allowed the area of the CA1 layer to be calculated from the transmission images taken at t=0. This could then be overlaid on the PI fluorescence images taken at t=24 hours and from this a percentage damage figure could be calculated. The amount of damage, across all the cultures for a particular treatment, was then averaged and t-test statistics were then carried out to see if there was any change in damage with the varying treatment groups.

4.3 Nociceptin effects on Synaptic Transmission

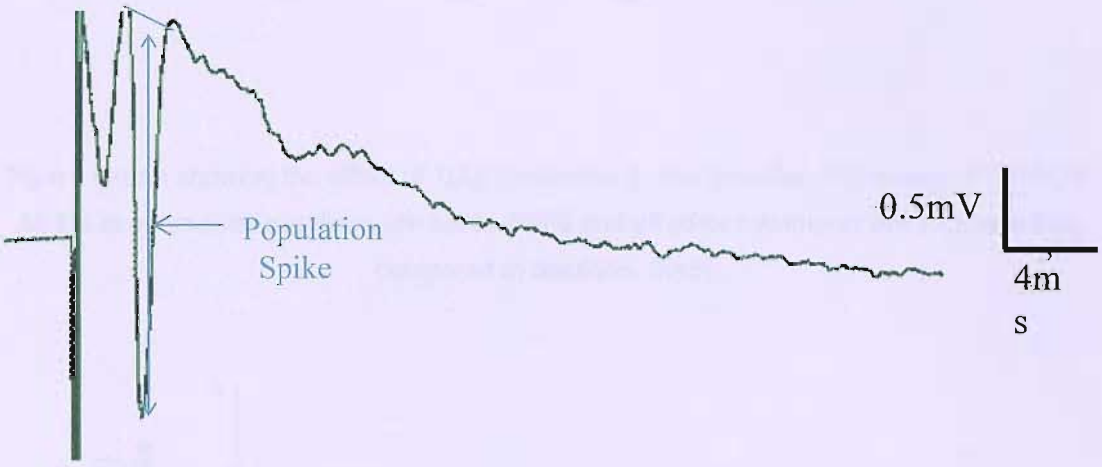


Fig 4.3 Illustration of how the population spike was calculated in the MEA setup.

4.3.1 Single Pulse

The first experiments I carried out were to look at the effect of 1μM Nociceptin on the synaptic transmission of an evoked population spike. The experiment was run so that a stimulus was given every 30 seconds and the resultant spike was recorded. The amplitude of the population spike was then measured during the analysis. After 30 minutes baseline, Nociceptin was added to the perfusion medium and left on for a further 30 minutes before being removed from the system. Figure 4.4 shows the effect of the Nociceptin on these population spikes. The data is analysed by comparing the last population spike, in the 30 minute treatment window, with relation to that observed at the same time during the baseline treatment (as a

percentage) and then average the values gained from all 5 experiments carried out.

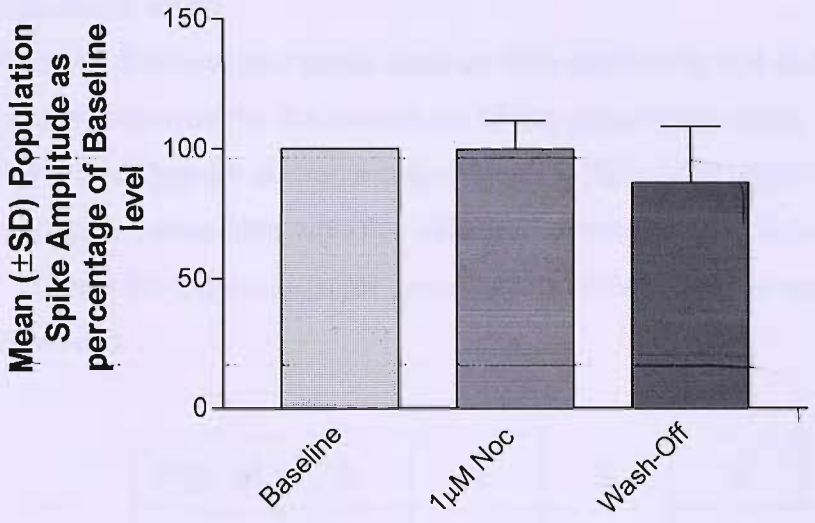


Fig 4.4 Graph showing the effect of 1µM Nociceptin on the Baseline Physiology of OHSC's. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=5).

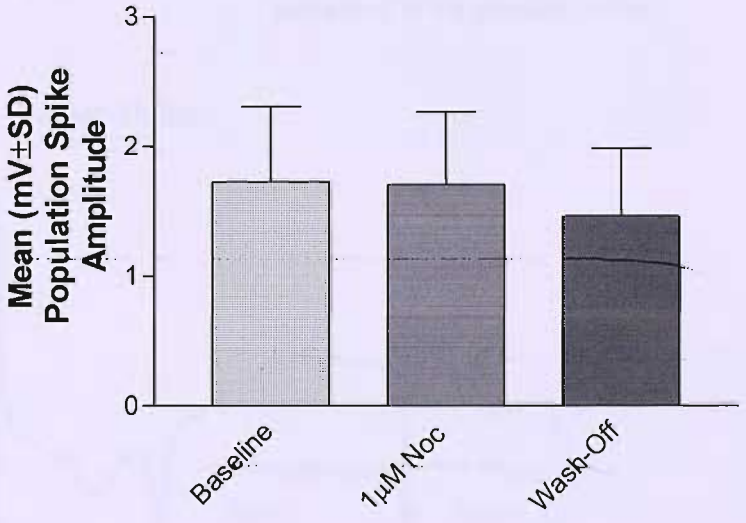


Fig 4.5 Graph showing the effect of 1µM Nociceptin on the Baseline Physiology of OHSC's. All values are those originally recorded, and there is no normalising to baseline. (n=5).

Figure 4.4 shows no effect when 1μM Nociceptin is washed onto the slice, and the wash-off causes little change in the amplitude of the observed population spike.

Figure 4.5 shows the same data as that seen in fig 4.4 but gives the original values recorded for the amplitude of the population spike. This shows that in the baseline an average response of 1.725mV is observed and this response varies little with the different treatments carried out. An average of 1.705mV for the Nociceptin group and 1.458mV for the wash off group was observed.

Exp. ID	A	B	C	D	E
Baseline	2.165 100%	2.084 100%	0.994 100%	2.191 100%	1.193 100%
1μM Noc	2.257 104.257%	1.755 84.189%	0.941 94.659%	2.210 100.890%	1.360 114.019%
Wash-Off	2.188 101.074%	1.163 55.814%	0.836 84.058%	1.764 80.523%	1.342 112.539%

Table 4.1 Effect of 1μM Nociceptin treatment on the Population Spike Amplitude in OHSC's. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

4.3.2 Paired Pulse

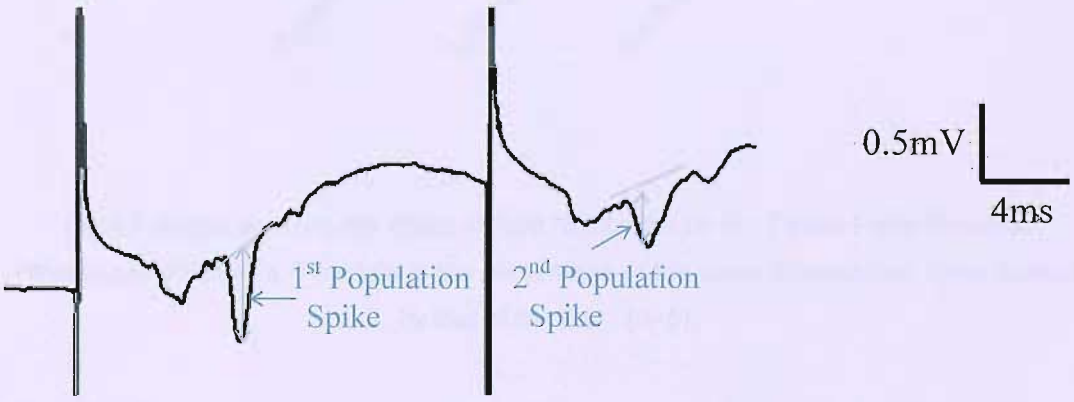


Figure 4.6 Illustration of how the two population spikes were measured in the paired pulse experiments. The second PS is calculated as a percentage of the first PS.

The next experiment I looked at was the effect of 1µM Nocicpetin on paired pulse experiments. These experiments were carried out in a similar way to those of the single pulse experiments except that instead of a single pulse two pulses, of the same amplitude, at an inter pulse interval of 20msecs was applied. 20msecs interval was used to ascertain the level of inhibitory action present within the slice, and any effects that the Nociceptin may have on this. The interval of 20msecs was chosen as it is one which is fairly robust at achieving paired pulse inhibition.

The data was then calculated as the amplitude of the second population spike as a percentage of the amplitude of the first population spike. Therefore a value of less than 100% would indicate that paired pulse inhibition is occurring.

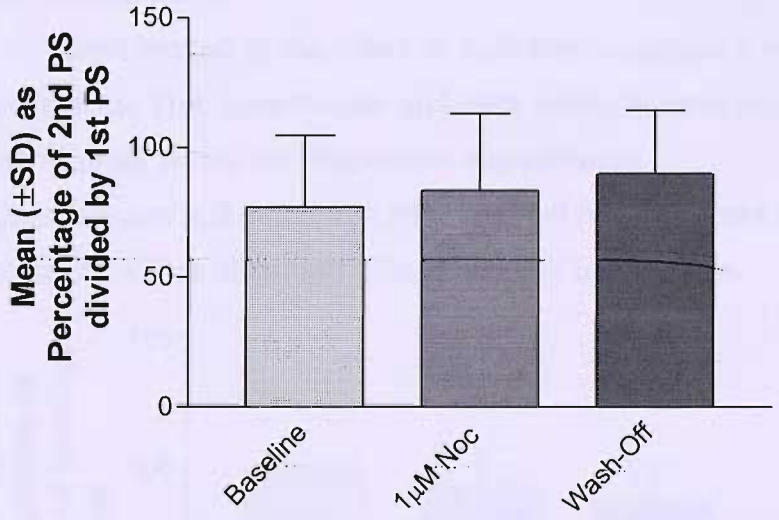


Fig 4.7 Graph showing the effect of 1µM Nociceptin on the Paired Pulse Baseline Physiology of OHSC's. The data is the percentage of the second population spike divided by that of the first . (n=5).

Figure 4.7 shows the result of 1µM Nociceptin on the paired pulse. This shows that there is no effect of the Nociceptin over the course of the experiment.

Exp. ID	A	B	C	D	E
Baseline	64.586%	101.792%	35.212%	98.432%	85.568%
1µM Noc	73.094%	111.140%	36.954%	104.991%	90.756%
Wash-Off	63.946%	111.523%	70.783%	119.361%	83.549%

Table 4.2 Effect of 1µM Nociceptin treatment on the population spike amplitude of paired pulse experiments in OHSC's. Values are in percent.

4.4 Neuropeptide Y effects on Synaptic Transmission

4.4.1 Single Pulse

I next looked at the effect of 1µM Neuropeptide Y in the single pulse experiments. The experiments and data analysis were carried out in the same manner as for the Nociceptin experiments.

Figures 4.8 and 4.9 show that NPY caused no significant changes in the population spikes observed throughout the experiment.

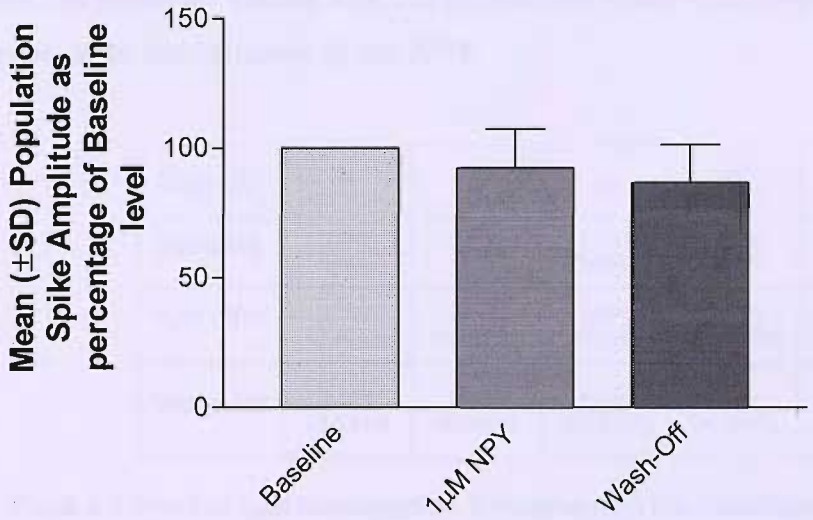


Fig 4.8 Graph showing the effect of 1µM Neuropeptide Y on the Baseline Physiology of OHSC's. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=5).

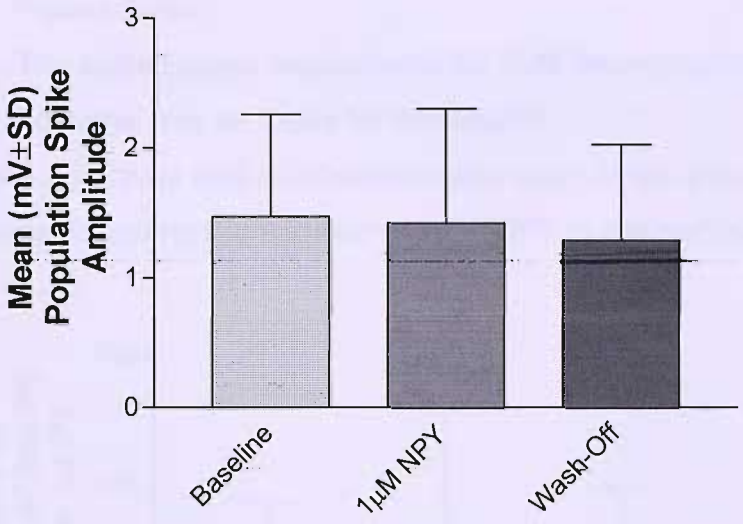


Fig 4.9 Graph showing the effect of 1µM Neuropeptide Y on the Baseline Physiology of OHSC's. All values are those originally recorded, and there is no normalising to baseline. (n=5).

Table 4.3 shows the individual results for this experiment. There is a decrease in some of the data points when NPY is administered to the slice but the wash-off shows that the population spike continues to decrease even after the removal of the NPY.

Exp. ID	A	B	C	D	E
Baseline	2.045 100%	0.695 100%	1.241 100%	2.526 100%	0.855 100%
1µM NPY	1.960 95.822%	0.486 69.954%	1.331 107.290%	2.618 103.678%	0.723 84.581%
Wash-Off	1.504 73.531%	0.558 80.312%	1.353 109.015%	2.377 94.104%	0.654 76.512%

Table 4.3 Effect of 1µM Neuropeptide Y treatment on the Population Spike Amplitude in OHSC's. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

4.4.2 Paired Pulse

The paired pulse experiments for 1μM Neuropeptide Y were carried out in the same way as those for Nociceptin.

Figure 4.10 shows that no changes were seen, in the paired pulse response, following the addition of 1μM NPY to the perfusion system.

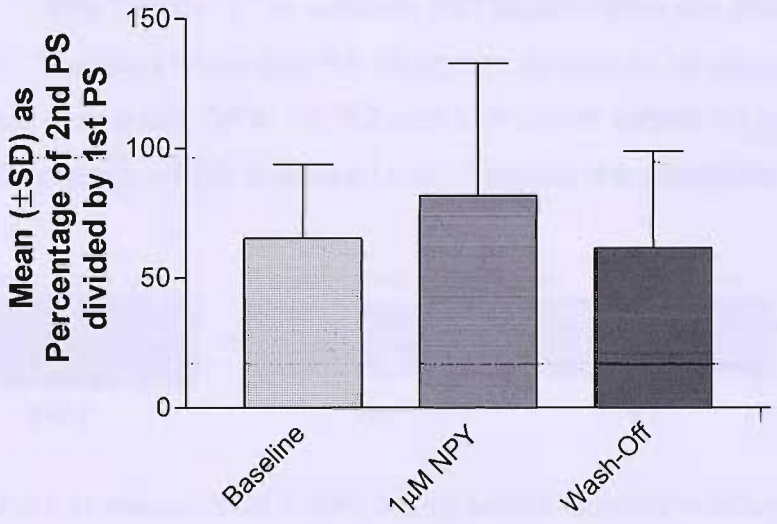


Fig 4.10 Graph showing the effect of 1μM Neuropeptide Y on the Paired Pulse Baseline Physiology of OHSC's. The data is the percentage of the second population spike divided by that of the first (n=5).

Exp. ID	A	B	C	D	E
Baseline	85.923%	81.536%	55.466%	19.175%	84.169%
1μM NPY	106.768%	49.559%	77.163%	21.761%	153.315%
Wash-Off	117.514%	51.900%	76.740%	20.135%	41.079%

Table 4.4 Effect of 1μM Neuropeptide Y treatment on the population spike amplitude of paired pulse experiments in OHSC's. Values are in percent.

The data presented above is at odds with that seen in the acute slices where both NPY and also Nociceptin have been shown to cause a

reduction in synaptic transmission (Colmers et al., 1985;Ikeda et al., 1997). Following these results I wanted to check whether or not the Nociceptin and NPY receptors were present in the cultures. I therefore carried out a western blot experiment to look for the OP4, Y1, Y2 and Y5 receptors.

4.5 Western Blots

The results of the western blot experiments are presented in figure 4.11. The blots show that the receptors appear to be present in all the tissue examined. OP4, Y1, Y2 and to a lesser extent Y5 show bands in the 40KDa range which is where I would expect the receptors to be found.

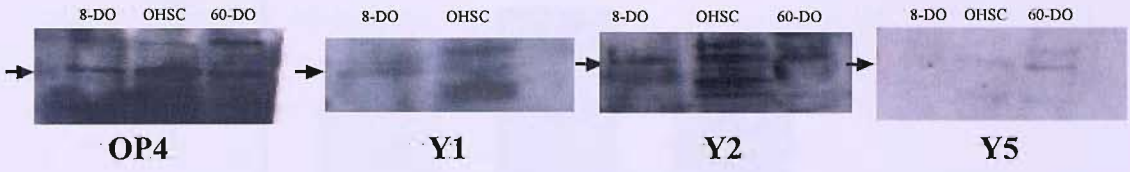


Fig 4.11 Western Blots of OP4, Y1, Y2 and Y5 receptors in 8-Day Old Rats (8-DO), Organotypic Hippocampal Slice Cultures (OHSC's) and 60-Day Old Rats (60-DO). Arrows indicate the receptor in question.

This confirms that the receptors are present in the cultures and therefore Nociceptin and Neuropeptide Y would be expected to perform in a similar way to that seen in acute slices.

4.6 Bicuculline Model

I first wanted to investigate the robustness of the Bicuculline induced bursting. In order to achieve this I carried out a change of medium, but the medium was swapped for an identical mixture of aCSF. This would allow me to find out whether there is a stable baseline throughout the experiment or if the convulsant activity varies during the duration of the investigation.

