The Role of VIP/PACAP Receptor Subtypes in Spinal Somatosensory Processing in Rats with an Experimental Peripheral Mononeuropathy

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DECLARATION

I hereby declare that the composition of this thesis and the work presented in it are entirely my own with the exception of the electrophysiological studies, which were carried out in collaboration with my supervisor, Dr. S. Fleetwood-Walker. Some of the studies have been published, reprints of which are included in an appendix.

Tracey Dickinson

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CONTENTS

ABSTRACT

Chronic constriction injury (CCI) of the rat sciatic nerve, produces an animal model of peripheral neuropathy exhibiting abnormal pain states similar to those seen in man, including behavioural signs of spontaneous pain, hyperalgesia and allodynia. Many components of neuropathic pain are resistant to classical opioid analgesics, and anaesthetic nerve blocks or surgical sympathectomies often provide only temporary relief. Since the expression of Vasoactive Intestinal Polypeptide (VIP) and Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is markedly up-regulated in dorsal root ganglia following peripheral nerve injury, we investigated whether VIP/PACAP receptors are important regulators of the amplified sensory responses which develop following neuropathy.

This study addressed the role of VIP_1, VIP_2 and PACAP receptors with regard to the responses of dorsal horn neurones in normal compared to CCI animals, using novel selective agonists and antagonists. In electrophysiological experiments on anaesthetised rats, the effects of ionophoretic application of VIP_1, VIP_2 and PACAP receptor antagonists were investigated on neuronal activity induced by innocuous brushing or cold stimulation of the cutaneous receptive field, or following peripheral application of the chemical algogen mustard oil. In normal rats, VIP_1 and PACAP receptor antagonists appeared to exert a general modification of dorsal horn neurone responses, inhibiting both brush- and mustard oil-induced activity to similar extents. In contrast, a novel VIP_2 receptor antagonist selectively inhibited mustard oil-evoked activity, whilst showing negligible effects on brush-evoked activity.

The effects of the VIP/PACAP receptor antagonists changed markedly in CCI animals so that antagonists for all three receptor subtypes showed negligible effects on brush-induced activity of dorsal horn neurones. In contrast, VIP_1/PACAP receptor antagonists significantly inhibited cold-induced activity, while a VIP_2 receptor antagonist had little effect. However, mustard oil-induced activity was significantly inhibited by all three receptor antagonists in CCI animals.

The activity of single, multireceptive dorsal horn neurones was markedly increased following ionophoretic administration of selective VIP_1, VIP_2 and PACAP receptor agonists both in normal and CCI rats. Following nerve injury however, two

main differences were apparent, and these may reflect changes in receptor expression: the number of dorsal horn neurones activated by the VIP_2 receptor agonist doubled (these neurones also showed a greater extent of cell activation than those excited in normal animals), while the percentage of neurones activated by the VIP_1 receptor agonist was seen to decrease. The proportion of cells activated by the PACAP receptor agonist remained unchanged.

In addition, *in situ* hybridisation histochemistry (ISHH) detection of mRNA for the three receptor subtypes, was employed to monitor any changes in receptor expression following nerve injury. This study revealed that CCI of the rat sciatic nerve produced a significant increase in the expression of VIP_2 receptor mRNA in laminae III/IV of the spinal dorsal horn. In contrast, VIP_1 receptor mRNA was seen to markedly decrease, while the expression of mRNA for the PACAP receptor appeared to be unchanged.

In conclusion, these results provide evidence that VIP/PACAP receptors may be important mediators/modulators of the transmission of sensory information at the spinal cord level, underlining the potential for VIP/PACAP receptor antagonists as new analgesics, particularly for use in currently intractable neuropathic pain states. These data demonstrate the involvement of the VIP_2 receptor in the transmission of nociceptive (C-fibre-mediated) information, both in normal and neuropathic animals. Although VIP_1 and PACAP receptor antagonists are rather non-selective inhibitors of sensory inputs in normal states, they may represent useful analgesics for certain aspects of allodynia (for example cold) as well as for polymodal C-fibre responses in neuropathy.

ABBREVIATIONS

[Arg^{16}] chicken secretin	VIP_1 receptor agonist
AC	adenylate cyclase
AMPA	α-amino-3-hydroyx-5-methyl-4-isoxazole proprionic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
bp	base pairs
BSA	bovine serum albumin
°C	degrees centigrade
cAMP	cyclic adenosine monophosphate
CCI	chronic constriction injury
CCK	cholecystokinin
cDNA	complementary DNA
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
ChAT	choline acetyl transferase
CNS	central nervous system
CS	conditioning stimulus
DAG	di-acyl glycerol
dATP	deoxyadenosine triphosphate
DEPC	diethyl pyrocarbonate
des(1-4) Arg^{16} Ro 25-1553	(des 1-4)[Glu^8, Lys^{12}, Arg^{16}, Nle^{17}, Ala^{19}] VIP(1-24) Asp, Leu, Lys, Lys, Gly, Gly, Thr NH_2 (lactam 21-25)
dH_2O	distilled water
DNA	deoxyribose nucleic acid
DRG	dorsal root ganglion
DTT	dithiothreitel
EAA	excitatory amino acid
EDTA	ethylenediamine tetra acetic acid
ER	endoplasmic reticulum
G protein	guanyl regulatory protein
GABA	γ-aminobutyric acid
GAL	galanin
GAP-43	growth associated protein-43
GDP	guanosine diphosphate
GLUT	glutamate
GTP	guanosine triphosphate
H Cl	hydrochloric acid
HRP	horse radish peroxidase
Hz	hertz
IP_3	inositol 1,4,5-triphosphate
i.p	intraperitoneal
ir	immuno-reactivity
i. t.	intrathecal
i.v.	intravenous

ISHH	*in situ* hybridisation histochemistry
KA	kainate
l; ml	litre; millilitre
LIF	leukaemia inhibitory factor
LTP	long term potentiation
mins	minutes
MΩ	mega ohms
mGluR	metabotropic glutamate receptor
M; mM	molar; milli molar
mg; kg	milli; kilogram
mm; μm	milli; micro metres
mRNA	messenger RNA
nA	nano amps
NaCl	sodium chloride
NGF	nerve growth factor
NK	neurokinin
NMDA	N-methyl-D-aspartate
nmol	nanomolar
NPY	neuropeptide Y
NRM	nucleus raphe magnus
$p \leq 0.05$	probability less than or equal to 0.05
PACAP	pituitary adenylate cyclase-activating polypeptide
PAG	periaqueductal grey
PBS	phosphate buffered saline
PF	paraformaldehyde
PG 97-269	[Ac-His^{1}, D-Phe^{2}, Lys^{15}, Arg^{16}, Leu^{17}] VIP(3-7)/GRF(8-27)
PK	protein kinase
PHA-L	*Phaseolus vulgaris* leukagglutinin
PIP_2	phosphatidyl inositol 4,5-diphosphate
PLC	phospholipase C
PNL	partial nerve ligation
PO	post operatively
PSB	pontamine sky blue
PSDC	postsynaptic dorsal column
PVP	polyvinyl pyrrolidone
RA	rapidly adapting
RNA	ribonucleic acid
RNase	ribonuclease
Ro 25-1553	[Ac-His^{1}, Glu^{8}, Lys^{12}, Nle^{17}, Ala^{19}] VIP (1-24), Asp, Leu, Lys, Lys, Gly, Gly, Thr NH_2 (lactam 21-25)
rTdT	recombinant terminal deoxyribonucleotide transferase
SA	slowly adapting
s.c.	subcutaneous
sec	seconds
SCG	superior cervical ganglia
SCT	spinocervical tract
SEM.	standard error of the mean
SMP	sympathetically maintained pain

SMT	spinomesencephalic tract
SNL	spinal nerve ligation
SNS	sympathetic nervous system
SOM	somatostatin
SP	substance P
SPET	suspended paw elevation time
SPN	sympathetic postganglionic neurones
SRT	spinoreticular tract
SSC	standard saline citrate buffer
STT	spinothalamic tract
TdT	terminal deoxyribonucleotide transferase
TE	Tris-EDTA buffer
TEA	Triethanolamine
tRNA	transfer RNA
VIP	Vasoactive Intestinal Polypeptide
vol	volume
WDR	wide dynamic range
wt	weight

CHAPTER 1 INTRODUCTION

pain - the range of unpleasant bodily sensations produced by illness or harmful bodily contact. *The Concise Oxford Dictionary.*

1.1 GENERAL INTRODUCTION

Pain is a normal consequence of acute and chronic damage or illness. Chronic pain due to nerve injury often persists long after the initial injury has healed, and the abnormal pain sensations which remain are thought to be indicative of a dysfunctional nervous system. Despite the prevalence of these symptoms, neuropathic pain remains a chronic condition that, as yet, has no clear physiological explanation nor a satisfactory treatment. The discovery of pain-modulatory systems within the central nervous system, and of the neurotransmitters that mediate their action, has contributed to our understanding of the complex pathways involved in the transmission of nociceptive information. Identifying the specific neurochemical alterations that arise in neuropathic conditions may provide important new insights into targets for the development of new and effective analgesic therapies.

1.2 ANATOMY OF THE SPINAL CORD

The spinal cord, located in the spinal canal of the vertebral column, begins as a continuation of the medulla oblongata, the inferior part of the brain stem. Somatosensory information is relayed from peripheral receptors to the spinal cord (or in certain cases the brain stem), via primary afferent fibres, and then on to the brain. So the processing of nociceptive information at the spinal cord level represents the first stage of effective control over its access to higher regions of the central nervous system (CNS).

1.2.1 Laminar Organisation of the Dorsal Horn

The grey matter of the spinal cord, which consists primarily of nerve cell bodies, fibres (both axons and dendrites) and associated non-neuronal cells, presents the general shape of an H within the white matter. This characteristic H shape, in

transverse section, can be subdivided into two main regions; the anterior or ventral horns and the posterior or dorsal horns.

The grey matter has a distinct architecture with a nomenclature which originated from the work of Rexed (1952), who divided the entire grey matter of the cat spinal cord and classified the laminae according to their cytoarchitectonic characteristics. Further anatomical studies have demonstrated a similar cytoarchitectonic scheme in the rat (Molander et al. 1984)(See Figure 1.1). In this scheme, the dorsal horn is divided into six layers or laminae:-

(a) Lamina I (The Marginal Zone): Lamina I is the thinnest and most superficial layer of the dorsal horn (Molander et al. 1984), characterised by the presence of the horizontally arranged, large marginal cells of Waldeyer (1888). Both unmyelinated C-fibres and myelinated Aδ fibres terminate in lamina I (but the myelinated Aδ primary afferent input is thought to predominate), and these afferents project to the thalamus, midbrain, or to other parts of the spinal cord (Willis and Coggeshall, 1991). There is no evidence that collaterals from large diameter primary afferent fibres enter lamina I (Brown, 1981). A number of early reports have suggested that lamina I contains primarily noci-specific neurones, which receive their projections from cutaneous, high-threshold Aδ mechanoreceptors and C-fibre thermal nociceptors (Cervero et al. 1976; 1979; Christensen, Perl, 1970; Light and Perl, 1979; Réthelyi et al. 1983), while more recent studies have revealed a large proportion of rat lamina I cells to be multireceptive (McMahon and Wall, 1983; Menétrey and Besson, 1981). So overall, the vast majority of dorsal horn neurones have the ability to respond to nociceptive stimuli, and this highlights the potential importance of lamina I in the transmission of noxious sensory information.

(b) Lamina II (the Substantia Gelatinosa): Lamina II is located directly beneath lamina I and also curves around the lateral edge of the dorsal horn. This lamina has a gelatinous appearance due to the large numbers of small cells and unmyelinated afferent fibres which it accommodates, hence the name 'substantia gelatinosa' (Willis and Coggeshall, 1991). Lamina II is concerned predominantly with the processing of sensory information from the skin and receives very little input from the non-cutaneous structures (muscle and viscera). This area displays an extensive unmyelinated primary afferent input, with perhaps some small, myelinated

afferents terminating here, while large myelinated afferents appear to be largely excluded (Willis and Coggeshall, 1991). The most superficial part of lamina II displays an extensively dense accumulation of very fine primary afferent fibres (C-fibres), and is filled with small, densely packed neurones, while the lower portion also has many small cells, which are much less densely packed (Light and Perl, 1979).

(c) Lamina III-IV (The Nucleus Proprius): Lamina III forms a broad band across the dorsal horn which is distinguished from lamina II by its slightly larger and more widely spaced cells (Light and Perl, 1979; Rexed, 1952). Intracellular injections and retrograde filling studies have demonstrated that significant populations of lamina III neurones project to the Spinocervical nucleus and into the dorsal columns (Brown, 1981; Brown et al. 1977). Lamina IV is a relatively thick layer which also extends across the dorsal horn, and is distinguished from lamina III by the heterogeneity of neurone size (ranging from very small, to quite large cells), and by the prominent presence of some very large cells (Molander et al. 1984). It is predominantly the intermediate to thick diameter A-fibres which innervate these deeper dorsal horn laminae, including non-nociceptive Aδ fibres, originating from D-type hair follicles, and the large diameter Aβ axons of sensory, cutaneous mechanoreceptors (Brown and Iggo, 1967; Light and Perl, 1979). In addition, many of the lamina IV neurones also receive a direct primary afferent input from their dorsal dendrites which project superficially into laminae I-III (Willis and Coggeshall, 1991).

(d) Lamina V-VI: Lamina V extends as a thick band across the narrowest part of the dorsal horn and contains many bundles of myelinated fibres as well as a large number of large diameter cells (Molander et al. 1984). Lamina VI only exists in the cervical and lumbosacral enlargements of the spinal cord where it represents the transitional layer between the primary afferent-dominated dorsal horn and the ventral horn. Very few primary afferents terminate here and this lamina is dominated by a large number of small neurones (Willis and Coggeshall, 1991).

The remaining laminae, laminae VII to X, represent the ventral horn and are beyond the scope of this study. Despite the obvious lamination pattern shown in Figure 1.1, there are no precise cytoarchitectonic borderlines within the spinal cord.

Laminae are recognised as zones of concentrations of particular cell types and tend to have intermingled edges. In addition, the borders between the various laminae differ slightly between different segmental sections (Molander et al. 1984).

1.2.2 Classification of Dorsal Horn Neurones

The spinal dorsal horn represents the first synaptic relay of the majority of afferents from skin, muscle and viscera. It is therefore an important site for the initial processing of signals from the periphery to the CNS with regards the transmission and modulation of pain. The dorsal horn is characterised by a wide range of cells of different sizes and morphological appearances (See Figure 1.2). This study will refer to three different types of dorsal horn neurone, found throughout the dorsal horn, which can be classified according to their response properties to cutaneous sensory stimuli:

(a) Non-nociceptive neurones (Class I): These cells are innervated by low-threshold mechanosensitive A-fibres, which respond only to innocuous mechanical stimulation of the cutaneous receptive field (Dubner and Bennett, 1983).

(b) Multireceptive neurones (Class II): Multireceptive, or wide-dynamic range (WDR) neurones (Mendell, 1966), have convergent inputs from both peripheral nociceptors and mechanoreceptors, enabling them to respond to both innocuous and noxious mechanical stimulation of the cutaneous receptive field and/or noxious thermal stimulation (Price et al. 1976; 1978). Electrical stimulation, either of the cutaneous nerve or transcutaneously, has revealed that quite often, the whole spectrum of primary afferents can activate multireceptive neurones, from large myelinated Aβ fibres to unmyelinated C-fibres (Besson and Chaouch, 1987; Willis and Coggeshall, 1991). Multireceptive neurones are most frequently found in laminae IV-VI of the dorsal horn, particularly in lamina V (Besson and Chaouch 1987). However they have also been recorded in the superficial laminae as well (Iggo, 1974; Menétrey and Besson, 1981; McMahon and Wall, 1983; Woolf and Fitzgerald, 1983).

(c) Nocispecific neurones (Class III): Nocispecific neurones are a well-defined population of neurones which appear to receive substantial inputs only from myelinated and unmyelinated nociceptive afferents (Aδ- and C-fibres) and therefore

respond only to high-threshold, noxious stimuli (Cervero et al. 1976). They have small cutaneous receptive fields and display little or no spontaneous activity (Perl, 1984). A relatively high proportion of nocispecific neurones has been reported in lamina I, including cells which project to thalamic and brain stem regions (Cervero et al.1976;1979; Christensen and Perl, 1970; Light and Perl, 1979). They are exclusively activated by noxious cutaneous stimulation, although some nocispecific neurones can also be driven by non-cutaneous inputs originating in the muscle or viscera (Cervero, 1983; Craig and Kniffki, 1983).

The transmission of nociceptive information will clearly be influenced then by differences in the distribution of these different types of neurone throughout the dorsal horn. In addition, the projection pattern of the different primary afferent fibre types will influence the type of response made by these dorsal horn neurones in different laminae, in particular the multireceptive neurones. Multireceptive dorsal horn neurones within the superficial layers of the dorsal horn are likely to be predominantly involved in mediating nociceptive information, as cutaneous Aδ nociceptors terminate primarily in lamina I, and C-fibres (mechanical, nociceptive and thermal) predominantly innervate lamina II. In contrast, Aβ fibres project into the deeper dorsal horn (laminae III-IV) (Willis and Coggeshall, 1991) where they have the ability to exert segmental control on the responses of spinal cord neurones (see Section 1.3.1). So the Aβ fibres may also directly influence the responses of both multireceptive and non-nociceptive neurones to cutaneous sensory stimuli.

Figure 1.1

Schematic diagram of the cytoarchitectonic organisation of segments L1-L6 of the rat spinal cord

Representation of the laminar divisions of Rexed (1952) as demonstrated in the lumbar (L) segments of the rat spinal cord.

Abbreviations:-

I - X - spinal cord laminae; **CC** - column of Clarke; **IL** - intermedio-lateral nucleus; **IM** - intermedio-medial nucleus; **LSN** - lateral spinal nucleus; **Liss** - Lissauer's tract; **LG** - lateral group of large cells in the dorso-lateral part of the ventral horn; **LM** - latero-medial nucleus; **MG** - medial group of large neurones in the intermediate zone; **Pyr** - pyramidal tract; **VM** - ventro-medial nucleus.
Note that LG, LM and VM are parts of lamina IX.

(Taken from Molander et al, 1984)

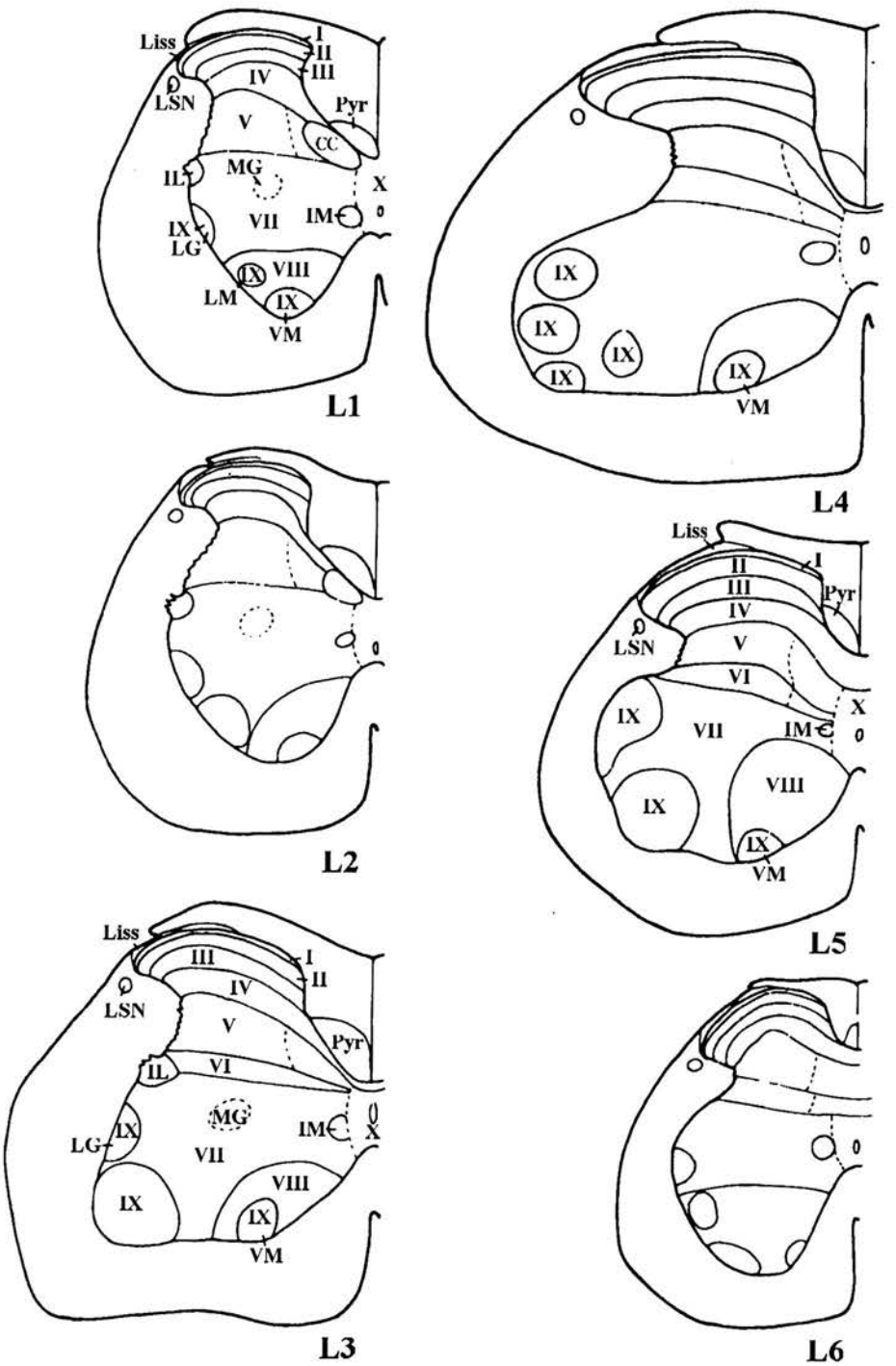

Liss
I
II
III
IV
V
Pyr
CC
LSN
IL
MG
X
IM
VII
IX
LG
VIII
LM
VM
L1
L2
L3
L4
L5
L6

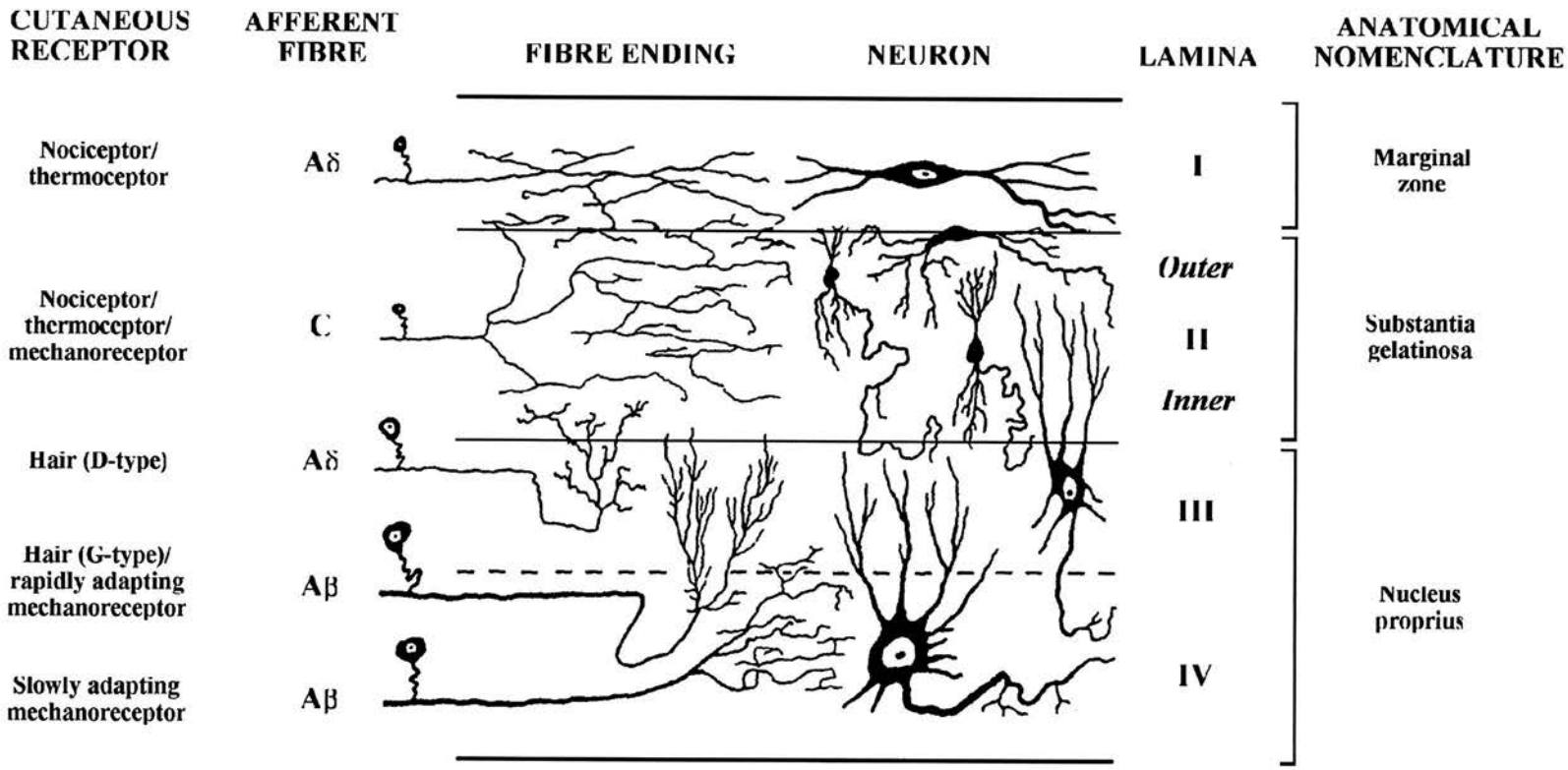
Figure 1.2

Schematic diagram of the cutaneous afferent input to, and neuronal organisation of the spinal dorsal horn

A hypothetical cross section of the spinal dorsal horn, illustrating the afferent fibres and neuronal elements present in the first four laminae. The laminar divisions of Rexed (1952) are indicated on the right. Afferent fibre types are listed to the left of the diagram, shown projecting onto neuronal types typical of laminae I-IV. The neurones illustrated are (from top to bottom): a marginal cell, an SG limiting cell, two SG central cells and two neurones of the nucleus proprius, the more superficial of which has dendrites penetrating lamina II.

(Taken from Cervero and Iggo, 1980)

High-threshold Aδ mechanoreceptors, with thin myelinated fibres, terminate predominantly in lamina I and II of the dorsal horn, as shown here, however they may also have scattered endings which terminate in lamina V of the deeper dorsal horn (Light and Perl, 1979). Similarly, the majority of C-fibre nociceptive afferents terminate in the superficial dorsal horn, particularly lamina II (as shown), but again may project their branches deep down into lamina V (Light and Perl, 1979).
In contrast, non-nociceptive Aδ axons from D-Type hair follicles, and large diameter Aβ myelinated afferent fibres (which innervate sensory cutaneous mechanoreceptors) distribute their axons in the deeper dorsal horn , laminae III-VI (Brown and Iggo, 1967; Perl, 1984), with some endings projecting to the inner portion of lamina II (Brown, 1981; Light and Perl, 1979).


CUTANEOUS RECEPTOR
AFFERENT FIBRE
FIBRE ENDING
NEURON
LAMINA
ANATOMICAL NOMENCLATURE
Nociceptor/ thermoceptor
Aδ
Nociceptor/ thermoceptor/ mechanoreceptor
C
Hair (D-type)
Aδ
Hair (G-type)/ rapidly adapting mechanoreceptor
Aβ
Slowly adapting mechanoreceptor
Aβ
I
Outer
II
Inner
III
IV
Marginal zone
Substantia gelatinosa
Nucleus proprius

1.2.3 Fibre Composition of Cutaneous Nerves

Peripheral nerves are composed of axons of sensory neurones and somatic and motor neurones. Primary afferent neurones and their associated fibres, represent the first step in the processing of sensory information from the periphery to the CNS. The large majority of peripheral nerve fibres terminate in the dorsal horn of the spinal cord. These afferent fibres can be divided into two main groups on the basis of their size, conduction velocity and whether or not they are myelinated.

The largest group of nerve fibres in cutaneous nerves is the myelinated A-fibre class. The rat sciatic nerve contains approximately twice as many of these myelinated, sensory axons compared to unmyelinated fibres (Schmalbruch, 1986). This group can be further subdivided according to their size and conduction velocity. The small diameter Aδ fibres conduct at 4-30m/s and supply hair follicle receptors or mechanical nociceptors. The large Aβ fibres have the fastest conduction velocities (30-100 m/s) and are mostly non-nociceptive, innervating corpuscular endings or hair follicle receptors (Willis and Coggeshall, 1991). The unmyelinated C-fibres have the smallest diameter and the slowest conduction velocities (< 2.5 m/s) (Gasser, 1950), and the majority of these fibres are nociceptive.

1.2.4. Classification of Afferent Fibre Receptors

The terminal regions of the primary afferent fibres, found in the peripheral tissue, constitute the receptive or dendritic part of the neurone, which have contact with well defined areas of skin from which the afferent fibres can be excited (receptive field). The cutaneous receptors transmit information about changes in the external and internal environment of the animal, and they are specialised to respond to specific stimuli (e.g. heat, pressure, chemicals). So clearly distinguishable classes of specialised nociceptors and mechanoreceptors exist in the periphery (Lynn, 1994), and these can be identified by experimentally applying a series of test stimuli to the receptive field area of the skin.

(a) Non-nociceptive Mechanoreceptors

Cutaneous mechanoreceptors are the most sensitive receptors, responding most readily to mechanical pressure stimuli of various intensity. They can be further subdivided according to their other characteristics:

Hair follicle receptors. These receptors, as their name suggests, are found in hairy skin and are activated by hair movement. They are the predominant class of units with myelinated Aδ axons within rat peripheral nerves (Lynn and Carpenter, 1982), and can be classified according to the type of hair which they innervate (Brown and Iggo, 1967). **D-hair** units tend to have relatively slowly conducting axons with large receptive fields, and respond well to slow movement of the fine down hairs, while **G-hair** units with their larger axons have relatively small receptive fields and tend only to be activated by fast movement of the guard hairs (Lynn and Carpenter, 1982). In addition **T-hair** units also exist, but are the least numerous, and these units can be excited by movement of the large tylotrich hairs.

Field receptors, rapidly adapting (RA) cutaneous receptors and Pacinian Corpuscles. These receptors also respond to light pressure, stroking or vibration of the receptive field area on the skin, but are not activated by hair movement. They are predominantly associated with the large Aβ fibres and generally transmit only non-nociceptive information.

Field receptors can only be activated by the brushing of large numbers of hairs along with direct stimulation of the underlying skin. RA receptors are found in glabrous skin. They signal the rate at which the skin is displaced (stimulus velocity), and are typically associated with Meissner's corpuscles. Pacinian corpuscles are subcutaneous receptors which respond to deformation of the cutaneous receptive field caused by firm pressure (Willis and Coggeshall, 1991)

Slowly adapting (SA type I or II) mechanoreceptors. These receptors are again associated primarily with large Aβ fibres, but tend not to innervate hair follicles. They detect both the displacement and velocity of mechanical stimuli, and tend to fire to maintained skin or joint displacements (Lynn and Carpenter, 1991). SA I mechanoreceptors are low threshold receptors associated with Merkel cell

complexes in the basal layer of the epidermis. SA II mechanoreceptors are identified with Ruffini endings located in the dermis and so respond to small displacements of the skin usually as a result of the skin stretching (Willis and Coggeshall, 1991).

C-mechanoreceptors. These sensitive mechanoreceptors with unmyelinated afferent fibres, are a distinct group of C-fibres with a high sensitivity to mechanical stimulation (Bessou and Perl, 1969). They constitute approximately 12-15% of all C-fibres within the saphenous nerve (Lynn and Carpenter, 1982), but have a slightly higher occurrence in the rat sural nerve (approximately 33%) (Leem et al. 1993), and tend to respond to slow brushing of the cutaneous receptive field, or to stretching of the surrounding skin. The receptive fields are generally small single points (Bessou et al. 1971) and are primarily found on hairy skin. These receptors rarely respond to warm thermal stimuli, although a large proportion have been reported to respond to cold stimuli (Bessou and Perl, 1969; Leem et al.1993; Lynn and Carpenter, 1982). The majority of these receptors are rapidly adapting, such that repeated forceful stimulation (for example probing or pinching the cutaneous receptive field) will result in "inactivation" of this group of fibres (Bessou and Perl, 1969).

(b) Non-nociceptive Thermoreceptors

Thermoreceptors signal innocuous changes in temperature, and respond poorly if at all to mechanical stimuli. They can generally be classified into two types:

(i) Cold thermoreceptors. Innocuous cool sensations and cold pain are mediated by different populations of primary afferent fibre. Cool sensations are signalled by activity in cold specific Aδ-fibres (and to a lesser degree cold-specific C-fibres) (Iggo, 1959;1969), with specific cutaneous receptors in both the hairy and glabrous skin (Iggo, 1969). Cold thermoreceptors are characterised by their high sensitivity to small falls in skin temperature (as little as 0.1deg.), and the most commonly studied in the rat are facial and scrotal thermoreceptors. The majority of these receptors have a relatively restricted range of innocuous cold temperatures (approximately 20 to 30°C) over which they give dynamic responses to small reductions in skin temperature (Heinz et al. 1990; Iggo, 1969).

(ii) Warm thermoreceptors respond to slight warming of the skin and are generally thought to be unmyelinated (Iggo, 1959). They are active at normal skin temperature (approximately 30°C) and are silenced by noxious levels of heat (48°C +).

(c) Nociceptors

Nociceptors were first described by Sherrington (1900) as sensory endings that respond to stimuli that threaten or actually damage tissue. There are two main groups of cutaneous nociceptors, the Aδ mechanical nociceptor and the C polymodal nociceptor.

(i) Aδ mechanoreceptors. These nociceptors are associated with Aδ fibres, exist in both glabrous and hairy skin, and are only excited by high-threshold, noxious mechanical stimulation. They are not normally activated by thermal stimuli (Besson and Chaouch, 1987; Burgess and Perl, 1967), but they may become sensitised upon repeated or long-lasting heat stimulation, after which they subsequently respond to future heat challenges (Burgess and Perl, 1973; Fitzgerald and Lynn, 1977; Perl, 1984). Approximately 20% of all A-fibre units within the rat saphenous nerve are high threshold mechanoreceptors, which display a wide range of conduction velocities and generally have small receptive fields (Lynn and Carpenter, 1982). The thresholds vary with the lowest thresholds found within the innocuous range. Electrophysiological recordings in conjunction with the use of neuronal tracing techniques based on the axoplasmic transport of marker substances (such as horse radish peroxidase (HRP) and the plant lectin *Phaseolus vulgaris* leukagglutinin (PHA-L)) have revealed that Aδ nociceptive primary afferents, originating from high threshold mechanoreceptors, terminate predominantly in Rexed laminae I, IV and V, with a few fibres branching in to lamina II and X (Cervero et al.1976; Light and Perl 1979; Perl 1984, Réthelyi et al.1983) (See Figure 1.2).

(ii) C polymodal nociceptor units are the most common form of primary C-afferent unit found within peripheral nerves of the rat (Lynn and Carpenter, 1982; Schmalbruch, 1986). They respond to multiple stimulus modalities hence the term polymodal, exist in both hairy and glabrous skin, and typically have small receptive fields. Effective activating stimuli include noxious thermal heat (> 45°C) and more

intense levels of mechanical pressure, as well as irritant chemicals (Perl, 1984). In undamaged skin, they generally do not exhibit any spontaneous activity in the absence of stimuli (Besson and Chaouch, 1987; Bessou and Perl, 1969). The vast majority of cutaneous C-fibre polymodal nociceptors have been shown to terminate in laminae I-II of the spinal dorsal horn (Cervero and Iggo, 1980; McMahon et al. 1984).

(iii) Cold nociceptors. Cold nociceptors may be associated with either Aδ- or C-fibres, and are excited by noxious cold stimuli. Due to the vascular problems incurred when attempting to stimulate the cutaneous receptive field or isolated nerve fibres at noxious cold intensities, the full range of cold temperatures able to excite cold nociceptors is not very clear. Saphenous nerve recordings in the rat have revealed however, that the cold threshold for many of these nociceptors is below 12°C, with a large proportion of Aδ nociceptors only firing at temperatures below 0°C (Simone and Kajander, 1996; 1997).

(iv) It has been reported that minor groups of nociceptors respond preferentially to **heat** or **chemical** irritants, or may be completely inactive until sensitised by the onset of inflammation or chemical irritants; the appropriately named **'silent' nociceptor** (Bessou and Perl, 1969; Besson and Chaouch, 1987).

1.3 Central Mechanisms of Sensory Transmission

Melzack and Wall (1965) were the first to describe the existence of a specific pain modulatory system within the CNS, with their "Gate Control Theory of Pain". They proposed that messages transmitted from the peripheral nerves to the brain, resulted from the convergence of more than one peripheral afferent impulse onto spinal cord neurones, and that these signals could be influenced by impulses from the brain which had the potential to exert a form of descending control. Many criticisms have been made of this model, resulting in modifications of many of the details of the gate control theory, but the hypothesis of a descending inhibitory control system, mediated by activity in large afferent fibres, is still an important consideration when investigating the transmission of nociceptive information at the spinal cord level.

1.3.1 Descending Control of Nociceptive Transmission

In addition to receiving afferent inputs from the periphery, spinal cord neurones also receive efferent influences from descending fibres. The effects of descending pathways from the brain on dorsal horn neurones may be direct or indirect, involving excitatory or inhibitory interneurones at the spinal cord level. These pathways are important in the maintenance of inhibitory control on dorsal horn neurones.

(a) Tonic Descending Inhibition

Many areas of the brain exert a potent descending inhibitory influence on spinal somatosensory mechanisms, in particular on nociceptive transmission in the spinal dorsal horn. An experimental model used to demonstrate tonic descending inhibition is reversible cold block, where the activity of a neurone, at a location caudal to the area to be blocked, is examined before, during and after cold block of the descending pathways (Brown, 1971). This technique has revealed that a number of different dorsal horn neurones are under a tonic inhibitory control. In decerebrate animals, cold block of the spinal cord at the thoracic level results in enhanced responses of Spinocervical tract (SCT) cells to peripheral stimuli (Brown, 1971), due to the blockade of descending inhibitory controls. Further studies have revealed that tonic descending inhibitory controls affect nociceptive transmission in lamina I as well as laminae IV-VI (Besson et al 1975; Brown, 1971; Duggan et al 1981), while cells in lamina II appear to have no such inhibitory influence (Cervero et al. 1979). The exact origin of tonic inhibition is unclear, but the lateral reticular nuclei and nucleus paragigantocellularis lateralis in the brain stem appear to contribute, as bilateral lesions in these areas appear to prevent such an inhibition (Foong and Duggan 1986; Morton et al. 1983).

(b) Supraspinal Modulation

Descending pathways from the higher centres of the CNS can also modify sensory or motor functions at the spinal level. A role for the brain stem in the modulation of pain is strongly supported by both behavioural and electrophysiological studies. A number of groups have investigated the neuronal effects induced by stimulating a variety of brain stem areas, and several areas have been shown to provide analgesia when stimulated: including the periaqueductal grey

(PAG) (Oliveras et al. 1974), the nucleus raphe magnus (NRM) (Guilbaud et al. 1977) and the reticular nuclei (Morton et al. 1983). Stimulation results primarily in an inhibition of activity evoked by noxious, rather than non-noxious stimuli applied to the periphery or the peripheral nerves (Duggan and Griersmith, 1979; Willis, 1977).

(c) Segmental Controls

The segmental controls are characteristically the inhibitory effects produced by the large diameter (Aβ) fibres on the responses of spinal neurones to nociceptive stimulation (Besson and Chaouch, 1987), and these inhibitory influences can be exerted on multireceptive and nocispecific dorsal horn neurones. A number of pharmacological agents have been found to affect the activity of dorsal horn interneurones. Excitatory amino acids (EAA) and neuropeptides have been proposed as excitatory neurotransmitters or modulators and will be discussed in more detail in Section 1.4. Inhibitory transmitters include the inhibitory amino acids γ-aminobutyric acid (GABA) and glycine and the endogenous opioids, which may be released secondarily to the activation of spinal interneurones by primary afferent fibres, or indirectly by the activation of pathways descending from the brain. In addition, monoamines such as, serotonin and noradrenaline, may be directly released by descending control systems to exert inhibitory effects (see Besson and Chaouch, 1987 for review).

GABA: GABA is an inhibitory neurotransmitter within the spinal cord, where it is widely distributed. GABA is found in approximately one-third of laminae I, II and III dorsal horn interneurones (Barber et al. 1982; Hunt et al. 1981; Todd and McKenzie, 1989), as well as in neurones of the rostral ventral lateral medulla which project to the spinal cord (Reichling and Basbaum, 1990). Ionophoresis of GABA results in inhibition of dorsal horn neurone activity including those in the substantia gelatinosa (Curtis et al. 1959;1977;Zieglgansberger and Sutor, 1983), implicating this amino acid in the control of sensory processing at the spinal level. Other evidence implicating a role for GABA in presynaptic inhibition is the blockage, at least in part, of primary afferent depolarization by the GABA antagonists picrotoxin and bicuculline (Besson et al. 1971; McLauchlin et al. 1975; Repkin et al. 1976).

GABA is thought to exert its inhibitory effects by influencing the influx of cations into the presynaptic cells (Robertson and Taylor, 1986).

Glycine: Glycine is also an important neurotransmitter with widespread distribution throughout the spinal cord. The highest concentrations of this amino acid appear to be found more ventrally in the spinal cord (Graham et al. 1967), but glycine-containing neurones and synaptic terminals are also found in the superficial layers of the dorsal horn (Todd, 1990). Electrophysiological experiments have revealed that ionophoresis of glycine into the spinal cord can inhibit the responses of dorsal horn neurones (Curtis et al. 1967a;1967b; Werman et al. 1968; Zieglgansberger and Sutor, 1983). However it is now commonly understood that glycine can have two opposing functions in nociceptive processing: an excitatory action via the glycine co-agonist binding site on the NMDA (N-methyl-D-aspartate) receptor (Gly_{NMDA}), or an inhibitory action via the strychnine-sensitive glycine receptor. Glycine is thought to be an important modulator of SP-evoked responses, as it has been shown to be released following microdialysis of SP in to the spinal cord *in vivo* (Smullin et al. 1990) and following bath application *in vitro* (Maehara et al. 1993). In addition, intrathecal glycine inhibits substance P (SP) evoked biting and scratching behaviours (Beyer et al. 1989), while intrathecal application of the glycine antagonist strychnine facilitates the nociceptive flexor reflex (Sivilotti and Woolf, 1994). The modulatory influences of glycine are therefore quite complicated, and can be exerted through several different pathways. The possible interactions between glycine and the NMDA and SP receptors will be discussed in more detail in section 1.4.

Endogenous Opioids: The functional importance of the spinal opioid system is well supported by the powerful and long-lasting analgesic effects seen in animals following the intrathecal-administration of morphine (Yaksh, 1981). These clinical effects clearly demonstrate the potential role for spinal opioid receptors in the inhibitory modulation of nociceptive messages. Several opioid peptides are known to be present within the interneurones and synaptic terminals of the spinal dorsal horn (Cruz and Basbaum, 1985; Glazer and Basbaum, 1981; Willis and Coggeshall, 1991), including enkephalin and dynorphin. Opiate receptors have been identified on primary afferent terminals, and μ-, δ- and κ- receptors have all been found in the

spinal cord (Atweh and Kuhar, 1977), particularly in the superficial dorsal horn, using specific radioligand binding assays. Ionophoresis of opioids in the substantia gelatinosa strongly inhibits the responses of dorsal horn neurones to noxious stimulation (Duggan and North, 1984; Fleetwood-Walker et al. 1988), this inhibitory action may be presynaptic or may be an indirect action following the activation of neighbouring interneurones.

1.3.2 Ascending Somatosensory Pathways

In addition to receiving inputs from descending controls, many dorsal horn neurones are projection neurones which relay sensory information to higher regions of the CNS, through tracts in the white matter. The main ascending tracts implicated in the transmission of nociceptive information in the rat are:

(a) Spinothalamic tract (STT): The STT transmits information from the spinal dorsal horn to the thalamic nuclei via the ventrolateral quadrant of the spinal cord. Anatomical studies based on the retrograde transport of HRP have revealed that the majority of STT neurones, projecting to the lateral part of the thalamus, are found in laminae I, III and IV of the rat spinal dorsal horn (Giesler et al. 1979). Willis et al. (1983) characterised the neurones of the STT in the monkey on the basis of their responses to peripheral stimulation to reveal that a high proportion of STT neurones respond to noxious stimulation (55% were multireceptive, while 32% were nocispecific). In the rat, STT cells usually respond to noxious mechanical stimulation of the cutaneous receptive field, but they can also respond to innocuous mechanical stimulation (Giesler et al, 1976), and so the STT is not solely involved in the transmission of nociception and pain. However, STT cells can be inhibited by stimulation of a number of different areas of the brain, including the NRM (Willis, 1977), PAG and the adjacent midbrain reticular formation (Gerhart et al. 1984; Hayes et al. 1979), and the periventricular grey (Ammons et al. 1986). The resulting inhibition following stimulation of these areas of the brain, is predominantly inhibition of the noxious responses of nocispecific and multireceptive neurones, therefore implicating the STT in the modulation and processing of nociceptive information, although responses to innocuous stimuli were also occasionally affected.

(b) Spinoreticular tract (SRT): The SRT projects from the dorsal horn to the brainstem reticular formation, again via the ventrolateral quadrant of the spinal cord. IIRP mapping has demonstrated that the majority of SRT cells are concentrated in laminae VII and VIII in the rat ventral horn (Chaouch et al. 1983), with very few cells found in the more superficial layers of the dorsal horn. Activation of SRT neurones via stimulation of the reticular formation (Menétrey et al. 1980) revealed that the majority of these cells could be activated by noxious sensory stimuli, with a large proportion being multireceptive neurones.

(c) Spinomesencephalic tract (SMT): The SMT projects to the mesencephalic reticular formation and lateral part of the PAG as well as to other sites in the midbrain (Mehler et al. 1960). SMT cells are predominantly found in lamina I as revealed by HRP tracing (Menétrey et al. 1982), as well as lamina V. Electrophysiological evidence has revealed a high proportion of the SMT neurones which originate in the marginal zone, to be nociceptive neurones (Menétrey et al. 1980), implicating the involvement of the SMT in the control of nociception and pain. However, there are also SMT cells which respond only to innocuous mechanical stimulation (Menétrey et al.1980).

(d) Postsynaptic dorsal column (PSDC): The PSDC originates primarily from neurones in lamina III of the rat spinal cord (Giesler et al. 1984), and contains ascending branches of primary afferent fibres which project through the dorsal funiculus to the nucleus gracilis and nucleus cuneatus (Giesler et al.1984). PSDC neurones may respond to either innocuous or to both innocuous and noxious stimulation of the cutaneous receptive field (Brown et al. 1983), however a few nocispecific neurones have also been reported (Angaut-Petit, 1975).