4.6.1 Control

The data presented in this series of experiments is the average frequency during a 5 minute period at the end of the treatment. Following a 30 minute baseline the medium is changed for an identical stock solution of aCSF for a further 30 minutes, and then the original aCSF is replaced for another 30minutes. The frequency during the five minute windows is worked out as a percentage of baseline and then averaged for all the experiments carried out.

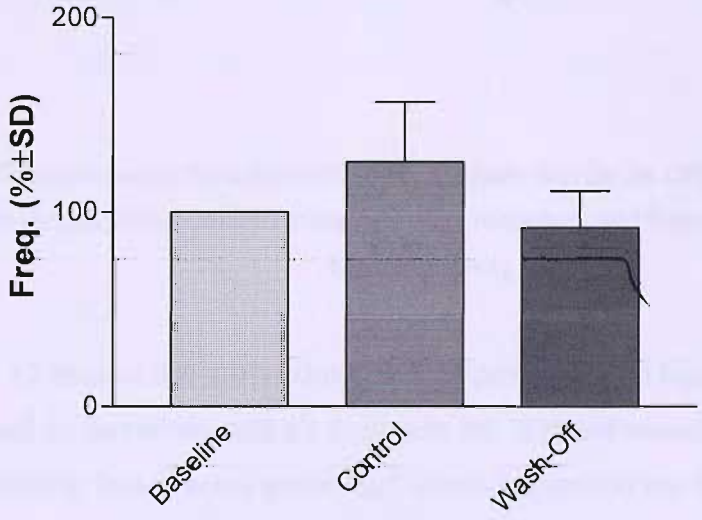


Fig 4.12 Graph showing the effect of Control medium change on OHSC's pre-treated with 10μM Bicuculline. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=4).

Figure 4.12 shows that throughout the experiment there is no decrease in the frequency observed. This confirms that the baseline remains stable for the duration of the period under investigation.

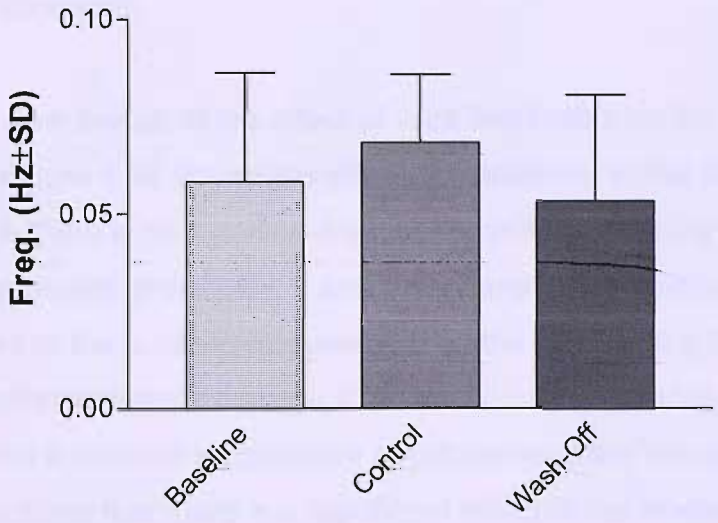


Fig 4.13 Graph showing the effect of Control medium change on OHSC's pre-treated with 10μM Bicuculline. All values are those originally recorded, and there is no normalising to baseline. (n=4).

Figure 4.13 shows the same data as that presented in figure 4.12 but is not normalised to baseline, and so contains the original values recorded.
The preceding two graphs show that when subject to control conditions the frequency stays fairly consistent throughout the time period under observation, in this case approximately 90 minutes. This means that I can say that any responses seen with the drug will be effects and should not be down to any background variation.

Exp. ID	A	B	C	D
Baseline	0.040 100%	0.047 100%	0.047 100%	0.100 100%
Control	0.067 166.667%	0.060 128.571%	0.053 114.286%	0.093 93.333%
Wash-Off	0.047 116.667%	0.040 85.714%	0.033 71.429%	0.093 93.333%

Table 4.5 Effect of Control treatment on 10μM Bicuculline generated spontaneous epileptiform activity in OHSC's. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

4.6.2 Nociceptin

I now looked at the effect of 1µM Nociceptin on the Bicuculline model. Figure 4.14 shows the effect of Nociceptin in the Bicuculline model. Although there is no significant effect of the Nociceptin by the statistical tests mentioned previously, it seems apparent that Nociceptin is causing a decrease in the bursting frequency. I ran the data for the Baseline and 1µM Nociceptin treatments through a further non-parametric test, in this case the t-test, and it showed a significant effect between the two groups. This would seem to show that there is a significant effect of the Nociceptin on this model in the culture system.

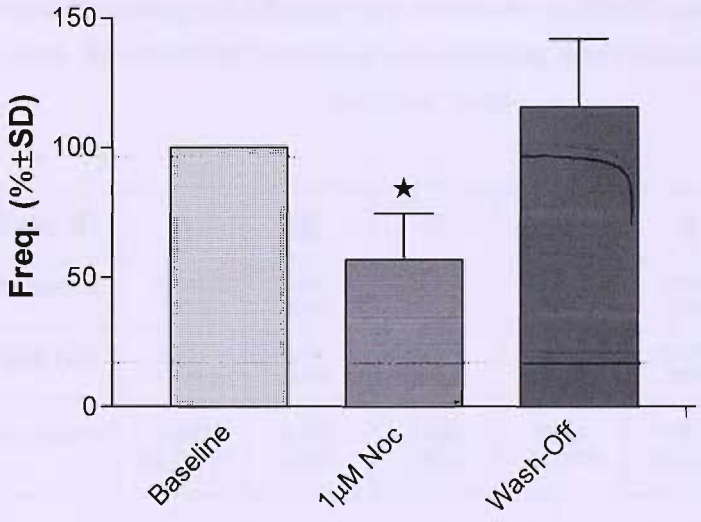


Fig 4.14 Graph showing the effect of 1µM Nociceptin on OHSC's pre-treated with 10µM Bicuculline. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=6). ★=$p<0.05$

Figure 4.15 shows the same data as previously shown in figure 4.14 but is not normalised to baseline. The data presented here is therefore the original values recorded for the frequency of the tissue in these experiments.

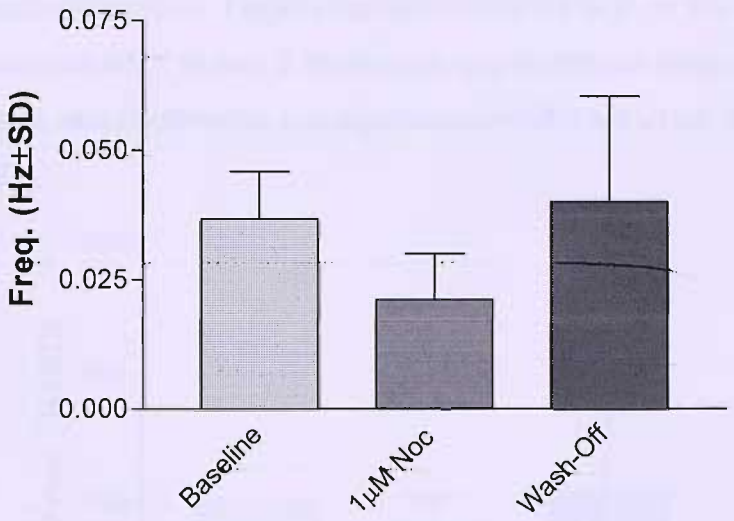


Fig 4.15 Graph showing the effect of 1µM Nocicpetin on OHSC's pre-treated with 10µM Bicuculline. All values are those originally recorded, and there is no normalising to baseline. (n=6).

Exp. ID	A	B	C	D	E	F
Baseline	0.047 100%	0.027 100%	0.033 100%	0.047 100%	0.040 100%	0.027 100%
1µM Noc	0.033 71.429%	0.007 25%	0.020 60%	0.027 57.143%	0.020 50%	0.020 75%
Wash-Off	0.040 85.714%	0.027 100%	0.080 240%	0.027 57.143%	0.033 83.333%	0.033 125%

Table 4.6 Effect of 1µM Nociceptin treatment on 10µM Bicuculline generated spontaneous epileptiform activity in OHSC's. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

4.6.3 Neuropeptide Y Results

I then went on to investigate the effects of 1µM Neuropeptide Y on the Bicuculline model. The data presented in Figure 4.16 was analysed in the same way as that presented in figure 4.14. Similarly to the data seen for the Nociceptin experiments, it appears that the Neuropeptide Y may cause a reduction in the bursting activity seen in the Bicuculline model, but that this effect is not significant by the statistical tests outlined in the materials

and methods section. I again decided to do a t-test on the data sets for the Baseline and NPY to see if there was any statistical effect. This showed that these two treatments are significantly different when this statistical test is used.

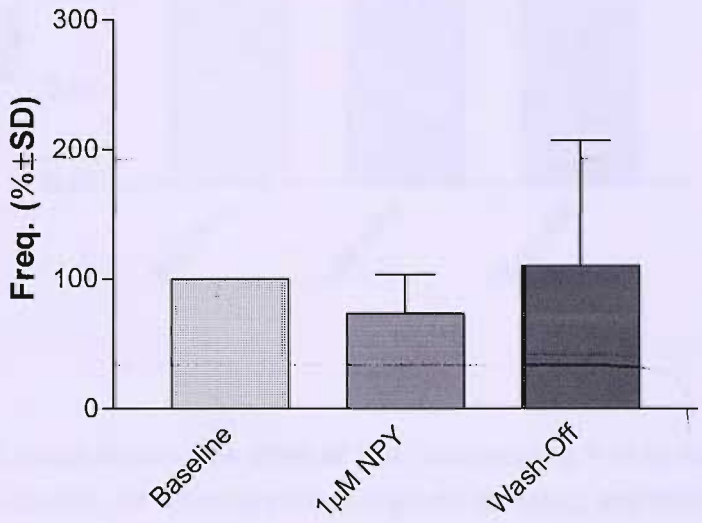


Fig 4.16 Graph showing the effect of 1µM Neuropeptide Y on OHSC's pre-treated with 10µM Bicuculline. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=6).

Figure 4.17 shows the same data seen in figure 4.16 but does not normalise the data to baseline and therefore shows the original values of the data recorded for this experiment.

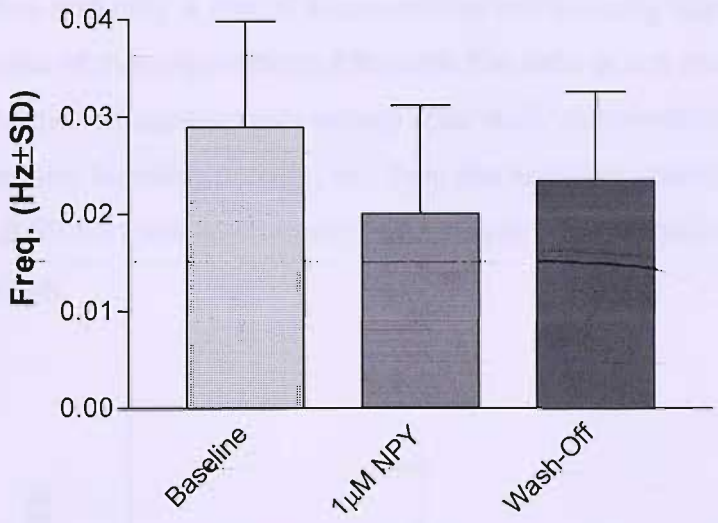


Fig 4.17 Graph showing the effect of 1µM Neuropeptide Y on OHSC's pre-treated with 10µM Bicuculline. All values are those originally recorded, and there is no normalising to baseline. (n=6).

Exp. ID	A	B	C	D	E	F
Baseline	0.033 100%	0.033 100%	0.033 100%	0.033 100%	0.033 100%	0.007 100%
1µM NPY	0.027 80%	0.033 100%	0.007 20%	0.027 80%	0.020 60%	0.007 100%
Wash-Off	0.027 80%	0.027 80%	0.007 20%	0.033 100%	0.027 80%	0.020 300%

Table 4.7 Effect of 1µM Neuropeptide Y treatment on 10µM Bicuculline generated spontaneous epileptiform activity in OHSC's. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

4.7 4-AP Model

4.7.1 Control

I carried out a control experiment to see the effects of time on the frequency recorded from the 4-Aminopyridine model of convulsant activity. Figure 4.18 shows that the level of bursting activity decreases throughout the experiment. The slices in this series of experiments were very difficult to

keep alive and only a few of those carried out actually survived for the full 90 minutes of the experiment. Although the data is not shown I carried out a large number of experiments where after 4-AP administration the culture initial caused bursting activity, but then the bursting activity dropped off after about 15-20 minutes even though there was no change in the experimental parameters.

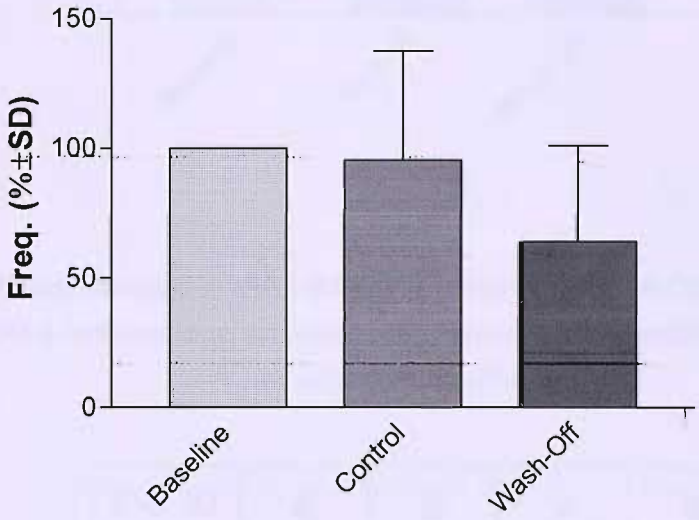


Fig 4.18 Graph showing the effect of Control medium change on OHSC's pre-treated with 100µM 4-Aminopyridine. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=4).

Figure 4.19 shows the same data presented in figure 4.18 but does not normalise the data to the baseline value.

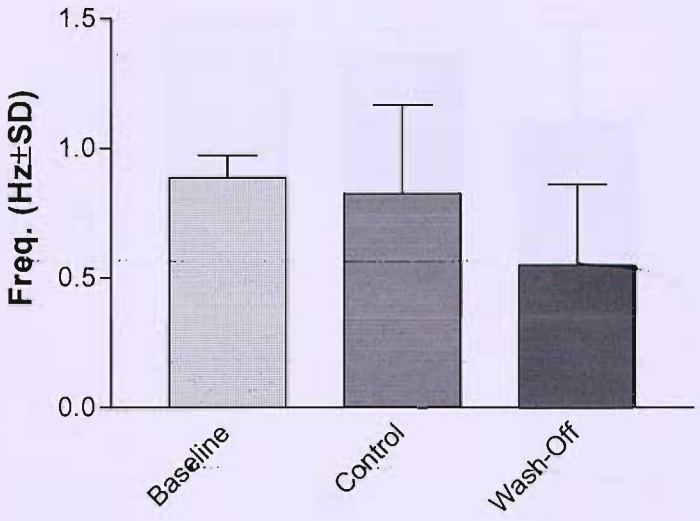


Fig 4.19 Graph showing the effect of Control medium change on OHSC's pre-treated with 100µM 4-Aminopyridine. All values are those originally recorded, and there is no normalising to baseline. (n=4).

Exp. ID	A	B	C	D
Baseline	0.787 100%	0.847 100%	0.933 100%	0.980 100%
Control	0.887 112.712%	1.080 127.559%	1.007 107.857%	0.327 33.333%
Wash-Off	0.573 72.881%	0.847 100%	0.660 70.714%	0.113 11.565%

Table 4.8 Effect of Control treatment on 100µM 4-Aminopyridine generated spontaneous epileptiform activity in OHSC's. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

4.7.2 Nociceptin

Figure 4.20 shows the data obtained for 1µM Nociceptin treatment of 100µM 4-aminopyridine induced bursting experiments. It was only possible to carry out 3 experiments for this treatment, as it was very difficult to get the experiment to last for the full 90 minutes. The culture tended to die much before this point.

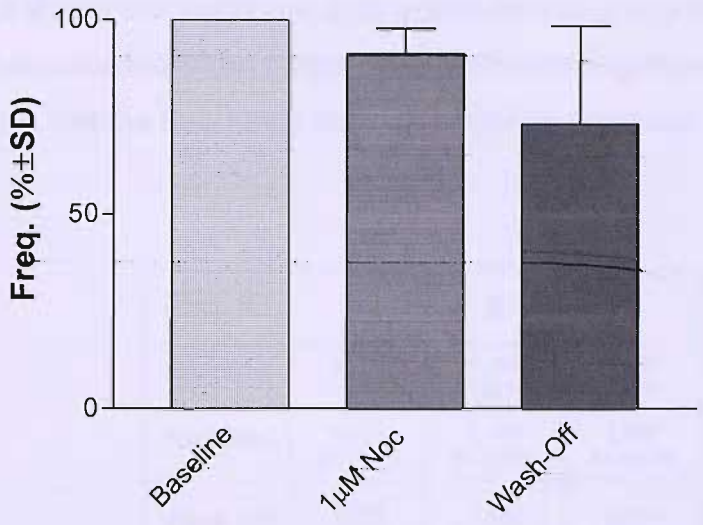


Fig 4.20 Graph showing the effect of 1µM Nociceptin on OHSC's pre-treated with 100µM 4-Aminopyridine. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. (n=3).

Figure 4.21 shows the same data as that presented in figure 4.20 but does not normalise to baseline.

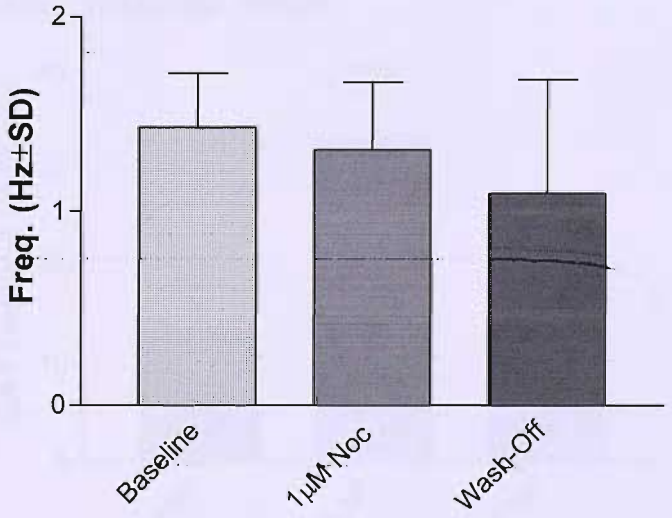


Fig 4.21 Graph showing the effect of 1µM Nociceptin on OHSC's pre-treated with 100µM 4-Aminopyridine. All values are those originally recorded, and there is no normalising to baseline. (n=3).

Table 4.9 shows the individual data points for this group of experiments. It shows that individually the frequencies decrease slightly when Noc is washed on, but the frequency also continues to decrease following removal of Noc.

Exp. ID	A	B	C
Baseline	1.753 100%	1.280 100%	1.260 100%
1μM Noc	1.713 97.719%	1.167 91.146%	1.067 84.656%
Wash-Off	1.753 100%	0.887 69.271%	0.633 50.265%

Table 4.9 Effect of 1μM Nociceptin treatment on 100μM 4-Aminopyridine generated spontaneous epileptiform activity in OHSC's. First values are Means (of frequency in Hertz); second value is the percentage as compared to the baseline value.

4.7.3 Neurotoxicity in Cultures

Following on from the studies previously carried out with 4-AP I decided to look at the neurotoxicity of 4-AP and Bicuculline in the cultures over a two hour incubation period.

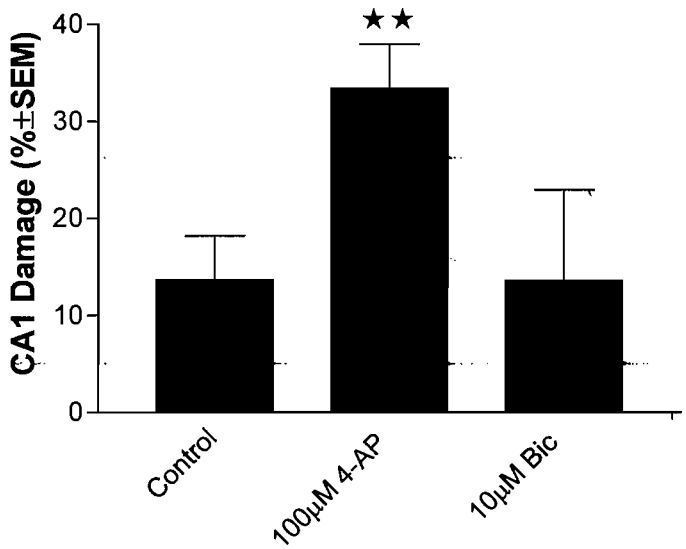


Figure 4.22 Graph illustrated the percentage of CA1 cell damage seen following 2 hour incubation with either Control, 100μM 4-AP or 10μM Bic aCSF. ★★=$p<0.01$ vs control.

Figure 4.22 shows that following 2 hours treatment, with the concentration of drug administered in the convulsant models, that the Control and Bicuculline treated cultures show a small amount of CA1 damage. However during this same time scale the 4-AP treatment results in a significantly larger amount of CA1 damage. This would seem to indicate that 100μM 4-AP was causing neurotoxicity in the cultures at a higher rate than that seen with 10μM Bicuculline or Control.

Figure 4.23 illustrates this damage for typical cultures for each treament.

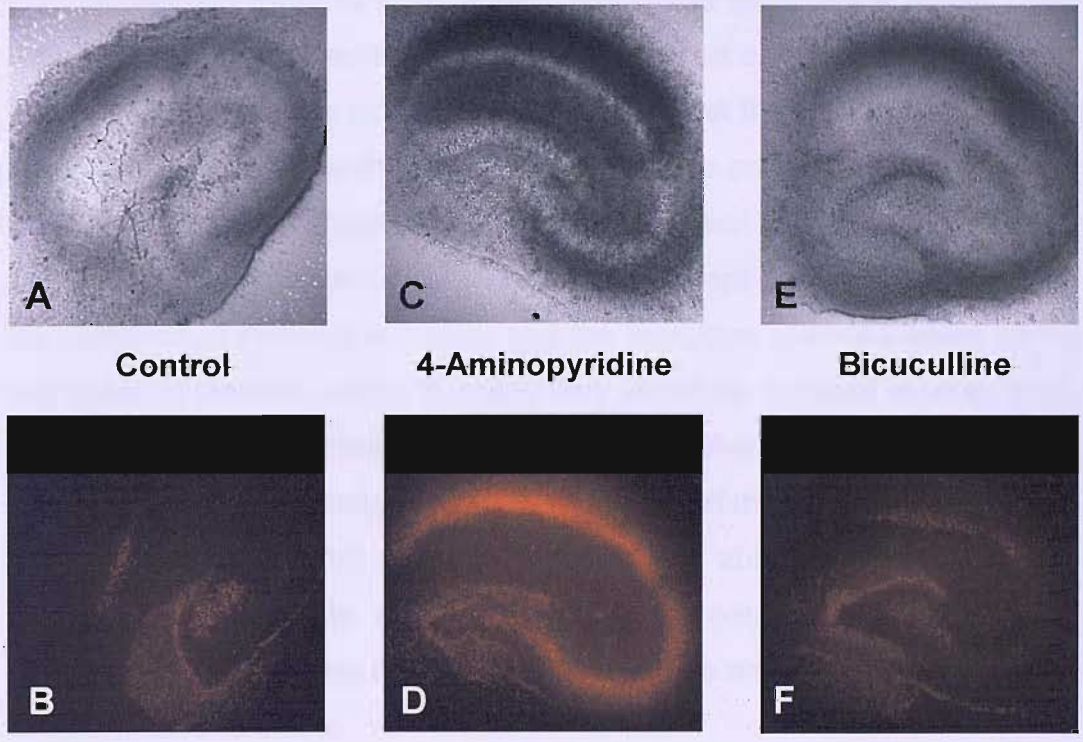


Figure 4.23 Pictures of the results seen following treatment with Control (A+B), 100μM 4-AP (C+D) or 10μM Bicuculline (E+F) aCSF. A, C and E show transmission images taken at t=0. B, D and F show images taken at t=24 hours when PI binds to the DNA of dead cells and fluoresces. n= 8 cultures (Control & Bic), 7 (4-AP)

The 4-AP picture (D) in figure 4.23 shows a noticeably larger amount of PI staining in the culture as a whole, and in particular in the CA1 area, than is seen with control (B) or Bicuculline (F) treatment. One of the 4-AP treated cultures was discarded because it was noticed that there was no CA1 layer present.

4.8 Discussion

Whereas both Neuropeptide Y and Nociceptin have been shown to inhibit synaptic transmission in acute slices (Colmers et al., 1987;Greber et al., 1994;Patrylo et al., 1999;Yu et al., 1997), this was not the case, seen here, in the organotypic hippocampal slice cultures. Although there are limited publications on the effect on synaptic transmission of Nociceptin and NPY in cultures, there is evidence that Nociceptin does not have an effect on the calcium efflux or glutamate-evoked currents in primary cultures of rat cortical neurons (Bianchi et al., 2004). This would back up the results I saw where NPY and Nociceptin did not have any effect on the synaptic transmission within the cultures. I have shown that the OP4, Y1, Y2, and Y5 receptors are present in the cultures, as they also are in the 8 day old and 60 day old rats. Therefore it raises the question that if the receptors are present then why is there no effect of the neuropeptides on synaptic transmission. A possible reason is that the receptors are not located on the pre-synaptic terminal, which is where they would be required in order to see an effect on the glutamatergic transmission. Another possibility is that the receptors are not properly coupled to their second messenger systems and so cannot exert an effect. I was unfortunately not able to carry out localisation experiments, to show where in the slices the receptors were, due to a lack of time and the incompatibility of the antibodies for performing immunohistochemistry.

The Nociceptin showed an inhibitory effect on the frequency of Bicuculline induced bursting activity, even though it did not inhibit synaptic transmission.

It would therefore appear that the anti-convulsant activity of Nociceptin in cultures acts independently of the ability to reduce synaptic transmission. Going back to the discussion in chapter 2, where I suggested that Nociceptin might be exerting its anti-convulsant action via a modulatory effect on the I_h potassium current. This would follow in this case as it has been shown that the current is present in dissociated rat hippocampal neurons (Simeone et al., 2005). It would seem sensible to conclude that although OP4 receptors may not be functional on the pre-synaptic terminal they are present on the post-synaptic membrane where they are coupled to

the G_i/G_0 second messenger G-protein pathway and bring about a reduction in bursting activity by reducing the rate of membrane repolarisation, thereby
prolonging the period between which busts could occur.
As NPY did not cause a reduction in bursting activity it may be the case that the anticonvulsant activities of NPY may be tied in to its ability to reduce synaptic transmission. Since the reduction of synaptic transmission by NPY in the cultures is not possible, then this would account for the inability to see an anti-convulsant effect in these experiments. Although the NPY did not cause any effects I do not believe this is due to any lack of potency of the peptide as the same batch was used by another member of the same division where it caused the expected effects (Howell et al., 2005).
The 4-AP model failed to produce a reliable baseline for experiments. It appeared that the 4-AP was causing the cultures to die during the experiment. From the toxicity studies it would appear that 4-AP is causing the CA1 area of the culture to die. Although I am not aware of any evidence that directly supports this there is contributing evidence (Kovacs et al., 1999a) that the $0Mg^{++}$ epileptiform model can result in cell death in OHSC's.

Chapter 5

Neuropeptide Y in Acute Slices

5.1 Introduction.

Neuropeptide Y has been shown to regulate excitatory synaptic transmission and hyperexcitability in the hippocampus (Baraban et al., 1997). Studies have shown that NPY inhibits glutamate release in rat hippocampal slices (Colmers et al., 1988;Klapstein and Colmers, 1993). This effect is most likely due to presynaptic actions on Y_2 receptors and appears to be selective, as NPY does not show an effect on inhibition in the hippocampus (Klapstein and Colmers, 1993).

NPY is a 36 amino acid peptide, which is one of the most abundant neuropeptides in the mammalian CNS. It has been linked to a large variety of physiological processes including feeding, memory, anxiety, circadian rhythms and blood pressure. NPY expressed in the CNS is most often co-localised within neurons along with classical neurotransmitters (most often noradrenaline (NA)). However it is not present in all NA containing vesicles within the presynaptic terminal. It is usually found in the vesicles that are released during high frequency stimulation of the nerve terminal.

NPY is constitutively expressed, in the hippocampus, in GABA interneurons.

NPY receptors, Y_1, Y_2, Y_4, Y_5 & y6 have been described (Wan and Benjamin 1995). All these receptors are G-protein coupled receptors. Y1, Y2 and Y5 receptor protein is found in the Stratum oriens, radiatum and pyramidale (Redrobe et al., 1999). Receptor protein is also found in the molecular layer, the highest concentrations of which are Y1, then Y5 followed by Y2; no Y4 receptor protein was found. A presynaptic Y2 receptor has been identified in the CA1 region (Weiser et al., 2000).

It has been shown previously (Whittaker et al., 1999) that *in vivo* NPY can attenuate the generation of long term potentiation. Lots of studies have investigated which receptor subtype, or combination of subtypes, may be responsible for its anti-convulsant activity. Recently it has been suggested that Y_2 alone is the most likely candidate (el Bahh et al., 2005).

It is interesting to note that the overwhelming amount of published data shows that NPY inhibits synaptic transmission and convulsant activity in acute hippocampal slices. As this was not what I saw in the organotypic

cultures I will show in this chapter the results when I investigated the effects of NPY on synaptic transmission and a convulsant model in acute hippocampal slices.

5.2 Materials and methods.

The preparation of acute hippocampal slices and the electrophysiology experiments presented in this chapter were largely carried out in the same way to those presented in chapter 2, and therefore most of the information regarding the methods can be found in that chapter.

In order to investigate the effects, of the neuropeptide, on the baseline physiology of the slices, responses were evoked. This was achieved by using a bipolar wire-wound stimulating electrode placed in the Schaffer collateral/commissural pathway, and recordings were taken from the CA1 area of the hippocampus (see Figure 5.1). Data was collected using a Labmaster TL-1 interface (Scientific Solutions, U.S.A.) linked to a computer running the LTP program (Anderson and Collingridge, 2001). Stimuli were generated by a constant voltage isolated stimulator (DS2A, Digitimer, UK). Stimuli were delivered at intervals of 30 seconds to prevent the development of long-term potentiation or depression (Stevens and Sullivan, 1998).

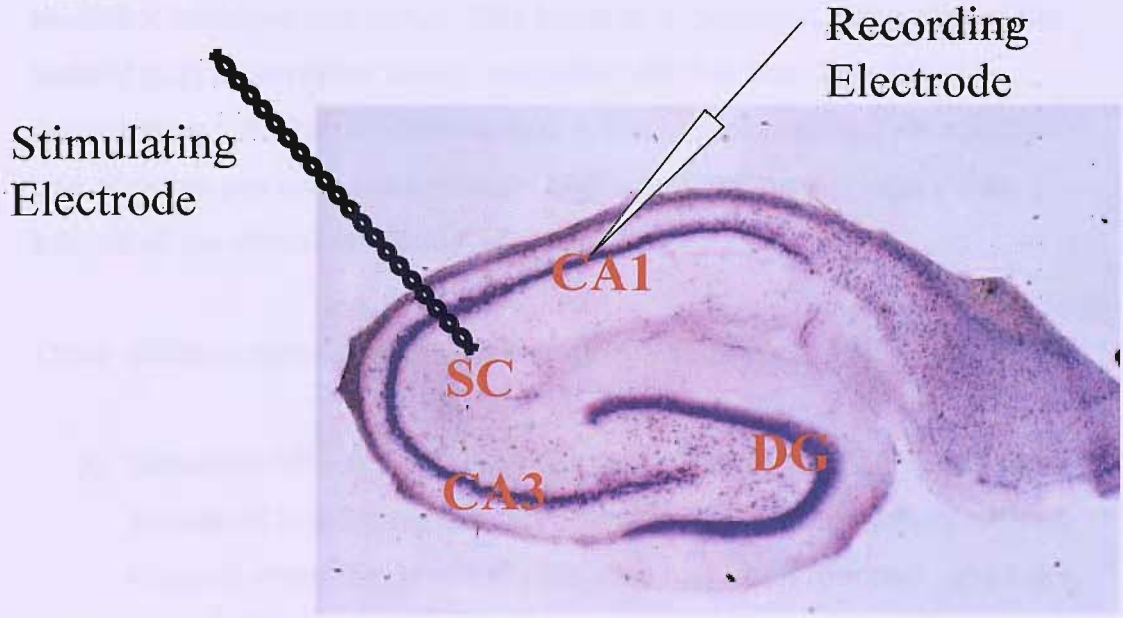


Figure 5.1 Diagram of Electrode Placement, SC Schaffer collateral/commissural pathway, DG Dentate Gyrus, CA1 Cornu ammonis area 1, CA3 Cornu ammonis area 3.

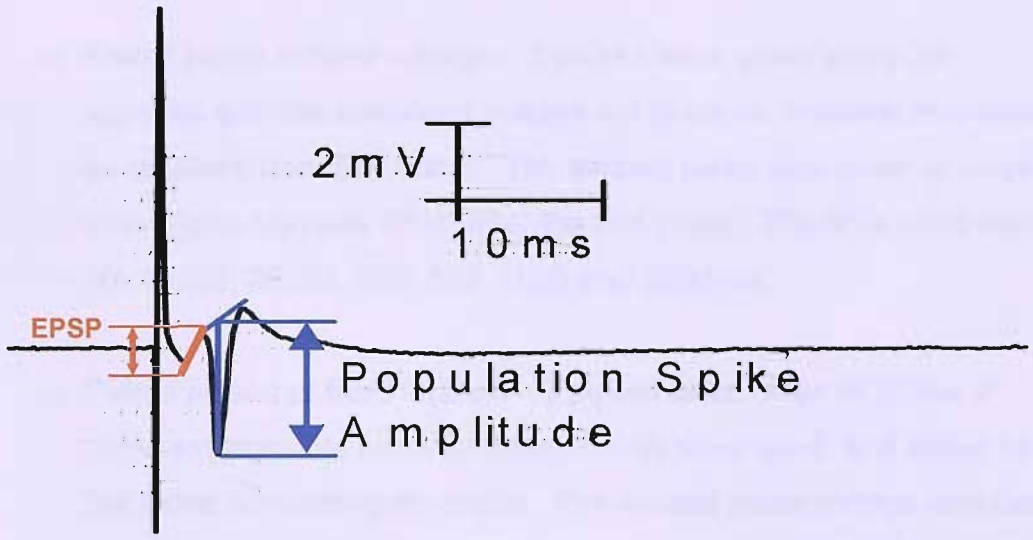


Figure 5.2 Diagram of Typical Population Spike. Shows the stimulus artefact, EPSP and Population Spike.

The population spike arises as a result of the stimulation of a group of neuronal cells, which results in axonal firing (see figure 5.2). The EPSP is the sum of the effects of the dendrites, and the Population Spike is the sum of the axonal discharges. The population spike is measured as indicated in figure 5.2.

When two stimuli (paired pulse) are given in quick succession then feedback inhibition can result. This leads to a reduction in the size of the second population spike when compared with the first. This effect is mediated by GABAergic interneurons in the hippocampus. This effect is described as paired pulse inhibition and is seen when the Inter Pulse interval of the stimuli is around 15-20ms.

Three different types of stimulation protocol were used: -

a) Stimulation/Response (S/R) Curves – The stimulation voltage was increased in increments of 2V, starting at 0V. The stimulation was stopped when the maximal response had been reached, and this level designated V_{MAX}. This was repeated for Baseline, Treatment, and Baseline (Wash-Off).

b) Paired pulse at fixed voltage – 2 pulses were given every 30 seconds with the stimulator voltage set to cause maximal response, as obtained from S/R curve. The second pulse was given at varying Inter-Pulse Intervals (IPIs) after the first pulse. The IPI's used were 10, 15, 20, 25, 50, 100, 500, 1000 and 2000 ms.

c) Paired pulses at fixed interval – 2 pulses were given at 20 ms IPI. In these experiments two stimulating boxes were used, and linked to the same stimulating electrode. The second pulse voltage was fixed at half the value that caused the maximal response. The first pulse was increased from 0V to maximal response voltage in increments of 2V.

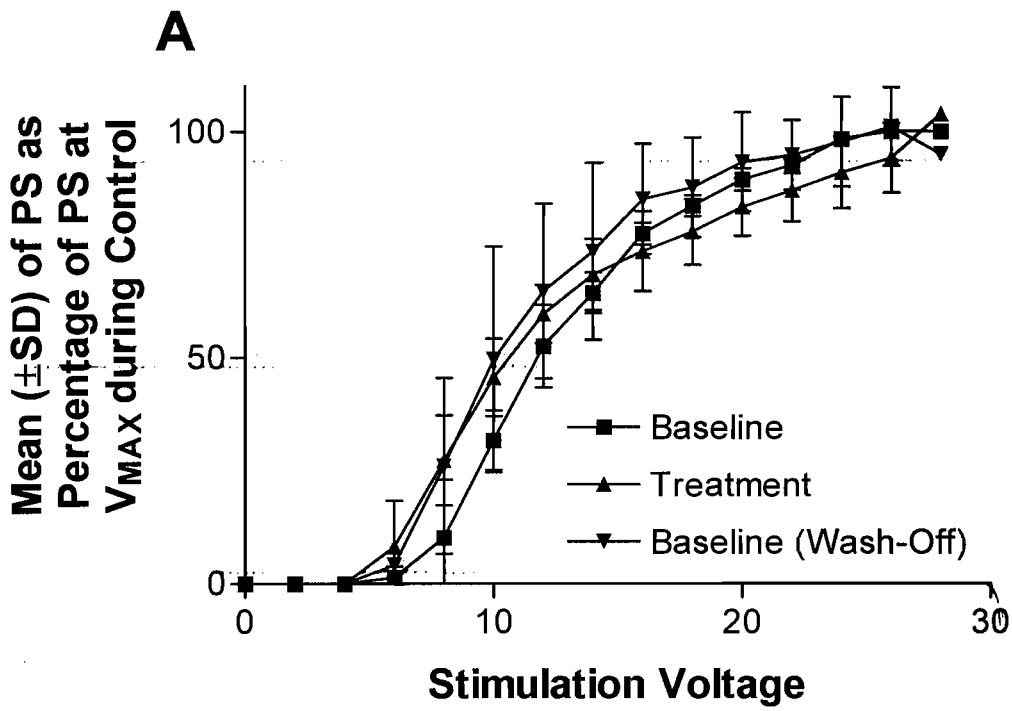
The stimulation protocols a-c where used in the same experiments. Following the S/R curve protocol, the paired pulse at fixed voltage protocol was repeated three times before the paired pulses at fixed interval protocol was applied. Once all these protocols had been completed in the presence of normal aCSF the recording system was perfused with qCSF containing varying concentrations of NPY. The protocols a-c were then repeated after a wash-on period of 30 minutes. The peptide was then washed out of the slice, and the protocols were repeated again after another period of 30 minutes.