1.4 Neurotransmitters of the Primary Afferent Nociceptor

Primary afferent nociceptors consist of a cell body (located in the dorsal root ganglia (DRG)) and adjacent nerve fibres which project both centrally and peripherally (Willis and Coggeshall, 1991). Activation of primary afferent nociceptors requires an intense mechanical, thermal or chemical stimulus at the peripheral terminal, resulting in the release of chemical substances from the nerve endings or 'synapses', which ultimately mediate the transmission of nociceptive

information centrally, as well as at the periphery. A greater knowledge of the identities and properties of these chemical transmitters released in the dorsal horn of the spinal cord and in the brain, would therefore be of great interest in the understanding of the processing of sensory information. Selective manipulation of those transmitters involved predominantly in the transmission of noxious information, being the prime targets for new analgesics.

A number of criteria have been proposed for the identification of neurotransmitters (Salt and Hill, 1983) summarised as follows:

1. the transmitter and the mechanism for its production should be present in the presynaptic neurone/terminal
2. the transmitter should be released upon physiological stimulation of the presynaptic neurone/terminal
3. exogenously applied transmitter candidates and the release of the endogenous transmitter should have the same effect on the postsynaptic neurone, and these effects should be attenuated following application of the appropriate pharmacological antagonist
4. an inactivation or removal mechanism for the putative transmitter should exist

A large number of pharmacological agents have been found to affect the responses of dorsal horn neurones (Willis and Coggeshall, 1991). Some of these, including the excitatory amino acids (EAAs) and several peptides, have been implicated in synaptic transmission via primary afferent fibres, and several neuroactive substances have been shown to co-exist within both primary afferents and sensory neurones (Ju et al. 1987; Smith et al. 1993). This highlights the potential for co-transmission of many substances, such that primary afferent fibres may affect various populations of dorsal horn neurones by releasing several different transmitters at the same time. In addition, a variety of transmitters acting at different receptors on the same postsynaptic neurone might have synergistic effects or may differentially modulate the threshold for neuronal firing. The dorsal horn is a primary receiving area for somatosensory input and contains high concentrations of a large variety of receptors for a number of different neurotransmitters, some of the principal ones are listed below:-

1.4.1 Glutamate

Glutamate is an extensively studied EAA which has been shown to be a neurotransmitter eliciting fast excitatory responses within the CNS (Watkins and Evans, 1981) and is believed to be involved in the transmission of nociceptive information. Curtis and colleagues first demonstrated the potential excitatory role for glutamate following its ionophoretic application onto spinal neurones (Curtis et al. 1959). Since then other studies have implicated this EAA as an important neurotransmitter. Electrical stimulation of dorsal column primary afferents has induced the release of glutamate *in vivo* (Roberts, 1974), while immunohistochemical and radiological studies have revealed the presence of glutamate in primary afferent fibres of the spinal dorsal horn (De Biasi and Rustioni, 1988), dorsal root ganglia (DRG) (Salt and Hill, 1983) and dorsal roots (Duggan and Johnston, 1970).

There are several receptor subtypes through which glutamate can mediate its cellular actions. The ionotropic group, which are receptor linked ion channels and comprise of the α-amino-3-hydroxy-5-isoxazole-4-propionate (AMPA), kainate (KA) and NMDA receptors, and also the metabotropic glutamate receptors (mGluR) which are coupled to various second messenger systems through GTP-binding proteins (Willis and Coggeshall, 1991).

NMDA receptors have been shown to be located throughout the brain and spinal cord (particularly in the superficial dorsal horn) (Greenamyre et al. 1984; Monaghan and Cotman, 1985), and there is compelling evidence to suggest that they play a role in excitability changes such as long term potentiation (LTP) (Collingridge et al. 1983; Morris et al. 1986) as well as in the generation of hyperalgesia (Dougherty et al. 1992b; Zhou et al. 1996). Intrathecal administration of NMDA has been shown to produce thermal hyperalgesia in the rat (Kolhekar et al. 1994), while NMDA receptor antagonists inhibit the responses of dorsal horn neurones induced by prolonged chemical nociception (Haley et al. 1990), joint inflammation (Neugebauer et al. 1993) or repetitive C-fibre conditioning stimuli ('wind-up') (Davies and Lodge, 1987). So the activation of NMDA receptors appears to contribute greatly to prolonged states of nociception.

The abnormal pain-related behaviours induced by peripheral nerve injury, such as hyperalgesia, allodynia and spontaneous pain, result from both increased

sensitivity of primary afferent fibres (peripheral sensitisation) (Campbell et al. 1988; Kajander and Bennett, 1992; Wall and Devor, 1983) and an increase in the excitability of spinal cord neurones (central sensitisation)(Coderre et al. 1993; Wall, 1991), and so the activation of NMDA receptors by EAAs may also be an important component in the development of neuropathic pain states. Indeed, in the chronic constriction injury model, pre- and post-treatments with the NMDA antagonist MK-801 have been reported to prevent or significantly reduce thermal and mechanical hyperalgesia (Mao et al. 1992a; 1992b; Smith et al. 1994). However, a more recent study has shown that although pre- and post-injury treatment with i.p. MK-801 suppressed the development of these pain states for a period of up to 7 days, the animals eventually became hyperalgesic by day 10-14 post injury (Kawamata and Omote, 1996). This suggests that activation of NMDA receptors appears to be strongly involved in the induction and maintenance of pain-related behaviours following nerve injury, but that other factors may also contribute especially in the longer term. Indeed, clinical trials in human patients have provided preliminary evidence that NMDA receptors play an important role in neuropathy. Intravenous infusions of the NMDA antagonist ketamine, reduced both allodynia and hyperalgesia in a number of patients with chronic causalgic pain (Byas-Smith et al. 1993). However, the concentrations required to provide relief also produced significant side effects, highlighting the need for the development of more effective therapeutic agents.

A number of regulatory sites exist on the NMDA receptor, including the glycine site (Kleckner et al. 1988), as mentioned earlier. Glycine has been termed a co-agonist of the NMDA receptor and appears to be very important in regulating a number of NMDA receptor-mediated responses. The selective Gly_{NMDA} site antagonist 7-chlorokynurenate, has been shown to inhibit NMDA-induced thermal hyperalgesia in the rat (Kolhekar et al. 1994), as revealed by an increased threshold to noxious radiant heat. Gly_{NMDA} site antagonists can also decrease the enhanced responses of spinal neurones resulting from repetitive C-fibre stimulation (Dickenson and Aydar, 1991). So occupation of the Gly_{NMDA} site appears to be crucial for the regulatory mechanisms and influences of the NMDA receptors expressed on dorsal

horn neurones, and highlights the importance of interactive effects of the many neuroactive substances found within the spinal cord.

The role of mGluRs within the CNS and their contribution to nociceptive processing in the spinal cord is becoming increasingly clearer, and a number of studies have provided evidence that mGluRs are involved in mediating noxious inputs to the spinal cord. Electrophysiological studies revealed that the mGluRs are important mediators in the sustained activation of dorsal horn neurones elicited by topical, cutaneous application of the chemical irritant mustard oil (Young et al. 1994), which produces a sustained activation of dorsal horn neurones analogous to 'wind up'. Further experiments have since revealed that it is the $mGluR_5$, and in particular the $mGluR_1$ receptor subtypes which are particularly important in mediating nociceptive transmission at the spinal cord level (Young et al, 1995a; 1995b; 1997).

So there are clearly a number of different pathways for glutamate to exert its function as a neurotransmitter within the CNS. In addition, glutamate has been shown to co-exist with SP in DRG neurones (Battaglia and Rustioni, 1988) and in primary afferent terminals within the superficial laminae of the dorsal horn (DeBiasi and Rustioni, 1988), and so it is highly likely that these compounds are co-released following sustained stimulation of nociceptors. There is indeed a wide range of evidence to implicate glutamate/SP interactions in the regulation of somatosensory processing within the CNS. Electrophysiological studies revealed that ionophoretic application of SP enhances the NMDA receptor-induced activity of primate STT neurones (Dougherty and Willis, 1990;1991), while ionophoresis of selective NK_1 receptor agonists increase the NMDA receptor-mediated response of rat dorsal and ventral horn neurones (Cumberbatch et al. 1995). More recently, Heppenstall and Fleetwood-Walker revealed that this NK_1 receptor facilitation of the NMDA receptor can be blocked by ionophoretic application of Gly_{NMDA} site antagonists (Heppenstall and Fleetwood-Walker, 1997a;1997b), thereby implicating an important interactive modulatory role for glycine, glutamate, SP and their receptors in the transmission of nociceptive information.

1.4.2 Substance P (SP)

SP was discovered by von Euler and Gaddam in 1931, and is the most extensively studied primary afferent neuropeptide. Its role as a neurotransmitter was first suggested by Lembeck (1953) who discovered, by means of a bioassay for the peptide, that the dorsal roots of the spinal cord contained more SP than the ventral horns. Gasparovic et al (1964), using a similar assay, went on to find that the levels of SP became depleted in the dorsal roots following pre-treatment with capsaicin, a neurotoxin which causes the destruction of the small diameter C-fibres the majority of which are nociceptors (Willis and Coggeshall, 1991). There is now a large body of anatomical, behavioural and electrophysiological evidence implicating SP and its receptors in sensory processing in the dorsal horn.

SP- immunoreactivity (-ir) and Preprotachykinin-A mRNA (the precursor gene for SP) have been detected in a number of different areas of the CNS known to be associated with the transmission of nociceptive information, including small diameter primary afferent neurones, the spinal dorsal horn and small diameter DRG cells (Gibson et al. 1981; Hokfelt et al. 1975;1977; 1980; 1993). SP is present in approximately 20% of all DRG neurones (Ju et al. 1987), where it has been shown to co-exist with a number of other substances including calcitonin gene-related peptide (CGRP), somatostatin (SOM) and glutamate (Battaglia and Rustioni, 1988; Ju et al. 1987), thus highlighting the potential for co-release, and therefore possible interactive roles, with other neuroactive substances. Within the spinal cord, the highest concentrations of SP-ir are found within the superficial layers of the dorsal horn (namely laminae I and II), but SP-ir has also been detected in laminae V-VII and X (Gibson et al. 1981; Hokfelt et al. 1975;1977; 1980). SP mediates its effects via the neurokinin$_1$ (NK_1) receptor, dense concentrations of which have been demonstrated within the superficial layers of the spinal cord (Helke et al. 1986; Näsström et al. 1992; Quirion et al. 1983; Yashpal et al. 1991a). In addition, at the electron microscope level, NK_1 receptor-ir has been found in major dendrites of dorsal horn neurones in laminae III, IV and to some extent V, dendrites which appear to extend into lamina II and presumably act as targets for SP released in this area (Brown et al. 1995; Naim et al. 1997).

Nerve section and dorsal rhizotomy largely, but not entirely reduce the distribution of SP-ir, and so it seems likely that SP is derived from primary afferents, intrinsic neurones and descending fibres (Jessell et al. 1979; Ogawa et al. 1985). Further studies have revealed that capsaicin treatment causes the depletion of SP from small primary afferents in the dorsal horn in conjunction with increasing chemical and mechanical nociceptive thresholds (Hayes et al. 1981; Nagy et al. 1981; Yaksh et al. 1979), further emphasising this fact. In fact it is now known that SP is synthesised within the cell bodies of primary afferents then transported to peripheral and central terminals of the sensory axon where it is released to exert its many biological and physiological effects (Harmar and Keen, 1982).

There is now a large body of both behavioural and electrophysiological evidence implicating a role for SP and NK_1 receptors in sensory processing in the dorsal horn. Radioligand binding assays have shown that the expression of NK_1 receptors within the dorsal horn is altered by peripheral stimulation. Brief noxious inputs decreased SP binding after 1 minute due to competition by the endogenous ligand (Yashpal et al. 1994) whereas sustained forms of nociception such as inflammation or peripheral nerve injury raised levels of bound SP presumably as a result of postsynaptic receptor upregulation (Aanonsen et al. 1992; Kar et al. 1994; Yashpal et al. 1991a). In addition, the upregulation of NK_1 receptors after inflammation and nerve injury was seen with immunohistological techniques (Abbadie et al. 1996), while *in situ* hybridisation studies have revealed that complete Freund's adjuvant (CFA) or formalin-induced inflammation caused a considerable upregulation of NK_1 receptor mRNA throughout the superficial and deeper layers of the spinal dorsal horn (McCarson and Krause, 1994; Schäfer et al. 1993) which was blocked by prior administration of opioid agonists (McCarson and Krause, 1995). Together these results implicate the involvement of SP and its receptors as important transmitters of nociceptive information within the CNS.

Direct evidence of a neurotransmitter role for SP has been revealed in a number of behavioural and electrophysiological studies. Behavioural studies have shown that intrathecal SP evokes scratching and biting behaviours in mice and rats, responses which are widely considered to reflect painful sensations (Hayes and Tyers, 1979; Hylden and Wilcox, 1981; Yashpal et al. 1982). While ionophoretically

applied SP has been shown to excite dorsal horn neurones (Henry, 1976; Zieglgansberger and Tulloch, 1979) and selectively activate high threshold and multireceptive laminae I and II neurones (Randic and Miletic, 1977). In addition, SP enhanced the responses of multireceptive and nocispecific neurones to repetitive C-fibre strength stimulation (Kellstein et al. 1990) which parallels the observed increase in duration and magnitude of the flexor reflex evoked by C-fibre conditioning stimuli (Wiesenfeld-Hallin, 1986). Furthermore, NK_1 receptor antagonists have been shown to block both the SP-induced (Wiesenfeld-Hallin, 1986; Kellstein et al. 1990) and electrically-conditioned elevated responses of the flexor reflex (De Koninck and Henry, 1991; Laird et al. 1993; Xu et al. 1992a). However, NK_1 receptor antagonists are generally not effective at reducing dorsal horn neurone responses to brief noxious stimuli (Fleetwood-Walker et al. 1987;1990). Similarly, neuronal and behavioural responses to more natural stimuli, such as brief noxious pinch and acute noxious thermal stimulation of the tail or paw, are generally not affected by NK_1 receptor antagonists (Couture et al. 1993; Garces et al. 1993; Malmberg and Yaksh, 1992; Picard et al. 1993; Seguin et al. 1995; Yamamoto and Yaksh, 1992). These results would suggest then, that SP and NK_1 receptors may have a more prominent role in sustained noxious states.

Similarly, release studies strongly support a role for SP in sustained nociception. SP is released from isolated rat spinal cord *in vitro* and from the cat spinal cord *in vivo*, following electrical stimulation of peripheral nerves at intensities sufficient to activate small afferent fibres (Aδ and C fibres) (Akagi et al. 1980; Klein et al. 1992; Yaksh et al. 1980), implying that the main release of SP is from nociceptive afferents. Microdialysis techniques revealed that noxious mechanical and severe thermal stimuli specifically evoked SP release (Kuraishi et al. 1989) and that greater increments in SP levels were recorded following stimuli which caused damage to the peripheral tissues. In addition, antibody microprobe techniques showed a release of SP within the superficial dorsal horn following noxious heat (52°C +), noxious mechanical or chemical stimuli (Duggan et al. 1987; 1988). So the main role for SP and NK_1 receptors in sensory processing therefore appears to be modulators of sustained nociception, due to inflammation.

1.4.3 Other Possible Neurotransmitters

It is apparent then that a number of different neurotransmitters and their receptors are important in the regulation of noxious sensory transmission within the CNS, and that different neuroactive substances may have synergistic or counter-active inhibitory effects on one another.

CGRP is yet another main constituent of primary afferent fibres, found in approximately 30% of all primary afferent axons (Levine et al. 1993). CGRP-containing fibres are largely unmyelinated (C) fibres or small diameter (Aδ) fibres, and have been shown to terminate predominantly in laminae I, II and V of the spinal cord (Carlton et al. 1988). In addition, the antibody microprobe technique revealed that noxious thermal, mechanical or electrical stimulation evokes the release of CGRP in the superficial dorsal horn (Morton et al. 1990), implicating this peptide as another important mediator of nociceptive transmission. A high proportion (approximately 80%) of SP-expressing DRG also contain CGRP (Battaglia and Rustioni, 1988; Ju et al. 1987), and so these two peptides will be predominantly co-released. CGRP exerts limited effects by itself but can dramatically potentiate the effects of SP. Ionophoresis of CGRP produces a slow-onset, long-lasting excitation of nociceptive dorsal horn neurones *in vivo* (Miletic and Tan, 1988). However, concentrations which have little or no consistent effect alone, synergistically increase the effect of SP on rat dorsal horn neurones (Biella et al. 1991), as well as enhancing the release of SP from spinal cord slices (Oku et al. 1987). In addition, CGRP has been found to inhibit the enzymatic degradation of SP (Le Greves et al. 1985); another biological property which would enhance the function of SP. So CGRP appears to be a neuromodulator rather than a neurotransmitter within the CNS, as it exerts limited effects by itself but dramatically potentiates the effects of other compounds (in particular SP).

Somatostatin (SOM) has also been implicated as a neurotransmitter within the CNS, as high levels of this peptide are found in a population of small diameter primary afferents, distinct from those which contain SP (Hokfelt et al. 1976; Nagy and Hunt, 1982). In addition, SOM-ir is especially dense in lamina II of the dorsal horn (Finley et al. 1981; Hokfelt et al. 1976). However, electrophysiological studies on spinal neurones *in vivo* and *in vitro* have produced a variety of results,

demonstrating both an inhibitory (Murase et al. 1982; Randic and Miletic, 1978) and an excitatory (MacDonald and Nowak, 1981b; Salt et al. 1982) role for this peptide within the CNS, so its functional role as a neurotransmitter is not very clear. It has been suggested that SOM may effect the release of other transmitters (MacDonald and Nowak, 1981a) which may explain the discrepancies in the electrophysiological studies and would suggest that the primary role for this peptide may be as a modulator rather than a transmitter.

Another two peptides which are thought to have an excitatory role within the CNS are vasoactive intestinal polypeptide (VIP) and cholecystokinin (CCK). These peptides also display properties which fit the criteria for a transmitter: They are both found within the CNS of a number of different mammalian species (Yaksh et al. 1988). They are expressed primarily in small diameter DRG, as well as in peripheral nerves, and the superficial layers of the spinal dorsal horn (Fuji et al. 1983;1985). However, the levels of these peptides are relatively low in thc spinal cord under normal circumstances (Noguchi et al. 1993) and so it is possible that their transmitter role becomes more important following nerve injury when their levels become markedly up-regulated (Hokfelt et al. 1994; Noguchi et al. 1989). The role of VIP in somatosensory transmission will be discussed in much more detail in the following section (1.5).

So a vast number of different neurotransmitters appear to be able to mediate the pain-modulatory systems within the CNS, and it seems likely that there is no one sole mediator of nociceptive pathways. Instead it seems much more likely that a variety of neurotransmitters interact within the CNS to mediate/regulate the transmission of nociceptive (and non-nociceptive) information. A better understanding of the complicated pathways involved in responses to noxious stimuli, and the transmitters which are involved, would therefore provide new insights into targets for novel, more effective analgesics.

1.5 VIP AND PACAP

1.5.1 Isolation

In 1970, work by Said and Mutt demonstrated the existence of a new polypeptide, which they named Vasoactive Intestinal Polypeptide (VIP), due to its potent vasodilatory activity. This peptide was isolated from porcine small intestine based on its ability to increase peripheral blood flow and decrease arterial blood pressure in dogs (Said and Mutt, 1970b). Further work revealed the peptide to consist of 28 amino acid residues (Mutt and Said, 1974) having a distinct chemical structure from the kinins; substance P, glucagon and secretin, despite showing some sequence homology. Although originally considered as being a gut hormone (Said and Mutt, 1970a; 1970b), radioimmunochemical and immunohistochemical studies have revealed a widespread distribution of VIP throughout the peripheral and central nervous systems of a variety of species (Gibson et al. 1981; Larsson et al. 1976; Yaksh et al. 1988), suggesting that it may also play an important role as a neurotransmitter.

Pituitary Adenylate Cyclase-Activating Peptide (PACAP) is a basic 38 amino acid neuropeptide originally isolated from ovine hypothalamus (Miyata et al. 1989), based on its ability to cause adenylate cyclase activation in rat anterior pituitary cell cultures. This peptide occurs as two variants, PACAP-38 (a 38 amino acid polypeptide) and the C-terminally truncated form, PACAP-27 (with only 27 residues) (Miyata et al. 1990). PACAP-38 and PACAP-27 are derived from the same precursor of 176 amino acids and have comparable biological activity (Miyata et al. 1990). PACAP is a member of the VIP/secretin/glucagon family of structurally related peptides, as it shows 68% homology with VIP at its N-terminal (Arimura, 1992)(See Figure 1.3). It also has a widespread distribution throughout the mammalian body and may also be an important neurotransmitter or modulator in the peripheral and central nervous systems (Arimura, 1992; Arimura and Shioda, 1995; Ghatei et al. 1993; Moller et al. 1993).

1.5.2 VIP/PACAP Receptor Subtypes

The sequence homology observed between VIP and PACAP means that they share the same receptor binding sites (Harmar and Lutz, 1994). VIP and the two

alternative processing forms of PACAP are recognised by a family of three receptors, namely the VIP_1 (Ishihara et al. 1992), VIP_2 (Lutz et al. 1993) and PACAP (Hosoya et al. 1993) receptors. All three receptors are G-protein-coupled receptors (Spengler et al. 1993), with seven conserved transmembrane domains, and there is a considerable degree of homology between the three receptor subtypes (Lutz et al.1993) (See Figure 1.4). The most notable features of the VIP/PACAP receptors are the relatively long amino-terminal extracellular tail, and the highly conserved cysteine residues within the extracellular loops (Hashimoto et al. 1993;1997).

Two specific forms of the VIP receptor have been identified so far, namely the VIP_1 (Ishihara et al. 1992) and VIP_2 (Lutz et al. 1993) receptors. They are both positively-coupled to the α-subunit of the adenylate-cyclase-stimulating G-protein (Gs), and so stimulate the activity of adenylate cyclase (See Figure 1.5) following their activation (Ishihara et al. 1992; Lutz et al. 1993). Both receptors bind VIP and PACAP with a similar high affinity, although they appear to be differentially expressed throughout the CNS (Cauvin et al. 1991; Usdin et al. 1994). The VIP_1 receptor was originally cloned from rat lung cDNA (Ishihara et al.1992) and is found predominantly in the liver, intestine and certain areas of the brain (notably the cortex, hippocampus and the olfactory bulb). The VIP_2 receptor was isolated from rat pituitary and olfactory bulb and is primarily expressed in the thalamus, hippocampus, suprachiasmatic nucleus and the hypothalamus (Lutz et al. 1993).

The PACAP receptor displays a much greater affinity for the two forms of PACAP (PACAP-27 and PACAP-38) than for VIP (Hashimoto et al. 1993; Shivers et al. 1991), and also has a widespread distribution throughout the body, including the CNS (Cauvin et al. 1991; Ghatei et al. 1993; Masuo et al. 1991). The PACAP receptor is coupled to dual signalling cascades involving the Gs and Gq proteins (See Figure 1.5), and so has the ability to stimulate both adenylate cyclase and phospholipase C (PLC) second messenger pathways (Spengler et al. 1993). The different second messenger pathways may be preferentially regulated by the two forms of PACAP, as both PACAP-27 and PACAP-38 can stimulate adenylate cyclase activity with similar EC_{50} values, but only PACAP-38 can stimulate PLC with high potency (Spengler et al. 1993). In addition, five subtypes of the PACAP receptor can be generated, by alternative splicing of the PACAP precursor, which

vary in the amino acid sequence within the third intracellular loop. These splice variants have a differential distribution within the peripheral and central nervous systems and show altered patterns of adenylate cyclase and PLC activation (Spengler et al. 1993). So variations in the expression of PACAP in its two amidated forms, and of the different VIP/PACAP receptor subtypes, may potentially result in diverse PACAP-mediated cellular activities within different areas of the body.

Comparison of the tissue distribution of the VIP_1, VIP_2 and PACAP receptors by *in situ* hybridisation has revealed remarkable differences: PACAP receptor mRNA is abundantly expressed in the brain but there is very little expression in peripheral tissue (Arimura and Shioda, 1995), whereas VIP_1 receptor mRNA is expressed in the lung, intestine and liver (Ishihara et al. 1992) while brain levels are much lower than that of PACAP. In contrast, mRNA for the VIP_2 receptor is found predominantly in the thalamus, hippocampus and suprachiasmatic nucleus, as well as in the stomach and testes (Harmar and Lutz, 1994; Usdin et al. 1994). This distinct distribution of the VIP/PACAP receptors within the CNS suggests that these receptors may have different physiological roles.

1.5.3 Anatomical Distribution of VIP/PACAP and their Receptors

VIP and PACAP are expressed widely in both the periphery and CNS of a variety of species (Arimura and Shioda, 1995; Ghatei et al. 1993; Gibson et al. 1981; LaMotte and de Lanerolle, 1986; Larsson et al. 1976; Vigh et al. 1991; Yaksh et al. 1988). Their distinct anatomical distribution within the CNS has led to the suggestion that these peptides may serve as sensory transmitters, and so may play an important role in the modulation of somatosensory processing.

Brain: Significant concentrations of VIP have been reported in many areas of the brain by a number of different laboratories, with the highest levels being found in the cerebral cortex, hippocampus and the hypothalamus (Besson et al. 1986; Larsson et al. 1976; Staun-Olsen et al. 1985). PACAP on the other hand, shows a much wider distribution with high levels demonstrated in the rat hippocampus, hypothalamus and anterior pituitary (Cauvin et al. 1991; Masuo et al. 1991; 1992). Within the rat brain, the specific binding of radiolabeled PACAP was shown to be around 10 times greater

than that of radiolabeled VIP, particularly at the level of the dorsal hippocampus (Masuo et al. 1993), and this may reflect a dominant presence of the specific PACAP receptor over the VIP_1 or VIP_2 receptors. More recent studies have revealed a distinct distribution of the VIP/PACAP receptor subtypes throughout the brain with only a few areas of overlap, notably the thalamus, hippocampus and olfactory bulb (See Figure 1.6), which may reflect the need for different biological functions in the different areas. The VIP_1 receptor is found predominantly in the cerebral cortex, hippocampus and the olfactory bulb of the brain, while the VIP_2 receptor is found primarily in the thalamus, hippocampus, hypothalamus, suprachiasmatic nucleus and the olfactory bulb (Harmar and Lutz, 1994; Usdin et al. 1994; Vertongen et al. 1997).

So, the mRNAs encoding the VIP_1 and VIP_2 receptors appear to have a complimentary distribution within the CNS. Even in areas of the brain where they are both present (for example the olfactory bulb and the cerebral cortex), they still appear to have distinct distributions: VIP_1 receptor mRNA is found within the external plexiform layer of the olfactory bulb while that of VIP_2 is generally found in the internal granular layer, similarly in the cortex, VIP_1 receptors are expressed in layers III and V while VIP_2 receptor expression appears to be exclusively in layer VI (Usdin et al. 1994; Vertongen et al. 1997).

Spinal cord:

Segmental localisation. Radioimmunoassays have revealed the presence of VIP and PACAP throughout all segments of the rat and cat spinal cord (Fuji et al. 1985; Moller et al. 1993; Mulder et al. 1994; Shehab and Atkinson, 1986a; 1986b; Yaksh et al. 1982). The expression of these peptides is broadly distributed throughout the full length of the spinal cord, but there is a clear rostro-caudal gradient of the autoradiographic binding sites for VIP, with the greatest concentrations generally found at lower lumbar to sacral levels (Gibson et al. 1981; Yashpal et al. 1991b).

Figure 1.3

Sequence Homology between VIP and the Two Amidated forms of PACAP

The aligned amino acid sequences of PACAP-38, PACAP-27 and ovine VIP, with the amino acid residues common to all three polypeptides marked in bold, to demonstrate the high degree of homology observed between the three peptides.

(Adapted from Arimura and Shioda, 1995)

PACAP-38:

His-Ser-Asp-Gly-Ile-**Phe-Thr-Asp**-Ser-**Tyr**-Ser-**Arg**-Tyr-**Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu**-Ala-Ala-Val-**Leu**-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH_2

PACAP-27:

His-Ser-Asp-Gly-Ile-**Phe-Thr-Asp**-Ser-**Tyr**-Ser-**Arg**-Tyr-**Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu**-Ala-Ala-Val-**Leu**-NH_2

oVIP:

His-Ser-Asp-Ala-Val-**Phe-Thr-Asp**-Asn-**Tyr**-Thr-**Arg**-Leu-**Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu**-Asn-Ser-Ile-**Leu**-Asn-NH_2

Figure 1.4

Sequence Homology Between the Rat VIP_1, VIP_2 and PACAP Receptors

Schematic model of the deduced amino acid sequence for the rat VIP_2 receptor, showing the areas of homology between the VIP_1, VIP_2 and PACAP receptors. All three receptors are G protein-coupled and possess 7 transmembrane domains with their associated intracellular and extracellular loops. Amino acids homologous to all three receptors are marked in red, while those common to both VIP_1 and VIP_2 receptors are coloured blue, VIP_2 and PACAP receptors are yellow, and those found in both VIP_1 and PACAP receptors are shown in green. The remaining, uncoloured amino acids are found only in the VIP_2 receptor. A similar degree of homology is also seen between the human VIP_1, VIP_2 and PACAP receptors (Pisegna et al. 1996).

(Adapted from Lutz et al.1993)

Several domains appear to be important for the selectivity of the ligand binding and subsequent receptor activation, including the amino-terminal extracellular domain, the transmembrane domains (I and II), and the C-terminal region (Hashimoto et al. 1997; Wulff et al. 1997), while the third intracellular loop is the area of the receptor associated with G-protein activation.

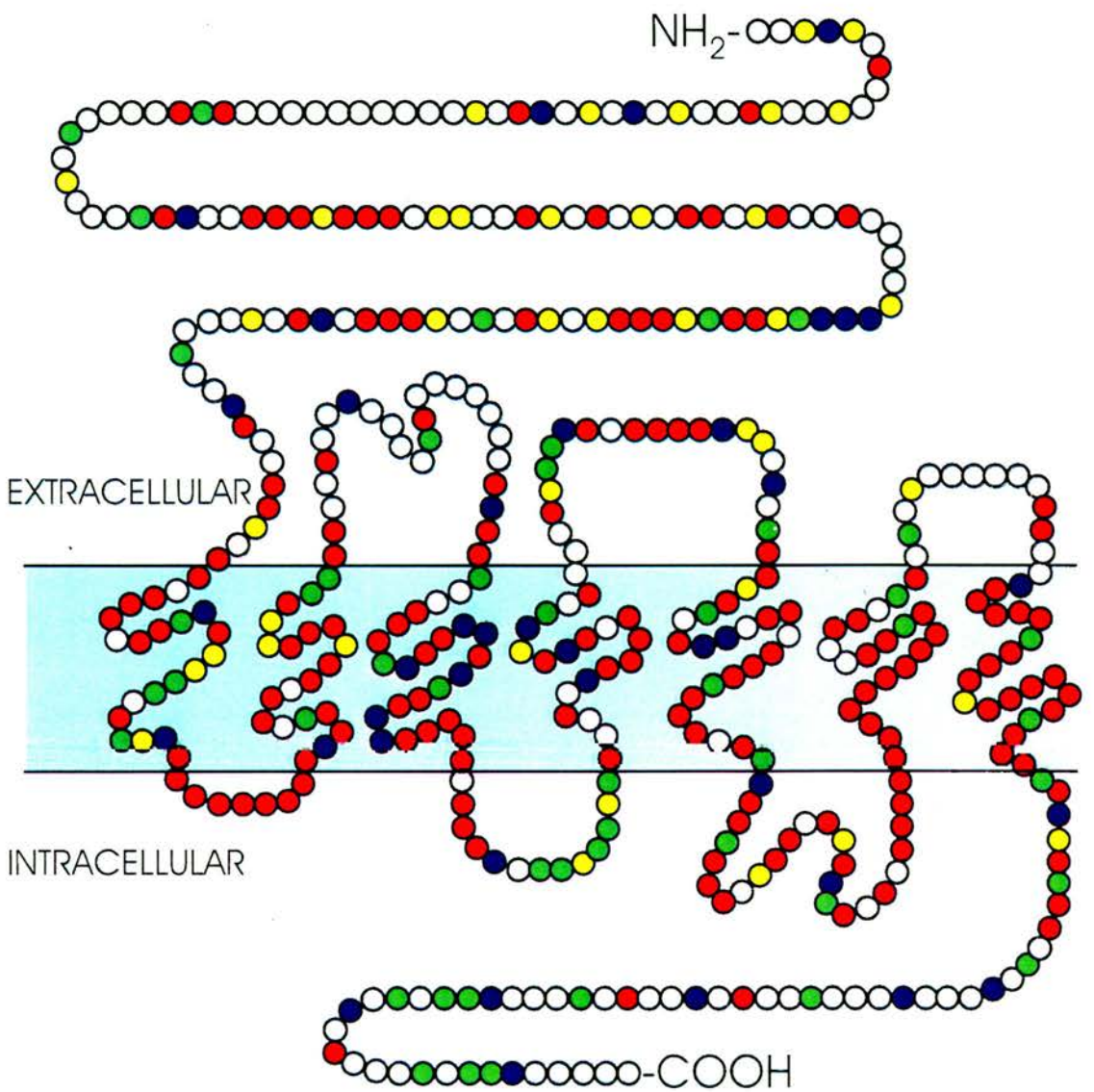
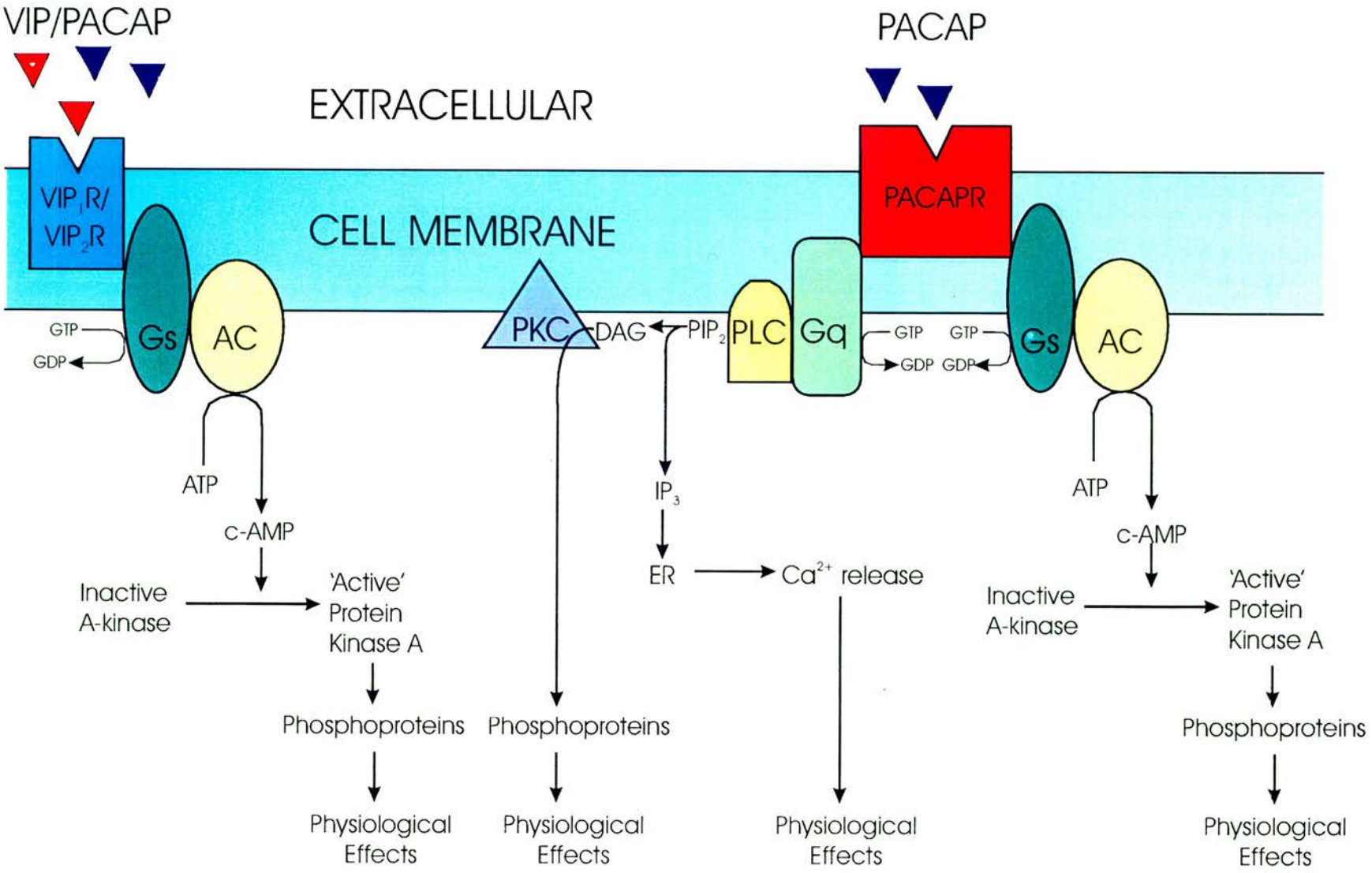

NH_2-
EXTRACELLULAR
INTRACELLULAR
-COOH

Figure 1.5

VIP/PACAP Receptor Activation of Second Messenger Pathways

Schematic representation of the VIP/PACAP receptors and the second messenger pathways associated with their activation. The VIP_1 and VIP_2 receptors are both positively-coupled to the α-subunit of the adenylate-cyclase-stimulating G-protein (Gs) which activates adenylate cyclase (AC) following the agonist-induced exchange of guanosine triphosphate (GTP) into guanosine diphosphate (GDP). Adenylate cyclase subsequently converts adenosine triphosphate (ATP) into cyclic-adenosine monophosphate (c-AMP), which in turn activates protein kinase A, to result in the phosphorylation of a number of proteins and receptors within the cell. Both VIP and PACAP can activate the VIP_1 and VIP_2 receptors with similar binding affinity. The PACAP receptor is coupled to dual signalling cascades involving the Gs protein, which activates adenylate cyclase as above, and the Gq protein, which activates the phospholipase C (PLC) second messenger pathway. Activation of the PLC pathway converts phosphatidyl inositol 4,5-diphosphate (PIP_2) into inositol 1,4,5- triphosphate (IP_3) which causes the intracellular release of calcium ions from the endoplasmic reticulum (ER), and di-acyl glycerol (DAG) which induces phosphorylation of intracellular proteins through activation of protein kinase C (PKC). Increases in the intracellular levels of calcium ions have been linked with a number of physiological effects including activation of immediate early genes and subsequently changes in receptor and gene expression within the cell, as well as enhancing the effects of various ion-channel gated receptors, including NMDA.


VIP/PACAP
PACAP
EXTRACELLULAR
VIP1R/
VIP2R
PACAPR
CELL MEMBRANE
GTP
GDP
Gs
AC
PKC
DAG
PIP2
PLC
Gq
GTP
GDP
GTP
GDP
Gs
AC
ATP
c-AMP
IP3
ATP
c-AMP
Inactive
A-kinase
'Active'
Protein
Kinase A
ER
Ca2+ release
Inactive
A-kinase
'Active'
Protein
Kinase A
Phosphoproteins
Phosphoproteins
Phosphoproteins
Physiological
Effects
Physiological
Effects
Physiological
Effects
Physiological
Effects

Lamina localisation. VIP and PACAP immunoreactivity can be seen throughout the spinal cord, but their highest levels are concentrated in the superficial laminae of the dorsal horn, laminae I and II (Dun et al. 1996; Kar and Quirion, 1995; Moller et al. 1993; Yashpal et al. 1991b). VIP-ir perikarya are found in the dorsal horn, lamina X and the intermediolateral nucleus (Fuji et al. 1985; Knyihar-Csillik et al. 1993; LaMotte and de Lanerolle, 1986; Yaksh et al. 1982), but the level of expression is very low in normal animals. PACAP-ir has been detected in numerous nerve fibres of laminae I and II of the dorsal horn (Dun et al. 1996; Moller et al. 1993), although some of these fibres also project into the deeper laminae of the dorsal horn (laminae III, IV and V) of all spinal cord segments (Dun et al. 1996). In the lower cervical, thoracic, lower lumbar and sacral segments, additional PACAP-ir fibres were also detected in the intermediolateral cell column (Dun et al. 1996). The mRNA for VIP_1 (Ishihara et al. 1992), VIP_2 (Lutz et al. 1993) and the PACAP receptors (Arimura and Shioda, 1995) is also expressed in spinal cord, particularly in the superficial dorsal horn.

Primary afferents. VIP-ir nerve fibres have been shown to be distributed within lamina I of the rat dorsal horn, with a few fibres projecting to lamina II (Fuji et al. 1985; Gibson et al. 1981; LaMotte and de Lanerolle, 1986). Dorsal rhizotomy produces an almost total depletion of VIP from the superficial laminae of the dorsal horn (Shehab and Atkinson, 1986a; 1986b; Yaksh et al. 1982), suggesting that the origin of VIP is attributed to fine primary afferent fibres. In addition, electrical stimulation of the sciatic nerve at or above C/Aδ fibre intensity, has been shown to produce a significant increase in VIP levels within the spinal cord, while stimulation at low threshold fibre intensity has no such effect (Yaksh et al. 1982). Suprisingly however, intrathecal capsaicin has no effect on the spinal cord levels of VIP (Yaksh et al. 1982) suggesting that the origin of this peptide is not likely to be C-nociceptors. Radioimmunological studies have revealed a dense accumulation of PACAP-ir nerve fibres in the superficial layers of the spinal cord (Dun et al. 1996; Moller et al. 1993), with a similar distribution to that of SP- and CGRP-ir fibres, but less numerous fibres. Capsaicin treatment results in the release of PACAP-27 and PACAP-38 from the rat spinal cord *in vivo* (Zhang YZ et al. 1997), as well as a significant decrease in

the number of PACAP-ir nerve fibres within the spinal cord (Moller et al. 1993) indicating that PACAP is present in a population of nociceptive C-fibres.

Dorsal Root Ganglia (DRG)

The intrinsic levels of VIP appear to be very low, as very little expression of mRNA for VIP can usually be seen in normal DRG (Noguchi et al. 1989; Yaksh et al. 1982), significant levels can normally only be viewed following colchicine treatment (Shehab and Atkinson, 1986a; Yaksh et al. 1982). Following axotomy however, there is a marked upregulation in the expression of VIP, such that approximately 40% of small to medium diameter DRG neurones now contain this peptide (Shehab and Atkinson, 1986a; 1986b). Unilateral rhizotomy performed at the same time as axotomy prevents this marked increase in VIP levels, and so the injured nerve fibres are most likely the source of VIP seen following nerve section (Shehab and Atkinson, 1986a; Shehab et al. 1986). PACAP mRNA on the other hand, is normally present in about 10% of rat DRG neurones (Mulder et al. 1994), and is predominantly expressed in small-medium diameter neurones (Dun et al. 1996; Moller et al. 1993; Mulder et al. 1994) suggesting that PACAP is a constituent of primary afferent fibres. Immunohistochemical studies have revealed that PACAP co-exists with CGRP and SP (Moller et al. 1993; Mulder et al. 1994), and so further implies a role for this peptide as a neurotransmitter.

Peripheral Distribution: As stated previously, the distribution of VIP/PACAP receptors is not solely confined to the CNS, a large number of peripheral tissues and organs also express these receptors, in particular the VIP_1 and VIP_2 receptors (Harmar and Lutz, 1994; Usdin et al. 1994). The VIP_1 receptor appears to have the widest distribution within the periphery, and is found in the liver, lungs, small intestine and the thymus (Ishihara et al. 1992; Usdin et al. 1994). In contrast, the VIP_2 receptor is present in a number of areas where VIP acts, but where VIP_1 receptor mRNA is absent, or is present at very low levels, including the stomach and the testes (Usdin et al. 1994). This again highlights the differential distribution of these receptors and suggests that they each have different biological roles within the body. On the other hand, there appears to be a very low level of expression of the specific PACAP receptor within the periphery, despite the clear biological roles

which PACAP has in a number of tissues and organs (Arimura, 1992; Hashimoto et al. 1993). PACAP-ir fibres have been demonstrated in the stomach, liver, duodenum and jejunum, but there appears to be no, or very little expression of the PACAP receptors here (Arimura, 1992; Hashimoto et al. 1993; Hosoya et al. 1993). This would suggest that any biological roles which PACAP has in these tissues are mediated by either the VIP_1 or VIP_2 receptors.

Thus, the wide distribution of VIP and PACAP within the CNS, in particular in primary sensory neurones of the spinal dorsal horn, suggests a possible role for these peptides in the transmission of somatosensory information. The majority of VIP- and PACAP-ir fibres are found in the superficial layers of the rat spinal cord, namely laminae I and II (Fuji et al. 1985; Moller et al. 1993; Yashpal et al. 1991b), an area known to be important in the transmission of nociceptive information (Cervero and Iggo, 1980).

However, the levels of these peptides, in particular of VIP, are relatively low under normal circumstances (Noguchi et al. 1993; Mulder et al. 1994), suggesting perhaps that they are not primary transmitters within the normal spinal cord. Following nerve injury however, the levels of these peptides are dramatically up-regulated within the spinal dorsal horn (Knyihar-Csillik et al. 1993; Shehab and Atkinson, 1986b; Zhang et al. 1996), as well as in small-medium diameter DRG neurones (Hokfelt et al. 1994; Zhang et al. 1995a; Zhang et al. 1996) and so it may be that VIP and PACAP only become important neurotransmitters in chronic pain conditions.

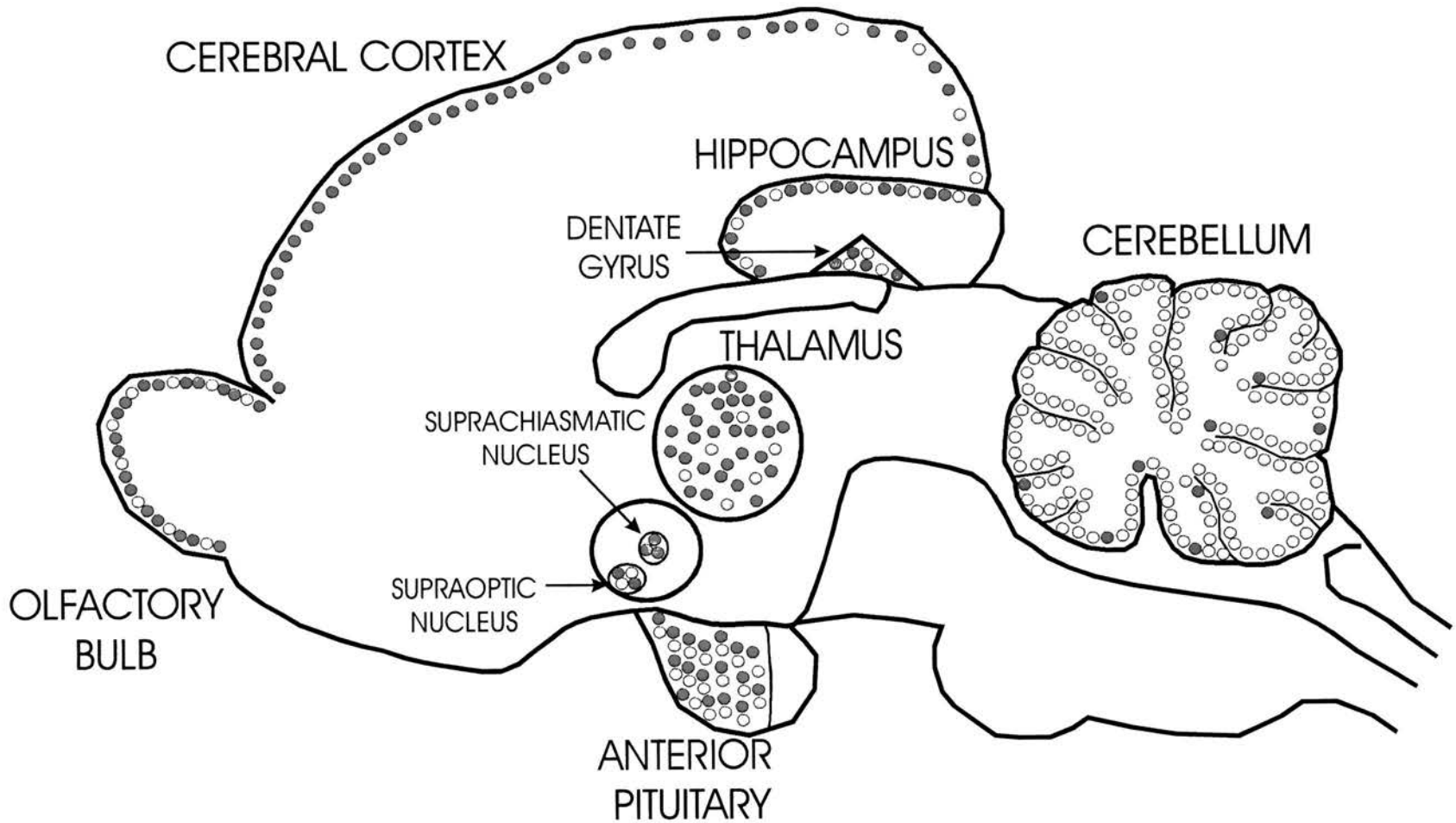
Figure 1.6

Differential Expression of the VIP_1, VIP_2 and PACAP Receptors Within the Rat Brain

Schematic representation of the rat brain showing the differential expression of VIP_1 (blue), VIP_2 (red) and PACAP (yellow) receptors, within the different brain areas.

All three receptors display a unique pattern of expression within the rat brain, with only a few areas of complete overlap, namely the olfactory bulb and the hippocampus. In the remaining areas there appears to be a preferential expression of one receptor subtype above the others, which may reflect a need for different physiological receptor properties within the different areas. The PACAP receptor is widely expressed throughout the rat brain, with the most abundant expression in the olfactory bulb, hippocampus, anterior pituitary and the cerebellum (where it is expressed almost exclusively). It is also co-expressed with the VIP_1 receptor in the dentate gyrus and the supraoptic nuclei of the hypothalamus. Relatively small amounts of expression are noted in the thalamus, which is predominated by the VIP_2 receptor, and the piriform layers of the cerebral cortex.
VIP_2 receptor expression is most abundant in the thalamus, anterior pituitary, olfactory bulb and the suprachiasmatic nucleus within the hypothalamus. It is also co-expressed with VIP_1 in the cerebral cortex and hippocampus.
There appear to be no areas where the VIP_1 receptor is the sole receptor expressed. It is found predominantly in the cerebral cortex, olfactory bulb and hippocampus, but can also be expressed with the PACAP receptor in the supraoptic nuclei, dentate gyrus and to a lesser degree within the cerebellum.

Based on information from (Harmar and Lutz, 1994; Hashimoto ct al. 1993; Ishihara et al. 1992; Lutz et al. 1993; Spengler et al. 1993; Vertongen et al. 1997)


CEREBRAL CORTEX
HIPPOCAMPUS
DENTATE
GYRUS
CEREBELLUM
THALAMUS
SUPRACHIASMATIC
NUCLEUS
SUPRAOPTIC
NUCLEUS
OLFACTORY
BULB
ANTERIOR
PITUITARY

1.5.4 VIP/PACAP-mediated Spinal Somatosensory Transmission

The availability of synthetic VIP and PACAP, and their analogues, has led to numerous *in vivo* and *in vitro* studies on the physiological properties of these two peptides. In addition to their anatomical distribution within areas of the CNS known to be associated with somatosensory processing (notably the superficial laminae of the dorsal horn), functional studies have provided strong evidence for a regulatory role of VIP and PACAP at the spinal cord level.

(a) Effects of VIP and PACAP on Behavioural Nociceptive Responses

Spinal reflexes have been extensively utilised in studies of pain mechanisms in animals and man, to study the role of a number of different neuroactive substances in modulating spinal cord excitability. The magnitude of the nocifensive hindpaw flexor reflex to electrical stimulation or to physiological stimuli, has been positively correlated to the activity of spinal dorsal horn neurones (Schouenborg and Sjolund, 1983), and is a graded response which is commonly used for both physiological and pharmacological studies.

A number of studies have been performed using this technique to determine the role of VIP at the spinal cord level, and to gain new insight into its proposed role as a neurotransmitter/neuromodulator. A brief conditioning stimulus (CS) train, applied to unmyelinated C-fibres has been shown to induce wind-up and facilitation of the flexor reflex (Wall and Woolf, 1984). Although VIP facilitates spinal cord reflex excitability in rats with intact peripheral nerves following its intrathecal application (Weisenfeld-Hallin 1987; 1989; Xu and Weisenfeld-Hallin, 1991), endogenous VIP does not appear to play a role in the C-fibre induced spinal sensitisation under normal conditions, as intrathecal application of the VIP antagonist (Ac-Tyr^{1}, D-Phe^{2})-GRF(1-29)-NH_2 had no effect on CS-induced reflex facilitation in normal animals (Wiesenfeld-Hallin et al. 1990a; Xu and Wiesenfeld-Hallin, 1991). However, within two weeks following sciatic nerve transection VIP becomes a major excitatory mediator of spinal reflex hypersensitivity, as the VIP antagonist now significantly inhibited C-fibre facilitation of the flexor reflex (Wiesenfeld-Hallin, 1989; Wiesenfeld-Hallin et al. 1990a), while the tachykinins (SP and CGRP) appeared to lose their excitatory role (Wiesenfeld-Hallin et al. 1990a). These results

suggest that there may be a switch in the role of excitatory neuropeptides following peripheral nerve injury from SP to VIP, which would parallel the corresponding changes in the expression of these peptides following axotomy.

The role of VIP in mediating nociceptive information at the spinal cord level was then considered in more detail, revealing that VIP appeared to be involved primarily in the transmission of thermal pain. Cridland and Henry revealed that intrathecal VIP produced a dose-dependent decrease in the withdrawal latency of the rat tail to noxious radiant heat, but showed no changes in the animal's withdrawal threshold to innocuous mechanical stimulation of the tail (Cridland and Henry, 1988). In addition, the spinal cord excitability following intrathecal VIP was considerably higher when the flexor reflex was evoked by noxious thermal stimulation of the ipsilateral foot, compared to that seen following cutaneous noxious mechanical pinch (Wiesenfeld-Hallin, 1987). These results are in contrast to those seen for SP and CGRP, which appear to be important mediators of both mechanical and thermal nociceptive information (Cridland and Henry, 1988), suggesting that specific peptides may be involved in the transmission of different types of pain modality.

The functional effects of PACAP are not quite as clear cut, and are currently under debate. Some groups have demonstrated an antinociceptive effect of PACAP at the spinal level, as intrathecal application of PACAP-27 was found to produce a significant and long-lasting suppression of the C-fibre evoked flexor reflex over the wide range of concentrations tested (Zhang et al. 1993). In addition, pre-treatment of rats with intrathecal PACAP was shown to decrease the number of formalin-induced instances of flinching behaviour, in a dose-dependent manner. However at the high doses used (approximately 15.5nmol) there was evidence of some motor defects, as tested using the placing/stepping reflex (Yamamoto and Tatsuno, 1995). However, on repeating these studies at lower doses, which showed no motor impairment, PACAP-27 was still found to depress both phases of the formalin responses, and therefore still suggests an antinociceptive role for this peptide.

Weisenfeld-Hallin's group however, have suggested a nociceptive role for PACAP. In decerebrate spinalised animals, they revealed that intrathecal PACAP, delivered over a wide low dose range (10ng to 10μg) produced a dose-dependent

facilitation of the flexor reflex, with no inhibitory effects observed at any of the doses (Xu and Weisenfeld-Hallin; 1996). This data implies that PACAP has an excitatory effect on the spinal cord function, similar to that seen for many other peptides investigated in this model, including SP, CGRP, VIP and SOM (Cridland and Henry, 1988; Wiesenfeld-Hallin et al. 1990b; 1991; Xu et al. 1990). In agreement with these findings, Narita et al.(1996) discovered that low doses of intrathecal PACAP (0.05-0.5μg) produced a dose-dependent decrease in tail-flick latency to noxious heat in mice, indicative of thermal hyperalgesia. Slightly higher doses (1-10μg) induced a dose-dependent increase in biting and scratching behaviours, indicative of pain behaviours (Narita et al. 1996), similar to those seen following intrathecal application of SP (Hayes and Tyers, 1979; Hylden and Wilcox, 1981). These results strongly support the idea that PACAP may be a sensory transmitter involved in nociceptive processing within the mouse spinal cord, and suggests that it may well have several distinct actions.

However, the different effects of PACAP observed in the results of the flexor reflex studies may be due to differences in the experimental set up between the two groups, including differences in the site of nerve stimulation and recording, the integrity of the spinal cord, and the presence or absence of anaesthesia, and so it is therefore important to consider other physiological effects of PACAP when trying to ascertain its role within the CNS.

(b) Effects of VIP and PACAP on Electrophysiological Responses

A number of electrophysiological studies have demonstrated the potential for VIP to act as a neurotransmitter of a number of different cell types within the CNS. Ionophoresis of synthetic VIP showed it to be a potent excitant of both nociceptive and non-nociceptive neurones throughout the rat trigeminal nucleus caudalis (Salt and Hill, 1981), while a similar non-selective excitatory effect was also observed on the vast majority of spinal cord neurones (laminae I-VII) in both the intact cat spinal cord and the rat spinal cord slice preparation (Jeftinija et al. 1982), despite the fact that the levels of VIP within the spinal cord appear to be lower than those seen in many other regions of the CNS (Emson, 1979; Loren et al. 1979). This excitatory role of VIP has also been demonstrated in different areas of the rat brain, including

the hippocampus (Dodd et al. 1979) and cerebral cortex (Phillis et al. 1978). In an *in vitro* slice preparation of the rat hippocampus, VIP was found to have an excitatory role on CA1 pyramidal neurones following pressure ejection from micropipettes. VIP application caused subsequent depolarisation of all the neurones tested, with an increase in the rate of discharge. The membrane potential and excitability usually returned to control resting levels within 60-180 secs of termination of VIP application, although repeated application was seen to cause an apparent desensitisation of VIP receptors (Dodd et al. 1979). In an *in vivo* study on the rat cerebral cortex, Phillis et al. recorded extracellularly from deep, spontaneously active neurones, including identified pyramidal cells. They found that ionophoretically applied VIP excited the vast majority of these cells, and that the membrane receptors for VIP appeared to undergo an apparent desensitisation following repeated application of VIP. However, within the cat visual cortex, VIP appeared to have the ability to increase or decrease both the spontaneous activity and the visual responses of the majority of cortical neurones tested (Murphy et al. 1993), suggesting that VIP may have dual modulatory roles within certain areas of the brain.

Extracellular patch-clamp recordings of cultured chick cortical neurones have revealed a direct modulatory effect of PACAP-38 (Liu and Madsen, 1997). This facilitatory effect appears to be independent of intracellular second messengers (such as cAMP, calcium ions and inositol phosphate), and it has been proposed that PACAP modulates this effect via the glycine co-agonist site(s) on the NMDA receptor (Liu and Madsen, 1997). The direct modulation of NMDA receptors via the glycine site has previously been suggested for the opioid peptides (Chen et al. 1995; Rusin and Randic, 1991) as well as for the tachykinins (Rusin et al. 1992; 1993). A similar facilitatory effect at the NMDA receptor has also been observed in patch-clamp recordings of rat sympathetic preganglion neurones (SPN), although in these experiments the effects of PACAP-38 were attenuated by pre-treatment of the cells with cAMP inhibitors (Wu and Dun, 1997), suggesting that in these cells, PACAP potentiates NMDA-receptor-mediated responses via second messenger systems (i.e. cAMP), and so may be a facilitatory effect following activation of the VIP/PACAP receptors (which are positively coupled to the adenylate cyclase pathway). PACAP has also been shown to have an excitatory effect in other regions of the brain,

including the magnocellular portion of the rat hypothalamic paraventricular nucleus. In a brain slice preparation, perfusion of PACAP-27 or PACAP-38 at low concentration (≤ 1μmol) produced prominent excitatory effects on a large proportion of the spontaneously firing neurones tested (Uchimura et al. 1996).

So VIP and PACAP both appear to play an important role as neurotransmitters/neuromodulators within the CNS. VIP appears to be a potent excitant in at least three different areas of the CNS (the spinal cord, cerebral cortex and hippocampus), and these effects are seen at low concentrations. Despite the variability in PACAP effects in some of the behavioural studies, an overall review of the current PACAP literature would suggest that its effect within the CNS is predominantly excitatory, particularly within the brain.

1.5.5 Other Physiological Roles of VIP and PACAP

(a) General Biological Actions

Both PACAP and VIP possess a number of physiological roles within the mammalian body. Although originally thought to be solely a gut hormone (Said and Mutt, 1970a; 1970b), the discovery of the wide distribution of VIP throughout the body has led to the revelation of numerous biological and physiological properties for this peptide.

Investigations into the effects of VIP, have shown an unusually wide range of biological activities for this peptide, including vasodilatory effects, increasing cardiac output, as well as causing hypotension and hyperglycaemia (Said and Mutt, 1970a). Within the systemic vascular system, VIP has been shown to have marked and prolonged effects, increasing blood flow while decreasing systemic blood pressure following intra-arterial infusion of the pure peptide (Said and Mutt, 1970b). In addition, VIP is a highly potent stimulant of vasodilation in many different organs, as well as playing a role in smooth muscle contraction (Piper et al. 1970) and so it appears to be an important regulator of blood flow to the digestive organs as well as being a mediator in various hypotensive states.

PACAP, as its name suggests, can stimulate the activation of adenylate cyclase in anterior pituitary cells (Miyata et al. 1989), and despite its considerable degree of homology with VIP (68% at its N-terminal), it can stimulate adenylate

cyclase in certain tissues with many fold greater potency than VIP. A dense network of PACAP containing fibres has been demonstrated in the gut wall of a number of different species, including human, rat, chicken and cat (Arimura, 1992; Sundler et al. 1992). These nerve fibres innervate longitudinal smooth muscle, blood vessels and Brunnel's glands in the intestine which suggests that PACAP has important regulatory actions within the gastrointestinal tract including motility, blood flow and secretory activity of the glands.

(b) Immunoregulatory Role of VIP/PACAP

The delivery of various mediators to immune cells, is an important regulatory aspect of the immune and inflammatory responses, and a substantial body of evidence exists to suggest that VIP and PACAP can modulate immune responses. As well as having the potential to exert an indirect influence on the immune response through its vasodilatory role (causing vascular smooth muscle relaxation and subsequently vasodilation at the site of injury/inflammation, thereby modulating lymphocyte trafficking), VIP can also act directly on a number of different cells of the immune system via specific receptors. Receptors for VIP and PACAP have been found on a number of different cells, including lymphocytes, macrophages and leukocytes (Goetzl et al. 1995; Gozes et al. 1991; Tatsuno et al. 1991; 1996; Xia et al. 1996), thereby enabling VIP and PACAP to modulate the proliferative responses of these immune cells and/or their release of various mediators. The expression of VIP_1 and VIP_2 receptors on human T and B lymphocytes (Gozes et al. 1991; Xia et al. 1996) enables VIP to have potent and apparently distinctive effects on T cell selection and T cell migration in the thymus, and so endogenous VIP may be one of the factors which regulates the negative selection of T lymphocytes during an immune response. In addition, VIP and PACAP can modulate the production of various cytokines from immune cells (Gottschall et al. 1994; Xin et al. 1997) as well as from epithelial cells *in vitro*, and so may indirectly influence immune and inflammatory responses at the site of injury. Other regulatory roles include the modulation of the growth rate of cultured lymphocytes, mediation of immunoglobulin production and secretion, and the regulation of adherence molecule expression which may ultimately effect immune cell migration (Goetzl et al. 1995;

O'Dorisio et al. 1981). These various immunoregulatory roles implicate VIP and PACAP in the modulation of a number of important biological processes within the body. The neurotrophic properties of VIP and PACAP, which appear to be vitally important in the regulation of immune and inflammatory responses, may therefore be equally important in the degenerative and regenerative processes which ensue from nerve injury.

(c) Neurotrophic Role of VIP and PACAP

There is a vast amount of evidence to suggest that VIP and PACAP may be important neurotrophic factors within the nervous system. VIP and/or PACAP have been shown to exhibit a number of distinct actions on the cell proliferation, survival and phenotypic expression of a number of well-characterised cell culture systems, including neural cells. These actions may be directly on the cells themselves or a result of the indirect activation of neighbouring cells.

VIP and PACAP appear to play an important role in the sympathetic nervous system, and have been shown to exert a number of effects on cultures of superior cervical ganglion (SCG) cells including increasing neurite outgrowth, promotion of cell survival and increasing cell proliferation (Colbert et al. 1994; Deutsch and Sun, 1992; Hernandez et al. 1995; Klimaschewski et al. 1995; Pincus et al. 1994). In addition, VIP and PACAP are also capable of inducing NPY gene expression in PC12 cell cultures (Colbert et al. 1994), and so their function in the sympathetic nervous system is not purely trophic. PACAP in particular, appears to play an important role preventing neuronal apoptotic death. This property was observed within physiological concentrations (10^{-10} to 10^{-8}M)and was considerably more pronounced than the effects of VIP (which was only effective in increasing cell survival at unphysiologically high concentrations (10^{-6})) (Deutsch and Sun, 1992; Tanaka et al. 1997). This suggests that PACAP-induced enhancement of PC12 cell survival is mediated primarily by the PACAP receptor, which has a high affinity for PACAP and a low affinity for VIP. Overall however, it is clear that PACAP and VIP are likely to play significant functional roles in the development and regulation of the sympathetic nervous system.

Within the CNS, immunoreactivity for VIP and PACAP has been demonstrated in early developing rat brain and spinal cord (Fuji et al. 1985; Hill et al. 1994), consistent with a neuronal growth factor property for these peptides. Indeed, in DRG cell cultures, VIP and PACAP have been shown to be important regulators of sensory neurone survival. PACAP appears to have a protective role, protecting developing DRG neurones from death in dissociated cell cultures and stimulating neurite outgrowth (Lioudyno et al. 1998). In addition, PACAP has the ability to induce the expression of CGRP-ir in DRG explants (Lioudyno et al. 1998), highlighting a potentially important role for PACAP in the phenotypic plasticity which follows nerve injury. PACAP can also promote cell survival and neurite outgrowth in cultured immature cerebellar granule cells (Gonzalez et al. 1997; Vaudry et al. 1988), implicating PACAP as an important mediator within the brain as well as the spinal cord. The majority of the literature however, suggests that VIP and PACAP influence the development of the nervous system in an indirect manner, by causing the release of trophic factors such as cytokines from non-neuronal cells (particularly astrocytes) (Brenneman et al. 1987;1990; Pellegri et al. 1998; Tatsuno et al. 1996; Waschek, 1996). Thus their neurotrophic property may not be their main function within CNS neurones of the normal adult. Even so, this still highlights a significant role for VIP and PACAP which may be extremely important in the survival of neuronal cells and the regeneration of damaged nerve fibres following nerve injury.

1.6 Pharmacology of Neuropathic Pain

The development of neuropathic pain may rely upon many different factors, and often the aetiology is not known. Possible causes include trauma or disease-evoked damage, ischaemia, inflammation, crushing or constriction to peripheral nerves, posterior roots, spinal cord or to certain regions of the brain, or may be a secondary result of specific disease processes or systemic disorders such as diabetes, rheumatoid arthritis, alcohol or viral infections (Scadding, 1984). The pathological changes which then occur both centrally and peripherally, lead to painful neuropathic symptoms which are characterised by several different types of pain sensation:

- Spontaneous pain: this may be a continuous superficial burning pain and/or deep aching pain, or it may be episodic attacks.
- Hyperalgesia: an exaggerated response to a painful stimulus whether it be mechanical, chemical or thermal in nature.
- Allodynia: pain in response to a normally innocuous mechanical or thermal stimulus.

The duration of these abnormal pain states can range from months to years or even decades (Scadding, 1994), as the pathological conditions often persist long after healing of the damaged peripheral tissue or nerve.

Empirical findings have strongly implicated an important role for the sympathetic nervous system (SNS) in the development of some forms of neuropathic pain (see (Bennett, 1991) for review). This has led to the commonly used term sympathetically maintained pain (SMP), as opposed to sympathetically independent pain which appears to have no sympathetic involvement. Neuropathic patients very often display signs of abnormal sympathetic activity such as abnormal skin temperature and trophic skin changes (Wakisaka et al. 1991). In addition, chemical or surgical sympathectomies often relieve the painful symptoms which ensue peripheral nerve injury (Kim et al. 1993; 1997; Shir and Seltzer, 1991). There is also a wide range of evidence for changes in sympathetic nerves following peripheral nerve injury, and possible interactions between the sympathetic nervous system and the development of chronic pain (See (McMahon, 1991) for review). Possible contributing factors from the SNS include:

- the influence of adrenergic receptors on sensory neurones which may enhance ectopic discharge
- indirect coupling of sympathetic and sensory neurones through sympathetic fibre sprouting
- sensitisation of nociceptive afferents and dorsal horn neurones by mediators of the SNS (e.g. noradrenaline)

These factors will be considered in more detail in section 1.6.3.

Cold allodynia appears to be a striking feature in neuropathic pain in humans (Engkvist et al. 1985; Frost et al. 1988), particularly in patients with SMP. Very little is known about the mechanisms underlying cold pain. It appears that cold pain sensation in humans can have various qualities including cold aching, burning and prickling sensations, which would suggest the involvement of multiple classes of nociceptors (Chery-Croze, 1983; Davis, 1988). In humans, it has been demonstrated that normally innocuous cool stimuli evoke a sensation of burning pain during ischemic nerve block of conduction in A fibres (Aβ and Aδ) (Wahren et al. 1989; Yarnitsky and Ochoa, 1990). This implies that at least some heat-sensitive C nociceptors are excited by innocuous cold stimuli but that this activity is normally inhibited by simultaneous activation of cold-sensitive A fibres. It is therefore likely that at least one mechanism underlying cold hyperalgesia associated with large fibre neuropathy, is the loss of inhibitory modulation at the central level normally provided by cold-sensitive A-fibres (Ochoa and Yarnitsky, 1994). In normal rats it is the small diameter nociceptive afferents which transmit cold sensory information, and saphenous nerve recordings have revealed that the cold threshold for many nociceptors is below 12°C, with a large proportion of nociceptors not being excited until stimulus temperatures approach 0°C and below (Simone and Kajander, 1996; 1997). In behavioural tests of conscious animals which have undergone a chronic constriction injury, it appears that this threshold has been altered as the majority of animals now show aversive behavioural responses to temperatures above 0°C (Attal et al. 1990; Bennett and Xie, 1988), signifying a change in sensitivity.

The mechanisms underlying chronic neuropathic pain states are poorly understood and so treatment is often unsatisfactory. Advances in the treatment of painful neuropathies have been slow to evolve and are often limited in success.

Classical opioid analgesics are often ineffective (Arner and Meyerson, 1988; Lee et al. 1994; Mao et al. 1995), and anaesthetic nerve blocks or surgical sympathectomies often provide only temporary relief (Dougherty et al. 1992a; Loh and Nathan, 1978; Luo and Wiesenfeld-Hallin, 1995). However, more successful methods of treatment do exist, including the use of anticonvulsant drugs (Fields et al. 1997; McQuay et al. 1995; Tanelian and Brose, 1991) and the tricyclic antidepressants (McQuay et al. 1996).

There appear to be many contributing factors to the development and maintenance of neuropathic pain states and each individual case may be subtly different in character. The efficacy of analgesic agents therefore may be highly dependent on the particular cause of the neuropathy, making the development of potential treatments even more complicated.

A large number of the painful neuropathies which afflict humans are a result of trauma or disease that produces only a partial nerve injury. Much of the present knowledge of neuropathic pain has come from the development of animal models of nerve injury which mimic the clinical conditions seen in man. These models have provided the means with which to analyse some of the mechanisms behind the hyperalgesia and allodynia induced by nerve injury, and therefore have greatly improved the understanding of the neural mechanisms underlying neuropathic pain states.

1.6.1 Animal Models of Neuropathic Pain

Neuropathies induced by damage to parts of the central nervous system are particularly common in humans, and a number of useful animal models have been developed which involve experimental damage to certain parts of the brain and/or spinal cord. One of the first models of peripheral nerve injury to undergo extensive investigation, was that of complete nerve section or axotomy (Wall et al. 1979). Axotomy of the rat sciatic nerve results in marked plasticity within the CNS including biochemical, structural and functional changes (Devor, 1994). However, the major disadvantage of this model is that it is very difficult to correlate the physiological changes with the occurrence of pain, as parallel behavioural testing can not be performed as the whole limb is denervated as a result of the nerve

manipulation. The degree of autotomy (where the animals gnaw at their denervated hindlimb) has been used as an indicator of pain, with a scoring system to grade the severity of self-mutilation. However there is much debate as to whether or not autotomy is a true representation of painful sensation, or if it is simply due to the animal ceasing to recognise its own limb due to the complete denervation (Coderre et al. 1986; Rodin and Kruger, 1984). Several animal models now exist using a partial nerve injury, which allows some correlation of the central changes which occur following nerve injury, with the severity of the pain-related behaviour. A variety of tests can be performed on the affected, ipsilateral hindlimb which measure differences in the altered behaviour of these animals to mechanical, thermal or chemical stimuli. Three primary models exist so far, all of which involve sciatic nerve manipulation and produce abnormal pain states in the ipsilateral hindlimb:-

1. The chronic constriction injury (CCI) model

The chronic constriction injury (CCI) model of Bennett and Xie (1988) is one of the more commonly used models in scientific investigations. This model is produced by loosely tying 4 chromic cat gut ligatures around the common sciatic nerve, at mid thigh level. Within one week the animals show altered spontaneous behaviours consistent with the presence of neuropathic pain. The affected limb is demonstrably hyperalgesic, and mechanical and thermal allodynia (both hot and cold) are also present (Attal et al. 1990; Bennett and Xie, 1988). In addition, the animals tend to hold their affected hindlimb in a protective position, with the toes ventroflexed. This abnormal behaviour is thought to be indicative of the presence of spontaneous pain, and a scoring index for the severity of this abnormal posture in relation to spontaneous pain has been used (Attal et al. 1990). These behavioural changes manifest within 36 hours of the nerve constriction, are most pronounced 10-14 days after induction of the injury and can last for up to three months. An important feature of this model is that the ligatures do not completely sever the nerve and so many of the axons within the nerve are left in continuity. There appears to be a partial, selective involvement of the SNS in this model, as chemical or surgical sympathectomies only appear to alleviate the increased responses to thermal stimuli, while having no effect on the lowered thresholds to mechanical stimuli or the

behavioural abnormalities relating to spontaneous pain (Desmeules et al. 1995; Neil et al. 1991; Perrot et al. 1993).

2. Partial Nerve Ligation (PNL)

Partial nerve ligation (PNL) (Seltzer et al. 1990) involves a tight ligation of approximately one third to one half of the sciatic nerve with an 8-0 silk suture, at a site just distal to the point at which the posterior biceps-semitendinosus nerve branches off. Within a few days mechanical allodynia and hyperalgesia, as well as thermal (heat) hyperalgesia develop in the ipsilateral hindlimb, and these abnormal pain states can last for up to 7 months (Seltzer et al. 1990). There is no evidence of cold allodynia in these animals. However, the animals do display abnormal grooming of the affected hindlimb, including licking and biting, as well as holding the affected hindlimb in a protective manner, which is thought to be indicative of the presence of spontaneous pain. No signs of autotomy are apparent in PNL animals. A marked feature of PNL, is the development of bilateral 'mirror' effects (Seltzer et al. 1990). There also appears to be an important contribution from the SNS in the development of these pain related behaviours, as chemical or surgical sympathectomy has been shown to alleviate or prevent the development of neuropathic pain (Shir and Seltzer, 1991).

3. Spinal Nerve Ligation (SNL)

Spinal nerve ligation (SNL) involves the tight ligation of either the L5, or the L5 and L6 spinal nerves contributing to the common sciatic nerve just distal to the DRG (Kim and Chung, 1992), thereby transecting all axons within the nerve. These animals develop abnormal grooming behaviours including licking and biting, which may be representative of spontaneous pain (Na et al. 1996). There is however, no sign of autotomy. The rats display a quick onset (within the first 1-2 days following surgery) of behavioural signs of mechanical and thermal (heat) allodynia, but no cold hypersensitivity in the ipsilateral hindlimb. These abnormal pain states can last for up to 5 months (Kim and Chung, 1992). The development of these abnormal pain behaviours is dependent on innervation by the sympathetic nervous system as surgical lumbar sympathectomy almost completely abolishes them (Choi et al. 1994;

Kim and Chung, 1991; Kim et al. 1993). This marked sympathetic involvement makes the SNL model a useful model for investigating the contribution of the sympathetic nervous system in the development of neuropathic pain. The main benefit of using this model is the fact that the surgery is standardised, with the same group of fibres being affected every time. However, the surgical procedure is considerably more extensive than that of the other two models.

Other Animal Models:-

A number of other neuropathic animal models exist but appear to be less extensively studied. Diabetic/neuropathic rodents are easily obtained and a number of different models exist (Watkins, 1990):

1. An inbred BB/Wistar strain of albino rats become spontaneously diabetic, and show behavioural signs of hyperalgesia and allodynia (Wuarin-Bierman et al. 1987).
2. Sand rats develop diabetes when fed ordinary rat lab chow, and again develop hypersensitivity to mechanical and thermal stimuli (Wuarin-Bierman et al. 1987).
3. Animals with experimentally-induced diabetes are produced following injection of B cell toxins, e.g. alloxan or streptozocin (Courteix et al. 1993), resulting in the development of mechanical and thermal hyperalgesia/allodynia.

However, the control of diabetes in these animals may also influence the development of neuropathic pain states directly or indirectly, making interpretation of experimental results more complicated. There are also animal models of peripheral nerve injury due to trauma, disease, metabolic disorders and toxins (Scadding, 1994), but none of these models appear to produce disorders of pain sensation like those which accompany the peripheral neuropathies in humans.

The Bennett and Xie model was chosen for use in the current investigations because it has been extensively studied and so allows the integration of the present results with previous findings. In addition, this model is relatively safe and simple to set up, with a low incidence of autotomy, wound infection or morbidity, and the affected hindlimb is easily accessible for behavioural studies.

1.6.2 Plasticity Following Peripheral Nerve Injury

1.6.2.1 Aetiology of Nerve Injury

Chronic constriction of the common rat sciatic nerve results in the development of a number of abnormal pain symptoms within a few days following nerve injury, including mechanical and thermal hyperalgesia and allodynia, as well as behavioural signs of spontaneous pain (Attal et al. 1990; Bennett and Xie, 1988). Several aspects of the nerve injury may contribute to the development of these abnormal pain symptoms.

The inflammatory response appears to play a major role in the development of neuropathic pain states, as daily injections of the anti-inflammatory agent dexamethasone, decreased the inflammatory response induced by chromic gut sutures, and subsequently blocked the development of guarding behaviour and thermal hyperalgesia in rats (Clatworthy et al. 1995). In contrast, application of cotton sutures soaked in Complete Freund's Adjuvant (CFA) resulted in augmentation of these abnormal pain behaviours (Clatworthy et al. 1995).

Changes in local blood flow through the injured nerve may also contribute to the sensory changes, as there is some evidence of decreased blood flow at the nerve injury site at a time when thermal hyperalgesia is evident (Myers et al. 1988).

Finally the type of suture material also appears to be important. Kajander et al. (1996) used chromic cat gut, plain gut or polyglactin sutures as ligatures in the chronic constriction injury model of neuropathy. They found that the subsequent abnormal paw position varied between the different test groups. Animals with chromic gut ligatures spent more time with their affected hind paw in abnormal positions than the other groups, and although the animals with plain gut or polyglactin ligatures spent more time with their hindlimb in abnormal positions relative to control animals, the affects on paw position were much greater in rats with chromic gut ligatures (Kajander et al. 1996). In addition, the use of chromic gut ligatures results in marked decreases in the levels of SP- and CGRP-ir in the spinal cord, while polygalactin or plain gut sutures appear to have no such effect on the expression of these peptides (Xu et al. 1996). As all types of suture appear to induce abnormal behavioural responses, the physical constriction of the nerve is likely to play an important part in their development. However, these results also suggest that

the chemical constituents of the chromic gut sutures may also have an effect, possibly on neuronal function and thereby influencing neuroma development and the inflammatory response. Indeed, Maves et al. (1995) have reported that providing an acidic environment around the rat sciatic nerve for 7 days will result in the progressive development of thermal hyperalgesia in these animals.

1.6.2.2 Morphological Changes Following CCI

The rat sciatic nerve originates from spinal segments L4-L6 and contains fibres of sensory, motor and sympathetic origin. At the mid-thigh level, it comprises of approximately 27 000 axons of which 6% are motor axons, 23% are myelinated sensory axons, 48% are unmyelinated sensory axons while 23% represent unmyelinated sympathetic axons (Schmalbruch, 1986).

There are marked morphological changes in the fibre composition of the sciatic nerve following CCI, and these changes have been well documented, allowing speculation as to the fibre type(s) involved in the development of the abnormal pain states which accompany peripheral nerve damage.

There are two main stages involved in the pathological changes of the sciatic nerve following CCI (Coggeshall et al.1993). The first is an early degenerative stage which is thought to be a direct result of the inflammation and swelling which occur at the site of nerve injury following application of the chromic cat gut ligatures, and appears to induce a slow strangulation of the axons underneath. This swelling can last up to 28 days post operatively (PO) and is maximal at day 3 PO, by which time a mass of connective tissue and a neuroma are often apparent (Coggeshall et al.1993). The sciatic nerve undergoes marked anatomical changes, distal to the site of nerve injury, at this time. From day 3 to two weeks after nerve ligation, there is a steady and extensive decrease in axon numbers of all types. The most prominent change is a profound loss of large myelinated axons, namely the Aβ fibres, distal to the lesion while the smaller myelinated and unmyelinated fibres appear to be less affected. At the electron microscope level, the few remaining large diameter Aβ fibres were seen to be in an advanced state of degeneration (Basbaum et al. 1991). This near complete loss of large myelinated fibres distal to the ligature, could result in the loss of central inhibitory controls which are normally exerted by the large diameter primary

afferents (see Section 1.3.1). However, the development of the chronic pain state can not be solely related to the loss of large diameter afferents, as all fibre types are affected to some degree. There is a significant decrease in the number of small myelinated Aδ fibres, although to a lesser extent than that of the Aβ fibres, in addition to the disruption and damage of a number of small unmyelinated fibres (i.e. the C-fibres). The effect of nerve ligation on the smaller myelinated fibres varied and was greatly affected by which fascicle was studied. It is possible that the whole population of Aδ fibres may not have been counted as some of these fibres may have been mistaken for unmyelinated C-fibres if they were seen at different stages of degeneration/regeneration and therefore had thin myelin sheaths. 1cm or more proximal to the nerve injury, the morphology of the nerve appeared normal (Basbaum et al. 1991; Coggeshall et al. 1993; Gautron et al. 1990), with no sign of degenerating fibres, which suggests that most of the damaged fibres had survived centrally.

The second stage of pathological change is the regenerative phase. This generally occurs from approximately day 28 PO onwards, when the sutures have been reabsorbed and the neuroma and swelling have subsided (Coggeshall et al. 1993). This coincides with fibre regeneration and the recovery of axonal numbers, and probably accounts for the eventual recovery of normal sensation within the affected limb (Guilbaud et al. 1993).

The time-course of these morphological changes within the injured nerve has been studied in parallel with the behavioural changes which develop. The associated behavioural studies have revealed that the onset of hyperalgesia is generally maximal at days 10-14 PO (Attal et al. 1990; Bennett and Xie, 1988), the time point which correlates with the initial fibre loss and predominant loss of large myelinated fibres (Basbaum et al. 1991; Coggeshall et al. 1993; Gautron et al. 1990). Guilbaud's group found that behavioural signs of hot and cold allodynia/hyperalgesia and mechanical hypersensitivity in the CCI model were maximal at week 2 PO, with a progressive recovery from week 3-4 PO onwards (Guilbaud et al. 1993). At week 2 there is a massive Wallerian degeneration of large myelinated fibres, with regeneration occurring from week 3 PO onwards (Ramer et al. 1997), which coincides with these behavioural changes. However the largest fibres still had not

completely recovered by week 15 PO and this contrasts with the disappearance of abnormal nociceptive behaviours and subsequent recovery from neuropathic pain states seen at approximately weeks 8-10 PO (Guilbaud et al.1993). So the damage to nerve fibres does appear to be of some importance, as the development of abnormal pain states appears to be linked to the presence of degenerating or regenerating A-fibres, however, there does not appear to be a strong correlation between the time course of morphological changes overall and neuropathic syndromes. It is likely that these morphological changes are an important link in the development and maintenance of neuropathic pain but that other additional factors are also involved.

1.6.2.3 Neuropeptide Plasticity

Investigations in animals with peripheral nerve injury have revealed a number of different neurochemical changes within the spinal cord and DRG that may be of important physiological significance (see figure 1.7). Following peripheral axotomy long-lasting changes in the production and expression of neuropeptides and their receptors are seen in primary afferent sensory neurones (Hokfelt et al. 1994). In addition, changes in the peripheral and central transport of these substances results in marked plasticity within the spinal cord as well. The main changes observed following complete nerve transection or partial nerve constriction include a down-regulation of the excitatory peptides SP and CGRP, as well as decreased SOM, while levels of VIP, PACAP, NPY, galanin and CCK are all seen to increase.

Substance P (SP): SP is found in approximately 30% of small to medium diameter DRG neurones normally (Ju et al. 1987; Smith et al. 1993). Following axotomy there is a significant decrease in the expression of SP mRNA in these DRG, which is most pronounced at day 10-14 PO and is accompanied by a parallel decrease in SP levels in the spinal dorsal horn (Barbut, 1981; Jessel et al. 1979; Noguchi et al. 1993; Shehab and Atkinson, 1986b). A similar marked decrease in SP production by primary afferent neurones is seen following CCI (Cameron et al. 1991;1997; Nahin et al.1994), with a significant decrease in the density of SP-ir fibres in the ipsilateral spinal cord (Kajander and Xu, 1995), but the reductions do not appear to be as great as those seen following axotomy. This is most likely due to the fact that CCI only produces a partial denervation and so fewer small diameter

primary afferents will be injured than in axotomy. This major decrease may reflect a lesser role for SP in nociceptive processing in neuropathy.

CGRP: CGRP is one of the most abundant neuropeptides in the DRG normally, where it is seen to co-exist with SP, SOM and galanin (Ju et al. 1987; Smith et al. 1993; Villar et al. 1989). Axotomy results in a marked decrease in the expression of CGRP mRNA in primary sensory neurones (Noguchi et al. 1989; 1990; 1993; Shehab and Atkinson 1986b), which is maximal between days 7-14 PO While decreased levels of CGRP-ir in the superficial dorsal horn are usually apparent by day 10 PO (Kajander and Xu, 1995; Shehab, and Atkinson, 1986b; Zhang et al. 1995b). A similar change has also been observed following chronic constriction of the sciatic nerve with approximately 50% reductions in the levels of CGRP mRNA in the DRG reported within 7-14 days following nerve injury (Nahin et al. 1994). However, subsequent decreases in the immunohistochemical staining for this peptide in the spinal dorsal horn have not been observed until approximately 60 days after the chronic constriction injury (Kajander and Xu, 1995).

Somatostatin (SOM): SOM is normally present in approximately 20% of small to medium diameter DRG (Ju et al. 1987; Smith et al. 1993). Nerve transection results in a marked decrease in the production of SOM by primary afferent neurones in rat and monkey DRG, with a corresponding decrease in SOM-ir in the superficial dorsal horn (Shehab and Atkinson, 1986b; Villar et al. 1989; Zhang et al. 1993b).

VIP: VIP-ir is present at relatively low levels in the superficial dorsal horn (Knyihar-Csillik et al. 1993) and primary afferent neurones (Fuji et al. 1985) of normal animals. Following axotomy there is a significant increase of VIP expression in small to medium diameter DRG neurones, where it is often co-localised with galanin (Nahin et al. 1994; Zhang et al. 1995a). There is also a corresponding increase in VIP-ir in the superficial dorsal horn (Knyihar-Csillik et al. 1993; Shehab and Atkinson, 1986a; 1986b). In double labelling studies using the retrograde transport of True Blue with VIP immunohistochemistry, it was revealed that VIP expression only occurs in cells which have had their axons cut in the peripheral part of the sciatic nerve (Shehab et al. 1986). This suggests that sensory neurones may express VIP as a direct response to axonal injury. Indeed VIP-ir has been shown to increase in the dorsal horn following nerve crush, but decreases to control levels as

soon as fibre regeneration has occurred (Knyihar-Csillik et al. 1991; 1993). VIP predominantly has an excitatory role (see section 1.5.4), and so it is possible that this peptide takes over the role of SP as a primary neurotransmitter within the CNS of neuropathic animals. In addition, it may also be an important neurotrophic factor and so may play a role in the regenerative processes which follow nerve injury.

PACAP: Abundant PACAP-ir fibres have been demonstrated in the normal superficial dorsal horn (Mulder et al. 1994; Zhang Q et al. 1995), and approximately 10% of DRG neurones express this peptide. Axotomy results in a rapid and prominent increase in PACAP mRNA in approximately 75% of DRG cells from as early as 15 hours PO, with a maximum increase at day 3 PO which begins to decrease by day 10 PO (Zhang Q et al. 1995). PACAP levels appear to return to control values by week 4 PO. PACAP is seen to co-exist with VIP, NPY and GAL to varying degrees (Zhang Q et al. 1995), but its time course of expression is considerably different to that of these other neuropeptides. The function of PACAP within the CNS is not altogether clear as it may have inhibitory or excitatory properties (see section 1.5.4), but the rapid onset of PACAP expression suggests that it may be important in the early phases of adaptation to nerve injury.

CCK: CCK is present in relatively low amounts in DRG normally (Fuji et al. 1985; Ju et al. 1987). Intrathecal CCK has been shown to have an excitatory role in flexor reflex studies (Weisenfeld-Hallin and Duranti, 1987). Following peripheral nerve injury the expression of CCK mRNA markedly increases in primary afferent neurones (Villar et al. 1989). The role of CCK in neuropathy is not clear (see (Wiesenfeld-Hallin and Xu, 1996a) for review) but it appears to decrease the analgesic effect of morphine and β-endorphin (Faris et al. 1983). In addition, antagonists of the CCK-B receptor (which is the prevalent form of CCK receptor within the rat spinal cord) potentiate opioid analgesics and prevent the development of morphine tolerance (Baber et al. 1989; Dourish et al.1990; Weisenfeld-Hallin et al. 1990d). This interactive role of CCK, and the fact that its levels are dramatically up-regulated following nerve injury may therefore help to explain the decreased efficacy of opioid treatment commonly observed in neuropathic patients.

Galanin: Galanin is a C-terminally amidated 29 amino acid peptide, the binding sites of which show dense labelling in laminae I-III and moderate labelling

in laminae IV-V of the rat dorsal horn (Kar and Quirion, 1994;1995). Galanin receptors are also widely distributed throughout the CNS, particularly in the superficial dorsal horn (Weisenfeld-Hallin et al. 1992; Zhang et al. 1995c). It is normally present in approximately 23% of small to medium diameter DRG neurones, where it co-exists with several other neurotransmitters including CGRP and SP (Ju et al. 1987; Zhang et al. 1993a; 1995c). Galanin levels have been shown to be decreased, at least in part, by neonatal capsaicin treatment, particularly in laminae I-II of the spinal dorsal horn (Skofitsch and Jacobowitz, 1985), and so galanin may be an important mediator of primary afferent C-fibres. Peripheral nerve injury leads to an increase in galanin mRNA expression within DRG cells within 24 hours (mainly in small to medium diameter neurones, but also in a few large cells), as well as in the ipsilateral dorsal horn (Ma and Bisby, 1997; Nahin et al. 1994; Romualdi et al. 1990; Zhang et al. 1995b). The pattern of co-expression of galanin and the other neuropeptides also changes following nerve injury with less CGRP/GAL co-expression and an increase in the co-existence of galanin with NPY and VIP (Nahin et al. 1994). The role of galanin within the CNS is not very clear as functional studies have provided variable results depending on the preparation and dose used, and the effect studied. In rats with an intact nerve, intrathecal galanin produces brief facilitatory effects at low doses, which become inhibitory as the dose increases, such that at very high doses galanin exerts a purely inhibitory effect (Weisenfeld-Hallin et al. 1988;89). In addition, in both electrophysiological and behavioural studies, galanin was seen to inhibit the analgesic effect of morphine on noxious thermal and mechanical stimuli (Weisenfeld-Hallin et al, 1990c), while having no effect alone on these nociceptive inputs. Galanin may also be an important modulator of excitatory neuropeptide action, as pre-administration of galanin intrathecally has been shown to antagonise the excitatory effects of SP and CGRP on the flexor reflex (Weisenfeld-Hallin et al.1990a; Xu et al. 1989; 1990). The inhibitory role of galanin is much more pronounced following nerve injury however, as the facilitatory effect of the galanin antagonist M-35 on CS-induced reflex facilitation is much more pronounced following sciatic nerve section (Wiesenfeld-Hallin and Xu, 1996b), with the magnitude of reflex depression being significantly greater, and with a more rapid onset than the effect seen in animals with an intact nerve. In addition, the VIP-

induced flexor reflex could be inhibited by galanin antagonists following axotomy (Xu et al. 1990), which corresponds with the increased expression of VIP- and GAL-ir in DRG following nerve injury, where these peptides are seen to co-exist in many cells. This suggests that galanin's functional role becomes more important following nerve injury, and that this peptide may play an important regulatory role trying to counteract the effects of excitatory neurotransmitters within the CNS.