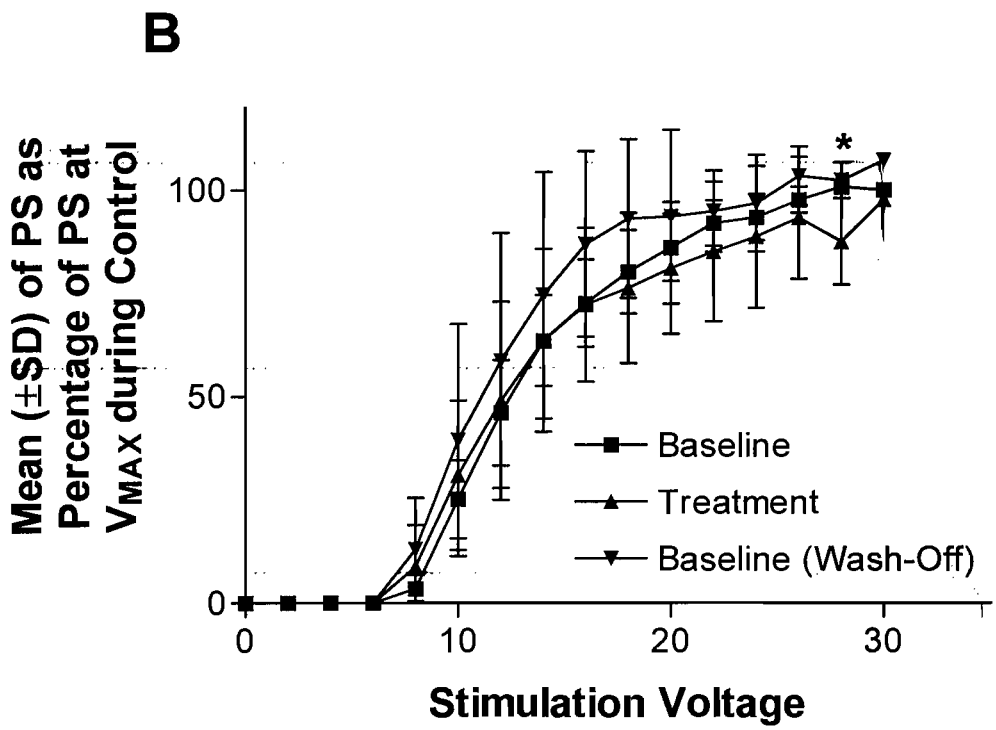
The data was analysed to find a value for each population spike (as in Figure 5.2) in mV. This data was then used to produce the graphs that follow. The experiments were then statistically analysed by carrying out analysis of variance, as compared to control, on the results. If a group of results showed significance then a Dunnett's *post-hoc* test was performed on the data.

5.3 Results

5.3.1 NPY effects on S/R curves

The following graphs show the effects of increasing concentrations of NPY on the synaptic transmission of the hippocampal slice.





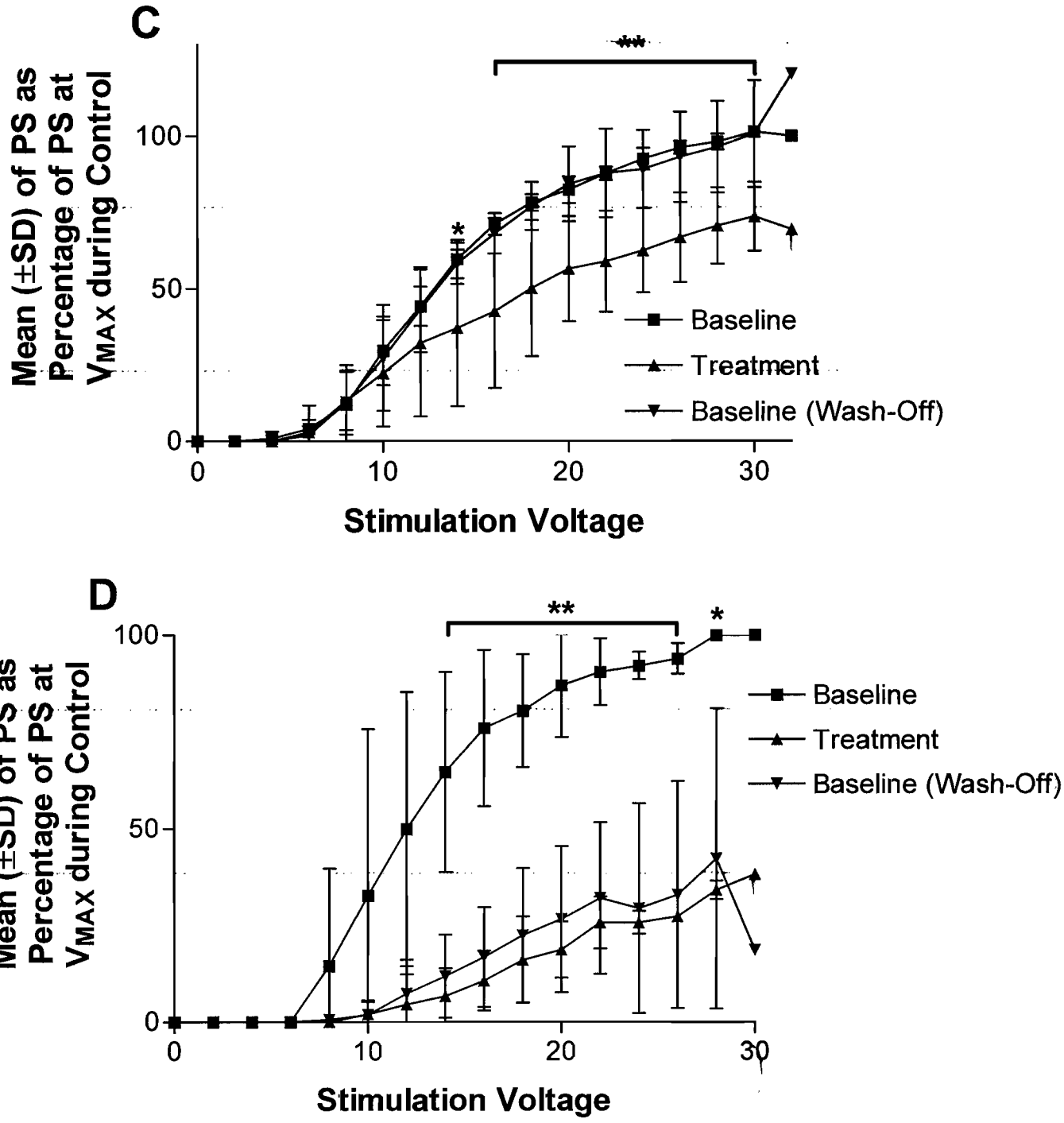


Figure 5.3 A-D Mean S/R curves showing the effect of A Control (n=3), B 100nM NPY (n=5), C 300nM NPY (n=6) and D 1μM NPY (n=3) on baseline physiology. The error bars are for Standard Deviation and *=$p<0.05$ & **= $p<0.01$.

The previous data shows that NPY has a dose dependent effect on the synaptic transmission of Acute Hippocampal Slices. In Fig 5.3C it can be seen that 300nM NPY causes a decrease in the response elicited by the

slice to a given level of stimulation. This concentration of NPY causes a 30% decrease in the level of response, as compared to that shown in the absence of the drug, and is significant at a level of $p<0.01$. This reduction in synaptic transmission was then reversed when the NPY was removed from the slice.

The inhibitory effect of NPY is also seen at a higher concentration of NPY (1µM Fig 5.3D), where it caused nearly a 60% decrease in response, however at this concentration the effect of the drug was not washed off within the time frame that was investigated.

The graphs in Fig.5.3 also show that with control treatment and with 100nM NPY there is little or no effect of the medium changes. And although 100nM NPY did cause a slight decrease in the level of synaptic transmission; this effect was not, on the whole, significant.

This data shows that NPY causes a dose dependent inhibition of synaptic activity within the hippocampus, and that at low concentrations (300nM) this effect is reversible.

5.3.2 Effects of NPY on Paired Pulse at Fixed Voltage.

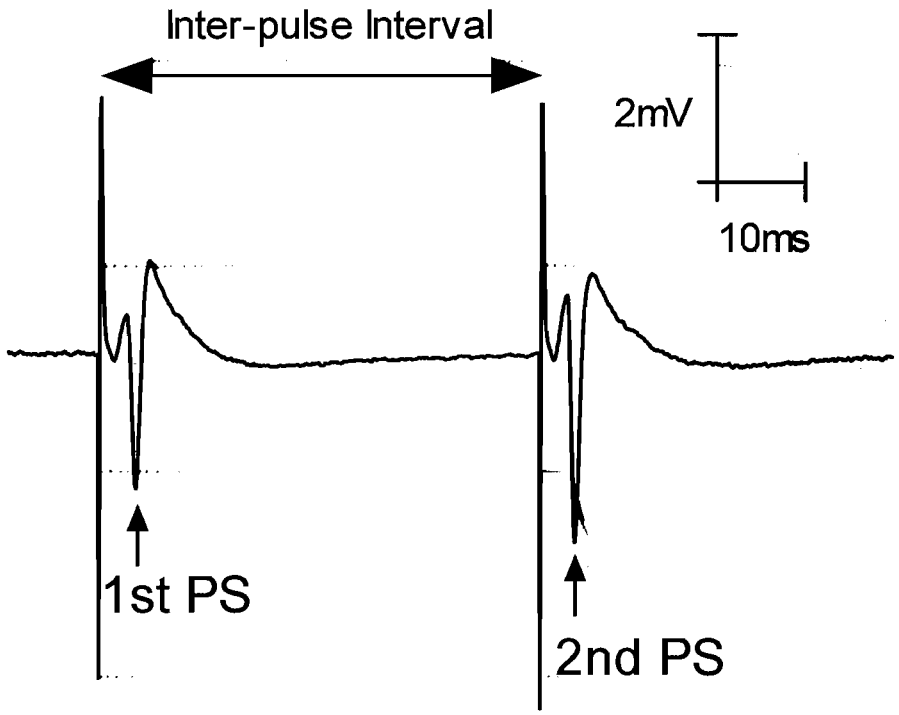
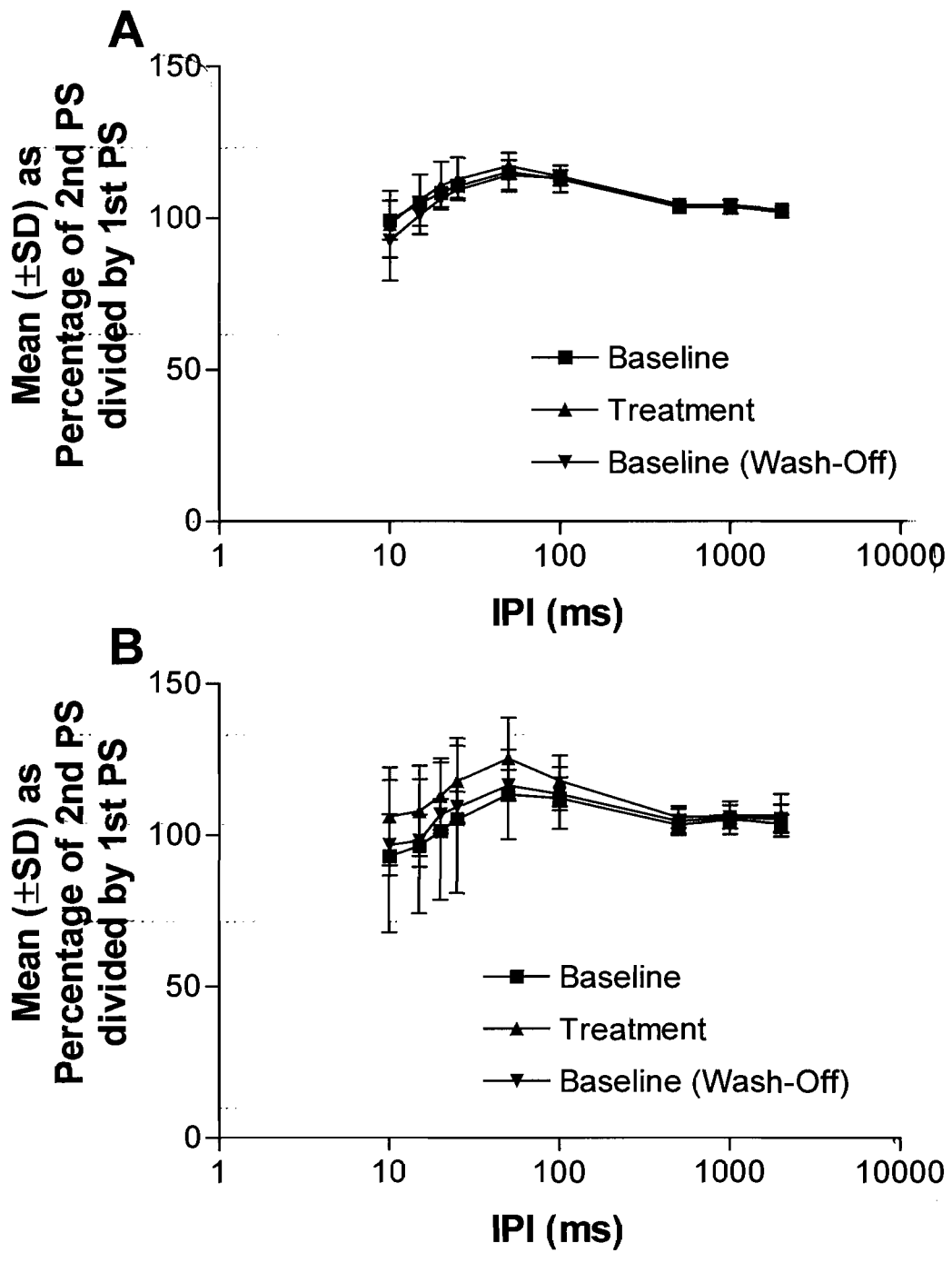


Figure 5.4 Schematic representation of the paired pulse protocol. (PS = Population Spike)

Figure 5.4 shows how the paired pulse protocol is carried out. In this series of experiments two pulses of equal voltage, equivalent to V_{MAX} (from the S/R curve protocol), are applied at various inter pulse intervals (IPI's). This investigates the effect of inhibition or facilitation that takes place within the hippocampus. If the 2nd population spike is smaller that the 1st then this demonstrates that feed-back inhibition is taking place, whereas if the 2nd PS is larger than the first then facilitation is occurring.

The following graphs show the effect of NPY on this effect.



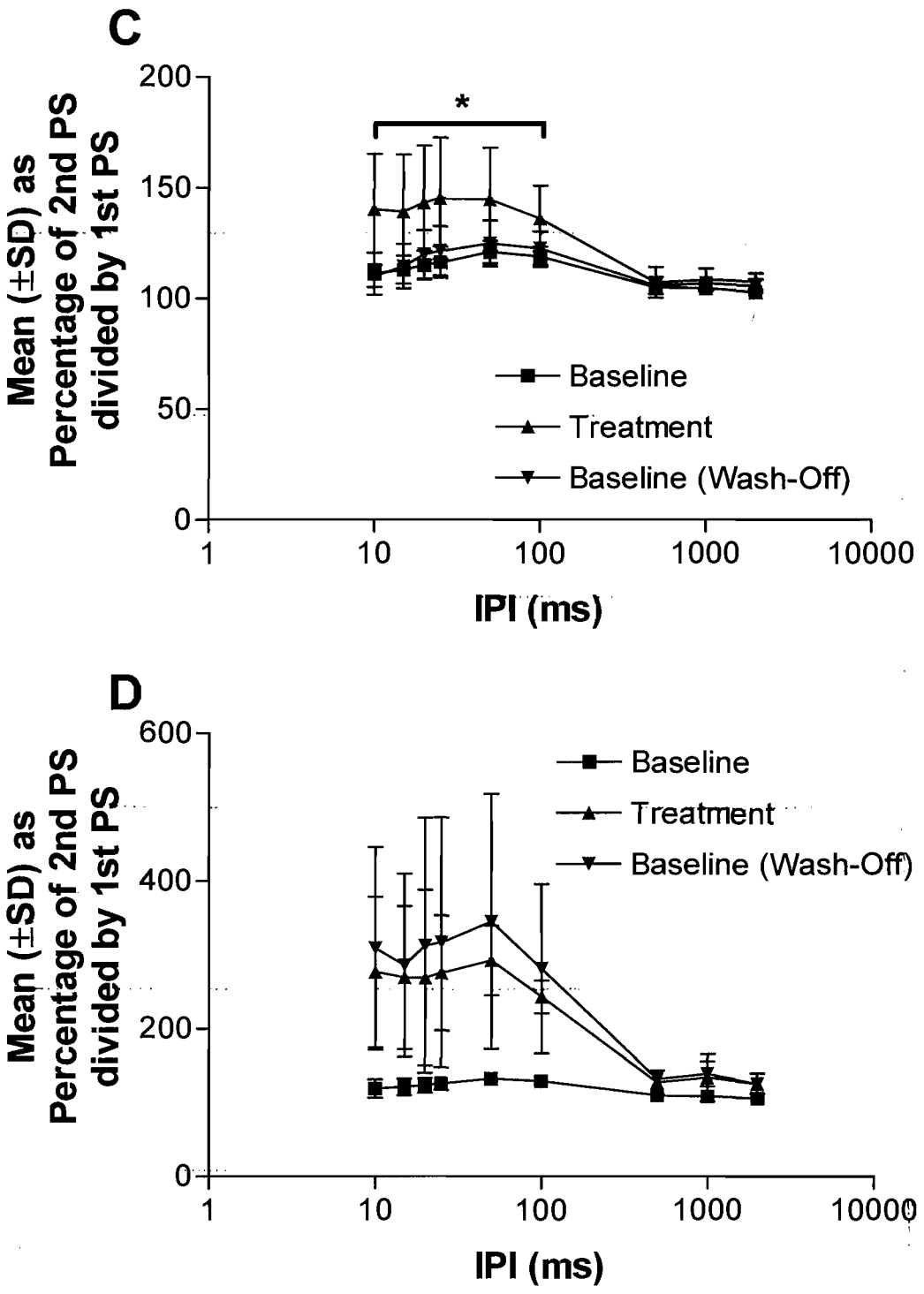
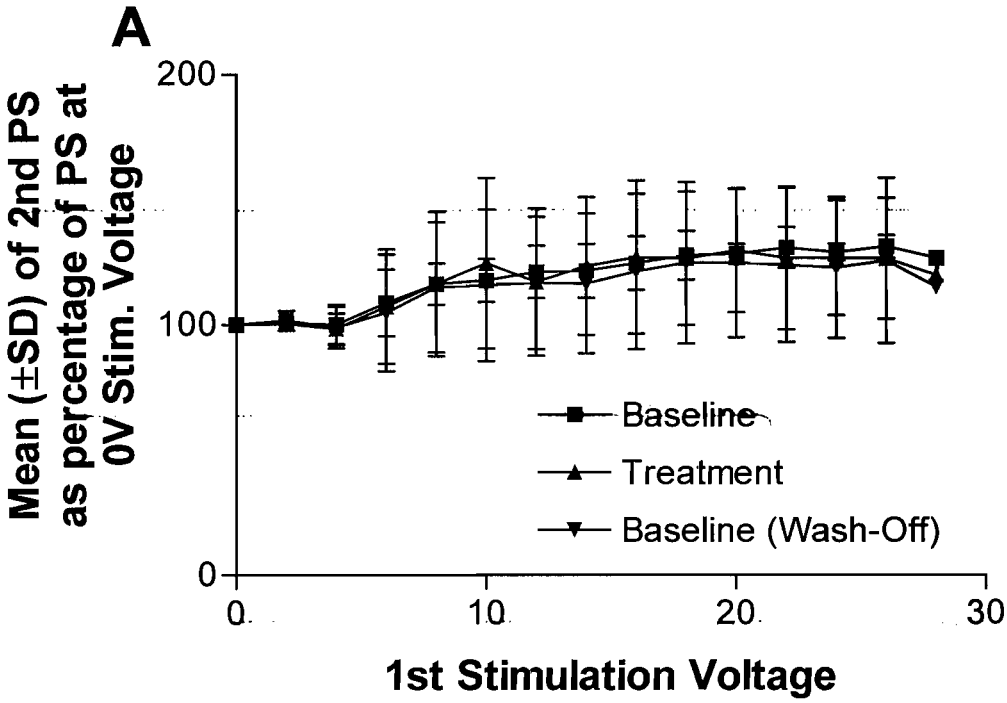