Neuropeptide Y (NPY): NPY is a 36 amino acid peptide with a large number of different NPY receptor subtypes, suggesting a diverse range of functional effects for this peptide within the body. NPY is one of the most abundant peptides in the mammalian peripheral and central nervous system (Allen et al. 1983; 1984). Within the CNS, NPY-ir fibres show dense labelling in the substantia gelatinosa, dorsolateral funiculus and the dorsal grey commisure in lumbosacral spinal cord (DeQuidt and Emson, 1986). This peptide is normally found predominantly within intrinsic neurones of the spinal dorsal horn (Gibson et al. 1984), where it may co-exist with galanin (Zhang X et al .1993b; 1995c) or even GABA (Laing et al. 1994; Rowan et al. 1993), and is virtually undetectable in normal DRG neurones. High levels of NPY are also found within sympathetic ganglia and tissue, with dense sympathetic innervation in guinea-pig, cat, pig and man (Lundberg et al. 1983), suggesting an important role for NPY in the regulation of the SNS. Following nerve injury there is a dramatic up-regulation of the levels of NPY in axons and varicosities of laminae III-V of the dorsal horn, as well as the induction of NPY-ir in many large or medium diameter primary afferent neurones (Kar and Quirion, 1992; Nahin et al. 1994; Zhang et al. 1995a). NPY is thought to play an inhibitory role predominantly within the CNS, as systemic administration of this peptide shows marked antinociceptive effects (Hua et al.1991). However, intrathecal NPY has been shown to have a dose-dependent biphasic effect on the spinal nociceptive flexor reflex, with brief facilitation of the flexor reflex at low dose (10/100ng) but intense and prolonged depression of the spinal mediated reflex at higher doses (1/10μg) (Xu et al. 1994).

Figure 1.8 represents a schematic illustration of the changes in the principal primary afferent neurotransmitters following peripheral nerve injury.

1.6.2.4 Factors Affecting Neuropeptide Plasticity Following Nerve Injury

Several different mechanisms may be involved in the marked changes in DRG gene expression following nerve injury. Prime candidates in this regulation are the neurotrophins. Peripheral nerve injury has also been shown to produce marked changes in the expression of neurotrophins and their receptors in DRG neurones, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and leukaemia inhibitory factor (LIF) (Cameron et al. 1997; 1991; Nahin et al.1994; Sebert and Shooter, 1993). Such factors may influence DRG cells and may be released from central processes to exert an effect in the CNS, or may be transported down peripheral axons to the site of injury, where they may regulate the activity of various non-neuronal cells.

Certainly in the case of VIP, there is strong evidence that the up-regulation of VIP expression is mediated by factor(s) carried by retrograde axoplasmic transport to DRG cells, as axotomy or nerve crush produce increased VIP levels which return to control values after nerve regeneration (Knyihar-Csillik et al. 1991; 1993).

The expression of SP by DRG neurones in vivo appears to depend strongly on the availability of NGF (Lindsay and Harmar, 1989; Otten et al. 1980; Verge et al. 1995). Intrathecal NGF has been shown to prevent the axotomy-induced decrease in levels of CGRP and SP (Fitzgerald et al. 1985) while application of anti-NGF to the cut end of the nerve encourages the change in SP/CGRP levels (Goedert et al. 1984; Schwartz et al. 1982). So it is possible that nerve injury interrupts the supply of retrogradely transported NGF, and this may directly or indirectly contribute to the observed decreases in SP and CGRP. The levels of expression of VIP however, appear to be under some other form of regulatory role, as changes in the density of VIP-ir in adult DRG neurones in culture appears to be independent of the presence or absence of NGF (Mulderry and Lindsay, 1990), contrasting with the very marked dependency on NGF observed for SP/CGRP.

Figure 1.7

Schematic of the Principal Changes within the CNS Following Peripheral Nerve Injury

Schematic drawing of a small and large primary sensory neurone in a dorsal root ganglion (DRG) sending a central branch to the dorsal horn of the spinal cord and with a peripheral branch which has been sectioned (axotomy). The main changes which occur within the central nervous system are highlighted in this schematic. The changes in levels of peptides and their receptors are indicated by arrows. Thus in small diameter DRG neurones the level of substance P (SP), calcitonin gene-related peptide (CGRP) and somatostatin (SOM) are decreased, along with neuropeptide Y (NPY) receptor mRNA. In contrast levels of vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP) and galanin (GAL) are markedly increased, with modest increase in cholecystokinin (CCK) and NPY. In the large diameter DRG there is a prominent increase in the levels of NPY and the NPY receptor, along with smaller increases in VIP and GAL, while the expression of CGRP is seen to decrease.
Structural and neurochemical reorganisation within the spinal cord and the damaged nerve, as indicated, ultimately contribute to the development of spontaneous ectopic firing in Afibres and an increased hyperexcitability of C fibres, as well as providing the potential for the development of abnormal Aβ fibre responses, including a loss of inhibitory properties.

(Adapted from Hokfelt et al. 1994)

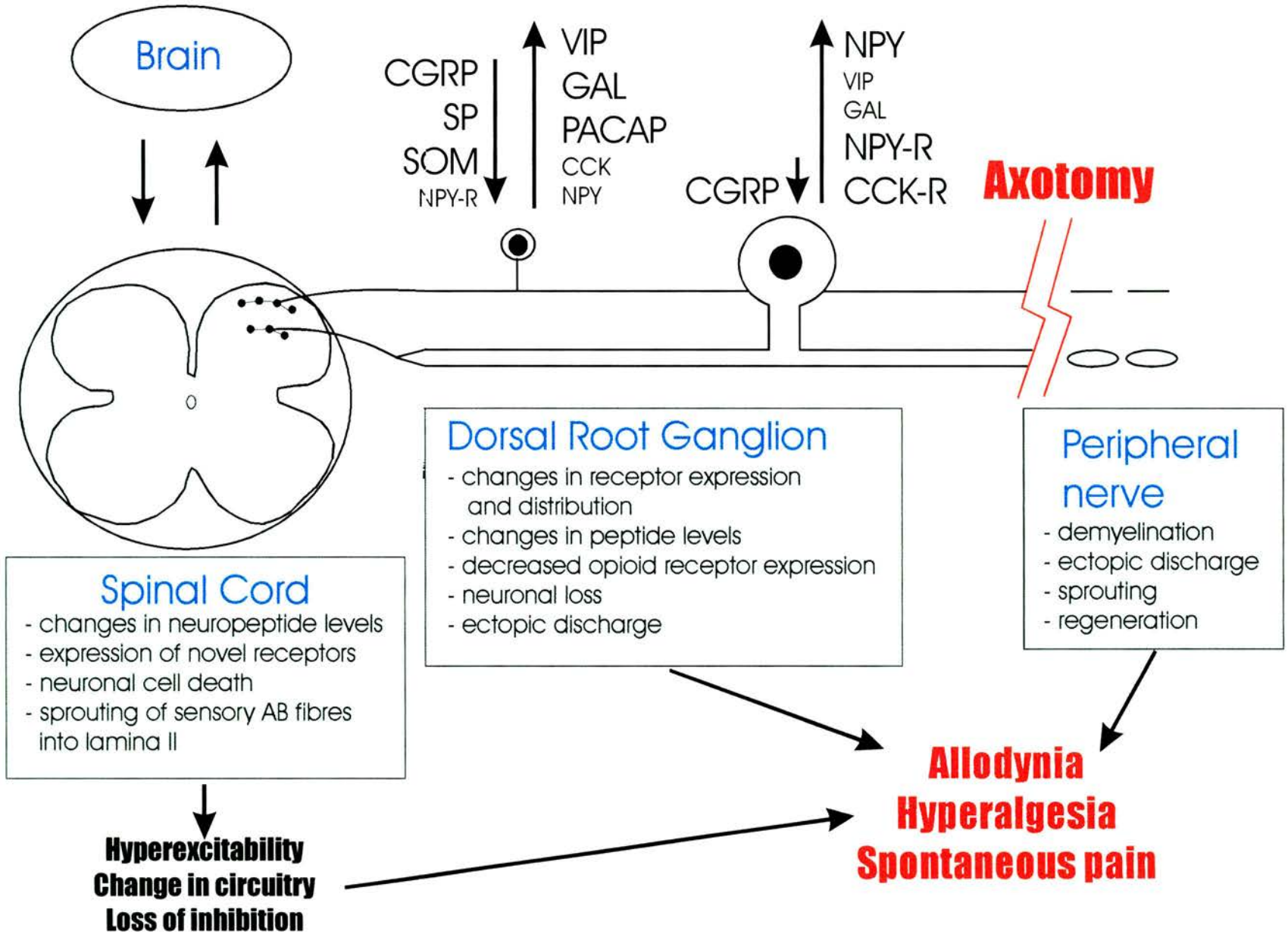

Brain
CGRP
SP
SOM
NPY-R
VIP
GAL
PACAP
CCK
NPY
CGRP
NPY
VIP
GAL
NPY-R
CCK-R
Axotomy
Dorsal Root Ganglion
- changes in receptor expression and distribution
- changes in peptide levels
- decreased opioid receptor expression
- neuronal loss
- ectopic discharge
Peripheral nerve
- demyelination
- ectopic discharge
- sprouting
- regeneration
Spinal Cord
- changes in neuropeptide levels
- expression of novel receptors
- neuronal cell death
- sprouting of sensory AB fibres into lamina II
Hyperexcitability
Change in circuitry
Loss of inhibition
Allodynia
Hyperalgesia
Spontaneous pain

Figure 1.8

Schematic Model of the Principal Primary Afferent Neurotransmitters

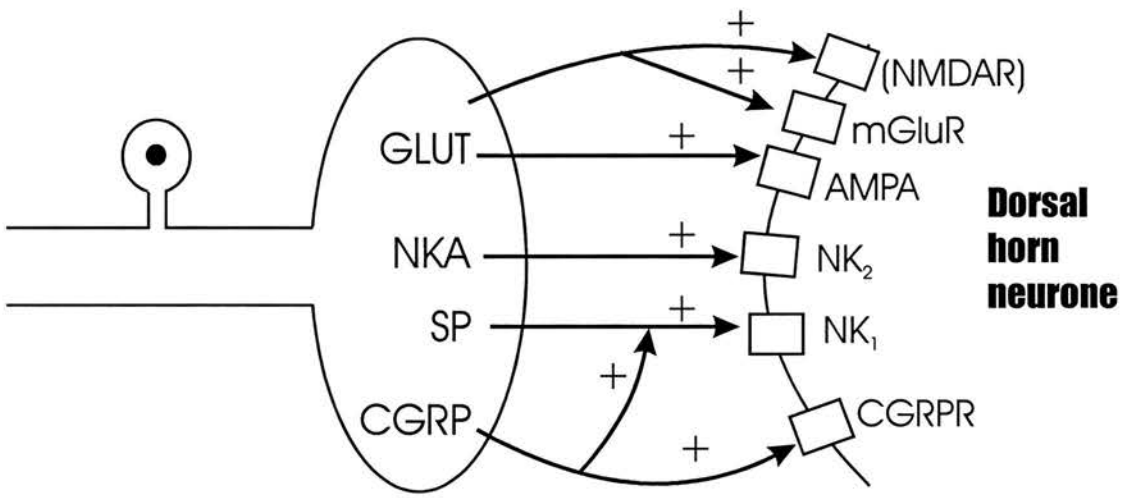
A schematic illustration of the major neurotransmitters/modulators released from intact and axotomised primary afferents.

Abbreviations:

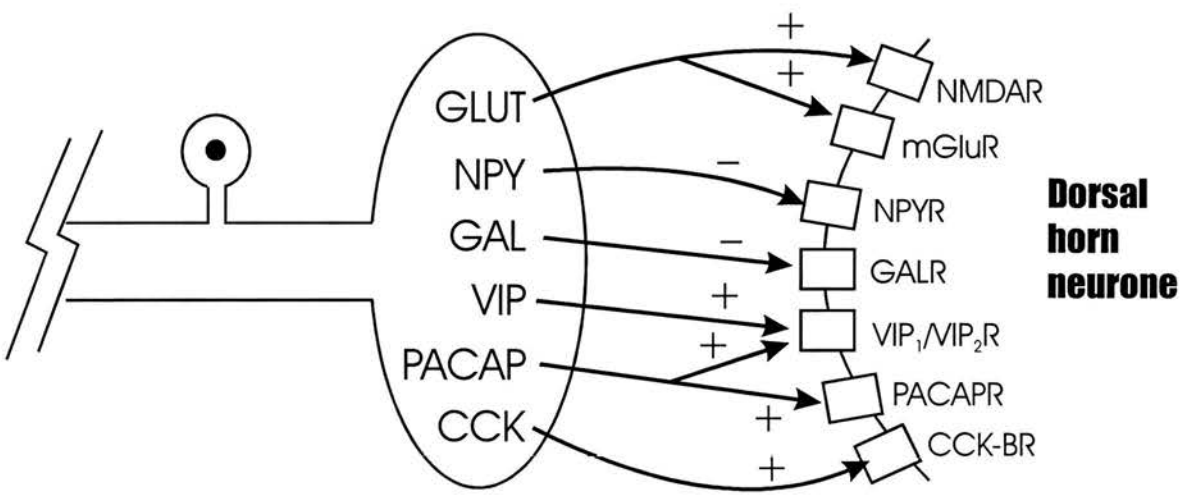
α-amino-3-hydroxy-5-isoxazole-4-propionate (AMPA); calcitonin gene-related peptide (CGRP); cholecystokinin (CCK); Galanin (GAL); glutamate (GLUT); metabotropic glutamate receptor (mGluR); neurokinin (NK); neurokinin A (NKA); N-methyl-D-aspartate (NMDA); neuropeptide Y (NPY); somatostatin (SOM); substance P (SP); pituitary adenylate cyclase activating polypeptide (PACAP);receptor (R); vasoactive intestinal polypeptide (VIP)

+ = excitatory; - = inhibitory

Intact Afferent



Axotomised Afferent



In contrast, the axotomy-induced increases in VIP and galanin appear to be mediated at least in part by LIF, particularly within the SNS, as mice in which the LIF gene has been disrupted by gene targeting show a marked impairment in the upregulation of VIP and galanin in the SCG and sensory neurones following axotomy (Sun et al. 1994b; Sun and Zigmond, 1996a; 1996b; Zigmond and Sun, 1997). Whether or not PACAP synthesis and receptor expression is also LIF-regulated remains to be determined.

Other factors which may play a role either directly or indirectly in nerve injury-induced neuropeptide plasticity include growth-associated protein 43 (GAP-43) and choline acetyl transferase (ChAT) which also appear to be up-regulated in the dorsal horn following nerve injury (Cameron et al. 1997). In addition ciliary neurotrophic factor (CNTF), which has been isolated from rat sciatic nerve extract (Manthorpe et al. 1986) has been shown to induce SP, and inhibit NPY synthesis in sympathetic ganglia cultures (Rao et al. 1992), and so may be another important regulatory factor in the alteration of DRG gene expression associated with nerve injury. It is clear however, that there are different controls for the expression of the different neuropeptides and it may be a combination of a variety of effects which finally determine the overall expression of primary afferent neuroactive substances following nerve injury.

1.6.3 Possible Factors Contributing to the Development of Neuropathic Pain

1.6.3.1 Sympathetic Nervous System (SNS)

There is a wide variety of evidence to suggest a strong link between the SNS and the pain related behaviours of neuropathy. Surgical or chemical sympathectomies (with guanethidine for instance) have been shown to significantly decrease the abnormal pain behaviours which develop following a number of different animal models of neuropathy (Kim et al. 1993; 1997; Kim and Chung, 1991; Shir and Seltzer, 1991), implying that the SNS may play an important role in the development of neuropathic pain states. The involvement of the SNS does appear to be quite variable however, and factors which appear to have an influence on this involvement include the site of nerve injury and distance from the spinal cord, and the type of injury (Kim et al. 1997). The variable degree of sympathetic

involvement in neuropathic pain may explain the variable effects of sympathetic manipulation in the treatment of neuropathic pain states (Loh and Nathan, 1978; Luo and Wiesenfeld-Hallin, 1995). Peripheral nerve injury has been shown to result in sprouting of sympathetic efferents into DRG neurones, from as early as day 4 PO in the CCI model, coinciding with the time when neuropathic pain behaviours are developing (Mclauchlan et al. 1993; Ramer and Bisby, 1997). Electrical stimulation of these sprouts subsequently evoked firing of myelinated primary afferent neurones, which was blocked following phentolamine treatment (Ramer and Bisby, 1997), and so these sympathetic efferent sprouts appear to represent a functional coupling of noradrenergic neurones with primary afferent fibres in the DRG. It is therefore possible that alterations in sympathetic innervation in DRG following nerve injury, may be involved in the generation of spontaneous activity in the injured nerve (see following section).

1.6.3.2 Changes in Primary Afferent Responsiveness Following Nerve Injury

It is possible that the spontaneous activity of injured primary afferents may greatly contribute to the induction and maintenance of the allodynic and hyperalgesic states which follow nerve injury, as well as contributing to the abnormal behaviours thought to be indicative of spontaneous pain.

Electrophysiological studies have revealed that following peripheral nerve injury there is an abnormally high level of spontaneous activity within primary sensory neurones (Laird and Bennett, 1993; Palecek et al. 1992). This abnormal activity appears to be principally mediated by Aβ and Aδ fibres (Kajander and Bennett, 1992; Kajander et al. 1992) which develop abnormal spontaneous activity from as early as day 1 and 3 PO. This spontaneous activity continues for at least several weeks post injury. Spontaneous discharges from C-fibres begin to contribute slightly later than those of A-fibres, from approximately day 10 PO onwards (Xie and Xiao, 1990), which corresponds with the maximal behavioural changes observed in the CCI model (Attal et al. 1990; Bennett and Xie, 1988).

The site of initiation of this abnormal activity is under debate. Electrophysiological recordings from primary afferents within the sciatic nerve have suggested that the spontaneous activity originates at the site of nerve injury, as the

majority of axons did not respond to electrical stimulation distal to the lesion. Xie and Xiao (1990) also report ectopic spontaneous discharge at the site of the constriction injury between days 6-44 PO. However other groups (Kajander et al. 1992; Zhang JM et al. 1997) believe that this ectopic discharge originates from the DRG, as transection distal to the DRG failed to halt the spontaneous activity while transection proximal to the DRG resulted in the abolition of this abnormal discharge. It seems likely that both the injury site and the DRG contribute to afferent ectopic firing (Tal and Eliav, 1996), and this subsequent hyperexcitability of neuronal elements involved in the processing of sensory information, may therefore contribute significantly to the production of spontaneous pain manifestations and hyperalgesia by triggering and maintaining central sensitisation.

As stated earlier, it appears that the SNS may play a major role in maintaining the ectopic firing of primary afferents through inappropriate functional connections following the sprouting of sympathetic efferents. McLauchlan et al. (1993) revealed that in axotomised animals, noradrenergic axons sprout into the DRG and greatly increase the number of basket-like endings around the neuronal cell body, thereby providing a coupling pathway for sympathetic-sensory fibres within the DRG. A similar, but slightly more rapid sprouting of sympathetic axons in DRG was also observed following CCI (Ramer and Bisby, 1997). Evidence for a direct coupling of these two systems has been shown using recording experiments where the ectopic discharge of injured axons could be altered by electrical stimulation of the sympathetic trunk (Devor and Janig, 1981; Devor et al. 1994; Habler et al. 1987; Korenman and Devor, 1981). Michaelis et al. (1996) found that the nerve injury-triggered sympathetic coupling within rat DRG changed with time, and that the excitatory coupling observed within the first few weeks of nerve injury, was subsequently replaced by an inhibitory coupling by day 60 PO. This would suggest then that the SNS may play an important role in the induction and maintenance of neuropathic pain states, but that this role may change following the regenerative changes which ultimately lead to the recovery of normal sensation

1.6.3.3 Degenerative Changes within the CNS Following Nerve Injury

Alterations in inhibitory influences

Chronic constriction of the rat sciatic nerve can result in a number of degenerative changes. As discussed previously, there are marked morphological changes within the sciatic nerve fibres, particularly in the Aβ range. But there is also some evidence that lesions of peripheral nerves can induce a series of complex changes centrally. Degeneration affecting the central terminals of primary sensory neurones may be initiated at different times and in different neurones of the affected population following nerve injury (Aldskogius et al. 1985). CCI has been shown to cause transsynaptic degeneration of spinal dorsal horn neurones in laminae I-III (Sugimoto et al. 1990) from as early as day 8 PO. This may be an excitotoxic effect due to excessive levels of postsynaptic depolarisation as a result of primary afferent ectopic discharge, or may be due to a disruption in the communication between the cell body and the central nervous system following axonal degeneration. This transsynaptic degeneration may have a direct influence on local and descending systems within the spinal cord, resulting in altered processing of sensory information.

There is currently conflicting evidence on the effects of CCI on the levels of inhibitory transmitters within the spinal cord. Immunohistochemical studies have revealed an almost complete loss of GABA-containing cells in laminae I-III of the ipsilateral dorsal horn following nerve injury (Ibuki et al, 1997), while radioimmunoassays have shown an increase in the levels of glycine and GABA within the spinal cord for up to 30 days PO (Satoh and Omote, 1996). In addition, this group also demonstrated a significant decrease in the paw withdrawal latency to thermal stimuli in CCI animals following intrathecal application of the GABA and glycine antagonists strychnine and bicuculline on day 7 PO (Satoh and Omote, 1996). This suggests that glycine and GABA still have the ability to exert intrinsic inhibitory modulatory effects in these animals. However, these tests were not repeated at week 2 PO, when the abnormal pain behaviours and morphological changes within the injured nerve are at their maximum, and so it is possible that these inhibitory effects of GABA and glycine may become altered as the neuropathic pain states develop. Subsequent alterations in the number of inhibitory neurones, the effects of ascending and descending systems to and from the brain, and changes in

the pattern of neurotransmitter co-existence, may all lead to impaired inhibitory function and central hyperexcitabilty following peripheral nerve injury.

Changes in Aβ fibre properties

There is strong evidence to implicate a role for Aβ fibres in the mediation of abnormal allodynic states. Selective blockade of Aβ fibres (but not of Aδ and C fibres) within the peripheral nerve of neuropathic patients, using local anaesthetic or ischemic nerve blocks, has been shown to markedly inhibit mechanical allodynia (Campbell et al. 1988; Gracely et al. 1992). This altered role of Aβ fibres is thought to perhaps be a result of altered central processing, such that the activation of the large myelinated afferent fibres now leads to painful sensations. Such central changes may be a result of the degeneration of large myelinated axons following nerve injury, which could then result in the loss of inhibitory control of these fibres at the spinal cord level. Indeed, electrophysiological studies have revealed that electrically stimulated Aβ fibres show a powerful inhibition of A- and C-fibre-evoked activity of dorsal horn neurones in normal animals. However in rats with a transected nerve the activation of Aβ afferents showed no inhibitory effects or weak, shorter effects on A- or C-fibre-evoked neuronal activity (Woolf and Wall, 1982). The degenerating fibres may even change properties following nerve injury. Na et al. (1993) detected an unusual type of mechanoreceptor in nerve-injured rats, which had characteristics similar to those of the rapidly adapting (RA) mechanoreceptor, but which produced an abnormal irregular discharge following activation. They named these receptors modified rapidly adapting mechanoreceptors, and suggested that they are abnormal RA mechanoreceptors, produced in response to nerve injury, which may play a contributing factor in the signalling of mechanical allodynia.

1.6.3.4. Regenerative Changes within the CNS Following Nerve Injury

Animals which undergo CCI show a gradual recovery, usually within 3 months of the original nerve injury. This suggests that nerve injury also induces a number of regenerative changes. Indeed, nerve injury has been shown to increase the expression of a number of factors associated with survival and regeneration including neurotrophic and other growth factors (Cameron et al. 1997; Ji et al. 1996; Sebert and

Shooter, 1993). As stated earlier, such factors include NGF, BDNF, LIF and GAP-43, all of which are important in the regulation of cell/fibre growth and survival.

A possible aversive reaction to the rapid up-regulation of growth factors to try and counteract the damaging effects of nerve injury, is the abnormal sprouting of axotomised large myelinated fibres. Woolf et al. (1992) provided the first evidence of Aβ-fibre sprouting following transection of the rat sciatic nerve. Using HRP-labelling, they demonstrated that a number of Aβ fibres sprouted, within a week of axotomy, from lamina III into lamina II of the spinal dorsal horn, an area where small diameter nociceptive afferents usually terminate, and that by week 2 there were dense extensions of these Aβ fibres (Woolf et al. 1992). This has since been confirmed by a number of other investigations (Koerber et al. 1994; Shortland and Woolf, 1993). A study into the time course of sprouting revealed that the effect was maximal at week 2 post injury, and persisted for over 6 months (Woolf et al. 1995), with a total recovery by 9 months. So this A fibre reorganisation is a prominent and long-lasting, but not permanent, feature of peripheral nerve injury.

Three different types of sprouting now seem to exist (Woolf et al. 1995):

1. Regenerative sprouting which involves the re-growth of an injured axon back to its own or new targets
2. Collateral sprouting, where the growth of the processes and terminals from an uninjured nerve grow into a denervated region
3. Conditioned collateral sprouting, which appears to be induced by injury to the peripheral processes of the sensory nerve and results in the sprouting of uninjured central sensory axons

This A fibre sprouting is clearly conditioned collateral sprouting and may result in low threshold mechanical fibres establishing functional contacts with nociceptive neurones in lamina II of the dorsal horn, thereby resulting in the production of inappropriate responses to innocuous stimuli. However, the fact that this sprouting is still evident up to 6 months after the original nerve injury, a time point when the abnormal neuropathic pain states no longer exist, suggests that this reorganisation is not the only contributing factor to the development and maintenance of neuropathic pain states.

The aetiology of neuropathic pain is clearly extremely complex and a wide variety of different factors appear to be involved in the induction and maintenance of the abnormal pain states. Much work still needs to be done to determine the principal contributing factors involved in neuropathy so that more effective and longer-lasting treatments can be evolved.

1.7 Aims of Current Work

Previous work indicates that VIP and PACAP may play a significant role in the regulation of nociceptive transmission at the spinal cord level. This study aimed to investigate the possible involvement of VIP/PACAP and their receptors in mediating the responses of dorsal horn neurones to a variety of sustained cutaneous stimuli in both normal and neuropathic animals, and to identify specific changes in the expression of the three VIP/PACAP receptor subtypes following a chronic constriction injury to the rat sciatic nerve. To do this:-

1. A behavioural model of neuropathy was produced by loose ligation of the rat common sciatic nerve, to provide a number of test animals which displayed positive signs of mechanical and thermal allodynia, as well as mechanical hyperalgesia, within their affected hindlimbs.
2. Electrophysiological techniques were employed to determine the role of VIP/PACAP in the transmission of sensory information by investigating the effects of selective VIP_1, VIP_2 and PACAP receptor antagonists on the sensory-induced responses of dorsal horn neurones, in both normal and neuropathic animals.
3. The responses of multireceptive dorsal horn neurones to selective VIP_1, VIP_2 and PACAP receptor agonists were then investigated to provide an insight into the expression of the three receptor subtypes and to try and ascertain any changes in receptor expression following nerve injury
4. The detailed distribution of VIP/PACAP receptor expression in the spinal cord was assessed for the first time, using *in situ* hybridisation histochemistry. The expression of VIP_1, VIP_2 and PACAP receptor mRNA was measured within the rat dorsal horn of normal and neuropathic spinal cord, enabling us to observe any changes in receptor mRNA expression following nerve injury.

CHAPTER 2 MATERIALS

All reagents used were of the highest analytical grade and were supplied by Sigma Chemical Company, Poole, UK., unless otherwise stated.

2.1 Anaesthetics

Urethane, α-chloralose, Halothane (Zeneca Ltd., Cheshire, UK), Sagatal (Rhône Mérieux Ltd., Essex, UK)

2.2 Sterile Surgery

- 4/0 sterile chromic catgut and 4/0 violet coated vicryl (Ethicon Ltd., Edinburgh, UK)
- Hibitane (Zeneca Ltd., Cheshire, UK)
- Sterile gowns, biogel gloves, hats, face masks and drapes (Hospital Management and Supplies, Glasgow, UK)

2.3 Electrophysiology

- Glass capillaries (Clark Electromedical Ltd., Reading, UK)
- Platinum wire (Goodfellow Metals, Cambridge, UK)
- Allyl isothiocyanate (mustard oil) (Aldrich Chemical Company, UK)
- Liquid Paraffin (Thornton and Ross, Huddersfield, UK)
- Agar (Unipath Ltd., Basingstoke, UK)

2.4 Drugs for Ionophoresis

Agonists: - **porcine VIP**

- **PACAP-38** (Bachem (UK) Ltd, Essex, UK)
- Selective VIP_1 receptor agonist **[Arg^{16}] chicken secretin**
- Selective VIP_2 receptor agonist [Ac-His^1, Glu^8, Lys^{12}, Nle^{17}, Ala^{19}] VIP (1-24), Asp, Leu, Lys, Lys, Gly, Gly, Thr NH_2 (lactam 21-25): **Ro 25-1553**

(The selective VIP_1 and VIP_2 agonists were synthesised by Philippe Gourlet, Ph.D., Laboratory of Biological Chemistry and Nutrition, Université Libre de Bruxelles, Belgium).

Antagonists: - **VIP (6-28), [p-Chloro-D-Phe^6, Leu^{17}]-VIP** and **PACAP (6-38)** (all Bachem (UK) Ltd, Essex, UK)

- Selective VIP_1 receptor antagonist [Ac-His^1, D-Phe^2, Lys^{15}, Arg^{16}, Leu^{17}] VIP(3-7)/GRF(8-27): **PG 97-269**
- Selective VIP_2 receptor antagonist (des 1-4)[Glu^8, Lys^{12}, Arg^{16}, Nle^{17}, Ala^{19}] VIP(1-24) Asp, Leu, Lys, Lys, Gly, Gly, Thr NH_2 (lactam 21-25): **des(1-4) Arg^{16} Ro 25-1553**

(The selective VIP_1 and VIP_2 antagonists were both synthesised by Philippe Gourlet, Ph.D., Laboratory of Biological Chemistry and Nutrition, Université Libre de Bruxelles, Belgium).

2.5 In Situ Hybridisation Histochemistry (ISHH)

- Oligodeoxyribonucleotide probes were supplied by Oswel Research Products, Southampton, UK. The probes were aliquoted into 200µl samples and stored at -70°C until required.
- Deoxyadenosine [α-^{35}S]-triphosphate ([α-^{35}S]-dATP: specific activity 1-1.3 x 10^3 Ci/mmol) was supplied by NEN Dupont (UK) Ltd., Hounslow, and was stored at -20°C prior to use.
- Recombinant terminal deoxynucleotidyl transferase (rTdt) enzyme and tailing buffer (GibcoBRL, Life Technologies Ltd., Paisley, UK)
- Nu-clean D25 spin columns (Scientific Imaging Systems Ltd., Cambridge, UK)
- Optiphase 'Supermix' liquid scintillation cocktail (Fisher Chemicals, Leics., UK)
- Absolute alcohol ANALAR grade (Hayman Ltd., Essex, UK)
- Acetic anhydride (BDH Laboratory Supplies, Poole, UK)

2.6 ISHH Stock Solutions

- **DEPC (Diethyl Pyrocarbonate) H_2O:** all solutions were made up with distilled water (dH_2O) which had previously been treated with the nuclease inhibitor DEPC, to minimise the risk of ribonuclease (RNase) contamination. 10-12 drops of diethyl pyrocarbonate were added per litre (l) of dH_2O and the solution was left to stir overnight under a fume hood before being autoclaved to destroy any last traces of DEPC.
- **0.1M Phosphate-Buffered Saline (PBS):** 100ml of 0.2M sodium phosphate, monobasic, anhydrous was mixed with 400ml 0.2M sodium phosphate, dibasic, anhydrous and 9g sodium chloride, made up to 1l total volume with DEPC H_2O, filtered and then autoclaved.
- **4% Paraformaldehyde (PF) in 0.1M PBS**: 20g of PF was dissolved in 400ml of 0.1M PBS at 60°C. After cooling to room temperature, the pH was adjusted to 7.4 before completing the volume to 500ml with 0.1M PBS.
- **De-ionised formamide**: formamide was de-ionised with 1g of mixed-bed anion exchange resin (Bio-RAD Laboratories, California, USA) per 10ml of formamide, for 1 hour at room temperature, filtered twice through Whatman No. 1 filter paper, then stored in sterile pots at -70°C in 20ml aliquots, ready for use.
- **Tris-HCl 1M**: 60.5g of Tris base was dissolved in 400ml DEPC H_2O, then adjusted to pH 7.6 before completing the volume to 500ml with DEPC H_2O.
- **Ethylenediamine tetra acetic acid (EDTA) 250mM**: 9.305g of EDTA (disodium salt) was dissolved in 80ml DEPC H_2O, adjusted to pH 7.6 and then made up to 100ml with DEPC H_2O.
- **Tris-EDTA (TE) Buffer**: 2.5ml 1M Tris-HCl, pH7.6 and 5ml 250mM EDTA, pH 7.6 were added to 20ml DEPC H_2O. The resulting solution was then aliquoted into 2ml Eppendorf tubes and stored at -20°C until required.
- **2x Hybridisation buffer** was made up to the following concentration prior to use and immediately stored at -20°C in 2ml aliquots, until use: dextran sulphate (10%; wt:vol), sodium chloride (600mM), Tris pH 7.6 (10mM), EDTA (1mM), Denhardt's solution (1% solution of Bovine Serum Albunim (BSA), Ficoll and Polyvinyl pyrrolidone (PVP))(0.1% ; wt:vol), salmon sperm DNA (0.01%;

wt:vol), Bakers yeast tRNA (0.005%; wt:vol) and glycogen (0.0005%; wt:vol)(Boehringer Mannheim, UK Diagnostics and Biochemical, Lewes, UK).

- **20x Standard Saline Citrate (SSC)**: 3M sodium chloride and 0.3M sodium citrate were made up in 800ml DEPC H_2O, the pH adjusted to 7 and the solution autoclaved before being stored at room temperature until required.
- **1M Dithiothreoitol (DTT)**: 1.54 g of DDT was dissolved in 10ml of DEPC H_2O, then stored in 100µl aliquots at -20°C until required.
- **Triethanolamine (TEA)**: 10M TEA (BDH Laboratory Supplies, Poole, UK) was diluted to a 1M solution with DEPC H_2O and the pH adjusted to 8 with NaOH. This solution was then further diluted to 0.1M on the day of acetylation.

2.7 Developer and fixer

- LM-1 Hypercoat nuclear emulsion and Hyperfilm β-max (Amersham International, Bucks, UK)
- Kodak D-19 developer powder (H.A. West (x-ray), Edinburgh, UK)
- Ilford Hypam K5 rapid fixer (diluted 1 in 5 in dH_2O) (Ilford Ltd., Cheshire, UK)

2.8 Staining solutions

- **Solochrome Cyanin**: 0.2g Solochrome cyanin RS (Edward Gurr) was dissolved in 96ml dH_2O with 4ml 10% iron alum and 0.5ml concentrated sulphuric acid.
- **10% Iron alum** (ammonium ferric sulphate): 10g of iron alum (Merk Ltd., Leics, UK) dissolved in 100ml dH_2O.
- Mayer's hematoxylin solution (0.1% certified hematoxylin with stabilisers) (Sigma Diagnostics, St. Louis, USA)
- Eosin (5% aqueous), Xylene and DePex mounting medium (BDH Laboratory Supplies, Poole, UK)
- Perfex mounting medium (Cell Path Plc., Herts, UK)

2.9 Miscellaneous

- Freezing spray (Greenhill Chemical Products Ltd., Burton-on-Trent, UK)
- Isopentane (Merk Ltd., Leics, UK)
- Decon 90 (Decon Laboratories Ltd., Hove, UK)

- Cryo-M-Bed embedding compound (Bright Instrument Company Ltd., Huntingdon, UK)
- Poly-L-lysine coated microscope slides, glass coverslips (22 x 50mm) and Silica gel (BDH Laboratory Supplies, Poole, UK)

CHAPTER 3 INVESTIGATION OF THE CHRONIC CONSTRICTION INJURY MODEL OF NEUROPATHIC PAIN - A BEHAVIOURAL AND HISTOLOGICAL STUDY

3.1 AIM

These experiments were carried out to produce an animal model of chronic, peripheral mononeuropathy which exhibits symptoms of neuropathic pain similar to those seen in man, and to investigate, using a variety of different quantifiable tests, the abnormal pain-related behaviours observed. The model under investigation is the chronic constriction injury (CCI) model of Bennett and Xie (1988) and involves loose ligation of the common sciatic nerve in the rat. The symptoms, which persist for up to three months, include spontaneous pain and lowered thresholds and exaggerated responses to mechanical and thermal stimuli (Attal et al. 1990; Bennett and Xie, 1988). It is a relatively simple surgical procedure and the affected hind limb is easily accessible for behavioural and experimental testing, therefore allowing investigation of the factors contributing to the development of the neuropathic pain states.

3.2 METHODS

3.2.1 Animals

Adult male Wistar rats were used (Charles River, UK). They were housed in standard laboratory cages in groups of 3-4, in a controlled environment with a natural 12 hour day/night cycle and temperature range 22-24°C. Food and water were supplied *ad libitum*.

3.2.2 Surgical Preparation of Animals with an Experimental Peripheral Mononeuropathy

The surgical procedure was carried out on 76 male Wistar rats weighing 200-250g on the day of surgery. Rats were initially anaesthetised with sodium pentobarbital (50mg/kg i.p.) and then supplemented as necessary with halothane. The right hind limb was shaved and the skin sterilised by wiping with Hibitane pre-

operative skin disinfectant (0.5% in 70% alcohol). Under aseptic conditions, the right sciatic nerve was exposed by blunt dissection at mid-thigh level, immediately proximal to the point of trifurcation, and approximately 1cm of nerve freed of adhering connective tissue. Four chromic cat gut (4.0) ligatures were then tied loosely around the nerve spaced at approximately 1mm intervals. Care was taken to tie the ligatures so as to barely constrict the nerve, as viewed under x40 magnification, thus ensuring that the circulation through the superficial epineurium was not completely blocked. The overlying muscle and skin were then closed in layers with sterile suture thread, and the wound re-sterilised with Hibitane to prevent any infection. The animals were then placed in recovery cages for 24 hours, with soft padding for bedding, before being returned to their normal cages. In the 5 sham-operated animals, the same exposure of the right sciatic nerve was made but no ligatures were applied. The animals were allowed three full days to recover from surgery before the behavioural testing was recommenced.

3.2.3 Behavioural Tests

Behavioural signs representing three different components of neuropathic pain were investigated: cold allodynia, mechanical allodynia and mechanical hyperalgesia. The animals were tested one day pre surgery then every 1 or 2 days post surgery, and the ipsilateral responses were compared to pre-operative baselines and/or the unaffected contralateral hind limb response.

(i) General observations

Daily inspections were made for signs of autotomy, and notes were also made of the animal's gait, and the posture and condition of the affected hind limb.

(ii) Cold allodynia

To detect the presence of cold allodynia, the rat's response to cold iced water was evaluated. This was determined using a cold water bath. Rats were placed in a clear plastic tank on an aluminium floor which was raised by a wire mesh stage and chilled by ice water. The ice water covered the metal plate by approximately 8mm, to ensure that both the glabrous and hairy skin of the animals' feet were in contact

with the cold water, and a constant temperature of 3-4°C was maintained. The floor felt distinctly cool to the human touch but did not feel painful over the period of time tested. The animals were given a few moments to acclimatise to their new surroundings and then a measurement was taken of the Suspended Paw Elevation Time (SPET) during a 20 second test period, that is the time the paw was elevated in a defensive posture following the presumably noxious cold stimulus. The animals were placed in the tank for 4 x 20 second intervals, with at least a 10 minute wait in between each test to allow the animal's paw to return to body temperature before the next test. The data was averaged and then pooled for each test day to produce a distribution curve showing the duration of suspended paw elevation in response to a cold stimuli with respect to the onset of neuropathy following sciatic nerve ligation.

(iii) Mechanical allodynia

To quantify the mechanical sensitivity of the foot, the withdrawal threshold in response to a normally innocuous mechanical stimuli was measured. Mechanical stimuli were applied using a set of calibrated von Frey filaments (a series of nylon monofilaments of increasing stiffness which exert defined levels of force as they are pressed to the point where they bend). We used the standard Semmes-Weinstein set of von Frey hairs (Stoelting, Wood Dale, Illnois, USA), in the range which produced bending forces of 0.41-125.89g. Each animal was placed in a wire mesh cage which allowed the rat to walk freely and the experimenter to reach the plantar surface of the foot from beneath, unobserved by the rat. Each filament was indented on the mid-plantar surface of the foot until it started to bend. This was repeated 8-10 times at a frequency of approximately 1Hz. The filaments were applied in ascending order starting from the weakest, and a response was characterised as a quick, robust paw flick. The response threshold was defined as being the lowest force of 2 or more consecutive von Frey filaments to produce a response.

(iv) Mechanical hyperalgesia

Mechanical pain thresholds were determined using the pin prick method (as previously described by Seltzer et al. 1990). Again the animals were placed in a wire mesh cage allowing them to walk freely, and a slightly blunted needle was gently

applied to the mid-plantar foot from beneath the cage, to elicit a single prick. The withdrawal response was graded accordingly: (1) a quick and robust paw flick; (2) a longer-lasting response, but one in which the animal replaced its paw within 2-3 seconds; (3) a prolonged response where the affected paw was held in the air for more than 5 seconds before being replaced, indicative of an exaggerated painful response. To ensure that this test did not have any long term effects on the receptive field properties of the rat paws, this test was only carried out once on the pre-operative day and once again when the animal displayed strong signs of mechanical and cold allodynia.

3.2.4 Statistics

In each behavioural study, data was pooled for each test day. Group averages are shown ± the standard error of the mean (SEM). The Mann-Whitney rank sum test was performed to determine any significant differences between ipsilateral and contralateral hind limb values ($p \leq 0.05$). A Kruskal-Wallis one way analysis of variance (ANOVA) on ranks, followed by Dunn's post-hoc pairwise comparison was then carried out ($p \leq 0.05$), to determine group values at different time points which differed from the pre-surgery control group.

3.2.5 Sciatic Nerve Dissection

Peripheral nerve histology was carried out on 6 neuropathic rats. Approximately 2 weeks after the ligatures were tied, when the animals showed clear signs of mechanical hyperalgesia and cold allodynia (as determined by the behavioural testing), the rats were administered a lethal dose of sodium pentobarbital. The sciatic nerve was then immediately exposed by blunt dissection and the nerve containing the ligatures was removed along with approximately 1cm of nerve proximal and distal to the ligatures. The excised nerves were immediately fixed in 10% formal saline. Two sciatic nerves were also removed from animals which did not have an experimental peripheral neuropathy, to act as controls. In these animals, euthanased as above, the sciatic nerve was exposed at mid-thigh level and approximately 2cm of nerve in length was freed of connective tissue and removed,

immediately proximal to the point of trifurcation. Once again the nerves were fixed immediately in 10% formal saline.

Wax-embedded, transverse sections (8μm thick) of the sciatic nerve proximal and distal to the ligatures, were mounted on glass slides. Sections were hydrated by immersing in 2 changes of xylene, followed by immersion in absolute alcohol, 70% alcohol and finally placing the sections in running tap water. The sections were then immersed in Solochrome cyanin for 10 minutes, rinsed in tap water and then differentiated in 10% iron alum for 5 minutes, to stain myelin a dark purple colour. The sections were then counterstained with eosin, dehydrated through ascending concentrations of alcohol (70%, 90% and absolute alcohol), then cleared in xylene before being mounted in Perfex mounting medium, for light microscopic evaluation.

3.3 RESULTS

3.3.1 General Observations

The majority of nerve-injured animals showed the behavioural alterations characteristic of the CCI model. All of the animals which underwent the surgery were very protective of their affected hind paw, guarding it from incidental contact and rarely, if ever, extending the ipsilateral limb. The affected paw was generally held awkwardly with the toes tightly drawn together and ventroflexed. The animals walked with a definite limp/hop and a number of rats walked without allowing the affected paw to touch the floor at all. These abnormalities in posture are thought to be indicative of spontaneous pain (Attal et al. 1990).

With these exceptions, the animals' health and behaviour were generally normal and there were no apparent signs of debilitating pain or distress. The level of general activity appeared to be normal, and the animals could be handled without evoking squealing or biting. In addition, the animals appeared to gain weight in accordance with control animals.

A small number of nerve-injured animals (n=5) exhibited autotomy, with distal phalange injury to one or more of the toes on the affected hind paw. These animals were immediately sacrificed and were not included in any of the studies.

3.3.2 Cold Allodynia

The quantitative assessment of cold allodynia, using a cold water bath, revealed a reduced threshold to the cold stimulus in the nerve injured hind paw of the majority of animals, while unoperated control rats showed no such response.

Unoperated control rats and sham treated animals, showed no pain-related behavioural responses when placed in the cold water bath, and explored the new territory without any apparent signs of distress. During their time in the tank, none of the animals lifted any of their paws out of the water either briefly or for an extended period of time, other than to walk about.

When the nerve damaged rats were placed in the cold water bath however, obvious behavioural changes were apparent. The increased sensitivity to cold was clearly visible from as early as day 4 post-operatively (D4 PO) when the majority of animals began to lift their affected paw out of the water for a few seconds for each test period. Maximal responses were observed from approximately D8 PO onwards, and were generally still high at D15 PO (Figure 3.1). Animals which displayed a marked response to the cold stimuli were generally used for electrophysiological or histological studies by D15 PO, and so the time course of development of cold allodynia measured in these animals does not extend past this time point. From D8 PO onwards, the animals which had developed cold allodynia displayed cold-evoked withdrawals which were significantly increased. Many of the animals did not place their affected paw in the cold water at all and the average SPET was 10.6 ± 1.0 sec. During the tests, several of the animals repeatedly licked the affected hind paw or were seen to flick their paw in the air as if they were trying to remove any traces of the cold water, indicating that the normally innocuous cold stimulus was in fact evoking a painful response. These abnormal pain behaviours were not accompanied by vocalisations however, and the general level of activity within the tank appeared to be similar to that of the control animals. The cold test did not appear to elicit any pain-related responses from the hind limb on the contralateral control side.

There did appear to be a degree of variability in the cold response of the nerve-injured animals, with a small proportion of the animals developing poor withdrawal responses of less than 5sec for the whole test period. These animals were not included in any of the electrophysiological or histological studies.

3.3.3 Mechanical Allodynia

Prior to the experimental nerve injury, the animals displayed an average withdrawal threshold of 62.8 ± 9.1g in the left paw and 53.9 ± 8.0g in the right hind paw, with no significant difference between right and left hind limb values (Figure 3.2).

After surgery however, there was a very clear decrease in the response threshold of the nerve-injured side with respect to time. From as early as D4 PO, there was a significant reduction in the withdrawal threshold of the ipsilateral hind paw (18.4 ± 3.0g) when compared with the contralateral hind limb value (56.8 ± 5.6g; Mann-Whitney rank sum test, $p \leq 0.05$) or pre-operative values (Kruskal-Wallis one way ANOVA, $p \leq 0.05$). The threshold continued to decline over the next few days, with maximal differences from D8 PO onwards when the animals displayed an average withdrawal threshold of 3.5 ± 1.4g in the nerve-injured limb. So forces that the rats rarely (if ever) responded to before the nerve injury now clearly evoked pain-related withdrawal reflexes, indicative of mechanical allodynia.

There were no significant differences between the threshold values of the contralateral hind limb in the nerve injured animals compared to pre-surgery values, over the two week test period.

3.3.4 Mechanical Hyperalgesia

When applied to the volar skin of the experimenter's hand, the pin prick stimulus produced a brief sensation that was sharp and barely painful. The normal response of control animals following a noxious pin prick to the plantar surface of their hind paws, was a quick, robust paw flick (Grade 1 response). This brisk withdrawal reflex was always of small amplitude and very short duration.

After surgery the response of the nerve-injured paw was obviously different from control withdrawal reflexes. Following the brief noxious stimulus, the nerve-injured animals held their affected paws in the air for an extended period of time (Grade 3 response), often for more than 15sec, while the response of the contralateral control paw was indistinguishable from that of control or pre-surgery (Grade 1 response). In addition, all the responses of abnormally long duration were also of abnormally great amplitude. In the normal reflex response the animals lifted their

paws approximately 1mm off the floor, while the abnormal reflexes brought the paw some 5-10mm off the floor. This test was used in a restricted fashion when there were clear signs of allodynia, as the animals appeared to show some signs of discomfort during these tests, and the exaggerated response was occasionally accompanied by vocalisation. For this reason there was not enough data to produce a time course of development of mechanical hyperalgesia. But it was clear that all of the animals which displayed strong signs of thermal and mechanical allodynia, also showed strong signs of mechanical hyperalgesia between D8-D15 PO.

3.3.5 Sciatic nerve histology

Histological sections of the rat sciatic nerve 2 weeks after a chronic constriction injury when behavioural changes are most prominent, revealed clear structural differences distal to the nerve ligature (Figure 3.3).

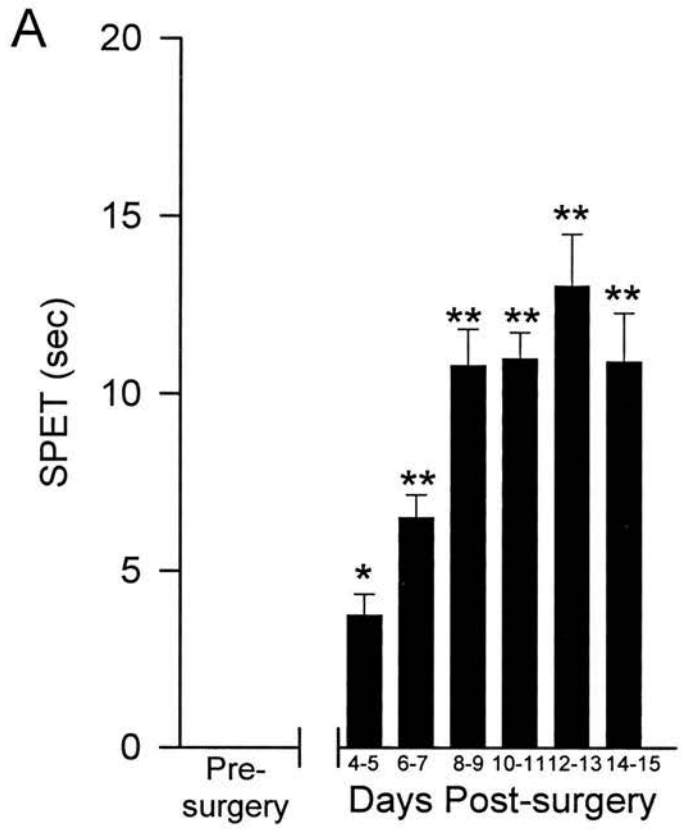
Light-microscopic examination of the cross-sectional area of the sciatic nerve distal to the ligatures showed a marked reduction in the number of large myelinated fibres compared to control nerve sections. The degree of severity of axonal degeneration appeared to vary slightly from one nerve to the next, but the general pattern of pathological changes within the sciatic nerve was clearly evident each time, that is a significant loss of large myelinated fibres distal to the ligatures.

The cross-sections of sciatic nerve taken 1cm or more proximal to the constriction injury, appeared morphologically similar to normal sections. The distribution of myelinated fibres did not appear to be affected in these sections when viewed under the light microscope.

Figure 3.1

Time course of the development of cold allodynia following an experimental mononeuropathy

Values represent the suspended paw elevation time (SPET) in response to the animal being placed in a cold water bath for a 20 second period. Responses were obtained for each animal, data combined, and the resultant group means utilised to produce a time course of the development of cold allodynia for the days following sciatic nerve injury. Part A shows the development of a marked cold allodynic response in neuropathic animals (n=50). Data was analysed using the Kruskal-Wallis one way ANOVA on ranks to determine any significant differences between post-operative and pre-operative values (* $p \leq 0.05$; ** $p \leq 0.01$). Part B shows the less marked cold allodynic response of a separate population of animals which did not appear to develop a prominent neuropathic pain state (n=20).


A
20
15
10
5
0
SPET (sec)
*
**
**
**
**
**
Pre-
surgery
4-5
6-7
8-9
10-11
12-13
14-15
Days Post-surgery

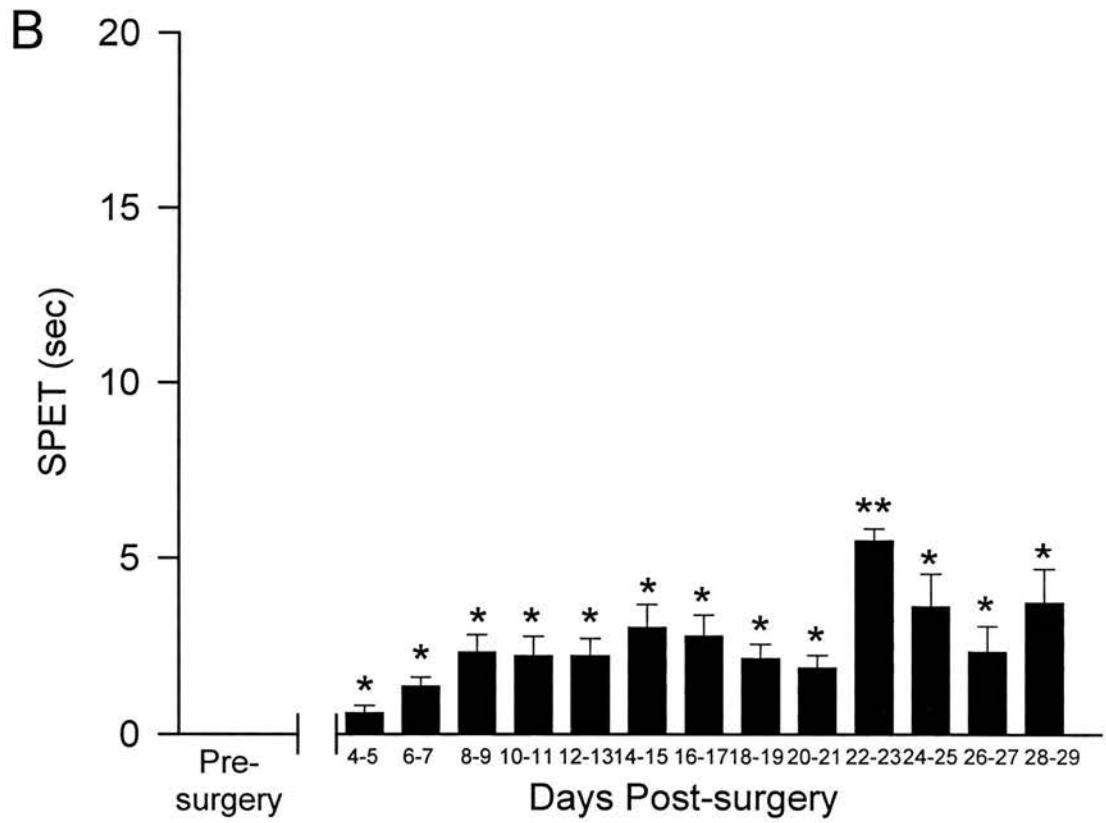

B
20
15
10
5
0
SPET (sec)
*
*
*
*
*
*
*
*
*
*
**
*
*
*
Pre-
surgery
4-5
6-7
8-9
10-11
12-13
14-15
16-17
18-19
20-21
22-23
24-25
26-27
28-29
Days Post-surgery

Figure 3.2

Time course of mechanical allodynia development following an experimental peripheral mononeuropathy

Data is plotted as the withdrawal threshold (g) to repetitive von Frey filament stimulation in the days following a chronic constriction injury, for 26 neuropathic animals. Group means ± SEM are plotted for each time course. Filled circles represent ipsilateral hind paw values while open circles represent the contralateral control data.

Data was analysed using the Kruskal-Wallis one way ANOVA to isolate groups that differ from the pre-operative control group ($p \leq 0.01$), and ** indicates significant differences between ipsilateral and contralateral values (Mann-Whitney rank sum test; $p \leq 0.01$).

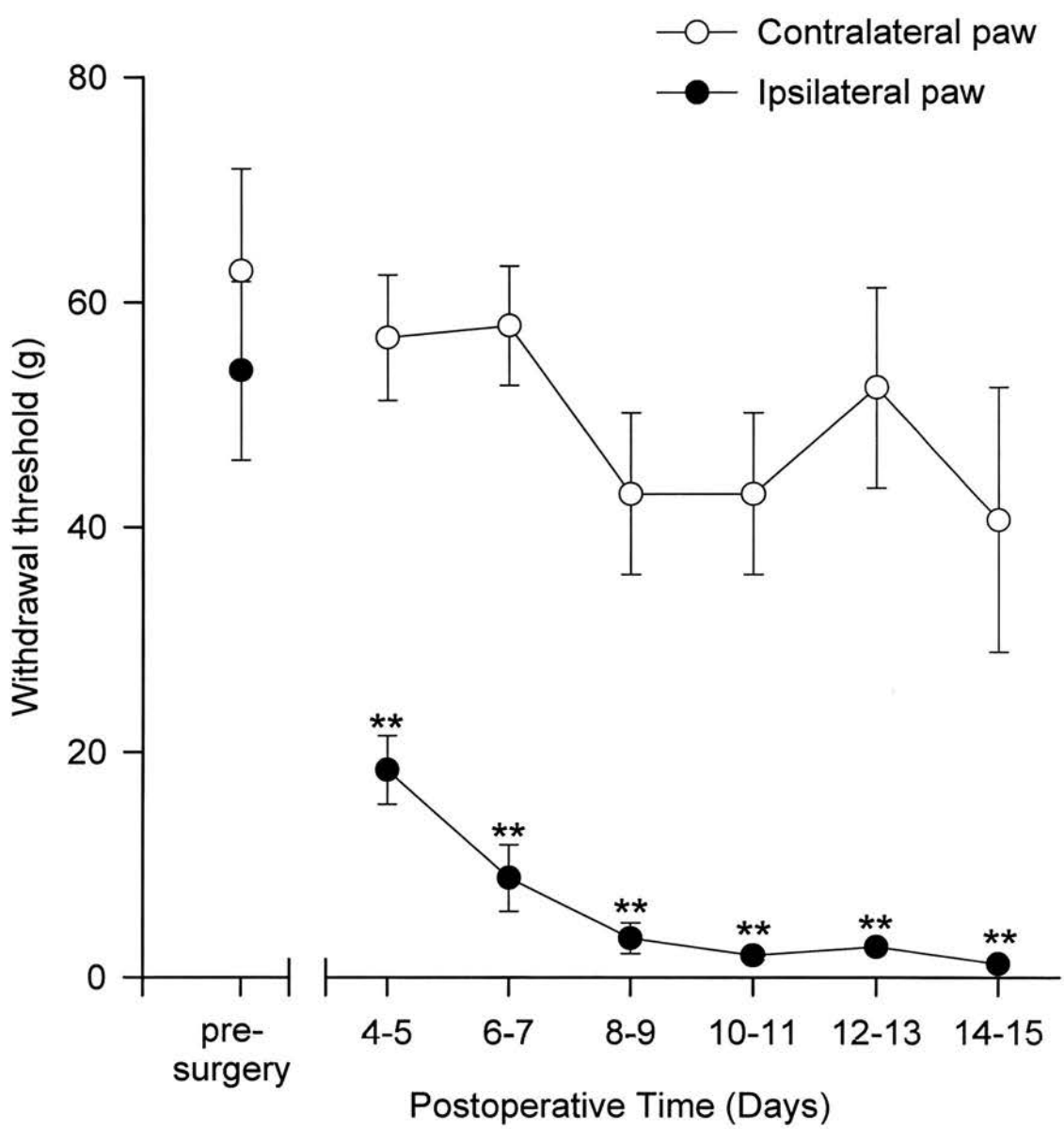
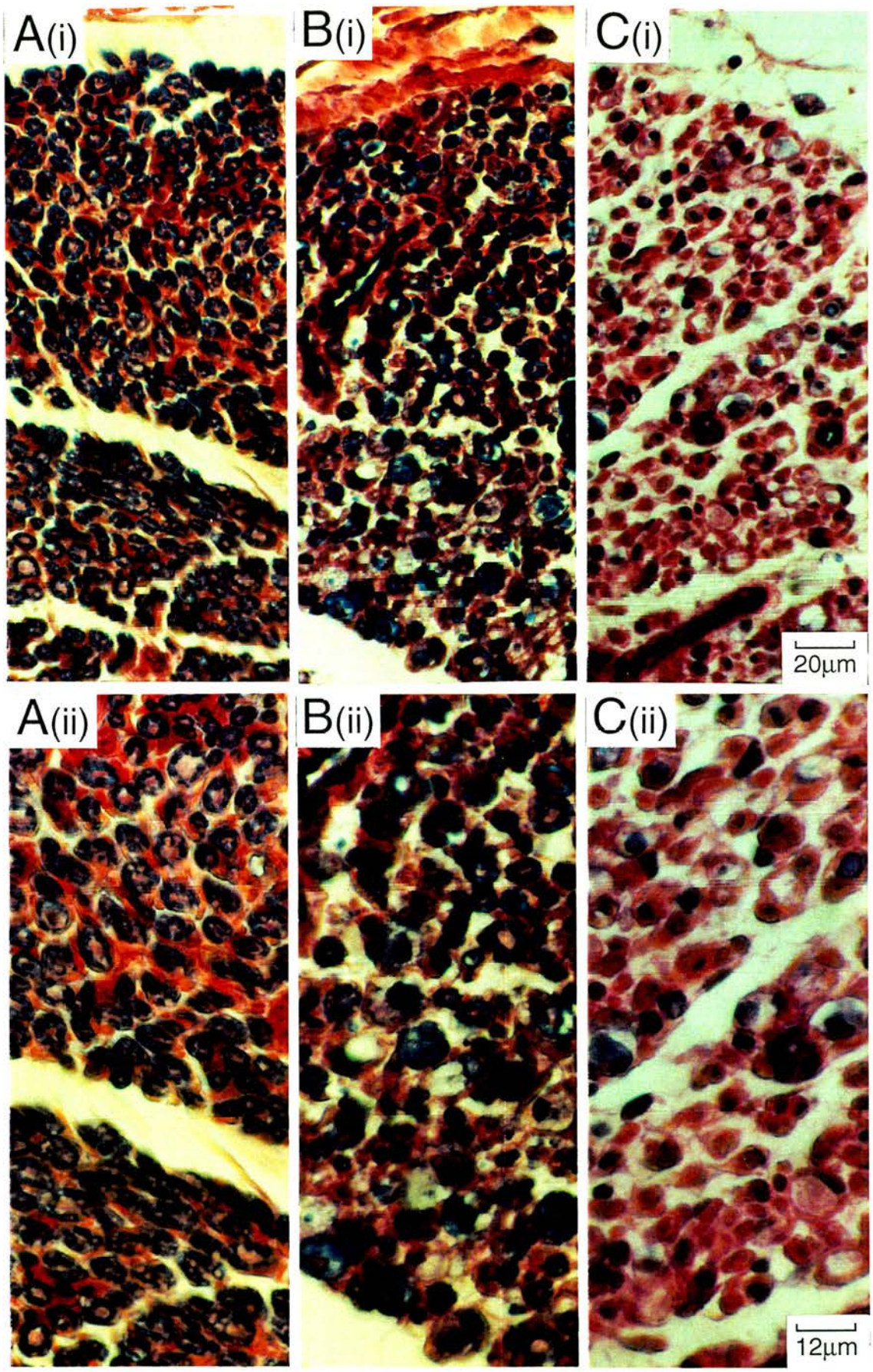

Contralateral paw
Ipsilateral paw
Withdrawal threshold (g)
80
60
40
20
0
**
**
**
**
**
**
pre-
surgery
4-5
6-7
8-9
10-11
12-13
14-15
Postoperative Time (Days)

Figure 3.3

Axonal pathology of the rat sciatic nerve following a chronic constriction injury as viewed under the light microscope

Transverse sections of rat sciatic nerve at the ligature level: Part A represents control sections from untreated animals, while B and C are proximal and distal sections respectively, from a ligated nerve at the second post-operative week. 8μm thin sections were stained with Solochrome cyanin and iron alum, then counter-stained with eosin. Note the significant loss of large myelinated fibres (myelin is stained a dark purple colour) distal to the ligature (C(i) and C(ii)) with respect to control (A(i) and A(ii)), while the sections proximal to the ligatures (B(i)and B(ii)) are almost indistinguishable from control sections.


A(i)
B(i)
C(i)
20μm
A(ii)
B(ii)
C(ii)
12μm

3.4 DISCUSSION

These results are broadly consistent with previous data which has shown the development of abnormal behavioural responses in animals which have undergone an experimental peripheral mononeuropathy (Attal et al. 1990; Bennett and Xie, 1988).

The data provide strong evidence that a chronic constriction injury produces cold allodynia in the affected hind limb of the rat. Exposure to the ice-cold water was not immediately noxious to the touch of uninjured humans and did not evoke any behavioural changes in the control or sham rats nor in the contralateral control limb of the nerve-injured animals. However, the majority of animals with an experimental neuropathy clearly behaved as if the cold water was eliciting pain in their affected hind limbs. As the neuropathy developed, the animals' threshold to the cold stimulus decreased, indicative of the development of cold allodynia.

There did appear to be some variability in the responses of individual animals to the cold stimulus, which has been reported previously (Bennett and Xie, 1988). Approximately one quarter of the nerve-injured animals failed to develop a strong cold allodynic response, that is to develop an average SPET of more than 5 sec for at least 3 consecutive days. It is not known why some animals appeared to be less responsive to the cold stimulus while mechanical allodynia was observed in all cases (see below). It has been speculated that the development of the abnormal pain behaviours following a CCI may result from the loss of inhibitory controls normally exerted by the large primary afferent fibres (Gautron et al. 1990), as it is the pathology of these fibres which is most greatly affected following nerve ligation (Basbaum et al. 1991; Coggeshall et al. 1993). The variability in behavioural patterns therefore may be due to slight differences in the "tightness" of the nerve ligatures, as it was difficult to ascertain a common "pressure" when tying the ligatures in individual animals. So it is possible that variations in ligation tightness may lead to differences in the degree of degeneration within the nerve and subsequently differences in the development of the chronic pain conditions.

The development of mechanical allodynia was revealed by testing the rat's withdrawal threshold following application of calibrated von Frey filaments in the innocuous range. The nerve-injured rats displayed a significantly lowered threshold to von Frey hair application from as early as D4 PO, again showing maximal effects

from D8 PO onwards. There appeared to be much less variation in the data collected for the mechanical testing as compared with the cold thermal test, with every nerve-injured rat showing a reduction in withdrawal threshold to some degree over the time period tested. Even those animals which produced a poor cold allodynic response, displayed a significantly reduced mechanical threshold.

In addition to the development of mechanical and thermal allodynia, the experimental neuropathy clearly produced a mechanical hyperalgesia, as application of a noxious pin-prick to the affected hind paw produced increased paw withdrawal latencies in all the animals tested. The magnitude and duration of the responses were obviously exaggerated in comparison to those of control rats; changes indicative of the development of hyperalgesia.

The abnormal position of the nerve-injured paw of the rat may reflect the development of another pain-state, as this presumably pain-relieving behaviour is thought to be indicative of spontaneous pain (Attal et al. 1990). It is possible that these abnormalities may be due to motor impairments following the nerve damage, but this seems unlikely as the rat holds the affected limb in particular protective positions rather than dragging the hind limb, as would be expected were it due to weaknesses in the dorsiflexor muscles of the hind limb. It seems more likely that the abnormal position of the paw predominantly reflects a reluctance of the rat to press its paw on to the ground, and that it is indeed protective guarding behaviour.

The time course of these pain-related disorders appears to be roughly comparable throughout the range of behavioural tests employed. Rats demonstrating both mechanical and thermal allodynia, as well as hyperalgesia, showed maximal responses over the second postoperative week, with obvious changes evident from as early as the fourth post-operative day. The histological alterations in the rat sciatic nerve following a CCI also appear to have a time course which parallels the development of the clinical signs (Coggeshall et al. 1993), and so we chose to look at the pathology of the nerve at D14 PO, the time of maximum behavioural changes.

The morphological changes which result within the sciatic nerve following CCI have been well documented, and allow us to speculate as to which fibre types are involved in the development of hyperalgesia and allodynia, the abnormal pain syndromes which accompany peripheral nerve damage.

Despite some variability between experimental animals in our sciatic nerve study, the dissections generally documented a dramatic loss of large myelinated fibres distal to the nerve ligatures 14 days after chronic constriction of the sciatic nerve, while there were no apparent signs of degeneration in the nerve 1cm or more proximal to the constriction. These results are in agreement with previous studies which have also shown that the predominant effect on nerve pathology following a chronic constriction injury is a massive degeneration of large myelinated axons (Basbaum et al. 1991; Gautron et al. 1990). The main consequence of these pathological changes could be a deficit in the control of noxious information at the spinal cord level, brought about by a lack of inhibitory influence from large diameter primary afferents due to their demyelination and loss. Indeed, the development of the behaviour pain states correlates with the initial fibre loss and predominant loss of large myelinated fibres (Coggeshall et al. 1993), but small myelinated and unmyelinated fibres are also affected to some degree (Basbaum et al. 1991; Carlton et al. 1991). So it is likely that the development of the abnormal pain states following a peripheral nerve injury, is the result of both afferent volleys in the small Aδ- and C-fibre afferents that are spared, as well as the loss of inhibitory control normally mediated by the large myelinated fibres. It is impossible from these anatomical studies alone, to speculate as to the predominance of one fibre type involvement above another in the production of the behavioural symptoms.

This study provides further behavioural evidence that the experimental peripheral neuropathy produced by sciatic nerve ligation, produces significant pain-related behavioural changes in the rat. These include hyperalgesia and allodynia to mechanical and thermal stimuli, as well as abnormalities in posture which may be considered as "spontaneous" pain-related behaviour. These symptoms appear to have a similar time-course of development showing maximal changes between D8-D15 post-surgery, a time when there are marked signs of degeneration within the affected nerve. The degree of variability case-to-case, in both the behavioural and histological studies, highlights the complexity behind the development of neuropathy. However, the pain-related behaviours documented here are also common in clinical causalgia in humans, making this CCI model of experimental

neuropathy a useful model for investigating the factors influencing the development and maintenance of neuropathic pain.

CHAPTER 4 THE EFFECTS OF VIP/PACAP RECEPTOR ANTAGONISTS ON SENSORY STIMULUS-INDUCED RESPONSES OF DORSAL HORN NEURONES IN NORMAL RATS

4.1 AIM

The purpose of these experiments was to investigate the role of VIP_1, VIP_2 and PACAP receptors in the transmission of sensory information within the spinal cord of rats. Selective antagonists for the three receptor subtypes were ionophoretically applied to the sustained activity of dorsal horn neurones induced by sensory stimuli, and their effects compared. Neuronal activity was induced by (i) innocuous brushing of the cutaneous receptive field or (ii) topical application of the chemical algogen mustard oil.

4.2 METHODS

4.2.1 Animals

As in section 3.2.1.

4.2.2 Surgical Procedure for Electrophysiological Preparation

Details of all reagents can be found in Chapter 2, sections 2.1 and 2.7. Experiments were carried out on 37 male Wistar rats (296g-394g), anaesthetised with intravenous α-chloralose (60mg kg^{-1}) and urethane (1.2g kg^{-1}) following induction with halothane. A tracheotomy was performed to maintain an unobstructed airway throughout the experiment, and the rat was allowed to breathe freely, although oxygen (0.1 $lmin^{-1}$) was passed over the end of the cannula to enrich the inspired air. The animal was mounted in a rigid, stereotaxic frame with its head stabilised using nose and ear bars, and the core body temperature was maintained at 37-38°C by means of a thermostatically controlled heated blanket and rectal probe. Spinal segment L2 was identified by locating the floating rib and the thoraco-lumbar spinal column was then supported using 3 pairs of swan-necked clamps on alternate segments, with the middle set of clamps supporting segment L2. A pool was made with skin flaps around the area of interest and a dorsal laminectomy performed under

X 12.5 magnification to expose segments L1-L4. To improve the stability of the preparation for extracellular recording, agar solution (2% in 0.9% saline solution) at 39°C was injected under the most rostral vertebrae and then poured over the entire pool, including the spinal cord. Once cooled, a core of agar was removed to expose the area of cord from which the recordings would be made, the dura carefully cut and a pool of 37°C liquid paraffin applied to the exposed area to prevent dehydration. Supplementary doses of α-chloralose were given in 0.3ml aliquots as required, with this need being determined by assessment of the animal's blink reflex.

4.2.3 Extracellular Recording and Ionophoresis of Drugs

Extracellular recordings were made from single dorsal horn neurones, using the central barrel (4M NaCl, pH 4.0-4.5) of 7-barrelled glass microelectrodes, with tip sizes 4.0-5.5μm. One side barrel contained 1M NaCl (pH 4.0-4.5) for automatic current balancing using a Neurophore BH2 Ionophoresis System (Medical Systems Corporation, New York). A second side barrel contained 2% Pontamine Sky Blue (PSB) in 0.5M sodium acetate for histological marking of recording sites (see section 4.2.8). The remaining barrels contained the drugs for ionophoresis.

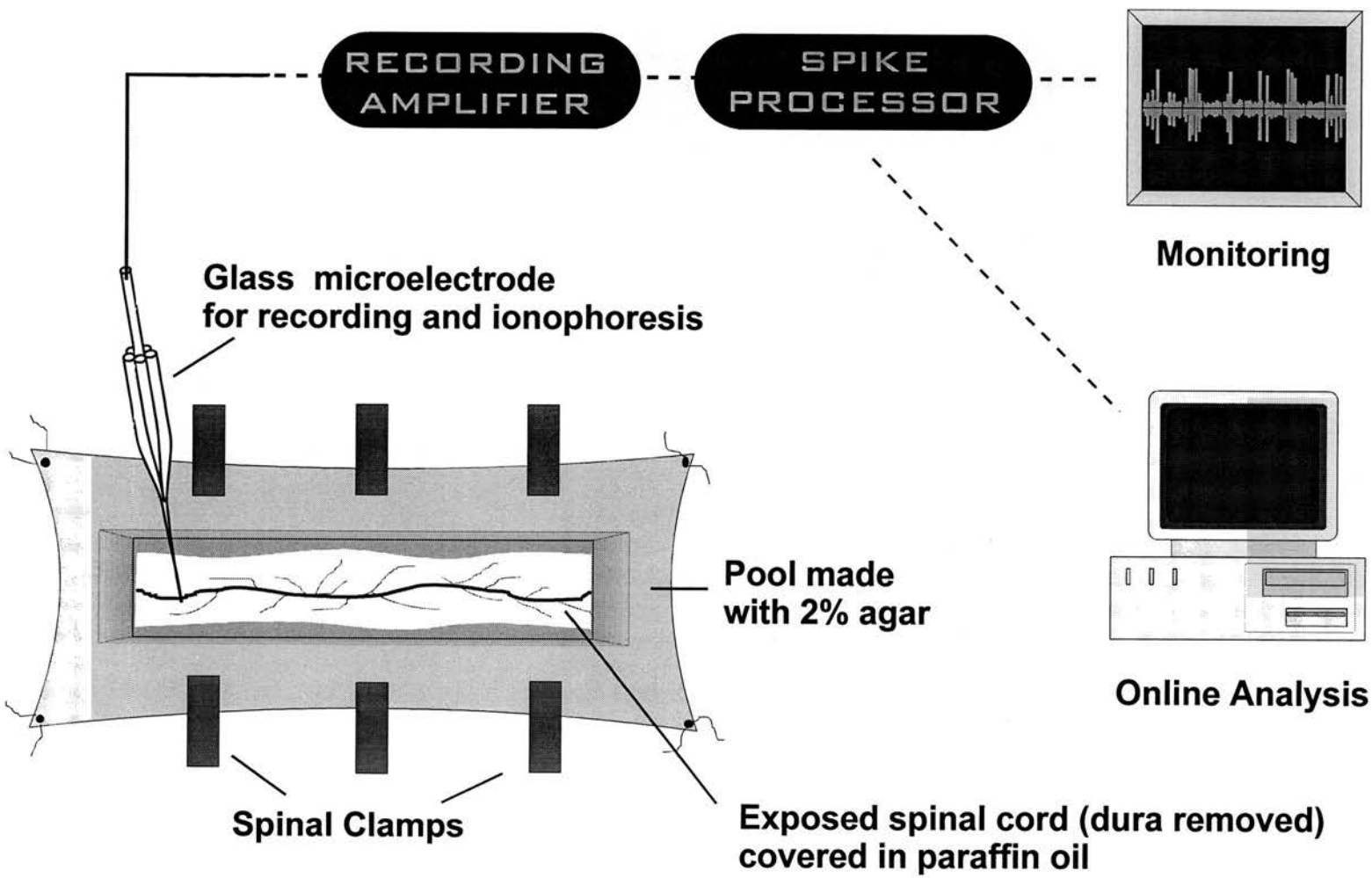
Recordings were made at depths of 0-1000μm from the spinal cord surface (as monitored by the microdrive reading), and cellular activity was observed on an oscilloscope screen (Tektronix). The output activity from the oscilloscope was continually monitored using a D.130 Digitimer Spike Processor, with the spike discriminator adjusted so that the activity of one neurone was isolated (as viewed by markers on the oscilloscope) for counting. Neuronal firing rate was continuously plotted on-line via a customised analysis program (Scap 90; Dr M Dutia, Department of Physiology, University of Edinburgh) on an IBM PS/2-70-121 computer. This program allowed markers to be added as data was collected, while an on-line analogue channel precisely marked changes in ionophoresis currents. (See Figure 4.1 for protocol schematic).

Figure 4.1

Schematic of the Protocol for the Electrophysiological Recording Experiments

Schematic representation of the recording set up for the electrophysiology experiments. The spinal cord vertebrae were stabilised using 3 pairs of swan necked clamps and a laminectomy performed to reveal lumbar segments L1-L4. Skin flaps were used to produce a pool around the area of interest, and this pool was filled with a 2% agar solution to provide stability during the recording. Following the removal of the agar core above the recording area, the dura was removed and the exposed spinal cord covered in 37°C paraffin oil.

Extracellular recordings were made via a 7-barelled glass micro-electrode, and the recording signal amplified 1000 times before being transmitted to a spike processor where the number of action potentials per second were counted. Neuronal firing rate, along with the analogue signal from the ionophoresis barrels, was continuously plotted on-line on an IBM computer. Neuronal activity was monitored on the oscilloscope screen.


RECORDING AMPLIFIER
SPIKE PROCESSOR
Monitoring
Glass microelectrode for recording and ionophoresis
Pool made with 2% agar
Online Analysis
Spinal Clamps
Exposed spinal cord (dura removed) covered in paraffin oil

4.2.4 Drugs for Ionophoresis

All solutions were 0.25mM in distilled water, pH adjusted to 4.5 with hydrochloric acid (HCl):

VIP_1 receptor antagonist: [p-Cl-D-Phe^{6},Leu^{17}]-VIP (Pandol et al. 1986)

General VIP receptor antagonist: VIP (6-28) (Fishbein et al. 1994)

VIP_2 receptor antagonist: (des 1-4)[Glu^{8}, Lys^{12}, Arg^{16}, Nle^{17}, Ala^{19}]VIP(1-24)(lactam 21-25) (**des (1-4) Arg^{16} Ro 25-1553**) (Gourlet and Robberecht - unpublished data)

PACAP receptor antagonist: PACAP (6-38) (Robberecht et al. 1992)

All peptides were ejected using positive currents and a retention current of -12nA was applied to each barrel when not in use. The resistance of the side barrels was monitored regularly and electrodes with resistance values exceeding 45MΩ were rejected. The antagonists were applied during sustained responses to sensory inputs, initially starting at 30nA and increasing to 45 or 60nA, until clear effects were seen. The antagonists were usually ejected for one and a half to two minutes in order to assess any maintained inhibition. If inhibition was present, ionophoresis of the peptide was stopped to look for recovery.

4.2.5 Controls

1M NaCl and pH 4.5 H_20 were ionophoresd from +30 to +60nA for current and vehicle controls respectively. These controls were carried out on both brush- and mustard oil-induced activity.

4.2.6 Identification of Neuronal Receptive Fields

Neuronal receptive fields were initially located by widespread manual brushing of the ipsilateral hind limb, while simultaneously lowering the electrode into the spinal cord using the microdrive. Neurones were then further examined with noxious pinch and heat (48°C, 10 sec). The heat stimulus was applied using a ramp-driven Peltier device (Medical Instruments, Yale University, USA) with a contact area of 1cm^2. The Peltier probe, while in contact with the animal's skin, was raised from a resting temperature of 32°C to 48°C (ramp rate 5°C/sec) for 10 seconds. The neurones were subsequently classified according to their sensory input with non-

nociceptive neurones being those which responded only to light brushing of the hairs of the cutaneous receptive field, while those which responded to brush, noxious pinch, and/or noxious heat inputs were classed as being multireceptive. No noci-specific neurones were investigated in this experiment due to the searching strategy used.

4.2.7 Quantification of Neuronal Responses to Cutaneous Sensory Stimuli

Following identification of the neurones (as described above), sustained sensory stimuli were applied to the cutaneous receptive field. Sustained innocuous inputs were provided by positioning a rotating, motorised brush, on to the centre of the receptive field. Noxious sensory inputs were produced by topical application of the chemical algogen mustard oil, allyl isothiocyanate (8% in paraffin oil).

Mustard oil is a chemical irritant which when administered topically, intra-articularly or intramuscularly will selectively activate C-afferent fibres (Woolf and Wall, 1986). Repeated applications of mustard oil were made to an area of approximately $2cm^2$ covering the identified neuronal receptive field, and after 2-7 applications, separated by 5 minute intervals, a steady elevated firing rate (3-45 Hz) was maintained. This sustained level of C-fibre activation is thought to be analogous to the ongoing C-fibre activity observed following electrical wind-up, and is a useful model to simulate the development of central sensitisation (Woolf and Wall, 1986). "Windup" is the term used to describe the increased responsiveness of a neurone due to repetitive stimulation of C-afferent fibres (Mendell, 1966), and such mechanisms are thought to contribute to the development of prolonged states of nociception (including hyperalgesia and allodynia) (Torebjork et al. 1992) as well as to the development of central hypersensitivity to sensory inputs (Davies and Lodge, 1987)

4.2.8 Histological Identification of Recording Sites

Following the completion of electrophysiological recording in a number of animals, the recording area was marked by ionophoretic ejection of Pontamine Sky Blue (PSB) (2% in 0.5M sodium acetate) to allow the histological identification of electrode placement. PSB was ejected at a current of 10μA for 10-15mins to mark the area of interest. The appropriate spinal cord section was then removed, rostro-

caudally marked then rapidly frozen with an aerosol spot freezing spray and fixed in 10% formal saline.

The tissue was mounted in 0.25% agar solution on a freezing microtome, and sections of 52μm cut. Sections with the blue spot were placed on poly-L-lysine slides and stained with 1% neutral red for light microscopic evaluation of the laminar position of the recording site.

4.2.9 Analysis of Results

Individual records were made for each neurone. Sustained firing was recorded as the action potentials per second (rate) integrated over 1000msec bins, plotted against time. Analysis was carried out off-line using the Scap-90 program. The inhibitory effect of the antagonists on evoked (brush or mustard oil) activity, was assessed by comparing the mean firing levels in the 30 sec period encompassing the greatest inhibition, to the mean evoked activity in the 30 sec period immediately prior to antagonist administration. The change in activity was expressed as a percentage of the control value for each cell, and then pooled to find the mean ± SEM. The raw data was analysed using the paired Student *t*-test to compare drug and pre-drug values. Values of $p \leq 0.05$ were considered significant.

4.3 RESULTS

4.3.1 Characterisation of Neurones

A total of 104 neurones from 37 rats were examined in this study. The vast majority of neurones were within laminae III-V of the dorsal horn, as determined by histological marking with PSB, and corresponding to microdrive electrode depth readings of 200-1000μm from the spinal cord surface. These microdrive readings have also been shown to correlate well with the depth of the electrode tip in the spinal cord in a number of other electrophysiological studies within our laboratory (Munro et al. 1993: Young et al. 1994; 1995b; 1997).

The 'receptive field' was defined as the total area in which a response from an isolated dorsal horn neurone could be elicited, following application of cutaneous sensory stimuli. All of the neurones investigated here responded readily to innocuous brushing of the cutaneous receptive field, and the majority of cells also

displayed nociceptive responses. 31 were classed as non-nociceptive neurones (responding to innocuous brush only) while 73 were classed as multireceptive. No noci-specific neurones were examined in this study. Only neurones with receptive fields on hairy skin were used, to facilitate classification and the reproducibility of responses to mustard oil application.

Prior to stimulation of the neurone by a sensory input, recording was started to determine the basal firing level. All neurones tested displayed a low basal firing rate of 0-1Hz.

4.3.2 Effects of VIP_1, VIP_2 and PACAP Receptor Antagonists on Sustained Neuronal Firing to Innocuous Brush Stimulation in Non-nociceptive Neurones

A total of 31 non-nociceptive neurones from 18 rats was investigated in this study. Neuronal firing was evoked by stimulating the cutaneous receptive field using a motorised, rotating brush. This activity was recorded for one minute before drug application, and control activity was in the range 5-28Hz. The effects of three VIP/PACAP receptor antagonists were assessed (See Figure 4.2): [p-Cl-D-Phe^6,Leu^{17}]-VIP, an antagonist with modest selectivity for the VIP_1 receptor (Usdin et al. 1994), VIP (6-28) which appears to show no preferential affinity to any of the VIP/PACAP receptor subtypes (Fishbein et al. 1994), and PACAP (6-38), a PACAP receptor- and, to a lesser degree, VIP_2 receptor-selective antagonist (Robberecht et al. 1992). All three antagonists were ionophoresed with ejection currents of +30 to +60nA.

No significant change ($p \leq 0.05$; paired Student *t*-test) was observed in neuronal firing for any of the three antagonists following their application to sustained neuronal firing, induced by innocuous brush. [p-Cl-D-Phe^6,Leu^{17}]-VIP showed a mean percentage inhibition of 8 ± 3% (n=14), VIP(6-28) exhibited 8 ± 5% inhibition (n=8), while PACAP(6-38) had a mean percentage inhibition of 9 ± 4% (n=11).

4.3.3 Effects of VIP_1, VIP_2 and PACAP Receptor Antagonists on Sustained Neuronal Firing of Multireceptive Neurones Induced by Brush or Mustard Oil

A total of 73 neurones from 32 rats were studied in this investigation, and as far as possible paired brush- and mustard oil-induced responses from the same neurone were investigated. Sustained neuronal firing was induced either by (i) stimulation of the cutaneous receptive field using a motorised, rotating brush, or (ii) topical application of the chemical algogen mustard oil. One minute of this evoked activity was recorded before applying any compounds. Four antagonists were assessed: [p-Cl-D-Phe^6,Leu^{17}]-VIP, VIP (6-28), PACAP (6-38), and the novel VIP_2 selective antagonist des(1-4) Arg^{16} Ro 25-1553 (Gourlet and Robberecht - unpublished data). All four antagonists were ionophoresed at ejection currents of +30 to +65nA.

Control, brush-induced activity (4-33Hz) was significantly inhibited by ionophoretic application of [p-Cl-D-Phe^6,Leu^{17}]-VIP by 56 ± 6 % (n=18), VIP (6-28) by 66 ± 5% (n=14) and PACAP (6-38) by 51 ± 8% (n=23)(mean ± SEM; $p \leq 0.01$, paired Student *t*-test on raw data). Recovery was observed in all cases, with the majority of cells returning to control firing levels within approximately 90sec. However, ionophoresis of the VIP_2 receptor selective antagonist, in the same current range, failed to inhibit such firing (n=10). Figure 4.3 shows typical examples.
In contrast, all four VIP/PACAP receptor antagonists significantly inhibited mustard oil-induced activity (3-45Hz) of dorsal horn neurones (see Figure 4.4). [p-Cl-D-Phe^6,Leu^{17}]-VIP showed 64 ± 8% inhibition (n=10), VIP(6-28) showed 69 ± 9% inhibition (n=10), PACAP (6-38) showed an inhibition of 63 ± 8%(n=12), while des(1-4) Arg^{16} Ro 25-1553 inhibited mustard oil-induced activity by 36 ± 7% (n=10). Again, the activity of all neurones returned to control levels within approximately 2 minutes of terminating drug application.

4.3.4 Current and Vehicle Controls

Ionophoresis of vehicle (pH 4.5 H_2O) or Na+ ions, in the range +30 to +60nA, had no detectable effect on the brush or mustard-oil induced activity of those neurones tested.

Figure 4.5 shows a summary histogram of the antagonist effects with respect to vehicle control.

Figure 4.2

Effects of ionophoretically-applied VIP/PACAP receptor antagonists on sustained brush-evoked responses of non-nociceptive dorsal horn neurones

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Parts (a), (b) and (c) show the generally-observed lack of effect of [p-Cl-D-Phe^{6},Leu^{17}]-VIP, VIP (6-28) and PACAP (6-38) respectively, when applied to the sustained innocuous brush-evoked activity of dorsal horn neurones. Each trace is from a different neurone and is entirely typical of the results obtained in the overall population sampled here.

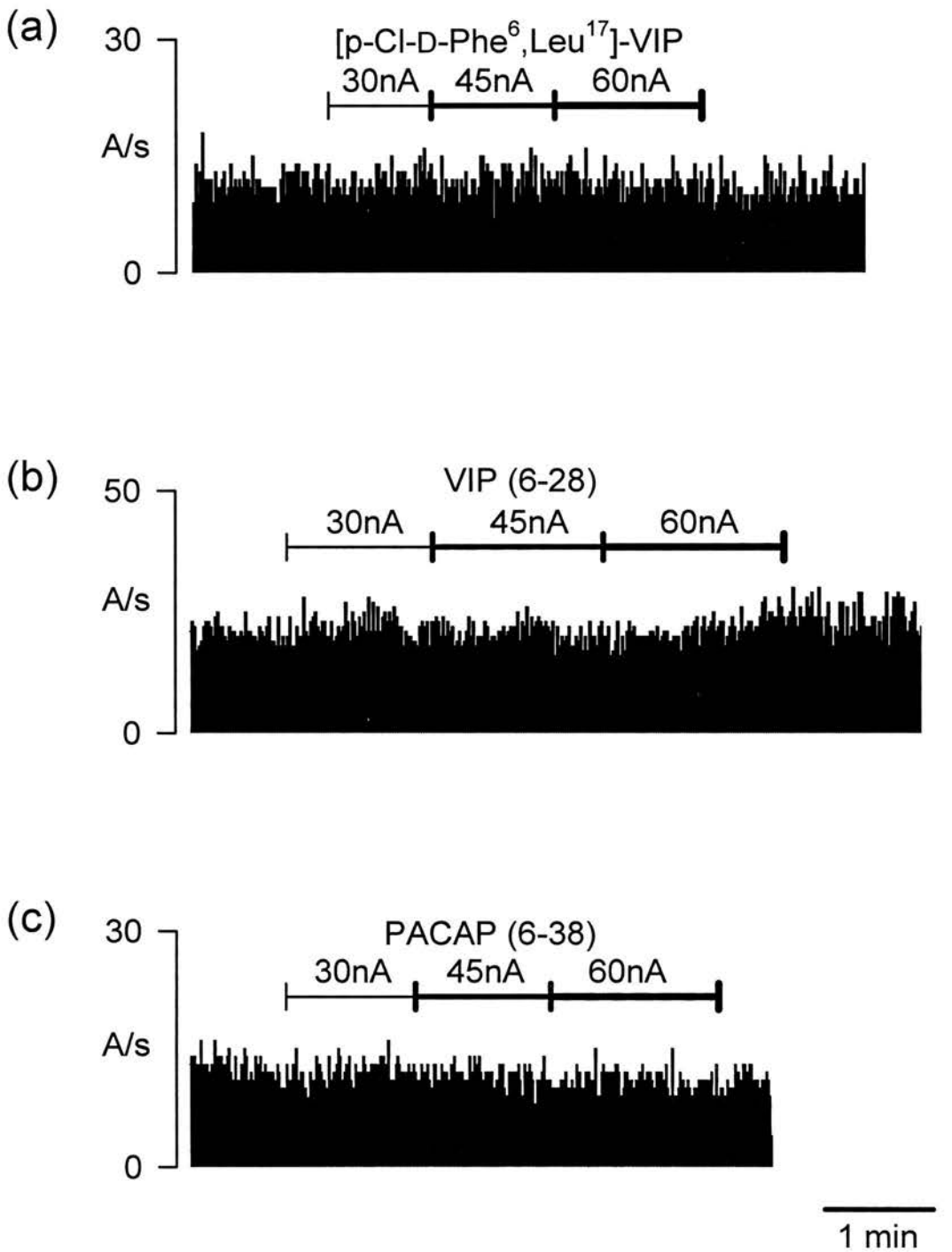

(a)
30
A/s
0
[p-Cl-D-Phe6,Leu17]-VIP
30nA
45nA
60nA
(b)
50
A/s
0
VIP (6-28)
30nA
45nA
60nA
(c)
30
A/s
0
PACAP (6-38)
30nA
45nA
60nA
1 min

Figure 4.3

Effects of ionophoretically-applied VIP/PACAP receptor antagonists on sustained brush-evoked responses of multireceptive dorsal horn neurones

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Parts (a), (b) and (c) show the marked inhibitory effects of [p-Cl-D-Phe^{6},Leu^{17}]-VIP, VIP (6-28) and PACAP (6-38) respectively, when applied to the sustained neuronal firing evoked by innocuous brushing of the cutaneous receptive field. Part (d) shows the generally-observed lack of effect of des(1-4) Arg^{16} Ro 25-1553 on brush-induced activity. These results are entirely typical of those obtained in the overall population sampled here.