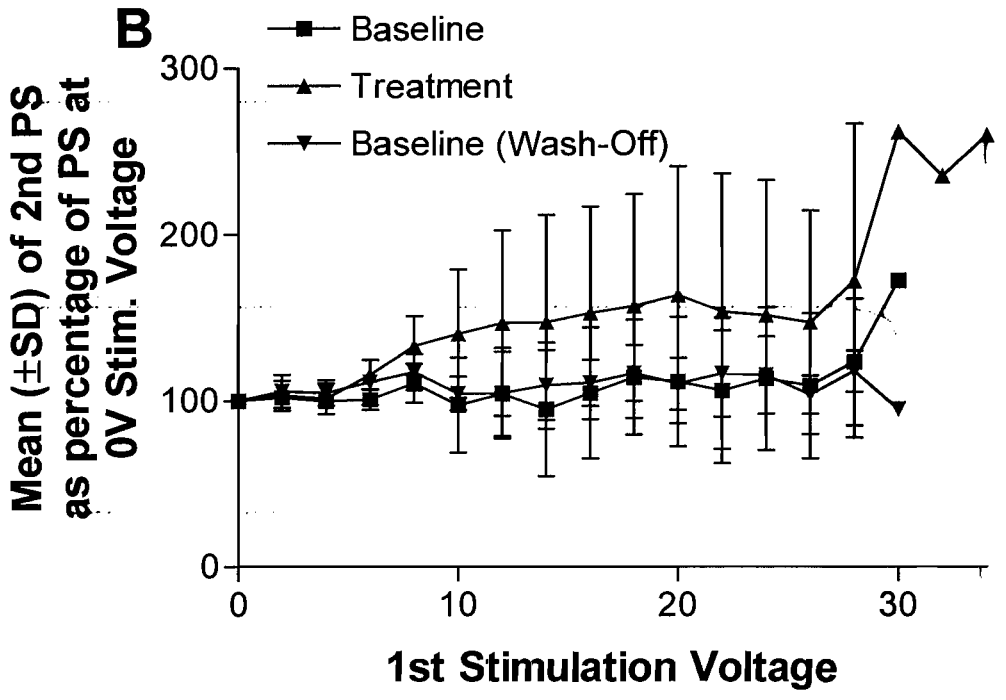
Figure 5.5 A-D Mean Inter Pulse Interval Graphs showing the effect of A Control (n=3), B 100nM NPY (n=5), C 300nM NPY (n=6) and D 1μM NPY (n=3) on paired pulse experiments. The error bars are for Standard Deviation and **= $p<0.01$.

The preceding graphs show that NPY affects the paired pulse experiments. At 300nM and 1μM NPY there is a marked increase in the percentage size of the 2nd PS at lower IPI's. This effect is, however, only significant in the

300nM experiment. At low IPI's (up to 100ms) the 2nd PS is significantly larger than the first, and this could be in part caused by the fact that the NPY causes the first PS to decrease considerably in size. Therefore even though the 2nd PS is smaller than occurs in the absence on NPY, the effect of the NPY on the 2nd PS is not as great as that of the first. However even taking this into account it does seem that NPY does cause an un coupling on the potential inhibition seen by the 1st PS upon the 2nd.

5.3.3 Effects of NPY on Paired Pulse At Fixed Interval.





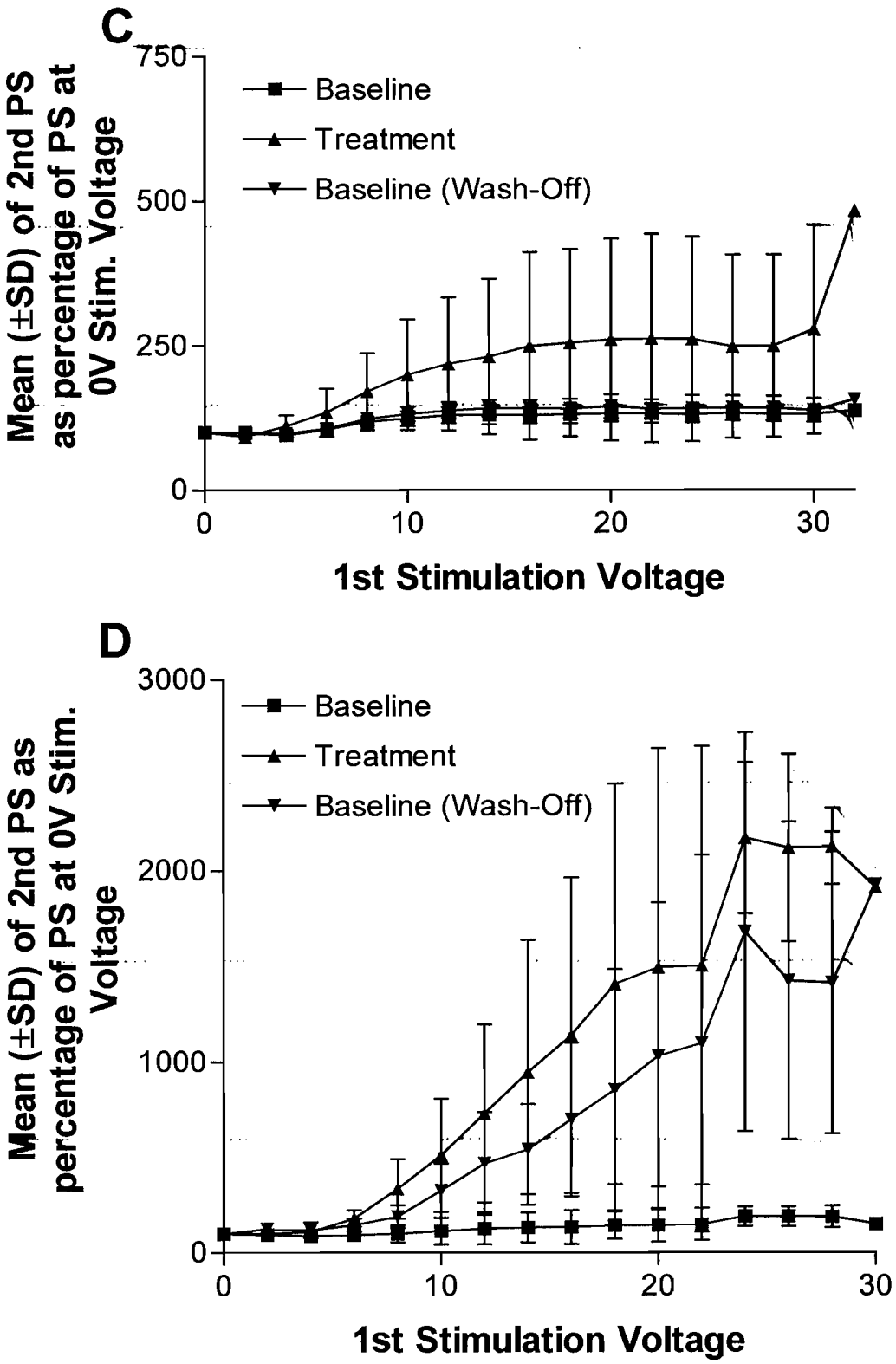


Figure 5.6 A-D. Mean Fixed Inter Pulse Interval Graphs showing the effect of A Control (n=3), B 100nM NPY (n=5), C 300nM NPY (n=6) and D 1μM NPY (n=3) on paired pulse experiments when the 1st Stimulation Voltage is variable and the 2nd Stimulus is fixed. The error bars are for Standard Deviation.

The previous graphs do not show any significant effects of NPY on the population spike at a fixed 15ms IPI, when the 1st stimulation voltage is gradually increased. Although there are no significant effects it is obvious from the data that NPY does have an effect on the 2nd population spike. At 300nM and 1μM concentration, the NPY causes an increase in the population spike as compared to that of control. Similarly to the paired pulse at varying IPI data, the NPY had greatly decreased the level of the 2nd population spike when the 1st stimulation voltage was set to 0V, and therefore the synaptic circuitry had a larger window of response available to it. This still does not negate the fact that exogenously applied NPY appears to upset the inhibitory circuitry within the hippocampus.

5.3.4 Effects on Epileptiform model.

In order to investigate the effects of Neuropeptide Y on epileptiform activity I wanted to carry out similar experiments to those already performed for Nociceptin in Chapter 2. I therefore decided to examine the effects of NPY in the 4-AP model.

Unfortunately I did not have the time to look at the NPY effects in the other epileptiform models .

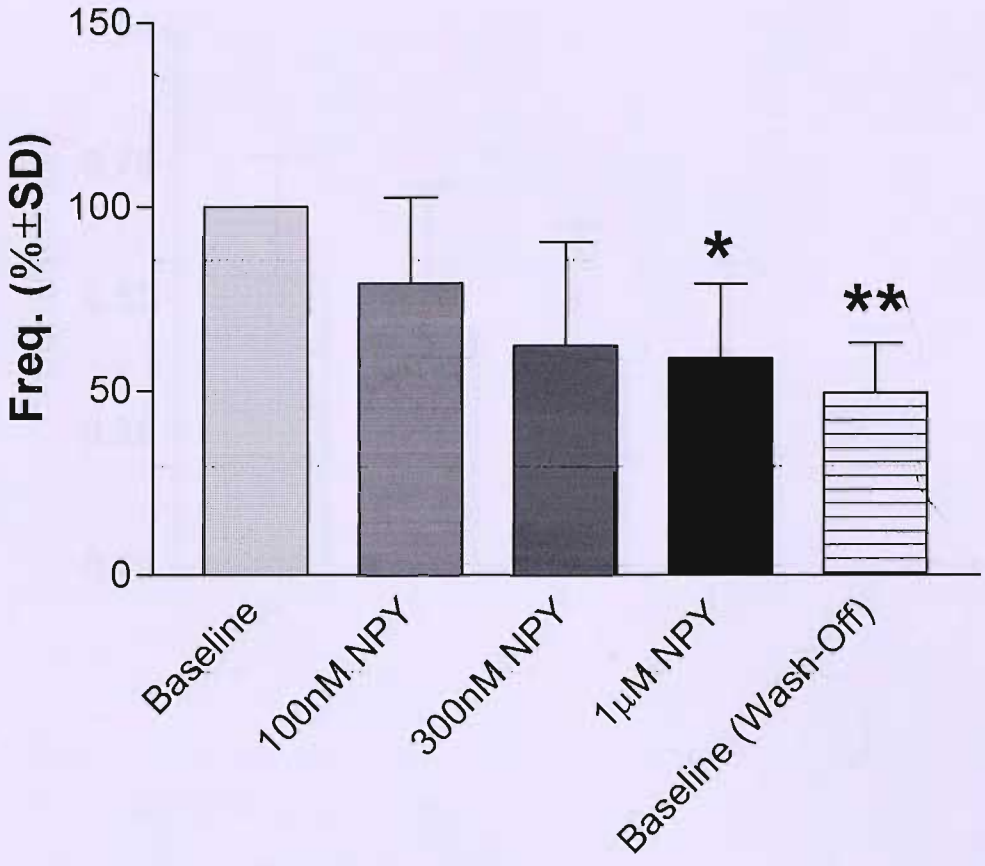


Fig 5.7 Graph showing the effect of varying doses of Neuropeptide Y on the frequency of spontaneous epileptiform discharges in Acute Hippocampal Slices pre-treated with 100μM 4-AP. All the experimental baselines are set to 100% and all other treatments are expressed as compared to baseline. *=$p<0.05$ & **=$p<0.01$ as compared to baseline (n=4).

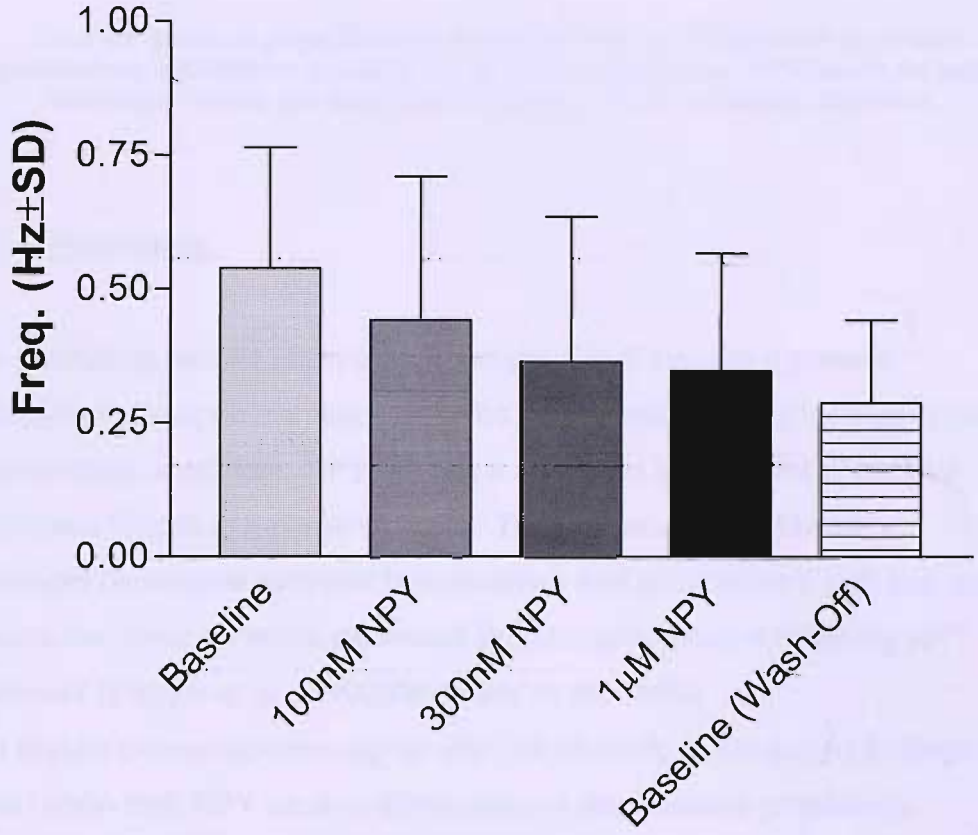


Fig 5.8 Graph showing the effect of varying doses of Neuropeptide Y on Acute Hippocampal Slices pre-treated with 100µM 4-AP. All values are those originally recorded, and there is no normalising to baseline. (n=4).

In the 4-AP model of epileptiform activity, NPY appears to be causing a dose-dependent decrease in the frequency of bursting, causing a 40% reduction in activity when 1µM NPY is administered ($p<0.05$). Unfortunately in the experiments carried out so far this effect is not reversed when the NPY is washed off.

Exp. ID	A	B	C	D
Baseline	0.6883 (±0.0879)	0.7183 (±0.0611)	0.5250 (±0.0869)	0.2267 (±0.0263)
100nM NPY	0.7667 (±0.0648)	0.5433 (±0.0492)	0.2933 (±0.1506)	0.1683 (±0.0146)
300nM NPY	0.6667 (±0.0484)	0.5150 (±0.0487)	0.1683 (±0.1104)	0.1100 (±0.0161)
1µM NPY	0.5800 (±0.0489)	0.4600 (±0.0540)	0.2667 (±0.0222)	0.0833 (±0.0539)
Baseline (Wash-Off)	0.4300 (±0.1033)	0.3583 (±0.0387)	0.2900 (±0.0086)	0.0700 (±0.0322)

Table 4.5 (previous page) Effect of Drug treatments on 10oμM 4-AP generated spontaneous epileptiform activity in Acute Hippocampal Slices. n=10 points for each treatment). Values are Means (of frequency in Hertz) ±Standard Deviation.

5.4 Discussion.

The preceding results show that Neuropeptide Y causes a general reduction in synaptic transmission. The first experiment shows that under physiological conditions NPY causes a reduction in the level of evoked responses following a given stimulus. This demonstrates that NPY somehow decreases synaptic transmission and is consistent with previous reports that have shown a decrease in glutamate release following NPY treatment (Patrylo et al., 1999;Whittaker et al., 1999).

The results presented here agree with the majority of published findings which show that NPY causes a reduction in the baseline physiology.

The next series of experiments showed that NPY does appear to affect the responses to paired pulse stimulation. The higher doses (300nM and 1μM) of NPY caused the experiments to reveal a larger ratio of second to first population spike at low inter pulse intervals. Although this response can in some way be explained as an effect of the large decrease in the first population spike due to the NPY action. It does not however explain why at the small IPIs the result is different to those at large IPIs. Somehow the NPY must be preventing the inhibitory neurons from re-polarising and therefore dampening the excitatory activity that is seen in the slice. This effect is similar for both the paired pulse experiments at fixed and varying IPIs, although it was not significant in the experiments at fixed IPIs.