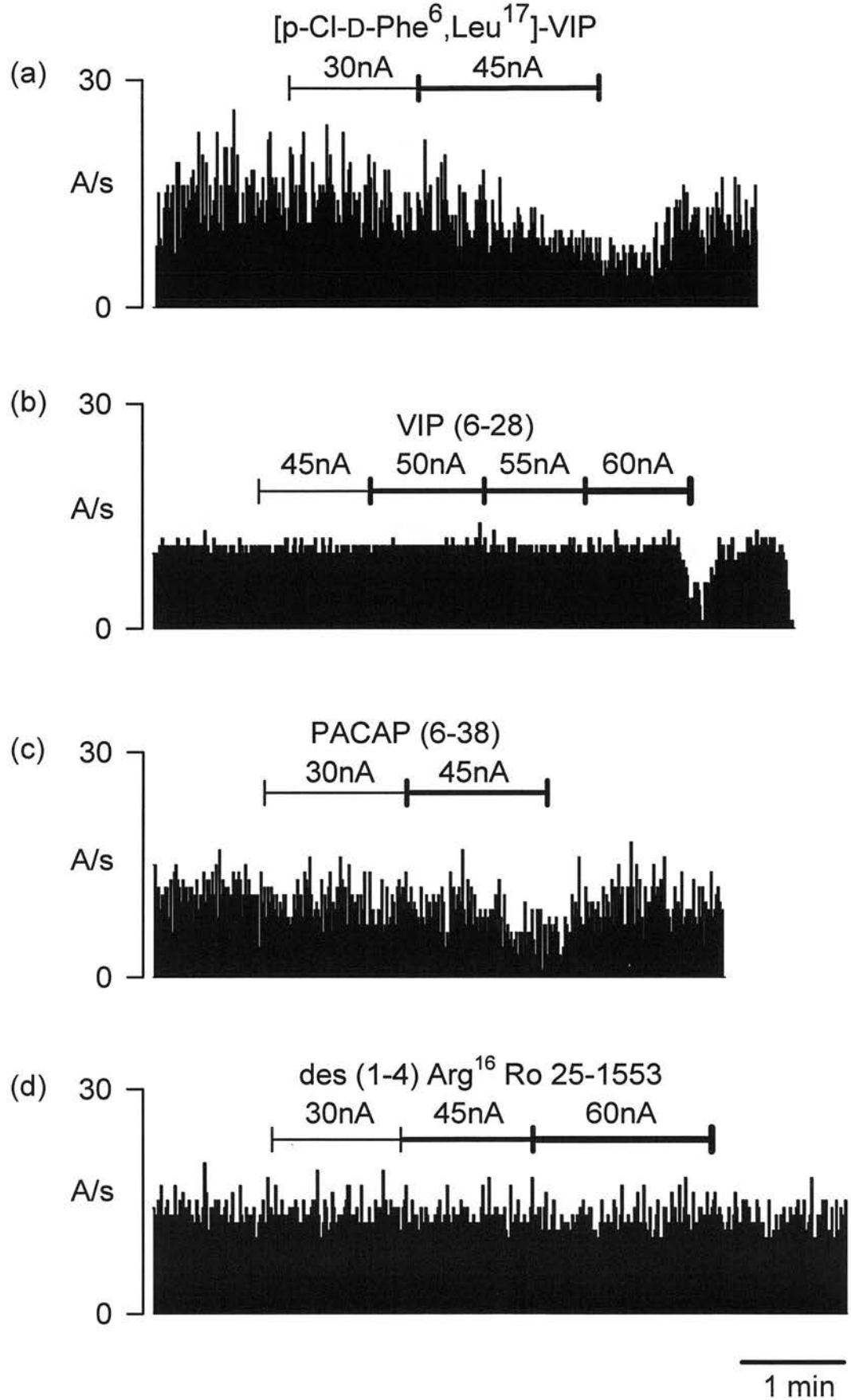

[p-Cl-D-Phe6,Leu17]-VIP
30nA
45nA
(a)
30
A/s
0
(b)
VIP (6-28)
45nA
50nA
55nA
60nA
30
A/s
0
(c)
PACAP (6-38)
30nA
45nA
30
A/s
0
(d)
des (1-4) Arg16 Ro 25-1553
30nA
45nA
60nA
30
A/s
0
1 min

Figure 4.4

Effects of ionophoretically-applied VIP/PACAP receptor antagonists on sustained mustard oil-induced activity of multireceptive dorsal horn neurones

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Parts (a), (b), (c) and (d) show the marked inhibitory effects of [p-Cl-D-Phe^{6},Leu^{17}]-VIP, VIP (6-28), PACAP (6-38) and des(1-4) Arg^{16} Ro 25-1553 respectively, when applied to the sustained neuronal firing evoked by noxious mustard oil application to the cutaneous receptive field. All examples show a significant inhibition of neuronal firing during antagonist application, with recovery to pre-drug control levels within approximately 1 minute of terminating antagonist application.

These traces represent paired responses from the neurones used in Figure 4.3; that is, both brush and mustard oil responses were tested in the same neurone for each antagonist. These results are entirely typical of those obtained in the overall population sampled here.

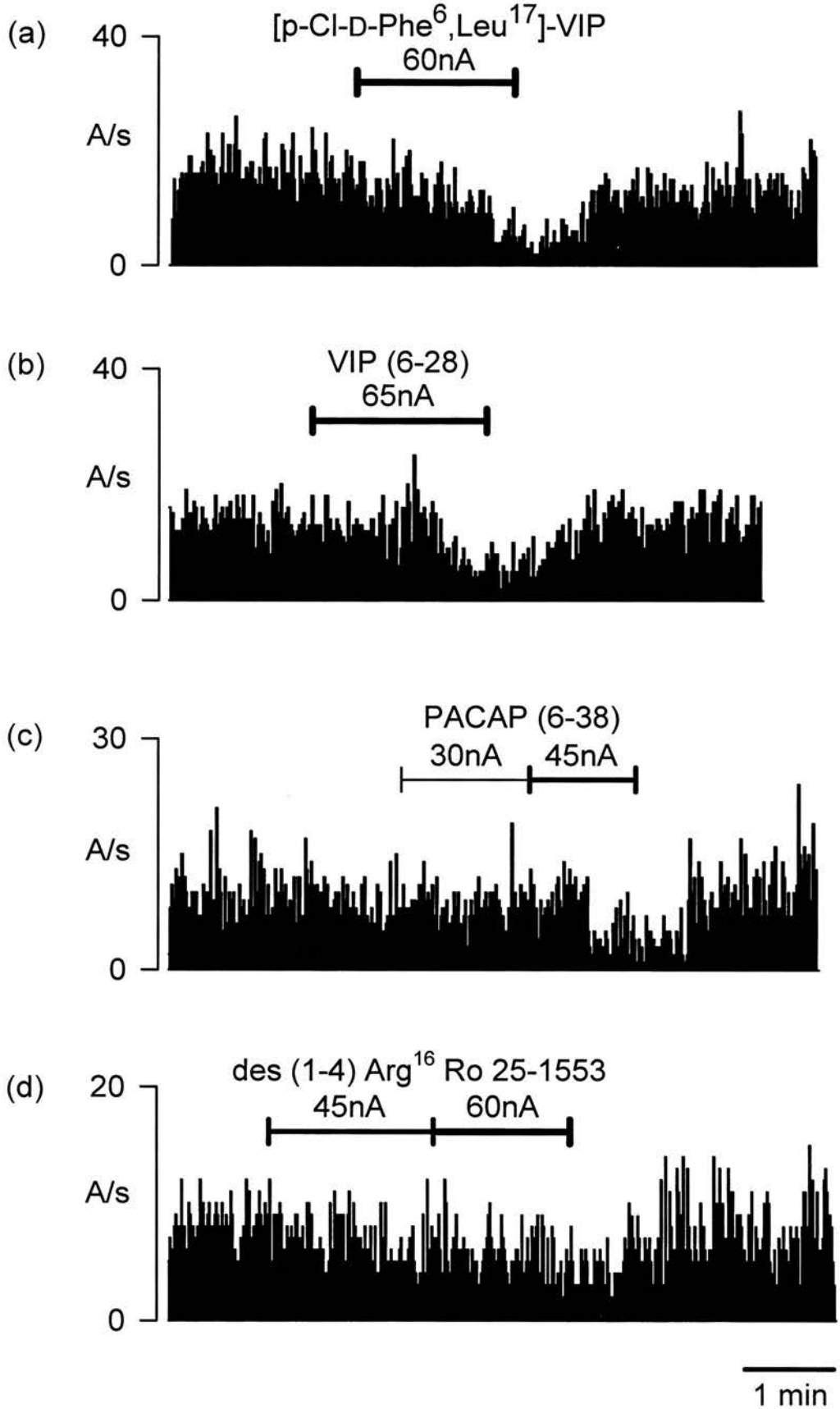
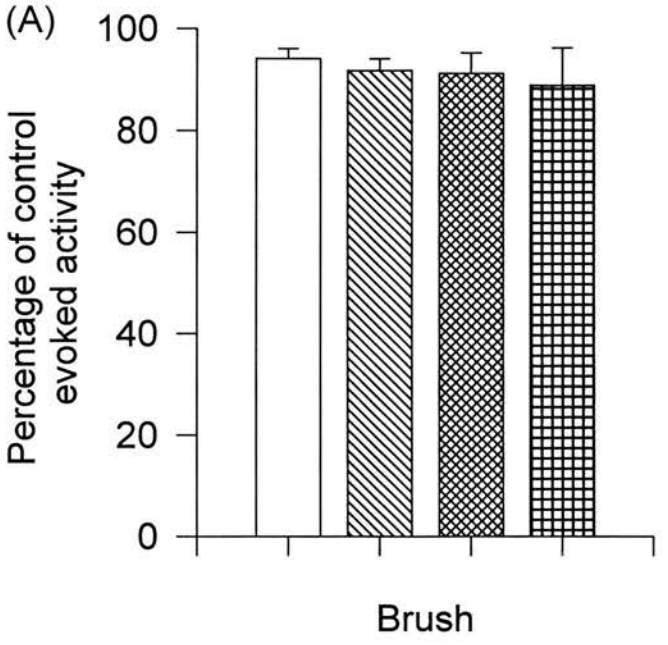

(a)
40
A/s
0
[p-Cl-D-Phe6,Leu17]-VIP
60nA
(b)
40
A/s
0
VIP (6-28)
65nA
(c)
30
A/s
0
PACAP (6-38)
30nA
45nA
(d)
20
A/s
0
des (1-4) Arg16 Ro 25-1553
45nA
60nA
1 min

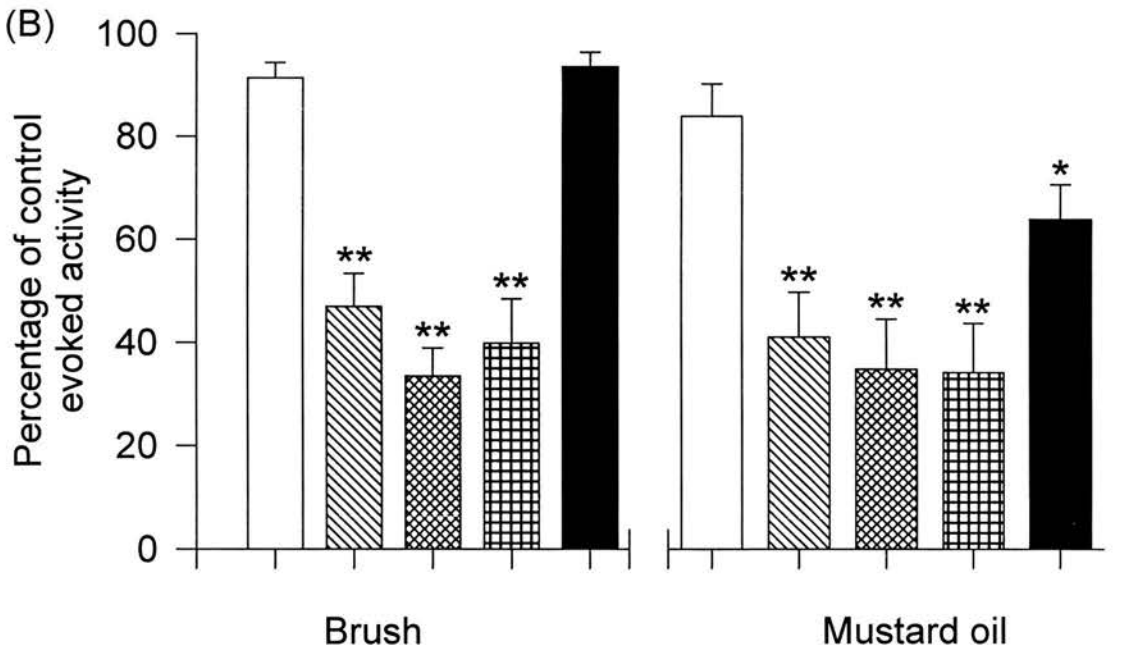
Figure 4.5

Summary histogram showing the overall effects of ionophoretically applied VIP/PACAP receptor antagonists on sustained sensory-induced responses of dorsal horn neurones

Summary histogram showing the mean percentage inhibition of the control neuronal firing rate of (A) non-nociceptive and (B) multireceptive dorsal horn neurones, in comparison with control vehicle application (pH4.5 H_20) ☐ Responses in the presence of ▨ [p-Cl-D-Phe^6,Leu^{17}]-VIP, ▩ VIP (6-28), ▦ PACAP (6-38) and ■ des(1-4) Arg^{16} Ro 25-1553 are shown.

The three antagonists tested showed negligible effects when applied to sustained neuronal activity of non-nociceptive neurones (A). Both brush and mustard oil-induced activity was significantly inhibited (paired student *t*-test on raw data; ** $p \leq 0.01$) by [p-Cl-D-Phe^6,Leu^{17}]-VIP, VIP (6-28) and PACAP (6-38), while only mustard oil-induced activity was inhibited by des(1-4) Arg^{16} Ro 25-1553 (* $p \leq 0.05$).


(A)
100
80
60
40
20
0
Percentage of control evoked activity
Brush


(B)
100
80
60
40
20
0
Percentage of control evoked activity
**
**
**
**
**
**
*
Brush
Mustard oil

4.4 DISCUSSION

VIP and PACAP are recognised by a family of three receptors, namely the VIP_1, VIP_2 and PACAP receptors. Binding sites for VIP and PACAP ligands have been shown to be present in the rat spinal cord, particularly in the lumbar and sacral segments, with the highest concentration in laminae I and II of the dorsal horn (Kar and Quirion 1995; Moller et al. 1993; Yashpal et al. 1991). The mRNA for VIP_1 (Ishihara et al. 1992), VIP_2 (Sheward et al. 1995) and PACAP receptors (Arimura and Shioda, 1995) is also expressed in spinal cord (particularly in the dorsal horn). These receptors appear to have distinct distributions throughout the peripheral and central nervous systems (Arimura and Shioda, 1995; Sheward et al. 1995; Usdin et al. 1994). This may suggest that the different receptor subtypes may each play specific functional roles within the CNS. The development of novel selective antagonists for the VIP_1, VIP_2 and PACAP receptors, has allowed us to address the role of each receptor subtype in the modulation of sensory processing within the spinal dorsal horn of normal animals.

The population of neurones investigated in this study comprised both non-nociceptive and multireceptive cells. From these studies it appears that there is a clear correlation between the type of dorsal horn neurone and the effect mediated by VIP/PACAP receptor antagonists in normal animals. None of the antagonists used here showed any inhibitory effects on the brush-induced activation of non-nociceptive dorsal horn neurones. It is not altogether surprising that the antagonists had little or no effect on neurones with a non-noxious input, i.e. those innervated by the larger Aβ fibres, as histological evidence so far has limited the presence of these peptides to small diameter nerve fibres (Fuji et al. 1983; Gibson et al. 1981; Staun-Olsen et al. 1985).

In contrast, all of the antagonists tested showed marked inhibition of at least one aspect of sensory stimulation of multireceptive neurones. This would suggest that VIP and PACAP may be largely restricted to modulating neuronal pathways concerned with the regulation of C-fibre-mediated transmission, and highlights the possibility that VIP/PACAP receptors may be important modulators in the transmission of nociceptive information.

Nociceptive afferents, including C-fibres, terminate in the superficial layers of the spinal dorsal horn (Cervero and Iggo, 1980), and previous evidence has already demonstrated that both VIP and PACAP are found in small diameter fibres (Fuji et al. 1983; Gibson et al. 1981; Staun-Olsen et al. 1985), inferred to be the Aδ- and C-fibres associated with polymodal and mechanical nociceptors. The majority of VIP- and PACAP-ir fibres are present in laminae I and II of the rat spinal dorsal horn (Moller et al. 1993; Yaksh et al. 1982), with additional fibres being found around the central canal and in lamina VII. These histological findings alone provide grounds for suggesting a role for these peptides in the control of sensory transmission, the present antagonist study has now provided physiological evidence.

Despite the fact that VIP and PACAP are present in relatively low abundance under normal circumstances (Noguchi et al. 1993; Mulder et al. 1994), the present results provide clear evidence for the presence of VIP/PACAP receptor subtypes on dorsal horn neurones under normal conditions. From these data we have shown that the activity of multireceptive neurones is clearly mediated or promoted, at least in part, by VIP/PACAP receptors. Variations in the effects of the antagonists for each of the three receptor subtypes, suggest differential roles for VIP_1, VIP_2 and PACAP receptors.

Antagonists selective for the VIP_1 and PACAP receptor subtypes showed a marked inhibition of the neuronal firing induced by either innocuous brush or mustard oil application, to a similar extent in each case. This non-selective effect, suggests that VIP_1 and PACAP receptors may exert a generalised modulation of multireceptive dorsal horn neurone responses in normal animals.

In contrast, the novel VIP_2 receptor selective antagonist examined here, appears to exert a much more specific regulatory role, showing negligible effects on brush-induced activity, while markedly inhibiting the cell firing induced by topical application of the chemical irritant mustard oil. This would imply that VIP_2 receptors may play a specific role in the modulation/mediation of nociceptive C-fibre dependent inputs, therefore implicating the VIP_2 receptor as a potential site to be targeted by novel analgesics.

Functional studies have already provided evidence for a regulatory role of VIP and PACAP in the transmission of nociceptive information at the spinal cord

level. Intrathecal administration of VIP is known to facilitate thermal but not mechanical nociceptive reflexes in the rat (Cridland and Henry, 1988; Wiesenfeld-Hallin, 1987), while an intrathecal injection of PACAP-38 has been shown to produce thermal hyperalgesia in the mouse (Narita et al. 1996).

The functional role of VIP is thought to become more apparent following nerve injury however, when the levels of this peptide are greatly increased within the spinal cord. Wiesenfeld-Hallin et al. (1991), demonstrated that in decerebrate, spinalised, unanaesthetised rats with an intact sciatic nerve, the SP antagonist Spantide II attenuated facilitation of the flexor reflex induced by either intrathecal application of SP or by a brief conditioning electrical stimulus, while the VIP antagonist (Ac-Tyr^1, D-Phe^2)-GRF (1-29)-NH_2 appeared to be ineffective. Following axotomy however, intrathecal application of (Ac-Tyr^1, D-Phe^2)-GRF (1-29)-NH_2 was shown to antagonise the conditioning induced facilitation of the flexor reflex, while the SP antagonist showed no such effect. So it would appear that after peripheral nerve section, the role of excitatory mediator of flexor reflex facilitation, is transferred from SP to VIP. This may reflect a transfer from SP to VIP as the most functionally important neuropeptide of C afferents following nerve injury, paralleling the decrease in SP and marked increase in VIP levels, that is observed within the spinal dorsal horn and small dorsal root ganglion cells (Hokfelt et al. 1994).

The role of PACAP in normal animals is a little less clear. The reported effects of intrathecal PACAP on spinal nociceptive reflexes are somewhat variable from different groups of investigators. Zhang et al. (1993) found PACAP-27 and PACAP-38 (0.63 -5pmol) to have anti-nociceptive effects on the C-fibre mediated flexion reflex, when given i.p. In addition they also found intrathecal PACAP-27 (up to 5pmol) to cause a dose-dependent suppression of formalin-induced (5% formalin solution s.c.) pain related behaviours (Zhang et al. 1996). However, even very high doses of PACAP-27 (approximately 15.5 nmol) could not completely abolish the response to formalin, and the high doses used appeared to cause some motor effects, as shown using the placing/stepping reflex (Yamamoto and Tatsuno, 1995). On the other hand, Weisenfeld-Hallin et al. (1996) have reported PACAP-27 to cause a dose-dependent facilitation of the flexor reflex, in decerebrate, spinalised rats at relatively low doses (10ng-10μg), and as stated previously, a single intrathecal

injection of PACAP-38 (0.05μg) can induce hyperalgesia in the mouse (Narita et al. 1996). The data obtained in our study are more consistent with the latter observations, as antagonists selective for the PACAP receptor were shown to have inhibitory effects on the transmission of sensory information within the dorsal spinal cord. We can only suggest that the variations in the results provided by Zhang et al. may be due to variations in the preparation, recording techniques and of course the high doses of PACAP used. In addition, it is important to consider that PACAP-38 and PACAP-27 are potent agonists for VIP_1 and VIP_2 receptors as well as the PACAP receptor.

The firing rate of the majority of neurones tested returned to control levels almost immediately the antagonist application was terminated. This suggests that the VIP/PACAP receptors may be expressed on the recorded neurones themselves, and that VIP and PACAP may act directly on dorsal horn neurones. However, we can not rule out the possibility that the peptides may be acting indirectly via neighbouring interneurones to regulate the recording neurones. The receptors may well be expressed on other generally excitatory neurones, or the peptides may even act via a cascade of inhibitory interneurones to exert a disinhibitory influence.

Our results further support the previous evidence that VIP/PACAP receptors may play important physiological roles as neurotransmitters within the CNS. From this study it is apparent that VIP/PACAP receptor antagonists have the ability to act selectively on the normal reception of sensory inputs by multireceptive neurones, and so have the potential to play important neurotransmitter roles at the spinal cord level. The receptor selectivity of the antagonists used, provides new insights into the type of VIP/PACAP receptors functionally important in regulating the transmission of sensory information by dorsal horn neurones, in normal rats.

It appears from these data, that the vast majority of multireceptive dorsal horn neurones are regulated by VIP_1, VIP_2 and PACAP receptors to some degree. The selective inhibitory effect of des (1-4) Arg^{16} Ro 25-1553 on mustard oil-induced activity of dorsal horn neurones, highlights a potentially important role for the VIP_2 receptor in the transmission of nociceptive sensory information in normal rats. This predicts that high affinity VIP_2 receptor antagonists could act as selective analgesics, particularly reducing C-fibre mediated pain. In contrast, the VIP_1 and PACAP

receptor antagonists are rather non-selective inhibitors of sensory inputs and so appear to be less promising as drug candidates in normal pain states.

CHAPTER 5 THE EFFECTS OF VIP/PACAP RECEPTOR ANTAGONISTS ON SENSORY STIMULUS-INDUCED RESPONSES OF DORSAL HORN NEURONES IN NEUROPATHIC RATS

5.1 AIM

The purpose of these experiments was to investigate the role of VIP_1, VIP_2 and PACAP receptors in the transmission of sensory information within the spinal cord of neuropathic rats. Selective antagonists for the three receptor subtypes, were ionophoretically applied to the sustained activity of rat spinal dorsal horn neurones, 2-3 weeks following sciatic nerve injury. Neuronal activity was induced by (i) light brushing of the cutaneous receptive field, (ii) cold stimulation via a thermal Peltier probe or (iii) topical application of the chemical algogen mustard oil.

5.2 METHODS

5.2.1 Animals

As in section 3.2.1

5.2.2 Surgical Preparation of Animals with an Experimental Peripheral Mononeuropathy

The animals used in this study underwent a unilateral hindlimb chronic constriction injury (CCI) as outlined in section 3.2.2. Only nerve-injured animals which showed strong signs of mechanical hyperalgesia and cold and mechanical allodynia (as determined by the behavioural testing (see section 3.2.3)) were used for electrophysiological recordings (n=33). All the animals used for electrophysiological recordings displayed an average suspended paw elevation time (SPET) of 8 seconds or more to innocuous cold stimulation on the day of recording, while the average withdrawal threshold to von Frey filaments was 2.7 ± 0.5g (compared to mean pre-surgery values of 53.9 ± 8.0g). In addition, the CCI animals displayed an exaggerated response to pin prick in the ipsilateral foot. That is they held their affected paw in the air for over 5 seconds following the noxious pin prick stimulus (grade 3 response). All contralateral responses were instantaneous, short-lived paw

flicks (grade 1 response), as were responses to pin prick challenges in normal animals

5.2.3 General Methods

Regarding surgical procedures, electrophysiological recording techniques, quantification of neuronal responses to evoked activity, histological identification of recording sites and analysis of results, please refer to section 4.2.

In addition to the sustained brush- and mustard oil-induced responses, pulses of potentially noxious cold inputs were applied by means of a ramp-driven Peltier device, with a contact area of $1cm^2$. The Peltier probe was placed in contact with the receptive field located on the hind limb of the animal; the probe was then periodically cooled from a base temperature of 32°C (ramp rate 5°C/sec) and maintained at the test temperature of approximately 5°C for 10 second intervals. The stimuli were applied every 2 minutes to give reproducible peaks of evoked activity (5.3-28.3 Hz), and the probe was maintained at 32°C in between each test. Because of the permanent contact between the probe and the receptive field, the neurones displayed a slightly higher basal firing rate than during the other tests (1.2-5.8 Hz), which was subtracted from the test firing rates during analysis. Where possible, all three sensory inputs were tested on the same neurone. This was the case for the majority of neurones tested. However in a small number only two of the three tests were carried out.

5.2.4 Drugs for Ionophoresis

All drug solutions were 0.25mM in distilled water, pH 4.5, and drugs were ejected using positive currents:

VIP_1 receptor antagonists: [p-Cl-D-Phe^6,Leu^{17}]-VIP (Pandol et al. 1986) and [Ac-His^1, D-Phe^2, Lys^{15}, Arg^{16}, Leu^{17}]VIP (3-7)GRF (8-27): PG 97-269 (Gourlet et al. 1997(c))

VIP_2 receptor antagonist: des (1-4) Arg^{16} Ro 25-1553 (Gourlet and Robberecht - unpublished data)

PACAP receptor antagonist: PACAP (6-38) (Robberecht et al. 1992)

5.2.5 Controls

1M NaCl and pH 4.5 H_20 were ionophoresed from +30 to +60nA for current and vehicle controls respectively. These controls were carried out on brush-, cold- and mustard oil-induced activity.

5.3 Results

5.3.1 Characterisation of Neurones

These results are based on data obtained from a total of 36 neurones in 33 neuropathic animals which had undergone sciatic nerve ligation 12-18 days previously. Extracellular recordings were made from single neurones located in the dorsal horn ipsilateral to the nerve injury, and in segments L4-L5. Microelectrode depths ranged from 200-1000μm from the surface of the spinal cord, corresponding to laminae III-V of the dorsal horn. All neurones tested displayed low basal firing rates of 0-1Hz.

Only truly multireceptive neurones were studied in this investigation, i.e. those neurones which responded strongly to light brushing, noxious pinch and/or noxious heat inputs to the cutaneous receptive field. No noci-specific or non-nociceptive neurones were investigated in these experiments. In general, the neurones investigated had receptive fields on the lower part of the ipsilateral limb (ankle and hairy foot) corresponding to the area innervated by the constricted part of the sciatic nerve (Schmalbruch, 1986). To facilitate classification and the reproducibility of responses to mustard oil application, only neurones with receptive fields on hairy skin were used.

5.3.2 Effects of VIP_1, VIP_2 and PACAP Receptor Antagonists on Sustained Neuronal Firing Induced by Brush, Cold or Mustard Oil Application

Neuronal firing was induced by stimulating the cutaneous receptive field using (i) a motorised, rotating brush, (ii) a thermally-maintained Peltier device (to produce approximately 10 second pulses of activity) or (iii) topical application of the chemical algogen mustard oil. Generally, one minute of this activity was recorded before application of the antagonists was commenced, or in the case of the cold

stimulus, at least two consecutive control pulses of similar magnitude. The antagonist effects were then assessed with respect to control, pre-drug firing rates.

(i) Brush-induced Activity

All four antagonists showed negligible effects when applied to the sustained-brush-evoked activity of dorsal horn neurones, ipsilateral to the nerve injury in CCI animals: [p-Cl-D-Phe^6,Leu^{17}]-VIP displayed 11 ± 3% inhibition (n=13), PG 97-269 inhibited brush-evoked activity by 10 ± 4% (n=7), des(1-4) Arg^{16} Ro 25-1553 displayed 6 ± 3% inhibition (n=10), while the mean percentage inhibition for PACAP (6-38) was 14 ± 7% (n=14) (See Figure 5.1).

(ii) Cold-induced Activity

Over half (56%) of the dorsal horn neurones tested responded strongly to a cold stimulus of approximately 5°C when applied to the receptive field via a Peltier device. There was some variability in the firing responses of the dorsal horn neurones tested, with some cells being more susceptible to the decreasing temperature while others had a greater firing activity during the sustained cold response. However, all neurones used produced consistent responses following each cold stimulus, giving reproducible peaks of activity of 5.3-28.3 Hz. A small proportion (9%) of cells showed only transient responses to the cold stimulus, while the remaining 35% appeared to have no response. The VIP_1 and PACAP receptor antagonists showed significant inhibitory effects of a similar magnitude when applied to the repeated cold-evoked activity. The VIP_1 antagonists [p-Cl-D-Phe^6,Leu^{17}]-VIP and PG 97-269 reduced the control firing rates by 25 ± 4% (n=10) and 25 ± 6% (n=7) respectively (see Figure 5.2a), while PACAP (6-38) inhibited the cold response by an average of 27 ± 8% (n=8) (Figure 5.2b). The selective VIP_2 receptor antagonist, des(1-4) Arg^{16} Ro 25-1553 however, showed little effect on cold-induced activity of dorsal horn neurones (12 ± 4% (n=10)) as shown in Figure 5.2c.

(iii) Mustard oil-induced Activity

Application of [p-Cl-D-Phe^6,Leu^{17}]-VIP or PG 97-269 significantly inhibited mustard oil-induced activity by 26 ± 6% (n=9) and 30 ± 6% (n=13) respectively.

Des(1-4) Arg^{16} Ro 25-1553 and PACAP (6-38) were slightly more effective, displaying mean percentage inhibitions of 44 ± 5% (n=12) and 40 ± 6% (n=12) respectively. See Figure 5.3.

Figure 5.4 is a summary histogram of the results, indicating the overall effects of all four peptide receptor antagonists on the stimulus-evoked activity of dorsal horn neurones in neuropathic animals.

Figure 5.1

Effects of ionophoretically-applied VIP/PACAP receptor antagonists on sustained brush-evoked responses of dorsal horn neurones in CCI animals

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

These traces show the generally-observed lack of effect of ionophoretic application of (a) [p-Cl-D-Phe^{6},Leu^{17}]-VIP, (b) PG 97-269 (a selective VIP_1 receptor antagonist), (c) des(1-4) Arg^{16} Ro 25-1553 (a selective VIP_2 receptor antagonist) and PACAP (6-38) on the sustained neuronal firing induced by a motorised rotating brush. These results are entirely typical of the whole population of neurones tested.

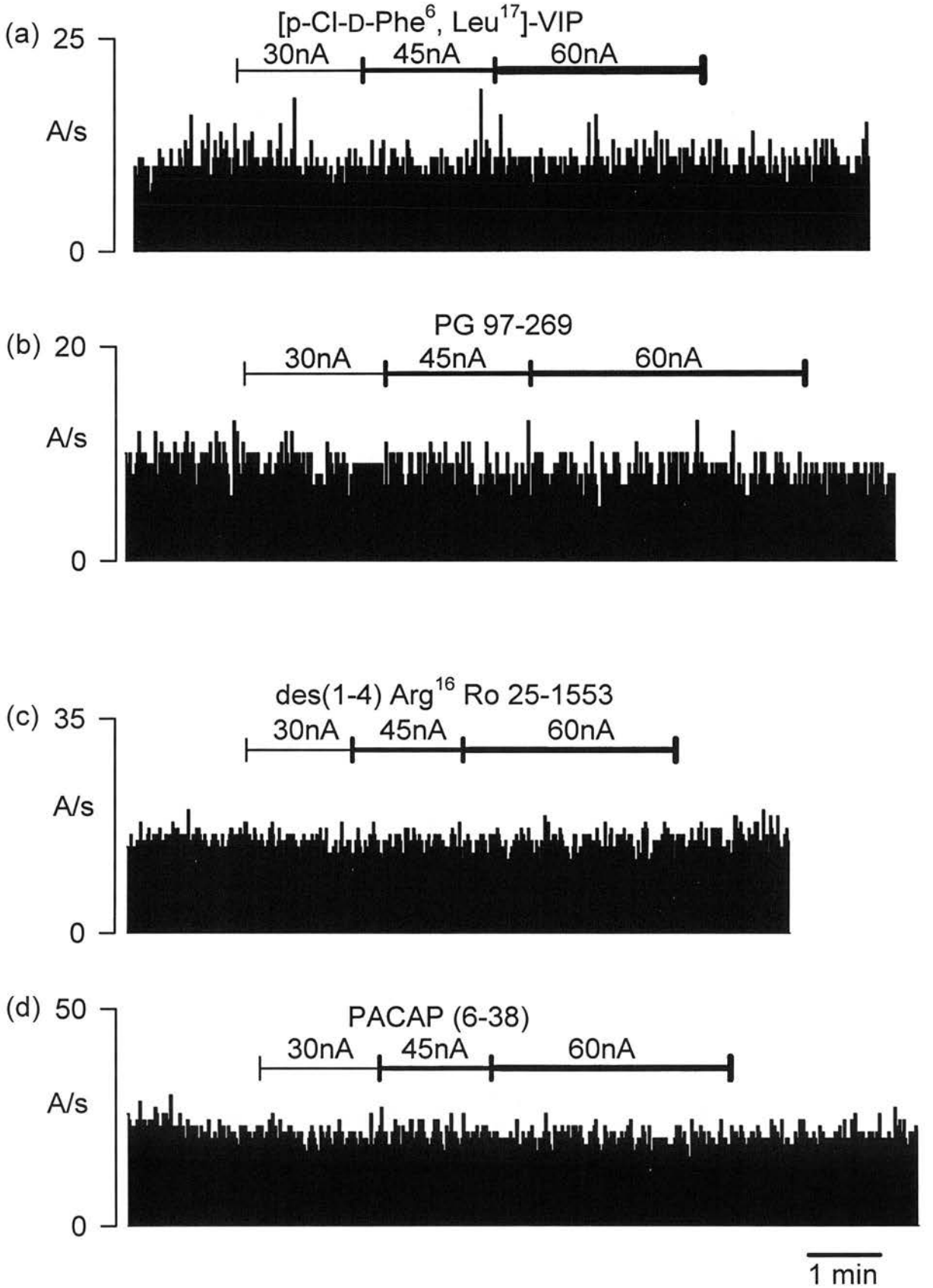

(a) 25
[p-Cl-D-Phe6, Leu17]-VIP
30nA
45nA
60nA
A/s
0
(b) 20
PG 97-269
30nA
45nA
60nA
A/s
0
(c) 35
des(1-4) Arg16 Ro 25-1553
30nA
45nA
60nA
A/s
0
(d) 50
PACAP (6-38)
30nA
45nA
60nA
A/s
0
1 min

Figure 5.2a

Effects of ionophoretically-applied VIP_1 receptor antagonists on cold-induced neuronal firing of dorsal horn neurones in CCI animals

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Parts (a)(i) and (ii) show the typical inhibitory effects of the VIP_1 receptor antagonists [p-Cl-D-Phe^6,Leu^{17}]-VIP and PG 97-269 respectively, on 10 second pulses of neuronal activity induced by repeated stimulation of the cutaneous receptive field by a cold Peltier probe at 5°C (▮). The x-axis breaks represent the 8 and 5 minute recovery periods required for neuronal firing rates to return to pre-drug control levels for [p-Cl-D-Phe^6,Leu^{17}]-VIP and PG 97-269 respectively. These results are entirely typical for the population of cells tested in this study.

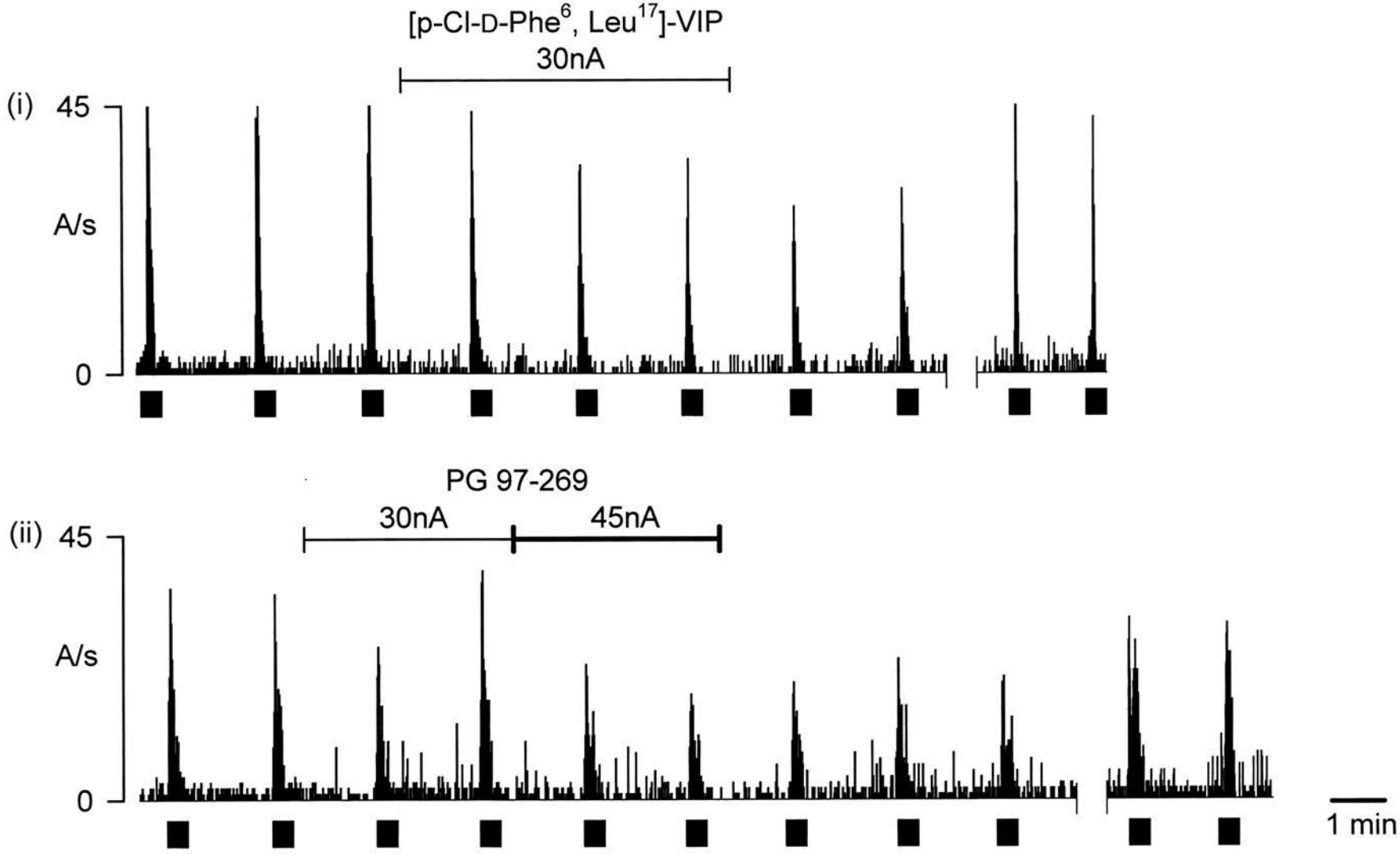

[p-Cl-D-Phe6, Leu17]-VIP
30nA
(i) 45
A/s
0
PG 97-269
30nA
45nA
(ii) 45
A/s
0
1 min

Figure 5.2b

Effects of ionophoretically-applied PACAP receptor antagonist on cold-induced neuronal firing of dorsal horn neurones in CCI animals

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Part (b)shows the marked inhibitory effect of ionophoretic application of PACAP (6-38) on the 10 second pulses of neuronal activity induced by repeated stimulation of the cutaneous receptive field by a cold Peltier probe at 5°C (■). These results are typical of those seen for the whole population sampled.

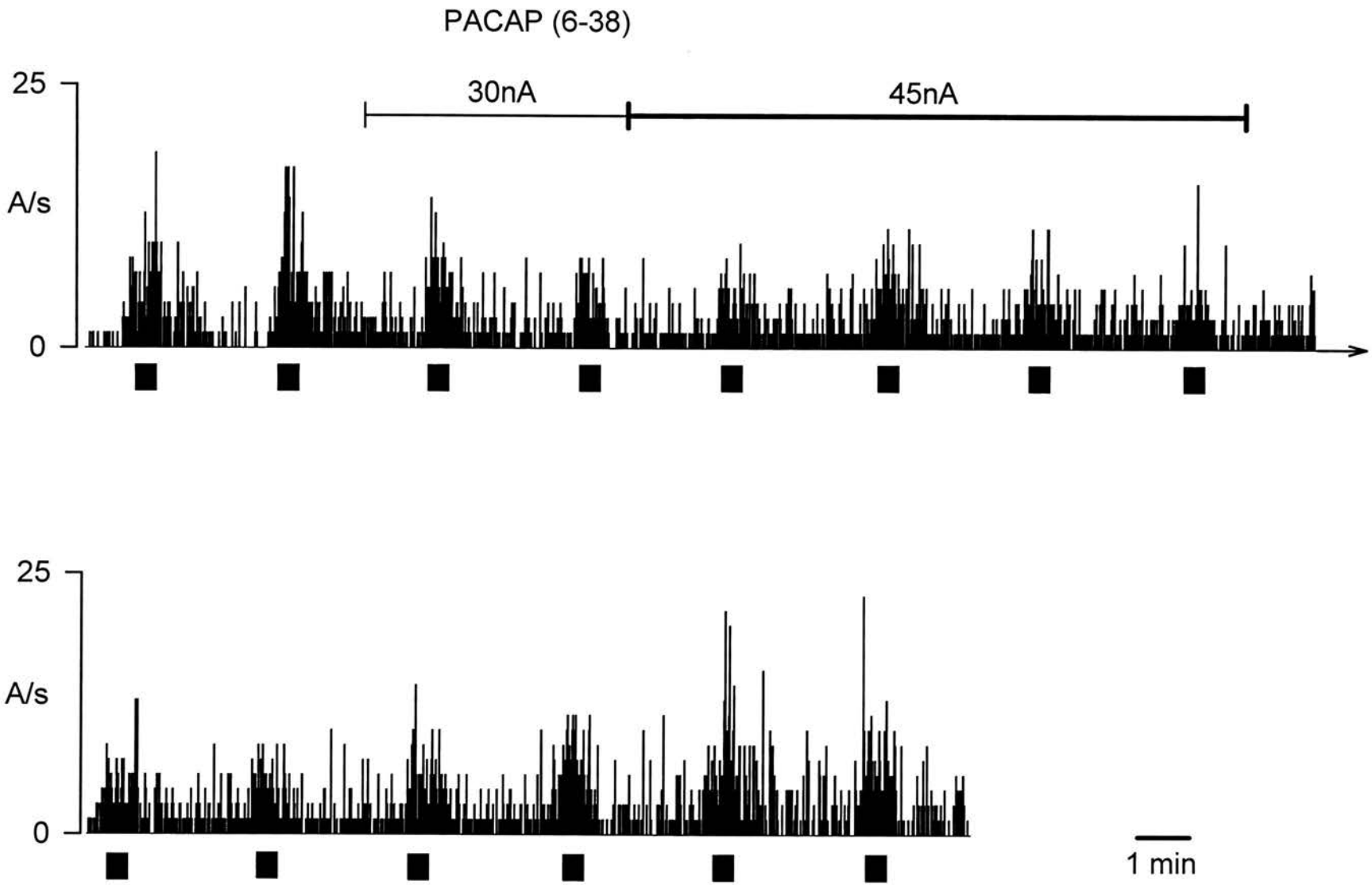

PACAP (6-38)
30nA
45nA
25
A/s
0
25
A/s
0
1 min

Figure 5.2c

Effects of ionophoretically-applied VIP_2 receptor antagonist on cold-induced neuronal firing of dorsal horn neurones in CCI animals

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Part (c) shows the generally observed lack of effect of the selective VIP_2 receptor antagonist des(1-4) Arg^{16} Ro 25-1553 on the 10 second pulses of neuronal activity induced by repeated stimulation of the cutaneous receptive field by a cold Peltier probe at 5°C (▮), through increasing currents of the antagonist.

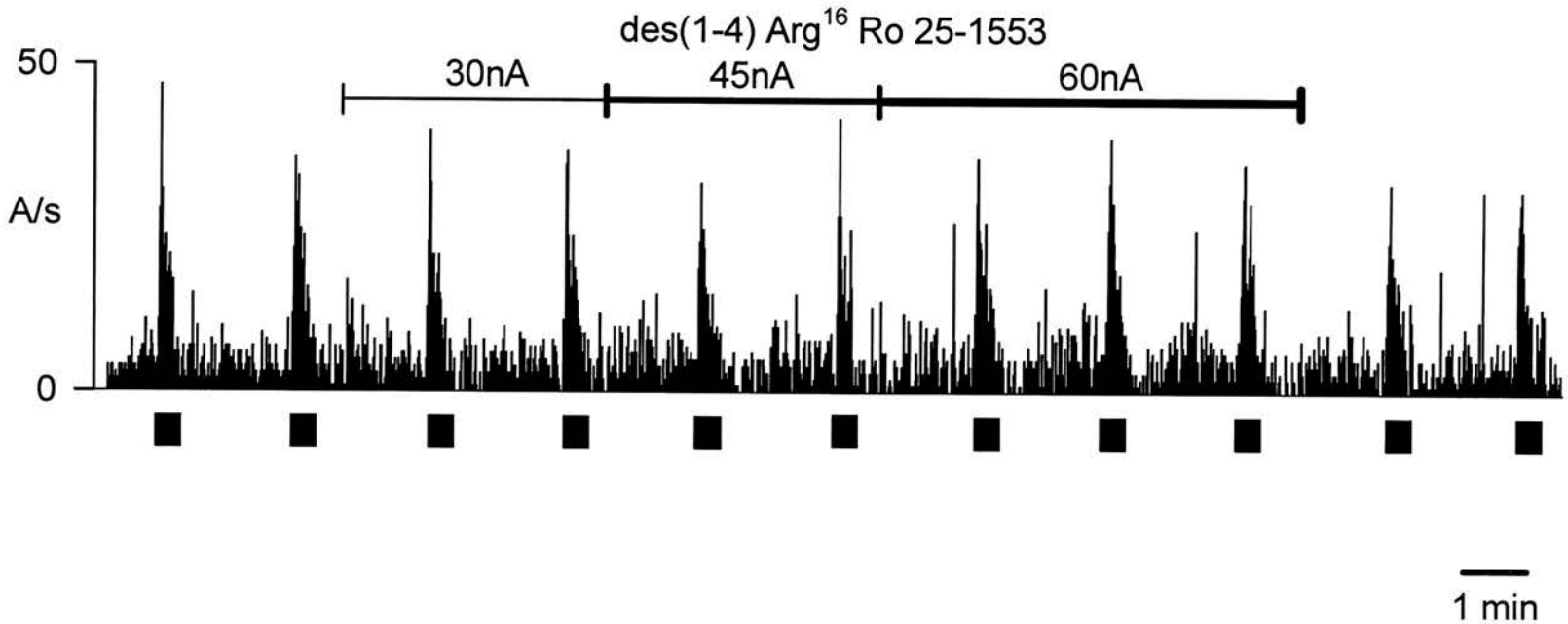

des(1-4) Arg16 Ro 25-1553
30nA
45nA
60nA
50
A/s
0
1 min

Figure 5.3

Effects of ionophoretically-applied VIP/PACAP receptor antagonists on the sustained mustard oil-induced activity of dorsal horn neurones in CCI animals

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

These traces represent the significant inhibitory effects of (a) [p-Cl-D-Phe^{6},Leu^{17}]-VIP, (b) PG 97-269, (c) des(1-4) Arg^{16} Ro 25-1553 and PACAP (6-38) on the sustained mustard oil-induced activity of multireceptive dorsal horn neurones. Each trace is from a different neurone and is entirely typical of the results obtained in the overall population sampled here.

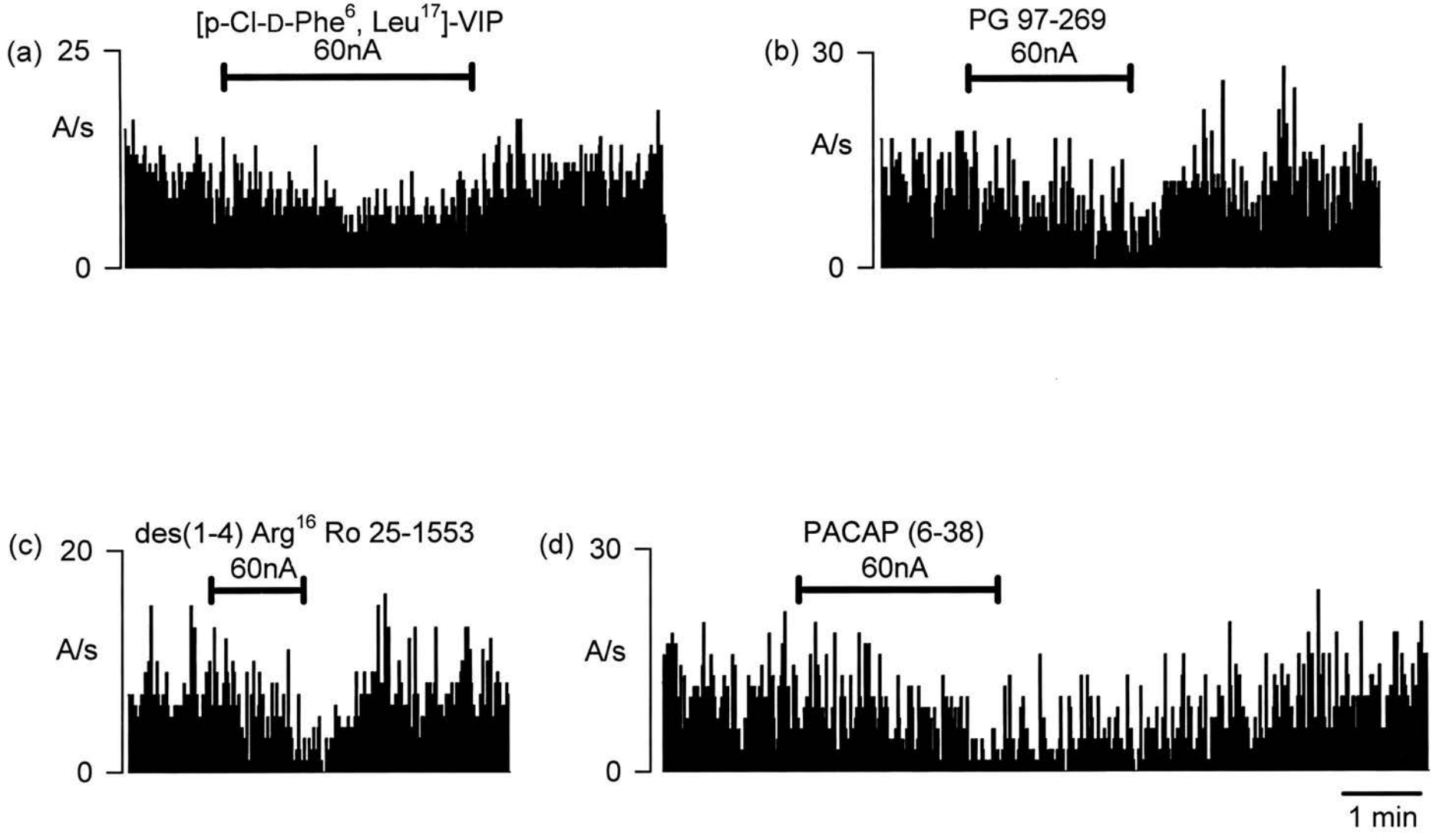

(a) 25
[p-Cl-D-Phe^{6}, Leu^{17}]-VIP
60nA
A/s
0
(b) 30
PG 97-269
60nA
A/s
0
(c) 20
des(1-4) Arg^{16} Ro 25-1553
60nA
A/s
0
(d) 30
PACAP (6-38)
60nA
A/s
0
1 min

Figure 5.4

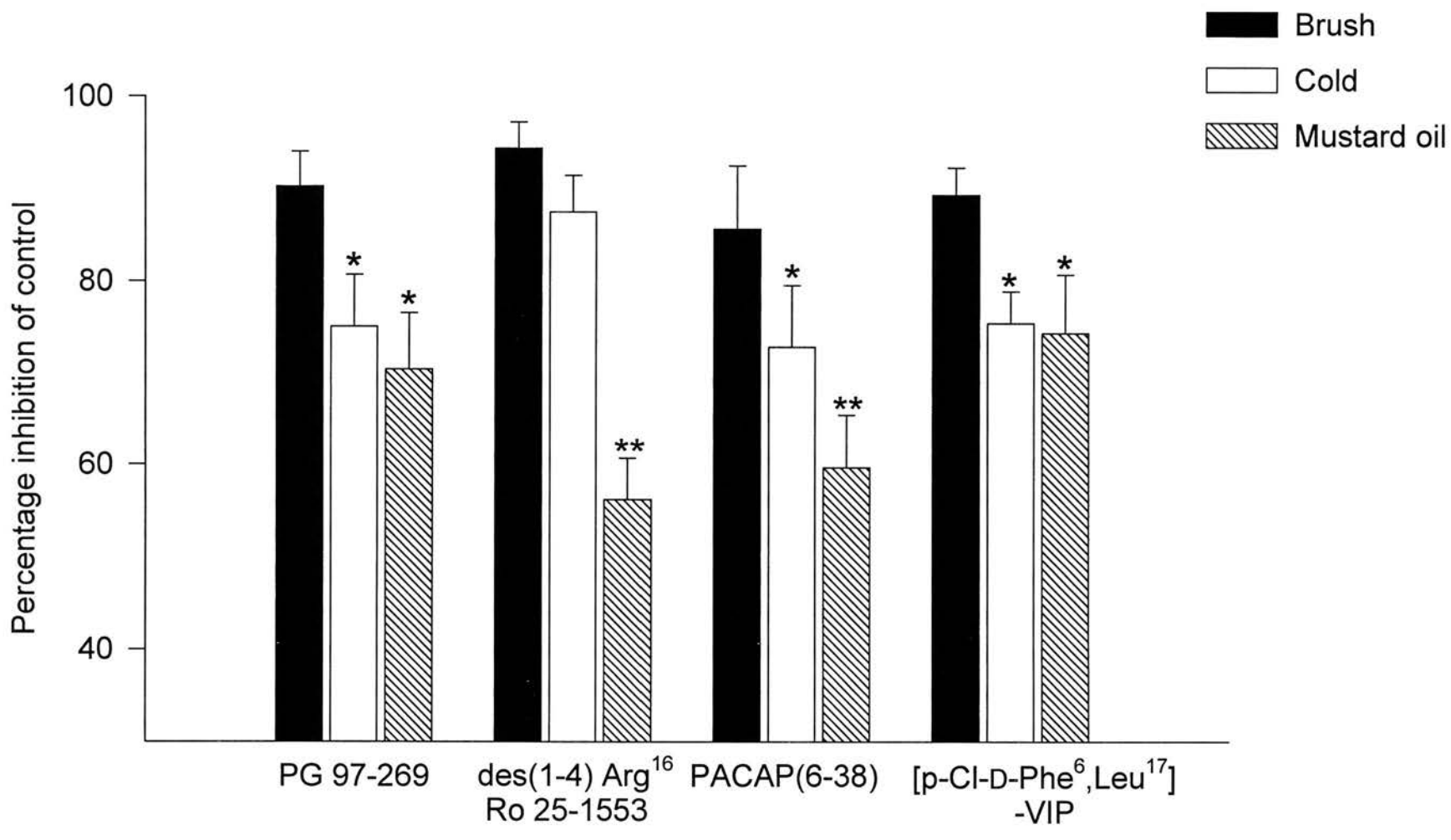
Summary histogram showing the overall effects of ionophoretically-applied VIP/PACAP receptor antagonists on sensory-induced responses of dorsal horn neurones in CCI animals

Summary histogram showing the mean percentage inhibition of control neuronal firing rate. These data are taken from animals which had undergone sciatic nerve constriction 2-3 weeks previously.

Effects of the antagonists [p-Cl-D-Phe^6,Leu^{17}]-VIP, PG 97-269, des(1-4) Arg^{16} Ro 25-1553 and PACAP (6-38) are shown on the multireceptive dorsal horn activity induced by () motorised, rotating brush, () stimulation of the receptive field with a cold Peltier probe or () topical application of the chemical irritant mustard oil.

All four antagonists showed negligible effects when applied to the sustained neuronal firing induced by a rotating brush. [p-Cl-D-Phe^6,Leu^{17}]-VIP and PG 97-269 inhibited cold- and mustard oil-induced activity to similar extents while PACAP (6-38) had a more marked inhibitory effect on mustard oil-evoked activity than on cold-evoked. In contrast, the novel VIP_2 receptor antagonist des(1-4) Arg^{16} Ro 25-1553, selectively inhibited mustard oil-induced activity only.

Raw data was analysed to determine significant differences in neuronal firing before and after drug application (paired Student *t*-test: * $p \leq 0.05$, ** $p \leq 0.01$).


Brush
Cold
Mustard oil
Percentage inhibition of control
100
80
60
40
*
*
**
*
*
**
*
*
PG 97-269
des(1-4) Arg16
Ro 25-1553
PACAP(6-38)
[p-Cl-D-Phe6,Leu17]
-VIP

5.4 DISCUSSION

These results provide further evidence of a modulatory role for VIP/PACAP and their receptors within the dorsal spinal cord, as well as providing new insight into the functional differences between the three receptor subtypes, VIP_1, VIP_2 and PACAP following an experimental peripheral mononeuropathy.

The profile of influence of VIP/PACAP receptors on sensory processing is clearly very different from that of other modulators such as the opioid and tachykinin peptides (Fleetwood-Walker et al. 1988;1993), since aspects of neuropathic pain appear to be resistant to classical opioid analgesics (Arner and Meyerson, 1988; Mao et al. 1995). Since VIP/PACAP expression is increased in dorsal root ganglia following peripheral nerve injury (Noguchi et al. 1989; Zhang Q et al. 1995), we investigated whether VIP/PACAP receptors might be critical regulators of the amplified sensory responses following neuropathy. Novel, selective antagonists for the VIP_1, VIP_2 and PACAP receptors were used to ascertain the role of each of the receptor subtypes in the modulation of sensory processing (particularly of noxious information) within the spinal dorsal horn of rats which had undergone a chronic constriction injury (CCI) of the sciatic nerve 2-3 weeks previously.

The population of neurones investigated in this study consisted solely of multireceptive neurones. No non-nociceptive neurones were studied as the vast majority of neurones examined appeared to be multireceptive, that is they responded to some form of noxious input. In addition, because of the reduced threshold observed in these animals to the von Frey filaments (indicative of mechanical allodynia), it is likely that the brush response could now be perceived as noxious and so the study concentrated on the responses of multireceptive cells. Of course it was not possible to determine what the properties of these recorded neurones were prior to nerve injury.

The effects of VIP_1, VIP_2 and PACAP receptor antagonists changed markedly in the nerve injured animals. The most obvious difference being the lack of effect of any of the four antagonists used on the brush-induced activity of dorsal horn neurones. This contrasts markedly with the prominent inhibitory effects of the VIP_1 and PACAP, but not the VIP_2 receptor antagonists in normal animals. As stated earlier, the behavioural changes exhibited in these animals make it difficult to

ascertain whether the brush stimulus is still innocuous, but the reduced threshold to von Frey hairs, indicative of mechanical allodynia, is consistent with the idea that this stimulus may now be noxious. From these experiments it is impossible to determine which fibre type is responsible for the transmission of brush-induced information. Although the CCI model causes more marked degeneration of Aβ than C or Aδ fibres (Basbaum et al. 1991; Coggeshall et al. 1993), a gradual recovery perhaps including regeneration and sprouting, does occur (Coggeshall et al. 1993; Koerber et al. 1994; Woolf et al. 1995). The neuropathy-induced increases in VIP and PACAP peptide expression are predominantly in small to medium diameter DRG cells, suggesting that VIP/PACAP are not likely candidates for direct mediators of the brush response in neuropathic animals unless new fibre types become involved in the input. It appears more likely that the mechanisms by which responses to low threshold inputs are amplified in neuropathy, act to curtail the inhibitory modulation normally exerted by VIP_1 and PACAP receptors on dorsal horn neurones. Whether this involves altered expression of these receptors or changes in their functional influence is not yet clear.

Cold allodynia appears to be a striking feature of neuropathy in humans (Frost et al. 1988; Engkvist et al. 1985), particularly in patients with sympathetically maintained pain (SMP). Very little is known about the mechanisms underlying this cold pain. In normal rats it is the small diameter nociceptive afferents which transmit cold sensory information, and saphenous nerve recordings have revealed that the cold threshold for many nociceptors is below 12°C, with a large proportion of Aδ nociceptors only firing at temperatures below 0°C (Simone and Kajander, 1996;1997).

The fibre types involved in the transmission of the cold response in neuropathic conditions are not clear. In humans, A fibre blocks have been shown to elevate the threshold temperature response of unmyelinated fibres such that cold stimulation now induces a noxious, burning sensation (Wahren et al. 1989). So it is possible that the exaggerated response to cold (allodynia) in neuropathic patients may be predominantly mediated by C-nociceptors. Furthermore, nerve recordings in the rat would suggest that activity induced by temperatures above 0°C, temperatures which now appear to elicit a withdrawal response indicative of cold allodynia in

nerve-injured animals, is transmitted primarily by C-fibres (Simone and Kajander, 1996). In the CCI model of neuropathy, C-fibre involvement is much more likely, due to the predominant loss and/or damage of myelinated fibres induced by the constriction injury (Basbaum et al. 1991; Gautron et al. 1990). In addition, an animal model of chronic pain due to ischemic spinal cord injury (Xu et al. 1992b) has shown that in these animals capsaicin-sensitive afferents mediate chronic cold allodynia (Hao et al. 1996), highlighting the involvement of C nociceptors.

Whatever the fibre type involved in the transmission of cold sensory information, there is a selective effect of the receptor subtype antagonists with regard to the regulation of the cold response in CCI animals. The cold-induced activity of multireceptive dorsal horn neurones was inhibited by only the VIP_1 and PACAP receptor antagonists, while the VIP_2 receptor antagonist showed negligible effects.

The sensitivity of the cold response in CCI animals to VIP_1 and PACAP receptor antagonists resembles that of the C-fibre (mustard oil) response (see below), and differs from the complete insensitivity of the antagonists on the brush-evoked response. This is consistent with the idea that the central changes induced by neuropathy act to rather selectively exempt low threshold brush responses from the normally significant modulatory influence of VIP_1/PACAP receptors. The VIP_2 receptor antagonist, in contrast, had no effect on cold responses of CCI animals, which would suggest that the VIP_2 receptor is not involved to any major extent in the regulation of cold allodynia in neuropathic animals.

All four antagonists significantly inhibited the mustard oil-induced activity of dorsal horn neurones of CCI rats. This implies that VIP_1, VIP_2 and PACAP receptors may all be involved with the transmission or modulation of nociceptive, C-fibre-mediated information at the spinal cord level. PACAP (6-38) and the VIP_2 receptor antagonist appeared to be the most effective, but the inhibitory effects of [p-Cl-D-Phe^6,Leu^{17}]-VIP and the more selective VIP_1 receptor antagonist were still significant. The inhibitory effect of the VIP_2 receptor antagonist on mustard oil, but not on cold responses of CCI animals, indicates a selective involvement of VIP_2 receptors in the former input pathway and suggests that the fibre types mediating these two inputs may be distinct sub-populations. The clear inhibitory effects of PACAP (6-38) correspond well to the observation that PACAP-like

immunoreactivity can be evoked in the rat spinal cord by capsaicin (Zhang YZ et al. 1997). This is consistent with the idea that any release of PACAP here may be from primary sensory afferents and highlights the possibility that this peptide may be involved in sensory neurotransmission or modulation.

In conclusion, these results provide evidence for regulatory roles of the VIP/PACAP receptors in the transmission of sensory information at the spinal cord level in neuropathic rats. The differential inhibitory effects of these receptor antagonists on cold- and mustard oil-induced activity of dorsal horn neurones in CCI animals highlights a possible role for specific VIP/PACAP receptor subtypes in the development of the abnormal sensory states which develop following nerve injury.

Importantly, these experiments underline the potential importance of VIP_1, VIP_2 and PACAP receptor antagonists as new analgesics for use in currently intractable neuropathic pain states. The results predict that VIP_2 receptor antagonists should act as selective analgesics, strongly reducing polymodal C-fibre-mediated pain (but not other responses) in neuropathy, where hyperalgesia (resistant to classic analgesics) is an important feature. In contrast, while VIP_1/PACAP receptor antagonists are rather non-selective inhibitors of sensory inputs in the normal state (see Chapter 4), they appear to have the useful property of attenuating certain aspects of allodynia (cold response) as well as polymodal C-fibre responses, while preserving low threshold mechanical responses. Drugs targeted against these receptors then, may prove to have novel unique profiles of effectiveness against the neuropathic pain states (hyperalgesia and cold allodynia) which develop following nerve damage or injury.

CHAPTER 6 - THE EFFECTS OF IONOPHORETICALLY-APPLIED VIP/PACAP RECEPTOR AGONISTS ON DORSAL HORN NEURONES IN NORMAL AND NEUROPATHIC ANIMALS

6.1 AIM

The aim of this study was to demonstrate the presence of VIP_1, VIP_2 and PACAP receptors within the dorsal spinal cord and to try to define any changes in responsiveness of dorsal horn neurones mediated by the three receptor subtypes in neuropathic, compared to normal rats. Electrophysiological studies were performed using novel selective agonists for the three receptor subtypes in control animals, and in those which had undergone sciatic nerve constriction 2-3 weeks prior to the recording experiments.

6.2 METHODS

6.2.1 Animals

Recordings were made from a total of 17 animals in this study (as in section 3.2.1).

6.2.2 Surgical Preparation of Animals with an Experimental Peripheral Mononeuropathy

The 8 neuropathic animals used in this study underwent a chronic constriction injury (CCI) as outlined in section 3.2.2. Only nerve-injured animals which showed strong signs of mechanical hyperalgesia in response to noxious pin-prick (Grade 3 response), cold allodynia (SPET of 8 sec or more) and mechanical allodynia (significantly reduced paw withdrawal threshold to von Frey filaments) were used for electrophysiological recordings, as determined by the behavioural testing (see section 3.2.3).

6.2.3 General Methods

Regarding surgical procedures, electrophysiological recording techniques, histological identification of recording sites and identification of neuronal receptive fields, please refer to section 4.2.

6.2.4 Drugs for Ionophoresis

All drug solutions were 0.5mM in distilled water and pH adjusted to 4.5 with HCl:

VIP_1 receptor agonist: [Arg16] chicken secretin (Gourlet et al. 1997a)

VIP_2 receptor agonist: [Ac-His^1, Glu^8, Lys^{12}, Nle^{17}, Ala^{19}] VIP(1-24), Asp, Leu, Lys, Lys, Gly, Gly, Thr NH_2 (lactam 21-25): Ro 25-1553 (Gourlet et al.1997b)

PACAP (and VIP) receptor agonist: PACAP-38 (Kimura et al. 1990)

All agonists were ejected using positive currents and a retention current of -12nA was applied to each barrel when not in use. The resistance of the side barrels was monitored regularly.

The agonists were ionophoresed at increasing currents from a starting current of 30nA and increasing to 45 and 60nA if necessary at 90 second intervals, until activation was achieved. A recovery period of at least 10 minutes was generally allowed before application of the next agonist, and the order of agonist testing was randomly varied fore each neurone. The percentage of dorsal horn neurones activated was then calculated for each receptor agonist.

6.2.5 Analysis of Results

Individual recordings were made for each neurone. The excitatory effect of the agonists was assessed by comparing the mean firing level in the 30 sec period encompassing the greatest activation, to the mean basal activity in the 30 sec period immediately prior to agonist administration. Activation was characterised as a significant increase in firing rate (paired Student's *t*-test: $p \leq 0.05$) from basal levels, which then returned to pre-drug levels following termination of peptide ionophoresis. The mean fold increase in firing rate of the activated cells was then calculated for each agonist. Statistically significant differences in the fold increase, between

neuropathic and normal animals, for each receptor agonist, were determined by the Mann Whitney U test (* $p \leq 0.05$).

6.3 RESULTS

6.3.1 Characterisation of Neurones

A total of 42 multireceptive neurones was tested in 9 control animals. Results in the neuropathic model were based on data obtained from 25 multireceptive neurones from 8 neuropathic animals. No non-nociceptive cells were examined in this study. All neurones displayed a low basal firing rate of 0-1Hz, and current control tests had no detectable effect on any of the neurones tested. In the vast majority of cases, the cells were tested with all three receptor agonists, with currents ranging from 30-65nA. The order of agonist testing was varied for each individual neurone. The vast majority of neurones were found within laminae III-V of the dorsal horn (corresponding to microdrive electrode depth readings of 200-1000μm from the spinal cord surface).

6.3.2 Effects of Ionophoretic Application of Selective VIP_1, VIP_2 and PACAP Receptor Agonists in Normal and CCI Animals

Excitation of the dorsal horn neurones was observed as the initiation of firing of a previously quiescent cell or as a significant increase in basal firing rates. The majority of cells that were activated responded within 3 minutes of agonist application but occasionally up to 8 minutes was needed to see a response.

In normal animals, approximately one third of the neurones tested (38%) could not be activated by any of the three receptor agonists. Of the remaining cells, [Arg^{16}] chicken secretin (the VIP_1 receptor agonist), Ro 25-1553 (the VIP_2 receptor agonist), and PACAP-38 were all found to have excitatory effects (see Figure 6.1), although on different proportions of neurones and to different extents. [Arg^{16}] chicken secretin, activated 34% of the total number of neurones tested with a mean 14-fold increase in cell activity, while Ro 25-1553 had a less marked effect, activating only 21% of neurones with an average 4-fold increase in firing. PACAP-38 had a much more marked effect, activating 58% of all dorsal horn neurones tested. Those neurones activated showed an approximate 22-fold increase in firing, often

with extremely long-lasting effects, with excitation often continuing for several minutes after application of the agonist was terminated.

In CCI animals (similar to results in controls) a subpopulation of all neurones tested (28%) could not be activated by application of any of the three agonists. However the proportions of cells activated by [Arg^{16}] chicken secretin and Ro 25-1553 were seen to change quite markedly. The number of dorsal horn neurones activated by [Arg^{16}] chicken secretin decreased slightly from 34% to 20%, while Ro 25-1553 was now seen to excite 44% of cells, double that seen in normal animals. These changes in the effectiveness of the selective receptor agonists, may reflect changes in the expression of the VIP_1 and VIP_2 receptors in the spinal dorsal horn following a neuropathic injury, or in factors able to modulate their cellular influence. As well as the changes in the proportion of cells activated by each of the receptor agonists, there were also notable changes in the course of activation for [Arg^{16}] chicken secretin and Ro 25-1553 (see Figure 6.2). Excitation caused by [Arg^{16}] chicken secretin in the neuropathic animals, was generally longer lasting, sometimes taking several minutes for the firing to return to control levels compared with the almost instantaneous recovery following activation of normal dorsal horn neurones. Ro 25-1553 also had a much more marked effect on dorsal horn neurones following neuropathy. The activation of the neurones often could not be stabilised due to the agonists pronounced excitatory effects (now showing an average 21-fold increase in activity compared to 4-fold at equivalent currents in normal control animals).

In comparison, the number of cells excited by PACAP-38 remained relatively unchanged at 61% (58% in controls), implying that expression of the PACAP receptor may not be significantly altered in neuropathic conditions. Similarly, those neurones activated showed an average 18-fold increase in firing (compared to the 22-fold increase seen in normals), and the course of activation by PACAP-38 also remained the same, with a number of the cells again being activated in a prolonged fashion.

Table 6.1 shows a direct comparison of the effects of the selective VIP/PACAP receptor subtype agonists on dorsal horn neurones in normal versus neuropathic animals.

In both normal and CCI animals, the vast majority of dorsal horn neurones were tested with all three receptor agonists, and the combinations of effective agonist-induced activation appeared to vary from one cell to the next. A small percentage of the neurones could be activated by all three receptor agonists, while others were activated by VIP_1 and VIP_2 receptor agonists only, VIP_1 and PACAP receptor agonists or VIP_2 and PACAP receptor agonists. This suggests that rat dorsal horn neurones may co-express the different receptor subtypes. Indeed, none of the cells tested appeared to be solely activated by either the VIP_1 or VIP_2 receptor agonists only, yet a small proportion of neurones were activated solely by PACAP-38. However, as this agonist has a high affinity for the VIP_1 and VIP_2 receptors, as well as the PACAP receptor, there could still be co-expression of VIP/PACAP receptor subtypes on these neurones.

Figure 6.1

Effects of ionophoretically-applied VIP/PACAP receptor agonists on spinal dorsal horn neurones in normal animals

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Parts (a) to (c) show the typical excitatory effects observed following ionophoretic application of the selective receptor agonists on multireceptive dorsal horn neurones in normal animals. Part (a) shows the typical neuronal response to application of the selective VIP_1 receptor agonist ([Arg^{16}] chicken secretin). Part (b) shows the commonly-observed effect of the VIP_2 receptor agonist (Ro 25-1553), which is small but significantly different to pre-drug values ($p \leq 0.05$; paired Student *t*-test), while (c) shows activation of the same neurone 10 minutes later by PACAP-38.

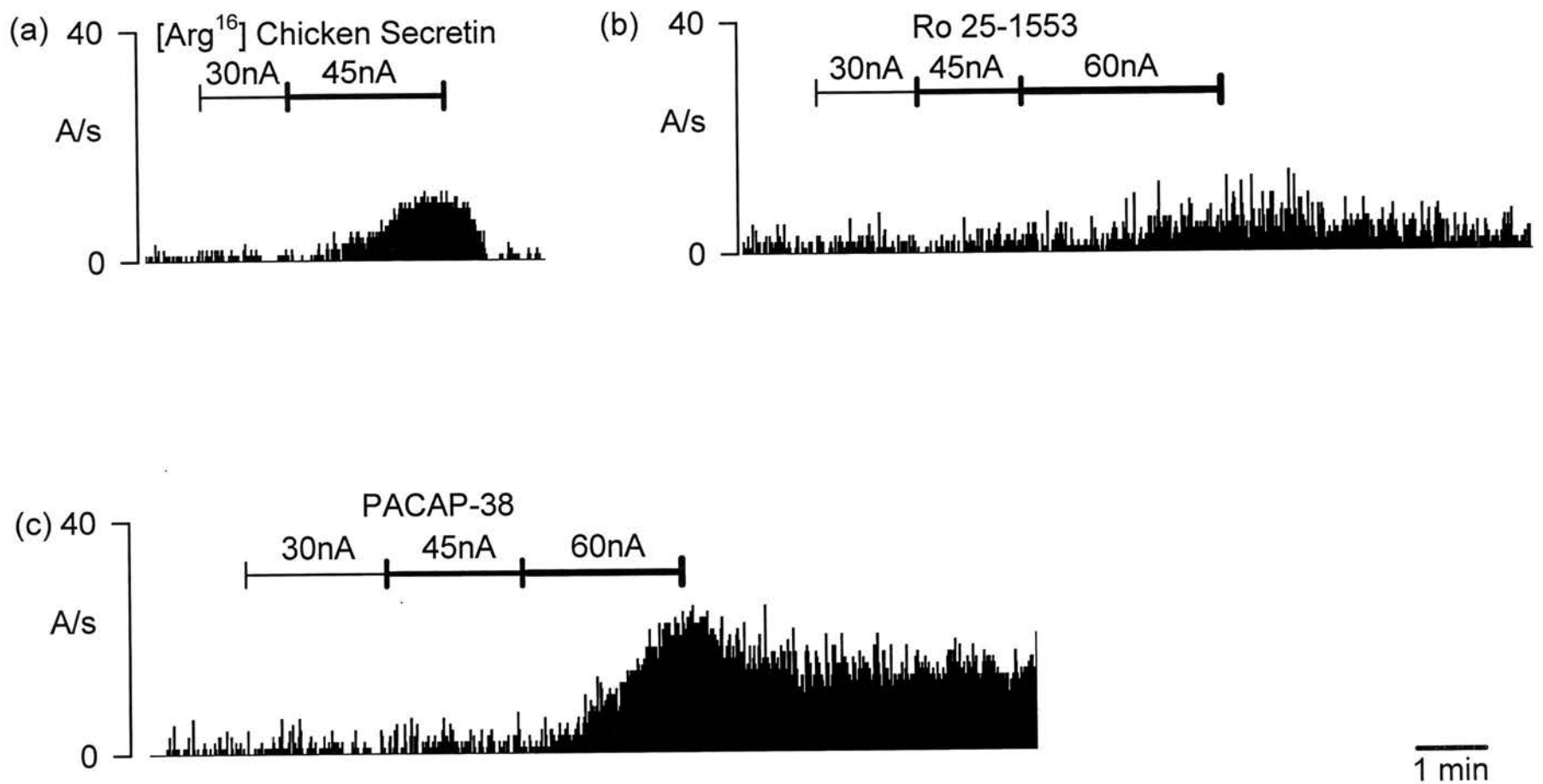

(a)
40
[Arg16] Chicken Secretin
30nA
45nA
A/s
0
(b)
40
Ro 25-1553
30nA
45nA
60nA
A/s
0
(c)
40
PACAP-38
30nA
45nA
60nA
A/s
0
1 min

Figure 6.2

Effects of ionophoretically-applied VIP/PACAP receptor agonists on spinal dorsal horn neurones in CCI animals

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Parts (a) to (c) show the typical excitatory effects observed following ionophoretic application of the selective VIP_1 ([Arg^{16}] chicken secretin), VIP_2 (Ro 25-1553) and PACAP (PACAP-38) receptor agonists respectively, on multireceptive dorsal horn neurones in animals which have undergone a sciatic nerve constriction injury 2-3 weeks previously. Note the marked excitatory effect of Ro 25-1553 (Part (b)) which is significantly greater than the response observed in normal animals ($p \leq 0.05$; Mann Whitney U-test). In contrast, the typical PACAP-38 mediated response is very similar to that observed in the normal animals, with a large proportion of the neurones continuing to fire for several minutes after application of the agonist was terminated.

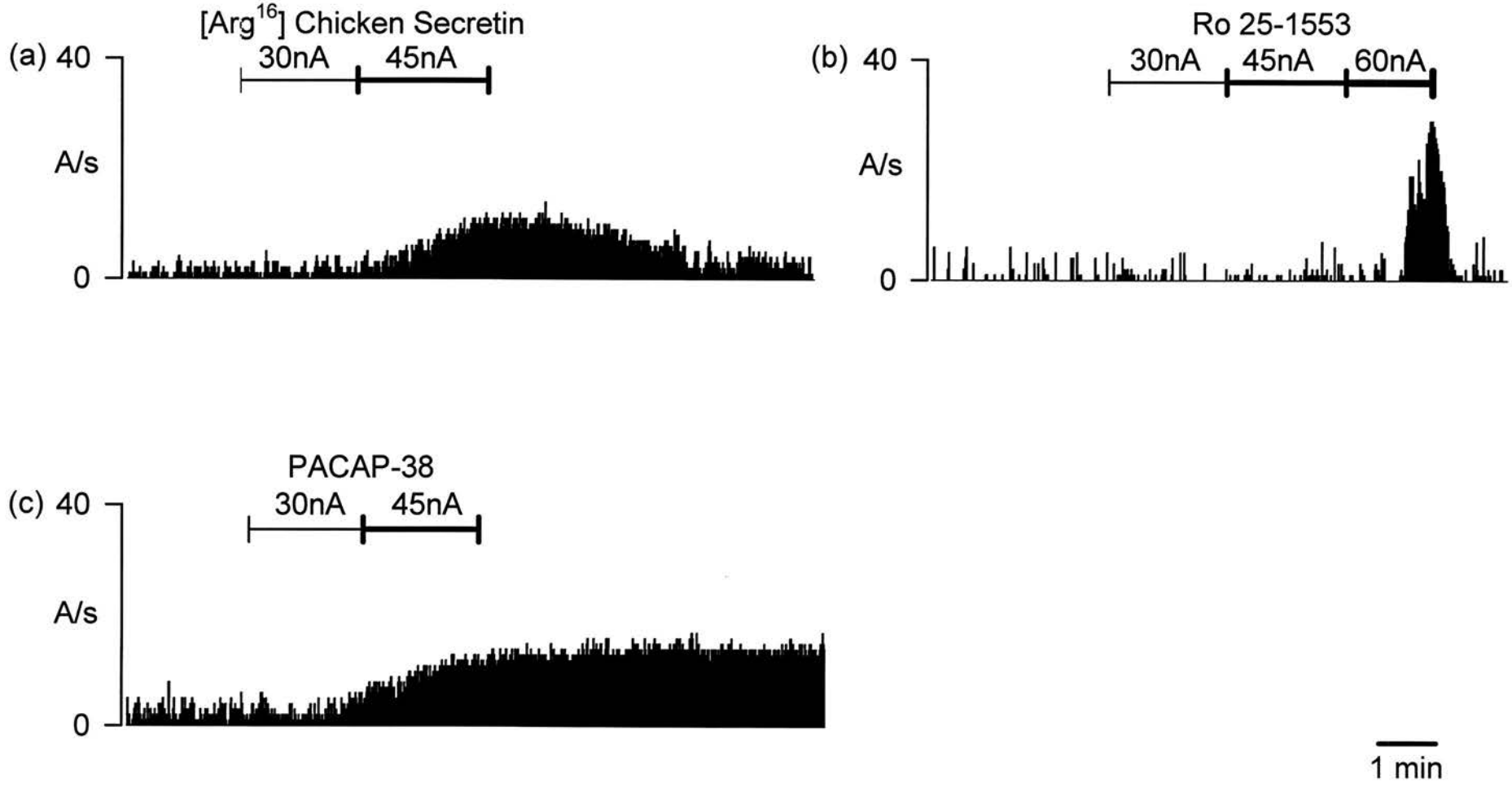

[Arg16] Chicken Secretin
(a) 40
30nA
45nA
A/s
0
Ro 25-1553
(b) 40
30nA
45nA
60nA
A/s
0
PACAP-38
(c) 40
30nA
45nA
A/s
0
1 min

Table 6.1

Summary table showing the overall effects of the selective VIP_1, VIP_2 and PACAP receptor agonists in the rat spinal dorsal horn

Summary table showing the percentage of dorsal horn neurones activated by [Arg^{16}] chicken secretin, Ro 25-1553 and PACAP-38 in the spinal cord of control animals (n=9) compared to CCI animals (n=8).

Also shown is the mean fold increase in neuronal firing rate following cell activation by each agonist. Statistically significant differences between normal and neuropathic animals were determined by the Mann Whitney U test (* $p \leq 0.05$).

		Receptor Agonist		
		[Arg^{16}] chicken secretin (VIP_1)	**Ro 25-1553 (VIP_2)**	**PACAP-38 (PACAP)**
Proportion of dorsal horn neurones activated	**Control**	34% (14/41)	21% (8/38)	58% (11/19)
	Neuropathic	20% (5/25)	44% (11/25)	61% (11/18)
fold increase in firing rate of activated cells	**Control**	13 ± 4	4 ±1	21 ± 6
	Neuropathic	8 ± 3	* 20 ± 7	17 ± 3

6.4 DISCUSSION

VIP and the two alternatively processed forms of the PACAP precursor (PACAP-27 and PACAP-38) are recognised by a family of three G-protein-coupled receptors (Harmar and Lutz, 1994), namely the PACAP receptor, which displays a much greater affinity for the two forms of PACAP than for VIP (Shivers et al. 1991; Hashimoto et al. 1993), and the VIP_1 (Ishihara et al. 1992) and VIP_2 (Lutz et al. 1993) receptors which display no marked selectivity for any one of the peptide ligands. It has been shown that these receptors have distinct distributions throughout both the peripheral and central nervous systems (Cauvin et al. 1991; Usdin et al. 1994), suggesting specific functional roles for each of the three receptor subtypes.

Using newly-developed, selective agonists for the three receptors, we investigated the responsiveness of multireceptive rat dorsal horn neurones in both normal and neuropathic animals, to try to elucidate any changes in receptor sensitivity before and after peripheral nerve damage.

The potential for VIP to act as a neurotransmitter has been shown on numerous occasions. Direct application of this peptide onto a variety of cells of the CNS (by ionophoresis or perfusion), has demonstrated an excitatory effect of VIP. Such cells include dorsal horn neurones (Jeftinija et al. 1982), cerebral cortex neurones (Murphy et al. 1993; Phillis et al. 1998) and neurones in the trigeminal nucleus caudalis (Salt and Hill, 1981). In addition, PACAP-38 has since been reported to cause a prominent increase in neuronal discharge of a number of sympathetic preganglionic neurones (SPN), during *in vitro* whole-cell recordings (Lai et al. 1997). In agreement with these original studies we have found both VIP and PACAP to be effective excitants of dorsal horn neurones.

Despite the relatively low abundance of VIP and PACAP in the spinal cord and DRG under normal conditions (Mulder et al. 1994; Noguchi et al. 1993), the present results provide clear evidence for the presence of VIP/PACAP receptor subtypes on dorsal horn neurones in normal rats. In addition we have shown differential responses to application of the selective agonists used for each of the receptor subtypes.

In normal animals, all three agonists showed excitatory effects to some degree. PACAP-38 appeared to be the most effective excitant, activating

approximately 60% of the dorsal horn neurones tested, when used at the same concentration and similar ionophoretic currents as the other receptor agonists. In the majority of cases, PACAP was also found to have a longer duration of action than [Arg^{16}] chicken secretin or Ro 25-1553, lasting for up to 20 minutes in some cases, despite terminating application of the agonist. This prolonged action of PACAP-38 may suggest a longer term regulatory role for PACAP, and is consistent with the idea that PACAP maybe involved in some aspects of the sensitisation of central sensory neurones. Therefore the PACAP receptor may be an important target for attenuating the development of the long-lasting pain states which ensue from nerve damage or injury.

The total population of neurones activated by PACAP-38 remained relatively unchanged in neuropathic animals as compared to normal, that is approximately two-thirds of the dorsal horn neurones tested. Similarly the degree of cell activation remained the same, suggesting that the expression of the PACAP receptor may not be markedly altered following nerve injury, despite the dramatic up-regulation of the peptide in sensory neurones and nerve fibres (Zhang et al. 1996; Zhang Q et al. 1995). However, since PACAP-38 is a potent agonist for all three receptor subtypes (Kimura et al. 1990), it cannot be concluded with certainty that its excitatory effects are solely PACAP-receptor-mediated, VIP_1 and VIP_2 receptor influences may well also contribute to the effects observed. Nevertheless, the effects of PACAP-38 were again, often much more pronounced than those of the VIP_1 and VIP_2 receptor agonists.

This long-lasting effect of PACAP-38 has similarly been reported during *in vitro* whole-cell SPN recordings, where PACAP-38 caused a prominent increase in neuronal discharge of a number of SPN, lasting over 1 hour in some neurones (Lai et al. 1997). Interestingly, it has been shown that PACAP-38 is much more resistant to degradation by neutral endopeptidases than VIP (Gourlet et al. 1997d). This could explain the longer duration excitation seen with PACAP-38 (as opposed to the effects of the VIP_1 and VIP_2 agonists), and would be consistent with a longer term modulatory role of PACAP *in vivo*.

The most notable difference in this agonist study was the doubling in the percentage of dorsal horn neurones activated by the selective VIP_2 receptor agonist

Ro-25 1553 in CCI compared to normal animals. Only one fifth of the neurones tested were activated by Ro-25 1553 in normal animals, with a 4 fold increase in firing from pre-drug levels. In contrast, more than 40% were activated in nerve-injured animals, and the degree of activation was significantly higher than that observed in normal animals. These results suggest that the expression of the VIP_2 receptor may well be up-regulated in the spinal dorsal horn following nerve injury.

In contrast, the proportion of dorsal horn neurones activated by the VIP_1 receptor agonist [Arg^{16}] chicken secretin was seen to decrease in the neuropathic compared to normal animals, suggesting that the expression of the VIP_1 receptor may be decreased following a CCI. The fold increase in the firing rate of activated cells was also seen to decrease, but this may simply be a direct result of a decrease in VIP_1 receptor expression. It is not clear why a small proportion of the cells appeared to have a longer-term activation following application of the VIP_1 agonist in CCI animals, it may be that the agonist has a slight cross-over action at the VIP_2 receptor, or it may be that the VIP degradation process is somehow delayed due to the marked upregulation of a number of different growth factors in the spinal cord following peripheral nerve injury, which would enable a longer-term activation of the receptors compared to normal conditions.

The differences in the proportions of neurones activated by the three receptor agonists, both in normal and neuropathic animals, implies that the expression of receptor subtypes is non-uniform throughout the cord, and that individual neurones may each express their own combination of receptor subtypes. Indeed, approximately 30% of the total number of dorsal horn neurones tested, in both neuropathic and normal animals, could not be activated by any of the three receptor agonists, suggesting that some dorsal horn neurones do not express the VIP/PACAP receptors at all. On the other hand, other neurones appeared to be responsive to all three agonists, which may indicate the expression of VIP_1, VIP_2 and PACAP receptors. It is not clear whether PACAP or VIP receptors mediate dual regulation of individual cells. Facilitatory or synergistic properties were not addressed in this study due to the relatively high currents which would be required for ionophoresis of two agonists simultaneously. It must be noted that this is a general overview of the responses of dorsal horn neurones, and may not necessarily be a direct representation

of the topography of receptor expression within the dorsal horn. The efficiency of electrophoretic transport out of the electrodes is likely to differ for the three receptor agonists, and as stated previously there may well be differences in the enzymatic degradation processes for the three agonists, so care must be taken when making comparisons.

However, in previous studies, the effects of the antagonists [p-Cl-D-Phe^{6},Leu^{17}]-VIP and PACAP (6-38) were assessed on a number of dorsal horn neurones activated by porcine VIP (n=6) or PACAP-38 (n=5), to try and ascertain the specificity of these receptor agonists. This study revealed that [p-Cl-D-Phe^{6},Leu^{17}]-VIP, but not PACAP (6-38), significantly inhibited VIP-induced neuronal firing by approximately 50%, while PACAP (6-38) selectively inhibited PACAP-38-induced activity by an average 30% (Dickinson et al. 1997). Unfortunately, time did not allow the repetition of these agonist/antagonist experiments on the novel selective VIP_1/VIP_2 receptor agonists.

In conclusion, VIP_1, VIP_2 and PACAP receptor agonists all have marked excitatory effects on multireceptive dorsal horn neurones, in both normal and neuropathic animals. The altered responsiveness of these neurones to the VIP_1 and VIP_2 receptor selective agonists following CCI, suggests that the spinal expression of these proteins may be altered following peripheral nerve injury. These data suggest that VIP_2 receptor expression increases following nerve injury, while expression of the VIP_1 receptor decreases. This altered expression of the receptor subtypes may contribute significantly to the modification of sensory processing that ensue in the dorsal horn following neuropathy, and in the pain states which subsequently develop.

CHAPTER 7 DIFFERENTIAL EXPRESSION OF VIP_1, VIP_2 AND PACAP RECEPTOR mRNA AFTER PERIPHERAL NERVE INJURY - AN IN SITU HYBRIDISATION STUDY

7.1 AIM

The object of these experiments was to evaluate any changes in the distribution of VIP_1, VIP_2 and PACAP receptor mRNA expression within the rat spinal cord following a peripheral mononeuropathy, using *in situ* hybridisation histochemistry (ISHH). Oligonucleotide probes, targeted at specific sequences of mRNA for the three receptor subtypes, were used to determine the distribution of VIP_1, VIP_2 and PACAP receptor expression in the dorsal horn of normal and neuropathic rats.

7.2 METHODS

7.2.1 Animals

4 rats with unilateral hindlimb CCI were prepared as outlined in section 3.2.2. Control samples were taken from 3 untreated male rats (see Section 3.2.1).

7.2.2 Tissue Removal and Sectioning

Details of all reagents can be found in Chapter 2, sections 2.1 and 2.5-2.9. A section of spinal cord, incorporating segments L2-L4, was removed from animals deeply anaesthetised with fluothane, using sterile surgical instruments. One sample was taken from each rat and then the animal was immediately killed by exsanguination. The spinal cord section was mounted vertically (rostral end down) on to a labelled, autoclaved metal chuck using Cryo-M-Bed embedding medium, and then rapidly frozen for 3-5 minutes in isopentane which had previously been cooled to between -40°C and -45°C on dry ice. The frozen cord and chuck were then wrapped in clingfilm and stored in a labelled Steriseal pot at -70°C until required for cutting.

Transverse sections of 12μm thickness were cut using a Cryotechnics Bright cryostat, which constantly maintained the uncut tissue and cut sections at

approximately -18°C. A new disposable blade was fitted for each new segment and cleaned regularly with ethanol to prevent ribonuclease (RNase) contamination of the tissue. Sections were thaw-mounted onto clean, poly-L-lysine coated glass slides, with each slide holding 10-12 sections.

At regular intervals (approximately 350μm) one or two sections were cut for histological examination under the light microscope, to verify tissue integrity and orientation. These sections were fixed in 10% formal saline for 5 minutes, stained with haematoxylin and eosin, dehydrated through increasing concentrations of ethanol, then mounted in DePeX mounting medium under a coverslip and retained for future histological reference.

In addition, two brains were obtained from untreated male rats, which were sacrificed by rapid decapitation following initial anaesthesia with fluothane. Brains were mounted transversely on the cerebellum/brainstem on autoclaved metal chucks using Cryo-M-Bed embedding medium, and then rapidly frozen for 5 minutes in previously cooled isopentane (as with the spinal cord sections). Tissue was sectioned coronally according to stereotaxic areas as defined by Paxinos and Watson (1986), at 12μm thickness on a cryostat, using the same method as outlined above for spinal cord tissue, but with two brain sections thaw-mounted per slide. Sections were taken which included brain regions of particular interest, including the dentate gyrus and hippocampus, areas found to contain high concentrations of PACAP-ir (Cauvin et al. 1991; Masuo et al. 1991; 1992).

All material was maintained at -18°C in the cryostat during the cutting session, and thereafter was stored in sealed Kartell boxes with dessicant at -70°C until required for hybridisation.