The convulsant model here shows a contradictory story to that seen in chapter 2 with the Nociceptin. Whereas Nociceptin had no effect on the 4-AP model the NPY does exhibit an effect. Although the NPY data is provisional in that it only has an n=4 and it would be a good idea to repeat the experiments so that the same models are tested with NPY as were examined in the Nociceptin data, the initials results would seem to indicate

that the mechanism of action of the NPY is different to that of Nociceptin based on the findings from this one model.

Chapter 6

Discussion

6.1 General Discussion

My project looked at the effects of Nociceptin on anticonvulsant models in acute hippocampal slice from adult rats and EL mice, and organotypic hippocampal slice cultures. My work was carried out in conjunction with Glaxo SmithKline and a portion of my experimental work was carried out at their laboratories in Harlow, Essex. Currently available treatments for epilepsy rely on targeting the convulsant activity that results during a seizure; but are also ineffective in a number of individuals, especially in TLE. Therefore there is a need to further investigate the aetiology of epilepsy and look at novel treatment mechanisms.

The work described in the preceding chapters shows that Nociceptin plays a significant role as an anticonvulsant in the models investigated. This indicates that the Nociceptin/OP4 pathway could provide a valuable avenue for future investigation of the causes and possible treatment targets for the pharmacological intervention in previously untreatable forms of epilepsy. Nociceptin showed a significant anticonvulsant action on acute hippocampal slices in which convulsant activity had been induced by 10μM Bicuculline or zero Mg^{++}. This effect was not seen in the High K^{+} or 4-AP bursting models. One possible mechanism of action which could account for these differences would be if stimulation of the OP4 receptor was linked to a decrease in the conductance of the HCN channels via adenyl cyclase (AC) and cyclic-AMP (cAMP), which would reduce the Ih current across the cell membrane and thus lead to a longer period of hyperpolarisation following ictal events. Opioids have been shown to affect the I_h current (Ingram and Williams, 1994) via an effect on AC, by blocking the forskolin induced upregulation of the current; and HCN channels have been shown to play a role in the modulation of nociceptive pathways (Chaplan et al., 2003) through an increase in spontaneous action potentials, following nerve injury, which is reduced by ZD7288.

This hypothesis could be further tested by looking at drugs that interact with the cAMP messenger system, such as forskolin, or directly with the HCN channels. It has been shown that CsCl blocks the HCN channels and ZD7288 is an antagonist of the channel. The I_h current has been implicated

as a target which if blocked could have potential antiepileptic effects (Kitayama et al., 2003), and specific blockade of the channel with ZD7288 in the zero Mg^{++} model has been shown to block spontaneous bursting in acute hippocampal slices (Arias and Bowlby, 2005).

There is evidence that the development of the I_h current does not alter when compared between that seen in cultures or *in vivo* observations (Vasilyev and Barish, 2004). Under normal conditions the HCN channels are formed from homotetramers of the four known HCN subunits (HCN1, HCN2, HCN3 and HCN4) (Ludwig et al., 1998;Robinson and Siegelbaum, 2003), but it has been shown that heterotetramers of HCN1 and HCN2 subunits can form in pyramidal neurons following seizure activity (Brewster et al., 2005).

As cAMP mediates a number of cellular processes, such as regulation of LTP within the hippocampus (Weisskopf et al., 1994;Frey et al., 1993;Bolshakov et al., 1997) and modulation of AMPA and KA activated receptors (Raymond et al., 1993;Michaelis, 1998;Yakel et al., 1995;Soderling, 1996;Barria et al., 1997) amongst numerous other things, it is reasonable to assume that the decrease in cAMP levels seen, following activation of the OP4 receptor, could cause a number of other effects. One of the major pathways by which cAMP acts is the promotion of protein kinase A (PKA) activity (Taylor et al., 1990), therefore a reduction in cAMP will lead to a down regulation of this enzyme and would lead to the termination of a number of processes that this messenger is involved in, such as LTP and sensitivity of AMPA/KA receptor. The potential actions of PKA could be investigated by utilising protein kinase inhibitor (PKI) which is a specific inhibitor of PKA (Zheng et al., 1993).

The action of Nociceptin in additional convulsant models could help to further define the mechanisms of action of this potentially beneficial pathway, and this could help to further the understanding, and treatment, of epileptiform activity in the human brain.

The models I have looked at show some differences depending on the system they are investigated in. The Bicuculline convulsant model showed an average bursting frequency of about 0.1Hz in the acute hippocampal slices taken from adult rats and EL mice. On the other hand the organotypic

cultures produced a frequency of about half this when exposed to an identical concentration of the drug (0.05Hz). One possible reason for this apparent difference could be due to the organotypic cultures showing an increase in the baseline level of inhibitory tone as compared to that seen in acute slices. This is illustrated in chapter 5 where the paired pulse experiments show that when a 20ms IPI is carried out under control conditions then a near complete blockade of the 2^{nd} population spike is achieved. This is compared to that seen in the acute slices, see chapter 6, where inhibition is not always seen, but when it is the maximum reduction seen in the 2^{nd} population spike is in the region of 20%-30% of the size of the 1^{st} population spike. This may also be a result of differences in the physiological makeup of the cells because of their age. The cultures are taken from 8 day old rat pups and kept for between 2-3 weeks, whereas the acute hippocampal slices are prepared from mature animals.
Within the individual experiments Nociceptin did not cause any observable change in the amplitude or envelope of the bursting events. The only change that was seen was in the frequency of the bursts.
The zero Mg^{++} model of epileptiform activity caused an average bursting frequency of around 0.15Hz in the acute rat hippocampal slices and around 0.4Hz in the EL mice hippocampal slices. The application of Nociceptin appeared to cause a change in the envelope as well as the frequency of bursting. During Nociceptin application the individual events show a decrease in the after-discharge seen from the slices. Whereas the baseline shows a concentrated bursting following the initial induction of an event, in the Nociceptin treated slices less individual bursts are seen, although the event may go on for slightly longer in the acute rat slices. When the Nociceptin is removed from the medium then the events return to the type seen before Nociceptin administration. In the slices from EL mice the bursts appear to spread out following administration of Nociceptin. The baseline effects seen following the removal of magnesium from the perfusion medium are in line with previously published observations (Arias and Bowlby, 2005).

Along with no effect on the frequency of 4-AP induced convulsant activity, Nociceptin does not appear to show any effect on the amplitude or envelope

of the bursting events and so confirms the lack of any activity in this model. Similarly no effect is seen in the High K^+ model on any of these parameters.

The results observed in the slices from sensitised EL mice confirm the previous observations seen in the acute slices from wistar rats, where Nociceptin blocks the convulsant activity caused by the Bicuculline and zero Mg^{++} models. Previous evidence exists for an anticonvulsant role of opioid agonists in EL mice (Koide et al., 1992). In this experiment two opioid agonists morphine and D-Ala2-D-Leu5-enkephalin, selective for OP3 (mu) and OP1 receptors (delta) respectively were given to EL mice; they both caused a reduction in the incidence of seizures and the seizure score in a dose-dependent manner. The action of both agonists was reversed by the application of the opioid antagonist naloxone. In this experiment interestingly the slices from non-sensitised mice did not show the same responsiveness to Nociceptin application. This could indicate that a process similar to kindling is going on in the mouse brain over a period of time. The sensitised mice are experiencing a large number of seizures over this period and it is possible that changes in receptor systems are observed. There is little information to show the changes that the OP4 undergoes during prolonged seizures but it is reasonable to deduce that similar changes to that seen with other neuropeptides, such as NPY, could be occurring. In the case of NPY up-regulation of receptors is seen in epileptic tissue taken from both animal models and humans. A possible way of determining this for sure could be to expose animals to the kindling model for a timescale similar to that seen in the EL mice, about 6 months, and then quantifying the levels of OP4 receptor seen in comparison to age matched controls. This would show whether the receptor numbers are being modified over the life of the animals.

The likelihood that OP4 receptor activation could result in a larger response to Nociceptin following prolonged exposure to epileptiform activity could play an important role if agonists of this receptor are developed for clinical application. Nociceptin may therefore cause a larger anticonvulsant action in epilepsy patients if the OP4 receptor shows a degree of up-regulation.

I have been unable to find any direct evidence of HCN channel modifications in the EL mouse model, but the evidence that seizure activity can alter HCN expression (Brewster et al., 2005;Bender et al., 2003;Brewster et al., 2002) leads to the conclusion that the levels of HCN channel expression in EL mice could well be altered by the continuous seizure activity that their condition involves. This could possibly be investigated by measuring the I_h current in slices from EL mice directly or by investigating the effect of the HCN channel antagonist ZD7288 in these slices. Another method would be to measure the levels of HCN channels (Brewster et al., 2005) found in EL mice and compare between 'sensitised' and 'non-sensitised' animals, and also maybe between the ddY background strain, if available, to see if there are any changes in the channel levels. It could be that the EL mouse has naturally elevated levels of these channels which may predispose it to epileptiform activity, this could be found if HCN channel levels are similar in the different EL mice types but different to the ddY mice. If the levels differ between 'sensitised' and 'non-sensitised' EL mice then this would indicate a likely change induced by the continual seizure activity which the 'sensitised' mice undergo throughout their lifespan.

The organotypic experiments raise some interesting points that require further investigation. Of most significance is the realisation that the anticonvulsant action seen, with Nociceptin, appears to be independent of its abilities to affect synaptic transmission. Evidence previously reported indicates that Nociceptin causes a reduction in glutamate release and inhibition of synaptic transmission (Meis and Pape, 2001;Kawahara et al., 2004;Gompf et al., 2005) in the CNS. At present there are no published reports of whether Nociceptin causes any reduction in synaptic transmission in organotypic cultures, or in fact any studies for Nociceptin in organotypic cultures. Without any contradictory evidence it would seem that I have shown a novel mechanism for the anticonvulsant action of Nociceptin that does not involve the inhibition of synaptic transmission.
The majority of work to this time, looking at neuropeptides as anticonvulsants, has been done on NPY, and in these cases the anticonvulsant actions seem to be closely related to the ability to cause a

reduction in glutamatergic neurotransmission. Within the hippocampus three NPY receptors Y1, Y2 and Y5 are found in large quantities. Y1 is found postsynaptically (Caberlotto et al., 1997) on dentate granule cells and in the hilar NPY-containing interneurons (Paredes et al., 2003). Y2 is found presynaptically in terminal regions of mossy fibers and schaffer collaterals (Jacques et al., 1997), and activation has been shown to cause inhibition of calcium mediated glutamate release (Colmers et al., 1987;Haas et al., 1987;Qian et al., 1997). Y5 has been found to be distributed in the CA3 subfield of the hippocampus (Dumont et al., 1998). There is some evidence though that NPY has actions in organotypic cultures. NPY has been shown to be neuroprotective against AMPA receptor induced neurotoxicity within a hippocampal organotypic culture model (Silva et al., 2003), it is also suggested in the same report that this effect is mediated through the Y2 receptor. Unpublished data from my department has been unable to confirm this finding (L.E.Sundstrom and A.K.Pringle).

It has also been shown that within organotypic cultures NPY containing neurons are present (Mitchell et al., 1996) and retain their basic morphological characteristics (Finsen et al., 1992).

This is supported by the evidence found here that NPY did not affect the Bicuculline model or the synaptic transmission in organotypic cultures. This evidence could further support a role for the I_h current where synaptic transmission is not directly involved in the anticonvulsant action. One possible way to investigate this would be to see if the Nociceptin could affect epileptiform activity in the Non-synaptic model of epilepsy in the organotypic cultures. This model relies on the epileptiform activity being propagated via gap junctions as Ca^{++} is removed from the extracellular fluid in order to prevent synaptic transmission. It has previously been shown that cAMP can cause increased conductance at gap junctions (Gladwell and Jefferys, 2001). If Nociceptin were able to block this model then it would support the hypothesis that its actions are independent of synaptic transmission.

4-AP showed neurotoxicity on the organotypic cultures but appeared to provide a stable model of convulsant activity when applied to acute hippocampal slices. This could be down to glutamate neurotoxicity following

the activation of NMDA receptors. As 4-AP application increases the resting membrane potential of the neuron then the Mg^{++} block will be lessened on the NMDA receptors. This will in turn lead to increased levels of Ca^{++} entry into the cell. This is a major cause of neurotoxicity within the CNS and could account for the cell death that is observed. Bicuculline does not affect the resting potential and so although glutamate is released from the presynaptic terminal it is moped up by the astrocytes present in the culture. This glutamate therefore never reaches a level where neurotoxicity could result.

I have shown that the receptors for OP4, Y1, Y2 and Y5 are all present in 8 day-old rats and in organotypic hippocampal slice cultures. This indicates that they all possess the possibility for NPY and Nociceptin to act on these tissues. Although the receptors are present the data does not show whether these are functional receptors and further studies could be useful to further the understanding of the receptors in cultures. Firstly it would be good to isolate where the receptors are expressed and secondly to examine whether they are coupled to their second messenger systems. This could be achieved by doing in-situ hybridisation studies to see where in the hippocampus the receptor mRNA is localised.
The activation of the second messenger pathways could be investigated and it could be seen whether there are any changes in cAMP levels following administration of the drugs.

Following the interesting observations that Nociceptin did not affect synaptic transmission in the cultures but did cause a reduction in convulsant activity, it was interesting to note that NPY, the most studied neuropeptide anticonvulsant, also did not affect synaptic transmission, but in this case it also did not cause an affect on convulsant activity in the Bicuculline model. This points to the possibility that the anticonvulsant actions reported for NPY may be mediated solely through its ability to affect synaptic transmission and so this provides a novel insight into the difference between Nociceptin and NPY as endogenous anticonvulsant agents.
Following these observations I outlined some work I did looking at the affects of NPY on synaptic transmission and one epilepsy model, 4-AP, in

acute hippocampal slices from wistar rats. These observations showed that the synaptic transmission and paired pulse inhibition were in line with what I would have expected to see from that which has been previously reported. Of most interest is the fact that NPY appeared to show an anticonvulsant action on the 4-AP model. This is different to the effect seen in the Nociceptin data, where Nocieptin did not show any effect on the bursting activity at the concentration tested. Incidentally the concentration used for Nociceptin was the same (1μM) as the upper dose used in the NPY experiments. This would further point to a difference between the methods of action of these two neuropeptides. It may be that due to its actions on synaptic transmission NPY would be seen to have anticonvulsant actions in the majority of models investigate within the acute slices. The Y2 receptor, which is the one that has been predominantly linked to a role in anti-convulsant behaviour (el Bahh et al., 2005), has been shown (Misra et al,, 2004) to be coupled to both the increase in G_i and G_q second messengers. This differs to the OP4 receptor where it is linked mainly to an increase in just G_i. The additional activation of Gq would lead to an increase in Phospholipase C (PLC) which would cleave PI 4,5-bisphospate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes an increase in intracellular calcium by stimulating the endoplasmic reticulum to release calcium, and DAG causes the activation of protein kinase C (PKC). PKC can, amongst other things, lead to the phosphorylation of ion channels and thus alter their properties and change the excitability of the neuronal membrane.

Nociceptin itself would not be a suitable future drug treatment as its peptide nature would make it difficult to target to the brain. Firstly, a peptide would be very difficult to administer as it would be unsuitable to give via the enteral routes and would have to be given by intravenous injection to ensure that it wasn't broken down. Once in the blood stream it would be unable to enter the brain as it could not cross the blood-brain barrier. Therefore any treatments that utilise the Nociceptin/OP4 pathway would need to be based around small molecule non-peptide ligands that can selectively activate the OP4 receptor.

6.2 Conclusions

This work demonstrates that the application of Nociceptin, to the perfusion medium of *in vitro* maintained acute slices and organotypic cultures, causes an anticonvulsant action as demonstrated in various models of epileptiform activity. This anticonvulsant action is also seen in *in vitro* induced convulsant activity in acute slices taken from a genetic mouse model of epilepsy. This action could be caused by the blockade of the I_h current in the hippocampus that could lead to a decrease in activity caused by the increase in the time taken to repolarise the membrane following an individual bursting event. The large amount of evidence linking the I_h current to a significant role in the development of epilepsy could potentially implicate the Nociceptin/OP4 pathway as an interesting target for future epilepsy treatments.

Appendix I

STOCK SOLUTIONS

Artificial Cerebrospinal Fluid (aCSF)

Compound	Concentration (mM)
NaCl	124
KCl	3
NaH_2PO_4	1
$MgSO_4$	1
$NaHCO_3$	25
Glucose	10
$CaCl_2$	2

Cutting aCSF

Compound	Concentration (mM)
Sucrose	189
Glucose	10
$NaHCO_3$	26
KCl	2.5
$MgCl_2$	5
$CaCl_2$	0.1
NaH_2PO_4	1.2

0 Mg^{++} aCSF

Compound	Concentration (mM)
NaCl	124
KCl	5.75
KH_2PO_4	1.25
$NaHCO_3$	26
Dextrose	10
$CaCl_2$	2

High K^+ aCSF

Compound	Concentration (mM)
NaCl	124
KCl	8.5
NaH_2PO_4	1
$MgSO_4$	1
$NaHCO_3$	25
Glucose	10
$CaCl_2$	2

Culture Medium

Compound	Quantity
Minimum essential medium + Earle's salts	50%
Heat inactivated horse serum	25%
Hank's balanced saline solution	25%
Glutamine	1mM
Glucose	4.5mg/ml

Serum Free Culture Medium

Compound	Quantity
Minimum essential medium + Earle's salts	75%
Hank's balanced saline solution	25%
Glutamine	1mM
Glucose	4.5mg/ml

Lysis Buffer – Stage 1

Compound	Concentration (mM)
NaCl	25
EDTA	2
DTT	0.5
HEPES	20
β-glycerophosphate	20

Make up 1l at the above concentrations and ensure that the pH is 8.0. If the pH is not correct then fluorine gas will be released during the next stage.

Lysis Buffer – Stage 2

Compound	Concentration (mM)
Sodium Fluoride	50
Sodium Vanadate	1
Triton X-100	0.1%

One tablet of 'mini-complete' (Boerhinger) protease inhibitors added to 10ml of lysis buffer

4x Separating Gel Buffer

1.2M TrisHCl, pH 8.8

2x Stacking Gel Buffer

0.25M TrisHCl, pH 6.8

30% Stock Acrylamide Solution

Acrylamide 37.5:Biscacrylamide 1

12.5% Acrylamide Solution

Compound	Quantity
30% Acrylamide Stock	50.3ml
4x Seperating Gel Buffer	30ml
20% SDS	0.6ml
H_2O	39.7ml

4% Acrylamide Solution

Compound	Quantity
30% Acrylamide Stock	16ml
2x Stacking Gel Buffer	60ml
20% SDS	0.6ml
H_2O	43.4ml

10x Electrode Buffer

Compound	Quantity
Tris	30.3g
Glycine	144g
20% SDS (in H_2O)	50ml

2x Sample Buffer

Compound	Quantity
2x Stacking Gel Buffer	25ml
20% SDS	10ml
20% Glycerol	10g
10% 2-mercaptoethanol	5ml
Bromophenol Blue (in IMS) 10mg/ml	200µl

Blotting Buffer

Compound	Quantity
10x Electrode Buffer	40ml
Methanol	100ml
H_2O	360ml

References

1. Amaral DG, Witter MP (1989) The three-dimensional organization of the hippocampal formation: a review of anatomical data. Neuroscience 31:571-591.

2. Andersen P, Soleng AF, Raastad M (2000) The hippocampal lamella hypothesis revisited. Brain Res 886:165-171.

3. Anderson P, Bliss TV, Skrede KK (1971) Lamellar organization of hippocampal pathways. Exp Brain Res 13:222-238.

4. Anderson WW, Collingridge GL (2001) The LTP Program: a data acquisition program for on-line analysis of long-term potentiation and other synaptic events. J Neurosci Methods 108:71-83.

5. Anton B, Fein J, To T, Li X, Silberstein L, Evans CJ (1996) Immunohistochemical localization of ORL-1 in the central nervous system of the rat. J Comp Neurol 368:229-251.

6. Arias RL, Bowlby MR (2005) Pharmacological characterization of antiepileptic drugs and experimental analgesics on low magnesium-induced hyperexcitability in rat hippocampal slices. Brain Res 1047:233-244.

7. Bahr BA (1995) Long-term hippocampal slices: a model system for investigating synaptic mechanisms and pathologic processes. J Neurosci Res 42:294-305.

8. Bahr BA, Abai B, Gall CM, Vanderklish PW, Hoffman KB, Lynch G (1994) Induction of beta-amyloid-containing polypeptides in hippocampus: evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. Exp Neurol 129:81-94.

9. Bahr BA, Kessler M, Rivera S, Vanderklish PW, Hall RA, Mutneja MS, Gall C, Hoffman KB (1995) Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. Hippocampus 5:425-439.

10. Baraban SC, Hollopeter G, Erickson JC, Schwartzkroin PA, Palmiter RD (1997) Knock-out mice reveal a critical antiepileptic role for neuropeptide Y. J Neurosci 17:8927-8936.

11. Barria A, Muller D, Derkach V, Griffith LC, Soderling TR (1997) Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. Science 276:2042-2045.

12. Bausch SB, McNamara JO (2000) Synaptic connections from multiple subfields contribute to granule cell hyperexcitability in hippocampal slice cultures. J Neurophysiol 84:2918-2932.

13. Beach RL, Bathgate SL, Cotman CW (1982) Identification of cell types in rat hippocampal slices maintained in organotypic cultures. Brain Res 255:3-20.

14. Bender RA, Soleymani SV, Brewster AL, Nguyen ST, Beck H, Mathern GW, Baram TZ (2003) Enhanced expression of a specific hyperpolarization-activated cyclic nucleotide-gated cation channel (HCN) in surviving dentate gyrus granule cells of human and experimental epileptic hippocampus. J Neurosci 23:6826-6836.

15. Benveniste M, Mayer ML (1991) Kinetic analysis of antagonist action at N-methyl-D-aspartic acid receptors. Two binding sites each for glutamate and glycine. Biophys J 59:560-573.

16. Bernard C, Wheal HV (1995) Plasticity of AMPA and NMDA receptor-mediated epileptiform activity in a chronic model of temporal lobe epilepsy. Epilepsy Res 21:95-107.

17. Bianchi C, Marani L, Barbieri M, Marino S, Beani L, Siniscalchi A (2004) Effects of nociceptin/orphanin FQ and endomorphin-1 on glutamate and GABA release, intracellular [Ca2+] and cell excitability in primary cultures of rat cortical neurons. Neuropharmacology 47:873-883.

18. Bingmann D, Speckmann EJ, Baker RE, Ruijter J, de Jong BM (1988) Differential antiepileptic effects of the organic calcium antagonists verapamil and flunarizine in neurons of organotypic neocortical explants from newborn rats. Exp Brain Res 72:439-442.

19. Bolshakov VY, Golan H, Kandel ER, Siegelbaum SA (1997) Recruitment of new sites of synaptic transmission during the cAMP-dependent late phase of LTP at CA3-CA1 synapses in the hippocampus. Neuron 19:635-651.

20. Brewster A, Bender RA, Chen Y, Dube C, Eghbal-Ahmadi M, Baram TZ (2002) Developmental febrile seizures modulate hippocampal gene expression of hyperpolarization-activated channels in an isoform- and cell-specific manner. J Neurosci 22:4591-4599.

21. Brewster AL, Bernard JA, Gall CM, Baram TZ (2005) Formation of heteromeric hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in the hippocampus is regulated by developmental seizures. Neurobiol Dis 19:200-207.

22. Buchs PA, Stoppini L, Muller D (1993) Structural modifications associated with synaptic development in area CA1 of rat hippocampal organotypic cultures. Brain Res Dev Brain Res 71:81-91.

23. Bunzow JR, Saez C, Mortrud M, Bouvier C, Williams JT, Low M, Grandy DK (1994) Molecular cloning and tissue distribution of a

putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type. FEBS Lett 347:284-288.

24. Caberlotto L, Fuxe K, Sedvall G, Hurd YL (1997) Localization of neuropeptide Y Y1 mRNA in the human brain: abundant expression in cerebral cortex and striatum. Eur J Neurosci 9:1212-1225.

25. Caeser M, Aertsen A (1991) Morphological organization of rat hippocampal slice cultures. J Comp Neurol 307:87-106.

26. Calo G, Rizzi A, Marzola G, Guerrini R, Salvadori S, Beani L, Regoli D, Bianchi C (1998) Pharmacological characterization of the nociceptin receptor mediating hyperalgesia in the mouse tail withdrawal assay. Br J Pharmacol 125:373-378.

27. Chan JS, Yung LY, Lee JW, Wu YL, Pei G, Wong YH (1998) Pertussis toxin-insensitive signaling of the ORL1 receptor: coupling to Gz and G16 proteins. J Neurochem 71:2203-2210.

28. Chaplan SR, Guo HQ, Lee DH, Luo L, Liu C, Kuei C, Velumian AA, Butler MP, Brown SM, Dubin AE (2003) Neuronal hyperpolarization-activated pacemaker channels drive neuropathic pain. J Neurosci 23:1169-1178.

29. Chen SF, Huang CC, Wu HM, Chen SH, Liang YC, Hsu KS (2004) Seizure, neuron loss, and mossy fiber sprouting in herpes simplex virus type 1-infected organotypic hippocampal cultures. Epilepsia 45:322-332.

30. Chesnut TJ, Swann JW (1988) Epileptiform activity induced by 4-aminopyridine in immature hippocampus. Epilepsy Res 2:187-195.

31. Clarke S, Chen Z, Hsu MS, Pintar J, Hill R, Kitchen I (2001) Quantitative autoradiographic mapping of the ORL1, mu-, delta- and kappa-receptors in the brains of knockout mice lacking the ORL1 receptor gene. Brain Res 906:13-24.

32. Clements JD, Westbrook GL (1991) Activation kinetics reveal the number of glutamate and glycine binding sites on the N-methyl-D-aspartate receptor. Neuron 7:605-613.

33. Colmers WF, Lukowiak K, Pittman QJ (1985) Neuropeptide Y reduces orthodromically evoked population spike in rat hippocampal CA1 by a possibly presynaptic mechanism. Brain Res 346:404-408.

34. Colmers WF, Lukowiak K, Pittman QJ (1987) Presynaptic action of neuropeptide Y in area CA1 of the rat hippocampal slice. J Physiol 383:285-299.

35. Colmers WF, Lukowiak K, Pittman QJ (1988) Neuropeptide Y action in the rat hippocampal slice: site and mechanism of presynaptic inhibition. J Neurosci 8:3827-3837.

36. Connor M, Vaughan CW, Chieng B, Christie MJ (1996a) Nociceptin receptor coupling to a potassium conductance in rat locus coeruleus neurones in vitro. Br J Pharmacol 119:1614-1618.

37. Connor M, Yeo A, Henderson G (1996b) The effect of nociceptin on Ca2+ channel current and intracellular Ca2+ in the SH-SY5Y human neuroblastoma cell line. Br J Pharmacol 118:205-207.

38. Cronin J, Dudek FE (1988) Chronic seizures and collateral sprouting of dentate mossy fibers after kainic acid treatment in rats. Brain Res 474:181-184.

39. Debanne D, Guerineau NC, Gahwiler BH, Thompson SM (1995) Physiology and pharmacology of unitary synaptic connections between pairs of cells in areas CA3 and CA1 of rat hippocampal slice cultures. J Neurophysiol 73:1282-1294.

40. Dhawan BN, Cesselin F, Raghubir R, Reisine T, Bradley PB, Portoghese PS, Hamon M (1996a) International Union of Pharmacology. XII. Classification of opioid receptors. Pharmacol Rev 48:567-592.

41. Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. Pharmacol Rev 51:7-61.

42. Dreifuss FE (1989) Classification of epileptic seizures and the epilepsies. Pediatr Clin North Am 36:265-279.

43. Dumont Y, Fournier A, Quirion R (1998) Expression and characterization of the neuropeptide Y Y5 receptor subtype in the rat brain. J Neurosci 18:5565-5574.

44. Duport S, Stoppini L, Correges P (1997) Electrophysiological approach of the antiepileptic effect of dexamethasone on hippocampal slice culture using a multirecording system: the Physiocard. Life Sci 60:L-6.

45. Egert U, Schlosshauer B, Fennrich S, Nisch W, Fejtl M, Knott T, Muller T, Hammerle H (1998) A novel organotypic long-term culture of the rat hippocampus on substrate-integrated multielectrode arrays. Brain Res Brain Res Protoc 2:229-242.

46. el Bahh B, Balosso S, Hamilton T, Herzog H, Beck-Sickinger AG, Sperk G, Gehlert DR, Vezzani A, Colmers WF (2005) The anti-epileptic actions of neuropeptide Y in the hippocampus are mediated by Y and not Y receptors. Eur J Neurosci 22:1417-1430.

47. el Bahh B, Cao JQ, Beck-Sickinger AG, Colmers WF (2002) Blockade of neuropeptide Y(2) receptors and suppression of NPY's anti-epileptic actions in the rat hippocampal slice by BIIE0246. Br J Pharmacol 136:502-509.

48. Engel J, Jr. (2001) Mesial temporal lobe epilepsy: what have we learned? Neuroscientist 7:340-352.

49. Feng Y, Chao DM, Li WM, Cao YX, Wang YQ, Wu GC (2004) Inhibition of nociceptin/orphanin FQ on penicillin-induced seizures in rats. Brain Res 1020:214-219.

50. Finsen BR, Tonder N, Augood S, Zimmer J (1992) Somatostatin and neuropeptide Y in organotypic slice cultures of the rat hippocampus: an immunocytochemical and in situ hybridization study. Neuroscience 47:105-113.

51. Florin S, Leroux-Nicollet I, Meunier JC, Costentin J (1997) Autoradiographic localization of [3H]nociceptin binding sites from telencephalic to mesencephalic regions of the mouse brain. Neurosci Lett 230:33-36.

52. Foddi MC, Mennini T (1997) [125I][Tyr14]Orphanin binding to rat brain: evidence for labelling the opioid-receptor-like 1 (ORL1). Neurosci Lett 230:105-108.

53. Fonnum F (1984) Glutamate: a neurotransmitter in mammalian brain. J Neurochem 42:1-11.

54. Fowler J, Crain SM (1984) Hyperexcitability in organotypic mouse hippocampal explants. Prog Neuropsychopharmacol Biol Psychiatry 8:507-514.

55. Francis PT, Sims NR, Procter AW, Bowen DM (1993) Cortical pyramidal neurone loss may cause glutamatergic hypoactivity and cognitive impairment in Alzheimer's disease: investigative and therapeutic perspectives. J Neurochem 60:1589-1604.

56. Frenk H (1983) Pro- and anticonvulsant actions of morphine and the endogenous opioids: involvement and interactions of multiple opiate and non-opiate systems. Brain Res 287:197-210.

57. Freund TF, Buzsaki G (1996) Interneurons of the hippocampus. Hippocampus 6:347-470.

58. Frey U, Huang YY, Kandel ER (1993) Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. Science 260:1661-1664.

59. Frotscher M, Gahwiler BH (1988) Synaptic organization of intracellularly stained CA3 pyramidal neurons in slice cultures of rat hippocampus. Neuroscience 24:541-551.

60. Frotscher M, Zafirov S, Heimrich B (1995) Development of identified neuronal types and of specific synaptic connections in slice cultures of rat hippocampus. Prog Neurobiol 45:143-164.

61. Fueta Y, Kawano H, Ono T, Mita T, Fukata K, Ohno K (1998) Regional differences in hippocampal excitability manifested by paired-pulse stimulation of genetically epileptic El mice. Brain Res 779:324-328.

62. Fukuda K, Kato S, Mori K, Nishi M, Takeshima H, Iwabe N, Miyata T, Houtani T, Sugimoto T (1994) cDNA cloning and regional distribution of a novel member of the opioid receptor family. FEBS Lett 343:42-46.

63. Furtinger S, Pirker S, Czech T, Baumgartner C, Ransmayr G, Sperk G (2001) Plasticity of Y1 and Y2 receptors and neuropeptide Y fibers in patients with temporal lobe epilepsy. J Neurosci 21:5804-5812.

64. Gahwiler BH (1984a) Development of the hippocampus in vitro: cell types, synapses and receptors. Neuroscience 11:751-760.

65. Gahwiler BH (1984b) Slice cultures of cerebellar, hippocampal and hypothalamic tissue. Experientia 40:235-243.

66. Gahwiler BH (1988) Organotypic cultures of neural tissue. Trends Neurosci 11:484-489.

67. Gahwiler BH (1981) Organotypic monolayer cultures of nervous tissue. J Neurosci Methods 4:329-342.

68. Gahwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM (1997) Organotypic slice cultures: a technique has come of age. Trends Neurosci 20:471-477.

69. Gariboldi M, Conti M, Cavaleri D, Samanin R, Vezzani A (1998) Anticonvulsant properties of BIBP3226, a non-peptide selective antagonist at neuropeptide Y Y1 receptors. Eur J Neurosci 10:757-759.

70. Gladwell SJ, Jefferys JG (2001) Second messenger modulation of electrotonic coupling between region CA3 pyramidal cell axons in the rat hippocampus. Neurosci Lett 300:1-4.

71. Gloor P, Fariello RG (1988) Generalized epilepsy: some of its cellular mechanisms differ from those of focal epilepsy. Trends Neurosci 11:63-68.

72. Goddard GV, McIntyre DC, Leech CK (1969) A permanent change in brain function resulting from daily electrical stimulation. Exp Neurol 25:295-330.

73. Gompf HS, Moldavan MG, Irwin RP, Allen CN (2005) Nociceptin/orphanin FQ (N/OFQ) inhibits excitatory and inhibitory synaptic signaling in the suprachiasmatic nucleus (SCN). Neuroscience 132:955-965.

74. Greber S, Schwarzer C, Sperk G (1994) Neuropeptide Y inhibits potassium-stimulated glutamate release through Y2 receptors in rat hippocampal slices in vitro. Br J Pharmacol 113:737-740.

75. Gulyas-Kovacs A, Doczi J, Tarnawa I, Detari L, Banczerowski-Pelyhe I, Vilagi I (2002) Comparison of spontaneous and evoked epileptiform activity in three in vitro epilepsy models. Brain Res 945:174-180.

76. Gutierrez R, Armand V, Schuchmann S, Heinemann U (1999) Epileptiform activity induced by low Mg2+ in cultured rat hippocampal slices. Brain Res 815:294-303.

77. Gutierrez R, Leff P, Romo-Parra H, Acevedo R, Anton B (2001) Orphanin-FQ/nociceptin inhibits kindling epileptogenesis and enhances hippocampal feed-forward inhibition. Neuroscience 105:325-333.

78. Haas HL, Hermann A, Greene RW, Chan-Palay V (1987) Action and location of neuropeptide tyrosine (Y) on hippocampal neurons of the rat in slice preparations. J Comp Neurol 257:208-215.

79. Harrison LM, Grandy DK (2000) Opiate modulating properties of nociceptin/orphanin FQ. Peptides 21:151-172.

80. Higashima M, Ohno K, Koshino Y (2002) Cyclic AMP-mediated modulation of epileptiform afterdischarge generation in rat hippocampal slices. Brain Res 949:157-161.

81. Howell OW, Doyle K, Goodman JH, Scharfman HE, Herzog H, Pringle A, Beck-Sickinger AG, Gray WP (2005) Neuropeptide Y stimulates neuronal precursor proliferation in the post-natal and adult dentate gyrus. J Neurochem 93:560-570.

82. Hughes J, Woodruff GN (1992) Neuropeptides. Function and clinical applications. Arzneimittelforschung 42:250-255.

83. Ikeda K, Kobayashi K, Kobayashi T, Ichikawa T, Kumanishi T, Kishida H, Yano R, Manabe T (1997) Functional coupling of the nociceptin/orphanin FQ receptor with the G-protein-activated K+ (GIRK) channel. Brain Res Mol Brain Res 45:117-126.

84. Ikeda K, Watanabe M, Ichikawa T, Kobayashi T, Yano R, Kumanishi T (1998) Distribution of prepro-nociceptin/orphanin FQ mRNA and its receptor mRNA in developing and adult mouse central nervous systems. J Comp Neurol 399:139-151.

85. Imaizumi K, Ito S, Kutsukaka G, Takizawa T, Fujiwara K, Tutikawa K (1959) The epilepsy-like abnormalities in a strain of mice. Jikken Dobutsu (Bull Exp Anim) 8:6-10.

86. Ingram SL, Williams JT (1994) Opioid inhibition of Ih via adenylyl cyclase. Neuron 13:179-186.

87. Ingram SL, Williams JT (1996) Modulation of the hyperpolarization-activated current (Ih) by cyclic nucleotides in guinea-pig primary afferent neurons. J Physiol 492 (Pt 1):97-106.

88. Iritani S, Niizato K, Nawa H, Ikeda K (2000) The distribution of neuropeptide Y and brain-derived neurotrophic factor immunoreactivity in hippocampal formation of the monkey and rat. Brain Res 852:475-478.

89. Jacques D, Dumont Y, Fournier A, Quirion R (1997) Characterization of neuropeptide Y receptor subtypes in the normal human brain, including the hypothalamus. Neuroscience 79:129-148.

90. Jefferys JGR, Mitchell P, O'Hara L, Tiley C, Hardy J, Jordan SJ, Lynch M, Wadsworth J (1991) *Ex vivo* release of GABA from tetanus toxin-induced chronic epileptic foci decreased during the active seizure phase. Neurochem Int 18:373-379.

91. Jenck F, Moreau JL, Martin JR, Kilpatrick GJ, Reinscheid RK, Monsma FJ, Jr., Nothacker HP, Civelli O (1997) Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress. Proc Natl Acad Sci U S A 94:14854-14858.

92. Kapusta DR, Sezen SF, Chang JK, Lippton H, Kenigs VA (1997) Diuretic and antinatriuretic responses produced by the endogenous opioid-like peptide, nociceptin (orphanin FQ). Life Sci 60:L15-L21.

93. Kawahara Y, Hesselink MB, van Scharrenburg G, Westerink BH (2004) Tonic inhibition by orphanin FQ/nociceptin of noradrenaline neurotransmission in the amygdala. Eur J Pharmacol 485:197-200.

94. King JT, Jr., LaMotte CC (1989) El mouse as a model of focal epilepsy: a review. Epilepsia 30:257-265.

95. Kitayama M, Miyata H, Yano M, Saito N, Matsuda Y, Yamauchi T, Kogure S (2003) Ih blockers have a potential of antiepileptic effects. Epilepsia 44:20-24.

96. Klapstein GJ, Colmers WF (1997) Neuropeptide Y suppresses epileptiform activity in rat hippocampus in vitro. J Neurophysiol 78:1651-1661.

97. Klapstein GJ, Colmers WF (1993) On the sites of presynaptic inhibition by neuropeptide Y in rat hippocampus in vitro. Hippocampus 3:103-111.

98. Klemp K, Woldbye DP (2001) Repeated inhibitory effects of NPY on hippocampal CA3 seizures and wet dog shakes. Peptides 22:523-527.

99. Knoflach F, Reinscheid RK, Civelli O, Kemp JA (1996) Modulation of voltage-gated calcium channels by orphanin FQ in freshly dissociated hippocampal neurons. J Neurosci 16:6657-6664.

100. Kohler C, Eriksson L, Davies S, Chan-Palay V (1986) Neuropeptide Y innervation of the hippocampal region in the rat and monkey brain. Comp Neurol 244:384-400.

101. Koide S, Onishi H, Yamagami S, Kawakita Y (1992) Effects of morphine and D-Ala2-D-Leu5-enkephalin in the seizure-susceptible El mouse. Neurochem Res 17:779-783.

102. Kovacs R, Gutierrez R, Kivi A, Schuchmann S, Gabriel S, Heinemann U (1999) Acute cell damage after low Mg2+-induced epileptiform activity in organotypic hippocampal slice cultures. Neuroreport 10:207-213.

103. Kunkel DD, Nguyen LB, Harrigan MR, Schwartzkroin PA, Malouf AT (1994) Somatostatin-containing neurons in rat organotypic hippocampal slice cultures: light and electron microscopic immunocytochemistry. Hippocampus 4:157-166.

104. Lahtinen H, Autere AM, Paalasmaa P, Lauri SE, Kaila K (2001) Post-insult activity is a major cause of delayed neuronal death in organotypic hippocampal slices exposed to glutamate. Neuroscience 105:131-137.

105. Lou LG, Ma L, Pei G (1997) Nociceptin/orphanin FQ activates protein kinase C, and this effect is mediated through phospholipase C/Ca2+ pathway. Biochem Biophys Res Commun 240:304-308.

106. Ludwig A, Zong X, Jeglitsch M, Hofmann F, Biel M (1998) A family of hyperpolarization-activated mammalian cation channels. Nature 393:587-591.

107. Marksteiner J, Ortler M, Bellmann R, Sperk G (1990) Neuropeptide Y biosynthesis is markedly induced in mossy fibers during temporal lobe epilepsy of the rat. Neurosci Lett 112:143-148.

108. Mayer ML, Westbrook GL, Guthrie PB (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. Nature 309:261-263.

109. McBain CJ, Boden P, Hill RG (1989) Rat hippocampal slices 'in vitro' display spontaneous epileptiform activity following long-term organotypic culture. J Neurosci Methods 27:35-49.

110. Meis S, Pape HC (2001) Control of glutamate and GABA release by nociceptin/orphanin FQ in the rat lateral amygdala. J Physiol 532:701-712.

111. Meunier JC (2003) Utilizing functional genomics to identify new pain treatments : the example of nociceptin. Am J Pharmacogenomics 3:117-130.

112. Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, Monsarrat B, . (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 377:532-535.

113. Michaelis EK (1998) Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. Prog Neurobiol 54:369-415.

114. Milner B (1972) Disorders of learning and memory after temporal lobe lesions in man. Clin Neurosurg 19:421-446.

115. Misra S, Murthy KS, Zhou H, Grider JR (2004) Coexpression of Y1, Y2, and Y4 receptors in smooth muscle coupled to distinct signaling pathways. J Pharmacol Exp Ther 311:1154-1162.

116. Mitchell J, Gatherer M, Best N, Sundstrom L, Wheal HV (1996) Hippocampal NPY neurons project to the fascia dentata in organotypic cultures. Hippocampus 6:173-182.

117. Mollereau C, Parmentier M, Mailleux P, Butour JL, Moisand C, Chalon P, Caput D, Vassart G, Meunier JC (1994) ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. FEBS Lett 341:33-38.

118. Moshe SL (2000) Mechanisms of action of anticonvulsant agents. Neurology 55:S32-S40.

119. Muller D, Buchs PA, Stoppini L (1993) Time course of synaptic development in hippocampal organotypic cultures. Brain Res Dev Brain Res 71:93-100.

120. Najlerahim A, Williams SF, Pearson RC, Jefferys JG (1992) Increased expression of GAD mRNA during the chronic epileptic syndrome due to intrahippocampal tetanus toxin. Exp Brain Res 90:332-342.

121. Naruse H, Kato M, Kurokawa M, HABAR, YABE T (1960) Metabolic defects in a convulsive strain of mouse. J Neurochem 5:359-369.

122. Neal CR, Jr., Akil H, Watson SJ, Jr. (2001) Expression of orphanin FQ and the opioid receptor-like (ORL1) receptor in the developing human and rat brain. J Chem Neuroanat 22:219-249.

123. Neal CR, Jr., Mansour A, Reinscheid R, Nothacker HP, Civelli O, Akil H, Watson SJ, Jr. (1999a) Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: comparison of ORL1

receptor mRNA expression with (125)I-[(14)Tyr]-orphanin FQ binding. J Comp Neurol 412:563-605.

124. Neal CR, Jr., Mansour A, Reinscheid R, Nothacker HP, Civelli O, Watson SJ, Jr. (1999b) Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat. J Comp Neurol 406:503-547.

125. Nicol B, Lambert DG, Rowbotham DJ, Smart D, McKnight AT (1996) Nociceptin induced inhibition of K+ evoked glutamate release from rat cerebrocortical slices. Br J Pharmacol 119:1081-1083.

126. Nishi M, Takeshima H, Mori M, Nakagawara K, Takeuchi T (1994b) Structure and chromosomal mapping of genes for the mouse kappa-opioid receptor and an opioid receptor homologue (MOR-C). Biochem Biophys Res Commun 205:1353-1357.

127. Noraberg J, Poulsen FR, Blaabjerg M, Kristensen BW, Bonde C, Montero M, Meyer M, Gramsbergen JB, Zimmer J (2005) Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair. Curr Drug Targets CNS Neurol Disord 4:435-452.

128. Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A (1984) Magnesium gates glutamate-activated channels in mouse central neurones. Nature 307:462-465.

129. Okawa H, Hirst RA, Smart D, McKnight AT, Lambert DG (1998) Rat central ORL-1 receptor uncouples from adenylyl cyclase during membrane preparation. Neurosci Lett 246:49-52.

130. Olsen RW (1981) The GABA postsynaptic membrane receptor-ionophore complex. Site of action of convulsant and anticonvulsant drugs. Mol Cell Biochem 39:261-279.

131. Ono T, Fueta Y, Janjua NA, Ohmori O, Ohno K, Murai Y, Mita T (1997) Granule cell disinhibition in dentate gyrus of genetically seizure susceptible El mice. Brain Res 745:165-172.

132. Paredes MF, Greenwood J, Baraban SC (2003) Neuropeptide Y modulates a G protein-coupled inwardly rectifying potassium current in the mouse hippocampus. Neurosci Lett 340:9-12.

133. Patrylo PR, Van Den Pol AN, Spencer DD, Williamson A (1999) NPY inhibits glutamatergic excitation in the epileptic human dentate gyrus. J Neurophysiol 82:478-483.

134. Peluso J, LaForge KS, Matthes HW, Kreek MJ, Kieffer BL, Gaveriaux-Ruff C (1998) Distribution of nociceptin/orphanin FQ receptor transcript in human central nervous system and immune cells. J Neuroimmunol 81:184-192.

135. Pomonis JD, Billington CJ, Levine AS (1996) Orphanin FQ, agonist of orphan opioid receptor ORL1, stimulates feeding in rats. Neuroreport 8:369-371.

136. Pomper JK, Graulich J, Kovacs R, Hoffmann U, Gabriel S, Heinemann U (2001) High oxygen tension leads to acute cell death in organotypic hippocampal slice cultures. Brain Res Dev Brain Res 126:109-116.

137. Pringle AK, Iannotti F, Wilde GJ, Chad JE, Seeley PJ, Sundstrom LE (1997) Neuroprotection by both NMDA and non-NMDA receptor antagonists in in vitro ischemia. Brain Res 755:36-46.

138. Qian J, Colmers WF, Saggau P (1997) Inhibition of synaptic transmission by neuropeptide Y in rat hippocampal area CA1: modulation of presynaptic Ca2+ entry. J Neurosci 17:8169-8177.

139. Raymond LA, Blackstone CD, Huganir RL (1993) Phosphorylation and modulation of recombinant GluR6 glutamate receptors by cAMP-dependent protein kinase. Nature 361:637-641.

140. Redrobe JP, Dumont Y, St Pierre JA, Quirion R (1999) Multiple receptors for neuropeptide Y in the hippocampus: putative roles in seizures and cognition. Brain Res 848:153-166.

141. Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ, Jr., Civelli O (1995) Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science 270:792-794.

142. Ribak CE, Bradurne RM, Harris AB (1982) A preferential loss of GABAergic, symmetric synapses in epileptic foci: a quantitative ultrastructural analysis of monkey neocortex. J Neurosci 2:1725-1735.

143. Ribak CE, Harris AB, Vaughn JE, Roberts E (1979) Inhibitory, GABAergic nerve terminals decrease at sites of focal epilepsy. Science 205:211-214.

144. Ribak CE, Hunt CA, Bakay RA, Oertel WH (1986) A decrease in the number of GABAergic somata is associated with the preferential loss of GABAergic terminals at epileptic foci. Brain Res 363:78-90.

145. Ribak CE, Joubran C, Kesslak JP, Bakay RA (1989) A selective decrease in the number of GABAergic somata occurs in pre-seizing monkeys with alumina gel granuloma. Epilepsy Res 4:126-138.

146. Robain O, Barbin G, Billette d, V, Jardin L, Jahchan T, Ben Ari Y (1994) Development of mossy fiber synapses in hippocampal slice culture. Brain Res Dev Brain Res 80:244-250.

147. Robinson RB, Siegelbaum SA (2003) Hyperpolarization-activated cation currents: from molecules to physiological function. Annu Rev Physiol 65:453-480.

148. Rossi GC, Perlmutter M, Leventhal L, Talatti A, Pasternak GW (1998) Orphanin FQ/nociceptin analgesia in the rat. Brain Res 792:327-330.

149. Routbort MJ, Bausch SB, McNamara JO (1999) Seizures, cell death, and mossy fiber sprouting in kainic acid-treated organotypic hippocampal cultures. Neuroscience 94:755-765.

150. Rubaj A, Zgodzinski W, Gustaw K, Sieklucka-Dziuba M (2002) Nociceptin, OP4 receptor ligand in different models of experimental epilepsy. Peptides 23:497-505.

151. Rudy B (1988) Diversity and ubiquity of K channels. Neuroscience 25:729-749.

152. Sandin J, Georgieva J, Schott PA, Ogren SO, Terenius L (1997) Nociceptin/orphanin FQ microinjected into hippocampus impairs spatial learning in rats. Eur J Neurosci 9:194-197.

153. Santoro B, Chen S, Luthi A, Pavlidis P, Shumyatsky GP, Tibbs GR, Siegelbaum SA (2000) Molecular and functional heterogeneity of hyperpolarization-activated pacemaker channels in the mouse CNS. J Neurosci 20:5264-5275.

154. Silva AP, Pinheiro PS, Carvalho AP, Carvalho CM, Jakobsen B, Zimmer J, Malva JO (2003) Activation of neuropeptide Y receptors is neuroprotective against excitotoxicity in organotypic hippocampal slice cultures. FASEB J 17:1118-1120.

155. Simeone TA, Rho JM, Baram TZ (2005) Single channel properties of hyperpolarization-activated cation currents in acutely dissociated rat hippocampal neurones. J Physiol 568:371-380.

156. Smialowska M, Bijak M, Sopala M, Tokarski K (1996) Inhibitory effect of NPY on the picrotoxin-induced activity in the hippocampus: a behavioural and electrophysiological study. Neuropeptides 30:7-12.

157. Smialowska M, Wieronska JM, Szewczyk B (2003) Neuroprotective effect of NPY on kainate neurotoxicity in the hippocampus. Pol J Pharmacol 55:979-986.

158. Soderling TR (1996) Modulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. Neurochem Int 28:359-361.

159. Somogyi P, Klausberger T (2005) Defined types of cortical interneurone structure space and spike timing in the hippocampus. J Physiol 562:9-26.

160. Sperk G, Bellmann R, Gruber B, Greber S, Marksteiner J, Roder C, Rupp E (1996) Neuropeptide Y expression in animal models of temporal lobe epilepsy. Epilepsy Res Suppl 12:197-203.

161. Stevens CF, Sullivan J (1998) Synaptic plasticity. Curr Biol 8:R151-R153.

162. Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37:173-182.

163. Stoppini L, Duport S, Correges P (1997) A new extracellular multirecording system for electrophysiological studies: application to hippocampal organotypic cultures. J Neurosci Methods 72:23-33.

164. Sundstrom L, Morrison B, III, Bradley M, Pringle A (2005) Organotypic cultures as tools for functional screening in the CNS. Drug Discov Today 10:993-1000.

165. Sundstrom LE, Brana C, Gatherer M, Mepham J, Rougier A (2001) Somatostatin- and neuropeptide Y-synthesizing neurones in the fascia dentata of humans with temporal lobe epilepsy. Brain 124:688-697.

166. Takahashi Y, Sadamatsu M, Kanai H, Masui A, Amano S, Ihara N, Kato N (1997) Changes of immunoreactive neuropeptide Y, somatostatin and corticotropin-releasing factor (CRF) in the brain of a novel epileptic mutant rat, Ihara's genetically epileptic rat (IGER). Brain Res 776:255-260.

167. Takahashi Y, Tsunashima K, Sadamatsu M, Schwarzer C, Amano S, Ihara N, Sasa M, Kato N, Sperk G (2000) Altered hippocampal expression of neuropeptide Y, somatostatin, and glutamate decarboxylase in Ihara's epileptic rats and spontaneously epileptic rats. Neurosci Lett 287:105-108.

168. Tallent MK, Madamba SG, Siggins GR (2001) Nociceptin reduces epileptiform events in CA3 hippocampus via presynaptic and postsynaptic mechanisms. J Neurosci 21:6940-6948.

169. Tallent MK, Siggins GR (1999) Somatostatin acts in CA1 and CA3 to reduce hippocampal epileptiform activity. J Neurophysiol 81:1626-1635.

170. Taylor SS, Buechler JA, Yonemoto W (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu Rev Biochem 59:971-1005.

171. Traub RD, Colling SB, Jefferys JG (1995) Cellular mechanisms of 4-aminopyridine-induced synchronized after-discharges in the rat hippocampal slice. J Physiol 489 (Pt 1):127-140.

172. Traub RD, Jefferys JG (1994) Are there unifying principles underlying the generation of epileptic afterdischarges in vitro? Prog Brain Res 102:383-394.

173. Vanderklish P, Neve R, Bahr BA, Arai A, Hennegriff M, Larson J, Lynch G (1992) Translational suppression of a glutamate receptor subunit impairs long-term potentiation. Synapse 12:333-337.

174. Vanderklish P, Saido TC, Gall C, Arai A, Lynch G (1995) Proteolysis of spectrin by calpain accompanies theta-burst stimulation in cultured hippocampal slices. Brain Res Mol Brain Res 32:25-35.

175. Vasilyev DV, Barish ME (2004) Regulation of the hyperpolarization-activated cationic current Ih in mouse hippocampal pyramidal neurones by vitronectin, a component of extracellular matrix. J Physiol 560:659-675.

176. Vaughan CW, Christie MJ (1996) Increase by the ORL1 receptor (opioid receptor-like1) ligand, nociceptin, of inwardly rectifying K conductance in dorsal raphe nucleus neurones. Br J Pharmacol 117:1609-1611.

177. Vaughan CW, Ingram SL, Christie MJ (1997) Actions of the ORL1 receptor ligand nociceptin on membrane properties of rat periaqueductal gray neurons in vitro. J Neurosci 17:996-1003.

178. Vezzani A, Schwarzer C, Lothman EW, Williamson J, Sperk G (1996) Functional changes in somatostatin and neuropeptide Y containing neurons in the rat hippocampus in chronic models of limbic seizures. Epilepsy Res 26:267-279.

179. Vezzani A, Sperk G, Colmers WF (1999) Neuropeptide Y: emerging evidence for a functional role in seizure modulation. Trends Neurosci 22:25-30.

180. Vitale M, Zamai L, Mazzotti G, Cataldi A, Falcieri E (1993) Differential kinetics of propidium iodide uptake in apoptotic and necrotic thymocytes. Histochemistry 100:223-229.

181. Wang JB, Johnson PS, Imai Y, Persico AM, Ozenberger BA, Eppler CM, Uhl GR (1994) cDNA cloning of an orphan opiate receptor gene family member and its splice variant. FEBS Lett 348:75-79.

182. Weiser T, Wieland HA, Doods HN (2000) Effects of the neuropeptide Y Y(2) receptor antagonist BIIE0246 on presynaptic inhibition by neuropeptide Y in rat hippocampal slices. Eur J Pharmacol 404:133-136.

183. Weisskopf MG, Castillo PE, Zalutsky RA, Nicoll RA (1994) Mediation of hippocampal mossy fiber long-term potentiation by cyclic AMP. Science 265:1878-1882.

184. Whittaker E, Vereker E, Lynch MA (1999) Neuropeptide Y inhibits glutamate release and long-term potentiation in rat dentate gyrus. Brain Res 827:229-233.

185. WHO (2003) Epilepsy: Epidemiology, Etiology and Prognosis.

186. Woldbye DP, Larsen PJ, Mikkelsen JD, Klemp K, Madsen TM, Bolwig TG (1997) Powerful inhibition of kainic acid seizures by neuropeptide Y via Y5- like receptors. Nat Med 3:761-764.

187. Woldbye DP, Nanobashvili A, Husum H, Bolwig TG, Kokaia M (2002) Neuropeptide Y inhibits in vitro epileptiform activity in the entorhinal cortex of mice. Neurosci Lett 333:127-130.

188. Yakel JL, Vissavajjhala P, Derkach VA, Brickey DA, Soderling TR (1995) Identification of a Ca2+/calmodulin-dependent protein kinase II regulatory phosphorylation site in non-N-methyl-D-aspartate glutamate receptors. Proc Natl Acad Sci U S A 92:1376-1380.

189. Yu TP, Fein J, Phan T, Evans CJ, Xie CW (1997) Orphanin FQ inhibits synaptic transmission and long-term potentiation in rat hippocampus. Hippocampus 7:88-94.

190. Zheng J, Knighton DR, Ten Eyck LF, Karlsson R, Xuong N, Taylor SS, Sowadski JM (1993) Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. Biochemistry 32:2154-2161.