7.2.3 Oligonucleotide Probes

Oligonucleotide probes for VIP_1, VIP_2 and PACAP receptor mRNA were synthesised and HPLC purified by Oswel Chemicals. Each probe was dissolved in approximately 1ml of sterile water, at a given concentration (μg/ml). The specificity of each probe was confirmed by homology screening of Genobase/EMBL sequences.

One 48mer VIP_1 receptor oligonucleotide probe was used, complementary to bases 1289-1336 of the rat VIP_1 receptor mRNA sequence (Ishihara et al. 1992). See Figure 7.1(a). The given concentration of this probe was 389μg/ml.

Two VIP_2 receptor probes were used (Figure 7.1(b)). A 47mer and a 44mer oligonucleotide complementary to nucleotides 163-209 and 1346-1389 of the rat VIP_2 receptor mRNA sequence respectively (Lutz et al. 1993).

Finally, to allow for increased sensitivity, a mix of five probes (was required for detection of PACAP receptor mRNA (Hosoya et al. 1993). These probes were 49, 45, 45, 42 and 48 bases long and were complementary to nucleotides 167-215, 544-588, 767-811, 1034-1075 and 1481-1528 respectively (See Figure 7.1 (c)).

Figure 7.1(a)

Amino Acid Sequence of Rat VIP_1 Receptor mRNA

DNA sequence (in black: bases 1151-1438) of rat VIP_1 receptor mRNA (Ishihara et al. 1992) showing the sequence (in red) chosen for the VIP_1 receptor antisense oligonucleotide probe. This complementary base probe is highly selective for rat VIP_1 receptor mRNA as determined by Genobase/EMBL screening.

1151 AACTTCAAGG CCCAGGTGAA AATGGTCTTC GAACTTGTCG TGGGCTCTTT

CCAGGGTTTC GTGGTGGCCA TCCTCTACTG CTTCCTCAAT GGTGAGGTGC

AGGCGGAGCT GCGGCGGAAG TGGCGGCGTT GGCATCTGCA GGGCGTCCTG
GT CCCGCAGGAC

GGCTGGAGCT CCAAATCCCA GCATCCGTGG GGAGGCAGCA ACGGCGCCAC
CCGACCTCGA GGTTTAGGGT CGTAGGCACC CCTCCG

ATGCAGCACG CAGGTATCCA TGCTGACCCG CGTCAGCCCG AGCGCACGCC

GCTCCTCCAG CTTCCAAGCG GAGGTCTCCC TGGTCTGA 1438

Figure 7.1(b)

Amino Acid Sequence of Rat VIP_2 Receptor mRNA

DNA sequence (in black: bases 91-310 and 1292-1480) of rat VIP_2 receptor mRNA (Lutz et al. 1993) showing the sequences (in red) chosen for the two VIP_2 antisense oligonucleotide probes. These complementary base probes are highly selective for rat VIP_2 receptor mRNA as determined by Genobase/EMBL screening.

91 ACCGAGGCGG CACGCTGAGC CCAGGAATGAG GGCGTCGGTG GTGCTGACCT

GCTACTGCTG GTTGCTGGTG CGGGTGAGCA GCATCCACCC AGAATGCCGG
CCACTCGT CGTAGGTGGG TCTTACGGCC

TTTCATCTGG AAATACAGGA AGAGGAGACA AAATGCGCAG AGCTGCTAAG
AAAGTAGACC TTTATGTCC

CAGCCAAATG GAGAATCACA GAGCTTGCAG CGGTGTCTGG GACAACATCA

CATGCTGGCG CCCTGCAGAC 310 ……..

1291 GTGCCTGACC CAGCCTGGGA GCCGGGACTA CCGGCTGCAC AGCTGGTCCA

TGTCCCGGAA TGGCTCAGAA AGCGCCCTAC AGATACACCG TGGCTCCCGT
GCCTT ACCGAGTCTT TCGCGGGATG TCTATGTGGC ACCGAGGGC

ACCCAGTCCT TCCTGCAGTC AGAGACCTCA GTCATTTAGC TGTGTCCCTC

ATACAGAGCT GACAGTGCTG CTGGGTTTGA CATATGTGTT 1480

Figure 7.1(c)

Amino Acid Sequence of Rat PACAP Receptor mRNA

Selected areas (bases 151-290, 501-640, 741-880, 1001-1100 and 1451-1580) of the DNA sequence (in black) for rat PACAP receptor mRNA (Hosoya et al. 1993) showing the sequences chosen (in red) for the five PACAP antisense oligonucleotide probes. These complementary base probes are highly selective for rat PACAP receptor mRNA as determined by Genobase/EMBL screening.

151 CCTGACTGCT CTCCTGCTGC CTGTGGCTAT TGCTATGCAC TCTGACTGCA
GACG GACACCGATA ACGATACGTG AGACTGACGT

TCTTCAAGAA GGAGCAAGCC ATGTGCCTGG AGAGGATCCA GAGGGCCAAC
AGAAGTTCTT CCTCG

GACCTGATGG GACTAAACGA GTCTTCCCCA GGTTGCCCTG 290……

501 CCTTCCCCCA CTACTTCGAT GCTTGTGGGT TTGATGATTA TGAGCCTGAG
CGGACTC

TCTGGAGATC AGGATTATTA CTACCTGTCG GTGAAGGCTC TCTACACAGT
AGACCTCTAG TCCTAATAAT GATGGACAGC CACTTCCG

CGGCTACAGC ACTTCCCTCG CCACCCTCAC TACTGCCATG 640 …….

741 AGGACTGGAT CTTGTAGCGC GAGCAGGACA GCAGTCACTG CTTCGTTTCC
CTGT CGTCAGTGAC GAAGCAAAGG

ACCGTGGAGT GCAAAGCTGT CATGGTTTTC TTCCACTACT GCGTGGTGTC
TGGCACCTCA CGTTTCGACA G

CAACTACTTC TGGCTGTTCA TTGAAGGCCT GTACCTCTTT 880 ……..…

1001 GATGCAGGAT GCTGGGATAT GAATGACAGC ACAGCTCTGT GGTGGGTGAT
CGAGACA CCACCCACTA

CAAAGGCCCC GTGGTTGGCT CTATAATGGT TAACTTTGTG 1090……..
GTTTCCGGGG CACCAACCGA GATAT

1451 TGGAGGAGCT GGAAGGTGAA CCGTTACCTC ACTATGGACT TCAAGCACCG
TGATACCTGA AGTTCGTGGC

GCACCCGTCC CTGGCCAGCA GTGGAGTAAA TGGGGGAACC CAGCTGTCCA
CGTGGGCAGG GACCGGTCGT CACCTCAT

TCCTGAGCAA GAGCAGCTCC CAGCTCCGCA 1580

7.2.4 ISHH Detection of VIP/PACAP Receptor mRNA

ISHH steps were carried out sequentially, covering a period of four days for each assay. Great care was taken to reduce the risk of contamination by RNases by using sterile glassware and using dH_2O treated with the nuclease inhibitor DEPC. Recipes for all stock solutions can be found in section 2.6. Laboratory coats and disposable gloves were worn at all times.

a) Probe labelling

All probes were labelled with [^{35}S]-dATP at their 3' ends using recombinant terminal deoxynucleotidyl transferase (rTdT) to yield a specific activity of approximately 2 x 10^6 cpm per 100µl of hybridisation buffer. The following reaction mix was incubated in a sterile Eppendorf tube at 37°C (with gentle agitation) for 1 hour: x µl of the appropriate oligonucleotide probe (calculated to ensure 3x more pmol [^{35}S]-dATP than oligonucleotide), 10µl of potassium cacodylate tailing buffer (TdT buffer), 4µl rTdT enzyme (15 units/µl), 4µl [^{35}S]-dATP (70pmol; 1-1.3 x 10^3 Ci/mmol) and enough DEPC H_2O to give a final volume of 48µl. Prior to incubation the mixture was vortexed briefly then spun down (13 000g, 2-3 secs).

Following the initial incubation period, a further 2µl of [^{35}S]-dATP and 2µl of rTdT enzyme were added to the reaction mixture to optimise conditions. Again the mixture was vortexed briefly then spun down (13 000g, 2-3 secs), before continuing the incubation for another hour at 37°C (with gentle agitation). The reaction was stopped by cooling on ice for 15 minutes.

At this time, 2 x 1µl pre-spun samples were removed and placed on to fibre-free filter paper in sterile Eppendorf tubes. 1ml of Optiphase scintillation cocktail was added to each sample for scintillation counting. The radiolabelled oligonucleotide probe was then separated from unincorporated nucleotides, by purification through a Nu-Clean D25 disposable spun column. Each sample was carefully pipetted on to the gel surface of individual columns, and then centrifuged for 4 minutes at 3 500g, with the labelled sample being collected in a fresh Eppendorf tube. 2 x 1µl post-spun samples were then taken as before and 1ml of Optiphase scintillation cocktail added to each sample for scintillation counting. Counts per minute (cpm) were read using a 1450 Microbeta plus scintillation counter

(Wallac, Finland) with customised software (Department of Veterinary Pathology, niversity of Edinburgh). The difference between the pre- and post-spun samples allowed the percentage incorporation of the radiolabel to be estimated (all probes showed between 50-60% incorporation). The labelled probe was then stored at -70°C until required (for a maximum of 14 days).

b) Post-fixing

Slides were brought to room temperature and immediately fixed in 4% paraformaldehyde in PBS (0.1M; pH 7.4) for 10 minutes, then rinsed in two washes of fresh PBS (0.1M; pH 7.4) of 5 minutes each. Non-specific binding of the probe to the glass and tissue was reduced by treating the slides for 10 minutes in ethanolamine solution (0.1M TEA and 0.026M acetic anhydride). Subsequently the sections were dehydrated through increasing concentrations of ethanol (50%, 70%, 80%, 90% and 100%) each buffered with ammonium acetate (0.3M) for 2 minutes each, with a final 2 minute stage in 100% unbuffered ethanol. After blotting the slides, they were then dried thoroughly with a hairdryer on cold setting for 1-2 hours.

c) Hybridisation

Previously-prepared stock solutions of hybridisation buffer and de-ionised formamide (see section 2.6) were brought to room temperature then aliquoted together to obtain a 1x hybridisation buffer/40% de-ionised formamide solution: dextran sulphate (10%; wt:vol), sodium chloride (600mM), Tris pH 7.6 (10mM), EDTA (1mM), Denhardt's solution (0.1% ; wt:vol), salmon sperm DNA (0.01%; wt:vol), Bakers yeast tRNA (0.005%; wt:vol) glycogen (0.0005%; wt:vol) and formamide (40%; wt:vol).

The radiolabelled probes were then added in a relative volume to give a final count number of 2×10^6 cpm/100µl hybridisation buffer (the optimum concentration of probe used was ascertained after carrying out a number of experiments with a range of conditions, to establish which gave the clearest signal to background ratio). The resulting hybridisation mixture was heated to 60-70°C for 10 minutes, to break any hydrogen bonds, then subsequently cooled on ice for 1 minute before adding the

reducing agent dithiothreitol (DTT; 10mM). This helped to protect the[^{35}S]-dATP from oxidation and ensured that the probe remained in a single stranded state even after the solution had been cooled. 100µl of this final solution was then carefully pipetted along the bottom edge of each slide and a DEPC-treated coverslip lowered gently onto the solution so that surface tension enabled the probe mixture to cover all the sections.

Hybridisation was carried out in sealed containers containing 2 sheets of Whatman (No. 1) filter paper saturated with a solution of 50% de-ionised formamide: 50% 4x SSC solution, at 37°C for 18 hours.

d) Post-hybridisation washes

Following the overnight incubation, the coverslips were removed and racks of slides were washed in decreasing concentrations of SSC solutions (2xSSC; 1xSSC; 0.5xSSC; pH 7) at 40°C for 2 hours each, to remove any excess hybridisation buffer solution and any non-specifically bound probe. The slides were then blotted with fibre-free paper and dehydrated through ascending concentrations of ethanol buffered with 0.3M ammonium acetate (50%, 70%, 80%, 90%, 100%) for 2 minutes each and lastly 100% absolute ethanol for 2 minutes, before being left to air dry overnight.

The following day, the slides were apposed to Hyperfilm β-max in a dark room and left to develop in a sealed autoradiographic cassette with intensifying screen for approximately 10 days at 4°C. The film was then developed using a standard X-ray Film developing machine (Small Animal Clinic, Summerhall, Edinburgh). The intensity of the latent image gave an insight as to how long to appose the slides to autoradiographic emulsion, before developing.

e) Emulsion coating

Under darkroom conditions, the dried slides were dipped in 20ml of Ilford K5 liquid autoradiographic emulsion at 43°C for approximately 5 seconds, blotted lightly then left to dry in an upright position overnight, in the dark, before being sealed in light-tight Kartell boxes containing silica gel desiccant, wrapped in black plastic and stored at 4°C for 6-12 weeks, depending on the intensity of the autoradiographic image developed previously.

f) Developing and staining

Following the assigned exposure time (6 weeks for the VIP_2 probes and 12 weeks for the VIP_1 and PACAP probes), the slides were brought to room temperature, and the exposed silver grains were developed at 15°C in Kodak D-19 developer for 4 minutes, rinsed in distilled water for 30sec, then fixed in Ilford Hypam K5 fixer (diluted 1:5 (vol:vol) with distilled water) for 5 minutes. The developed slides were then rinsed in copious amounts of distilled water (4x 10 minute washes) to remove all traces of the fixer . The slides were lightly counter-stained with Mayer's haematoxylin (4 minutes) and 5% eosin (2-3secs), with distilled water washes in between each stain, before being dehydrated through 50%, 70%, 90% and 100% ethanol (2 minutes each). The slides were then placed in fresh xylene solution (2 minutes) and finally mounted in DePeX mounting medium.

7.2.5 Controls

Data for each rat were obtained from a number of parallel assays to minimise experimental variation, and hybridisation using coronal brain sections acted as positive controls to verify the sensitivity of the ISHH methodology. In addition, control experiments for the specificity of hybridisation were carried out in parallel, as follows:

(a) RNase Control

In order to destroy all RNA in the cut spinal cord sections, 100µl of RNaseA solution (1mg/ml in PBS) was pipetted along the bottom edge of 3 slides and a DEPC-treated coverslip lowered gently onto the solution so that surface tension enabled the mixture to cover all the sections. The slides were then incubated at 37°C for one hour, in boxes humidified with PBS. Following the incubation period, the coverslips were carefully removed and the slides washed in 2x DEPC H_2O for five minutes each, before being blotted dry and then hybridised as normal with radiolabelled probe. To reduce the risks of contamination however, the remaining steps were carried out in Kartell and hybridisation boxes which were always kept separate from the rest of the assay.

(b) "Cold" Probe Controls

Radiolabelled probe for each receptor was mixed with a 10-fold Molar excess of corresponding "cold" unlabelled probe, and the amount of hybridisation buffer adjusted accordingly for each resulting hybridisation solution. The rest of the assay was then continued as normal.

7.2.6 Analysis of Results

(a) Cell Counts

Positively-labelled cells were counted under the light microscope at x 40 magnification using a Whipple grid graticule (total area 30 625μm^2). Cells were considered to be positively-labelled if the silver grains showed a dense pattern over and around the cell nucleus, and if this pattern was obviously more dense than the background silver grain expression.

Cell counts were carried out in two separate regions of the spinal dorsal horn, namely the lateral edge of lamina II and the lateral edge of laminae III/IV. Counts were made for each side of the neuropathic spinal cord sections (i.e. ipsilateral and contralateral to the nerve injury), while for normal control tissue, counts were taken randomly from either side of each section.

Raw data were collected as the number of VIP_1/VIP_2/PACAP receptor mRNA expressing cells on the ipsilateral (experimental) side versus the number on the corresponding contralateral (control) side for each section. In addition, contralateral and normal control values were also compared. At least four spinal cord sections per slide were counted, with one slide taken from each of 4 neuropathic or 3 control animals. Mean cell counts per animal were then calculated for each area of analysis (i.e. lamina II or III/IV) for ipsilateral and contralateral neuropathic, and control dorsal horn.

(b) Silver Grain Density

The silver grain density of the total area over and around the nucleus of each positively-labelled cell, was measured using Image 1.49 software from Improvision, on an Apple Macintosh computer. Silver grain measurements were taken from positively-labelled cells in the lateral edges of lamina II and laminae III/IV. This

area value, referred to as the silver grain density (μm^2 per cell) reflected the degree of VIP_1/VIP_2/PACAP receptor mRNA expression.

Again, counts were made from the ipsilateral and contralateral sides of the neuropathic spinal cord sections, and from normal control tissue. The same sections used for the cell count measurements were correspondingly used in the silver grain density analysis. Analysis was carried out by firstly capturing one image field in the appropriate laminar area, using a CCD video camera (Sony, Japan) mounted on a Zeiss Axioscope Microscope (x 40 magnification), with a Hamamatsu C2400 CCD camera control. Each image was blank field adjusted to remove any artefacts present within the camera set-up. The region of interest (ROI) was standardised for lamina II and laminae III/IV measurements, as a circle with the average diameter of the dorsal horn neurone nuclei in the area of interest. The density threshold was then selected by determining the grey scale range which highlighted all silver grain particles, including a small proportion of those seen as background expression (this threshold fluctuated very little throughout each analysis session). Values were taken as pixel counts and then converted to silver grain measurements by calculating the mean number of pixel grains per silver grain, in each area of analysis. Silver grain density measurements were taken from 10 lamina II neurones and 6 large laminae III/IV neurones. In all cases, the most highly-expressing cells in each area were utilised.

In addition, control background densities were measured from a non-expressing cell within each area of analysis. These values were always approximately one-fifth or less of the density values for positive cells.

Results were entered into a spreadsheet for statistical analysis, and the mean number of silver grains per positively-labelled cell per section calculated. Background values were subtracted from the total count values to give the net density of expression per lamina II or laminae III/IV positively-labelled neurone. Finally, the mean silver grain density of expressing cells per animal was calculated.

7.2.7 Statistics

Data are presented as the mean ± SEM for neuropathic (n=4) or control (n=3) animals. Group means were analysed using the Jandell Scientific Sigmastat program, and the data was tested for normal distribution. A two-tailed pairwise

comparison *t*-test was used to determine ipsilateral versus contralateral differences in the number of expressing cells as well as in the density of silver grains per positively-labelled cell. The unpaired student *t*-test was used to test the significance of contralateral versus control animal values. $P \leq 0.05$ was considered significantly different.

7.3 RESULTS

7.3.1 Distribution of VIP_1, VIP_2 and PACAP Receptor mRNA Expression within the Normal Spinal Dorsal Horn

ISHH, using oligonucleotide probes complementary to specific areas of VIP_1, VIP_2 and PACAP receptor mRNA, revealed that the basal expression of mRNA for all three receptors was differentially distributed throughout the spinal cord of normal animals (See Figure 7.2).

The mRNA for the VIP_1 receptor appeared to show the most widespread basal expression in the dorsal horn of normal animals, particularly in lamina II where the average cell count for positively-labelled cells was 36 ± 5, compared to mean cell counts of 8 ± 1 and 9 ± 3 for VIP_2 and PACAP receptor mRNA respectively (See Table 7.1 for raw data). However, in terms of hybridisation per cell (represented by silver grain density per cell), VIP_1 receptor mRNA expression appeared to be greater than that seen in the more superficial laminae, with an increased average silver grain density of 22 ± 1 compared to 10 ± 1 in lamina II. Again, in laminae III/IV the expression of mRNA for the VIP_1 receptor was higher than that seen for VIP_2 and PACAP receptors, with respect to both mean number of positively labelled cells (13 ± 1 compared to 8 ± 1 and 6 ± 1 respectively) and average silver grain density (22 ± 1 compared to 15 ± 2 or 13 ± 4).

As mentioned, in contrast to VIP_1 receptor mRNA expression, there appeared to be very few VIP_2 positively-labelled neurones in lamina II of the lumbar spinal dorsal horn. The average silver grain density for those which were apparently expressing was only 4 ± 1, not quite 5-times the level of background silver grain expression, the criteria set for identifying positively-labelled cells. From these data then it would seem there is normally very little, if any VIP_2 receptor expression in lamina II. In contrast, the density of silver grain expression in laminae III/IV was

much higher (average 15 ± 2), suggesting that there may be differential expression of VIP_2 receptor mRNA in the deeper laminae of the normal spinal dorsal horn, but as stated previously, the number of positively-expressing cells was still slightly less than that seen for VIP_1.

The overall density of silver grain deposition appeared to be very low for the PACAP receptor mRNA. This low level of silver grain expression made it very difficult to distinguish expressing, from non-expressing neurones within the spinal cord. Similar to the VIP_2 receptor expression, there were very few PACAP receptor expressing neurones within lamina II of the dorsal horn, and those which did appear to be positively-labelled displayed a very low silver grain density (mean 7 ± 1), indicative of a low level of expression. The density of expression in laminae III/IV was slightly higher (average 13 ± 4), but there was also a greater degree of variability within the values.

7.3.2 Distribution of mRNA for VIP_1, VIP_2 and PACAP Receptors within the Spinal Dorsal Horn of CCI Animals

The expression of VIP_1, VIP_2 and PACAP receptor mRNA appeared to be differentially regulated after peripheral nerve injury. Following CCI of the rat sciatic nerve, there are visibly obvious changes in the expression of mRNA for VIP/PACAP receptor subtypes, in the dorsal horn ipsilateral to nerve injury, as revealed by ISHH.

When compared to control and contralateral values, the number of neurones expressing mRNA for the VIP_1 receptor appeared to decrease in the ipsilateral dorsal horn following CCI. These decreases were significantly different ($p \leq 0.05$) in the lateral edges of laminae III/IV (Figure 7.3(a)) with a decrease in the average cell count from 15 ± 1 to 12 ± 1 (contralateral) or 13 ± 1 (control value). The expression of VIP_1 receptor mRNA, represented by silver grain deposition, also appeared to show a decrease (25 ± 2 to 19 ± 1: contralateral versus ipsilateral values). Although there was no significant difference between the contralateral and control values for either cell counts or silver grain densities (two-tailed unpaired student *t*-test: $P \leq 0.05$), the overall expression of silver grains, including background expression, was slightly higher in the neuropathic sections. In contrast, the ipsilateral cell count and silver grain density values in lamina II remained not detectably changed from either

control or contralateral values (Figure 7.4(a)), that is a large proportion of lamina II neurones still appeared to be expressing mRNA for the VIP_1 receptor.

On the other hand, the number of cells expressing mRNA for the VIP_2 receptor, markedly increased in laminae III/IV, in the same lateral edges where the expression of VIP_1 receptor mRNA was seen to decrease (Figure 7.3(b)). This significant increase was highly consistent for both cell counts and absolute silver grain counts. The average number of positively-expressing cells within the area of analysis increased in the ipsilateral spinal cord to a value of 11 ± 1 from a contralateral value of 7 ± 1 cells per 30 625 μm^2. Similarly, the average silver grain density increased markedly from a contralateral value of 16 ± 2 to 25 ± 1 in the ipsilateral dorsal horn. As with the VIP_1 receptor, there were no apparent changes in the expression of mRNA for the VIP_2 receptor in lamina II of the dorsal horn following CCI (Figure 7.4 (b)). Raw data in Table 7.1. Again, there were no significant differences between the contralateral and control values for either mean cell counts or silver grain densities (two-tailed unpaired student *t*-test: $P \leq 0.05$),

As with the control sections, the expression of silver grains, indicative of expression of PACAP receptor mRNA, was consistently low throughout the entire spinal cord of all sections analysed, including background expression. This made it difficult to distinguish between expressing, and non-expressing dorsal horn neurones. From the data obtained, the levels of mRNA expression for the PACAP receptor appeared to be much lower than those observed for the VIP_1 and VIP_2 receptors, in the ipsilateral dorsal horn of neuropathic animals. No significant changes were seen in the low number of cells expressing (approximately 7-8 per 30 625μm^2), or the density of expression of PACAP receptor mRNA in contralateral versus ipsilateral dorsal horn, nor in contralateral versus control values, in either laminae III/IV (Figure 7.3 (c)) or lamina II (Figure 7.4 (c)). As the PACAP hybridisation observed here is equivalent to background levels, this suggests that there may be very little PACAP receptor mRNA expressed in the spinal cord of normal and neuropathic animals.

Data showing the average number of positively-labelled mRNA-expressing cells (per 30 625μm^2) and the average silver grain density for these expressing cells,

are displayed as summary histograms for all three receptor subtypes in Figures 7.5 and 7.6 respectively.

7.3.3 Controls

To verify the sensitivity of the PACAP receptor mRNA hybridisation, the constitutive expression in untreated rat brain was assessed. Positively labelled cells were observed in the dentate gyrus and CA1-CA3 layers of the hippocampus, as well as throughout the thalamus (data not analysed).

Control experiments with RNase A pre-treatment prior to hybridisation, or using hybridisation with an excess of unlabeled probe, failed to give convincing specific hybridisation:

(a) RNase controls

No positively-labelled cells were detected in the spinal cord sections which had been pre-treated with RNase A prior to hybridisation. This treatment produced a low, even distribution of silver-grains equivalent to the non-specific background observed in positive controls, when using the VIP_1/VIP_2/PACAP probes (See Figure 7.1).

(b) "Cold" Probe Controls

The specificity of the ISHH technique was further demonstrated by diluting the radiolabelled probe with a 10-fold molar excess of "cold" unlabelled probe. This resulted in a significant decrease in the density of silver grain expression in the treated sections when compared to positive control values.

Table 7.1

Raw Data

Tables showing the mean number of positively-labelled cells per 30 625μm^2 (A) and the average number of silver grains per positively-labelled cell (B), for each of the three receptor subtypes, in lamina II and laminae III/IV of normal and neuropathic rats, as revealed using *in situ* hybridisation histochemistry.

Data is expressed as the mean ± the standard deviation for each test animal.

Table A	Control		Neuropathic			
Receptor mRNA			Ipsilateral		Contralateral	
	LII	LIII/IV	LII	LIII/IV	LII	LIII/IV
VIP1	41 ± 11	14 ± 1	32 ± 9	15 ± 6	43 ± 11	15 ± 2
	41 ± 5	13 ± 1	30 ± 10	12 ± 1	31 ± 7	15 ± 5
	27 ± 6	12 ± 1	16 ± 3	10 ± 6	18 ± 2	14 ± 6
			41± 8	8 ± 2	39 ± 1	13 ± 1
VIP2	10 ± 6	9 ± 1	20 ± 5	14 ± 2	16 ± 7	8 ± 2
	6 ± 4	8 ± 3	11 ± 1	10 ± 1	6 ± 1	7 ± 1
	8 ± 3	7 ± 2	5 ± 2	8 ± 3	3 ± 2	7 ± 2
			3 ± 1	10 ± 4	4 ± 1	6 ± 1
PACAP	15 ± 1	6 ± 1	8 ± 1	9 ± 2	7 ± 3	7 ± 3
	7 ± 4	5 ± 2	5 ± 2	8 ± 2	5 ± 3	7 ± 2
	6 ± 1	6 ± 1	12 ± 3	7 ± 1	8 ± 4	9 ± 2

Table B	Control		Neuropathic			
Receptor mRNA			Ipsilateral		Contralateral	
	LII	LIII/IV	LII	LIII/IV	LII	LIII/IV
VIP1	10 ± 2	23 ± 4	12 ± 2	18 ± 2	13 ± 1	20 ± 1
	10 ± 1	22 ± 1	9 ± 3	18 ± 2	11 ± 3	24 ± 4
	10 ± 3	20 ± 1	12 ± 1	20 ± 2	13 ± 2	25 ± 2
			14 ± 1	19 ± 6	17 ± 2	29 ± 1
VIP2	6 ± 4	19 ± 4	5 ± 2	23 ± 9	5 ± 2	13 ± 6
	3 ± 1	12 ± 2	10 ± 1	26 ± 1	7 ± 1	21 ± 1
	5 ± 1	15 ± 3	6 ± 3	25 ± 7	6 ± 2	15 ± 7
PACAP	6 ± 1	9 ± 1	8 ± 3	13 ± 7	8 ± 1	15 ± 9
	10 ± 1	20 ± 1	7 ± 2	21 ± 1	8 ± 1	20 ± 5
	6 ± 2	10 ± 2	6 ± 1	15 ±5	6 ± 1	11 ± 3

Figure 7.2

Photomicrographs Showing Areas of Analysis Within the Spinal Dorsal Horn, and RNase Treated Control Tissue

Part (A) shows a low power lightfield, black and white photomicrograph of the dorsal horn of normal rat lumbar spinal cord (outlined in white), as revealed by ISHH (scale bar 150μm). This photomicrograph represents tha basal expression of VIP_1 receptor mRNA within the normal dorsal horn, although it is easier to see the expression of individual silver grains at higher power (see figures 7.3/7.4). The black boxed areas show the regions of analysis for Lamina II (upper box) and Laminae III/IV (lower box).

Parts B, C and D represent high power, light field, black and white photomicrographs showing the typical levels of background hybridisation with probes for VIP_1, VIP_2 and PACAP receptor mRNA respectively, in laminae III/IV of control dorsal horn, following pre-treatment of the sections with RNaseA prior to hybridisation (scale bar 30μm). Note the absence of silver grain clustering around the cell nuclei (a characteristic of labelled cells). The non-specific silver grain expression here is similar to the low level of even background expression seen in control regions within untreated sections in control and CCI experiments (see figures 7.3/7.4 for comparison).

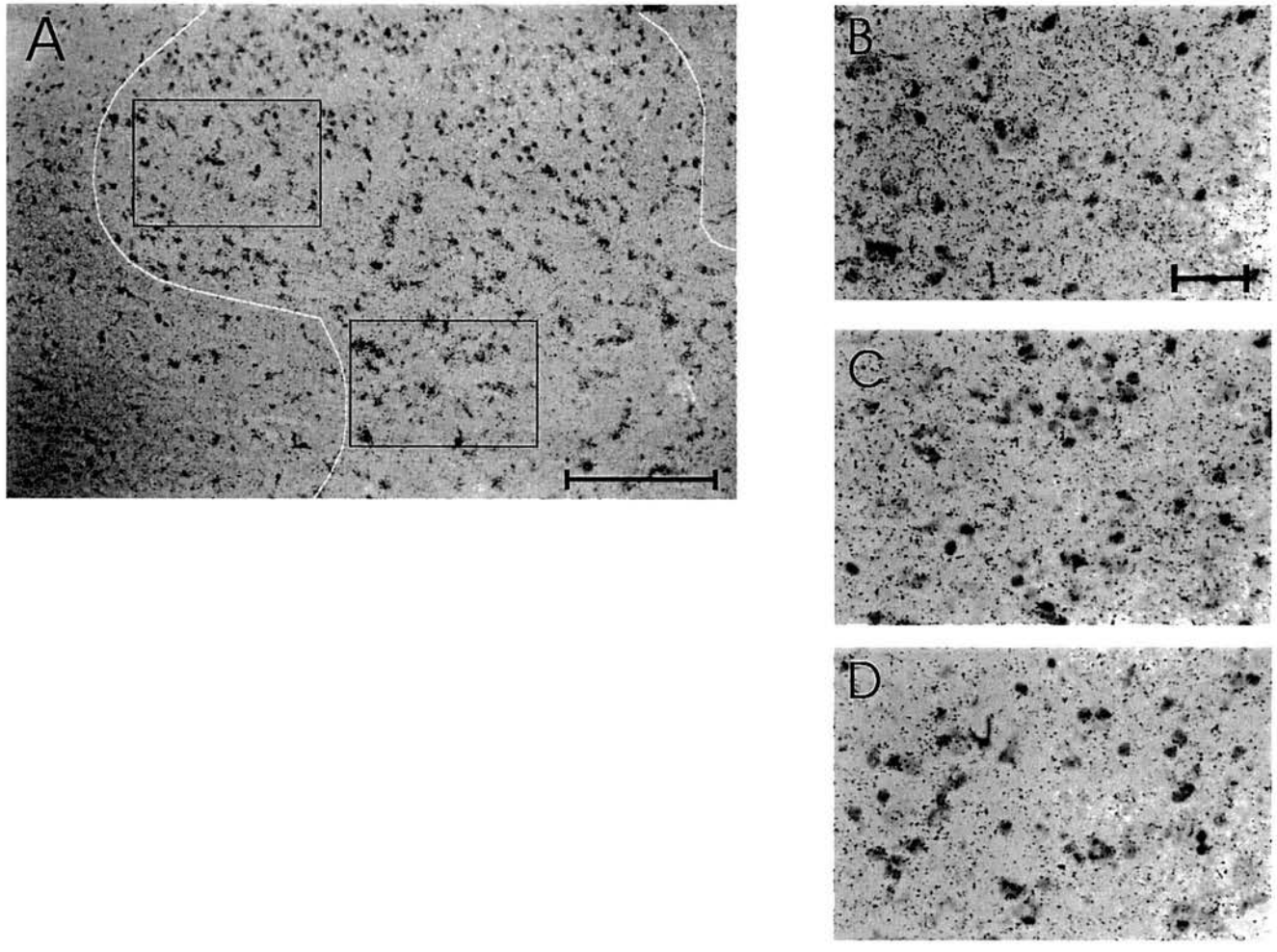

A
B
C
D

Figure 7.3

VIP_1, VIP_2 and PACAP Receptor mRNA Expression in Laminae III/IV of the Rat Spinal Dorsal Horn Before and After CCI

Highpower lightfield, black and white photomicrographs showing the typical levels of (A) VIP_1, (B) VIP_2 and (C) PACAP receptor mRNA expression in the lateral edges of Laminae III/IV of rat lumbar spinal cord (scale bar 30μm). Parts (i) show the receptor mRNA expression ipsilateral to a chronic constriction injury of the rat sciatic nerve, while parts (ii) and (iii) show typical examples of the expression contralateral to nerve injury and in control tissue respectively. Positively-labelled neurones where identified by a dense accumulation of silver grains (approximately > 5 times background expression) over and around haematoxylin stained nuclei.

Despite the higher levels of background silver grain expression, a significant decrease in the expression of VIP_1 receptor mRNA ipsilateral to the nerve injury (Part A(i)) when compared to contralateral (A(ii)) tissue can clearly be seen. In contrast, the expression of VIP_2 receptor mRNA is seen to significantly increase ipsilateral to nerve injury (B(i)) from control (B(iii)) or contralateral (B(ii)) levels. While the ipsilateral expression of mRNA for the PACAP receptor (C(i)) remains relatively unchanged from contralateral (C(ii)) or control (C(iii)) levels.

There were no significant differences between contralateral and control levels of mRNA expression, as revealed by cell counts or silver grain density, for any of the three receptors.

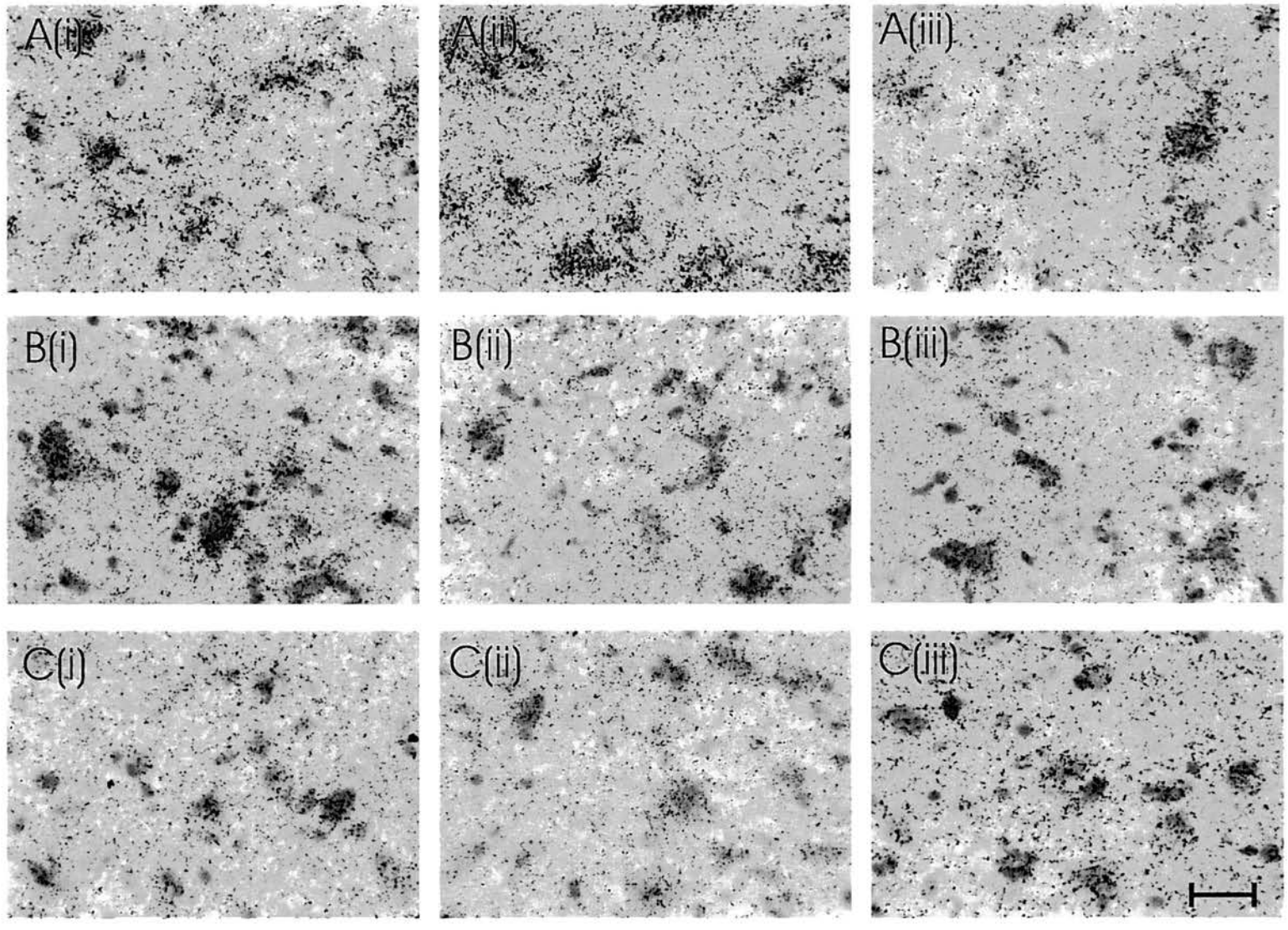

A(i)
A(ii)
A(iii)
B(i)
B(ii)
B(iii)
C(i)
C(ii)
C(iii)

Figure 7.4

VIP_1, VIP_2 and PACAP Receptor mRNA Expression in Laminae II of the Rat Spinal Dorsal Horn Before and After CCI

Highpower lightfield, black and white photomicrographs showing the typical levels of (A) VIP_1, (B) VIP_2 and (C) PACAP receptor mRNA expression in the lateral edges of Lamina II of rat lumbar spinal cord (scale bar 30μm). Parts (i) show the receptor mRNA expression ipsilateral to a chronic constriction injury of the rat sciatic nerve, while parts (ii) and (iii) show typical examples of the expression contralateral to nerve injury and in control tissue respectively. Positively-labelled neurones where identified by a dense accumulation of silver grains (approximately > 5 times background expression) over and around haematoxylin stained nuclei.

Note the higher number of positively-labelled cells for VIP_1 receptor mRNA (Parts A), compared to VIP_2 and PACAP (Parts B and C respectively). This was consistent throughout the whole of lamina II in all the sections analysed. There were no significant differences due to CCI between average values of the number of positively-expressing cells, or the density of mRNA expression for either VIP_1, VIP_2 or PACAP receptors in lamina II.

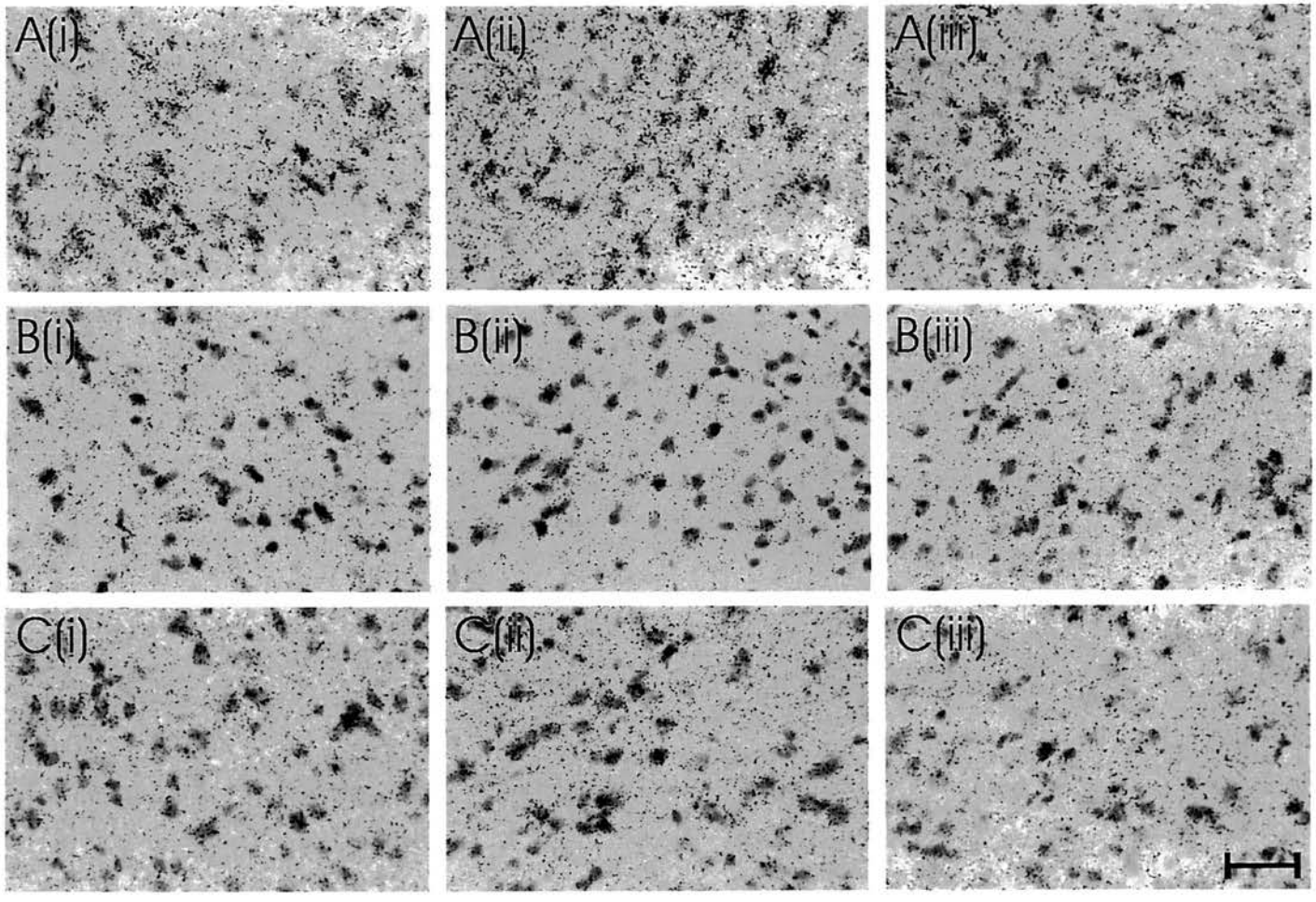
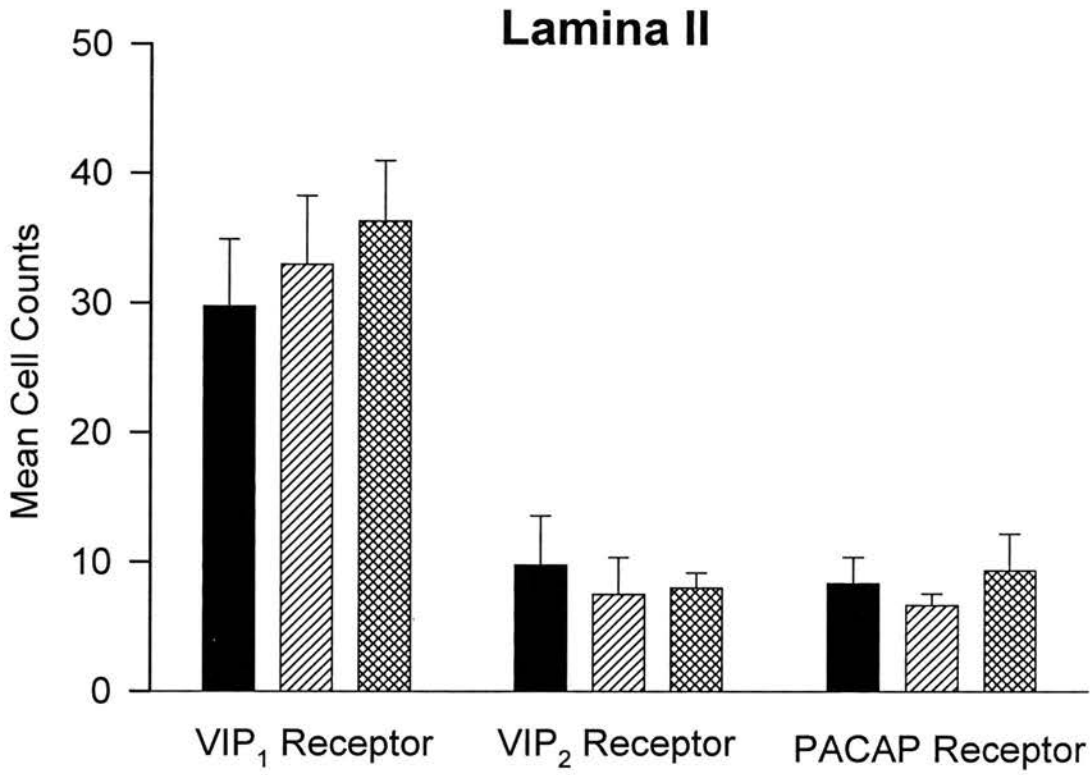

A(i)
A(ii)
A(iii)
B(i)
B(ii)
B(iii)
C(i)
C(ii)
C(iii)

Figure 7.5

Mean number of Positively-Expressing Dorsal Horn Neurones for VIP_1, VIP_2 and PACAP Receptor mRNA

Summary histogram showing the average number of dorsal horn neurones (per 30 $625\mu m^2$) positively-expressing VIP_1, VIP_2 or PACAP receptor mRNA in Lamina II and Laminae III/IV of the lumbar spinal cord. Values ipsilateral to the nerve injury (■) are shown with respect to contralateral (▨) and control (⊠) values.

The average number of cells expressing mRNA for any of the three receptors was relatively unchanged in Lamina II in normal compared to neuropathic spinal cord. In laminae III/IV however, the number of cells expressing VIP_1 receptor mRNA was seen to markedly decrease ipsilateral to nerve injury compared to contralateral values, while those expressing VIP_2 receptor mRNA significantly increased (two-tailed paired student *t*-test: * $P \leq 0.05$). There were no significant differences between contralateral and control values for any of the three receptors (two-tailed unpaired student *t*-test: $P \leq 0.05$).


Lamina II
Mean Cell Counts
50
40
30
20
10
0
VIP1 Receptor
VIP2 Receptor
PACAP Receptor

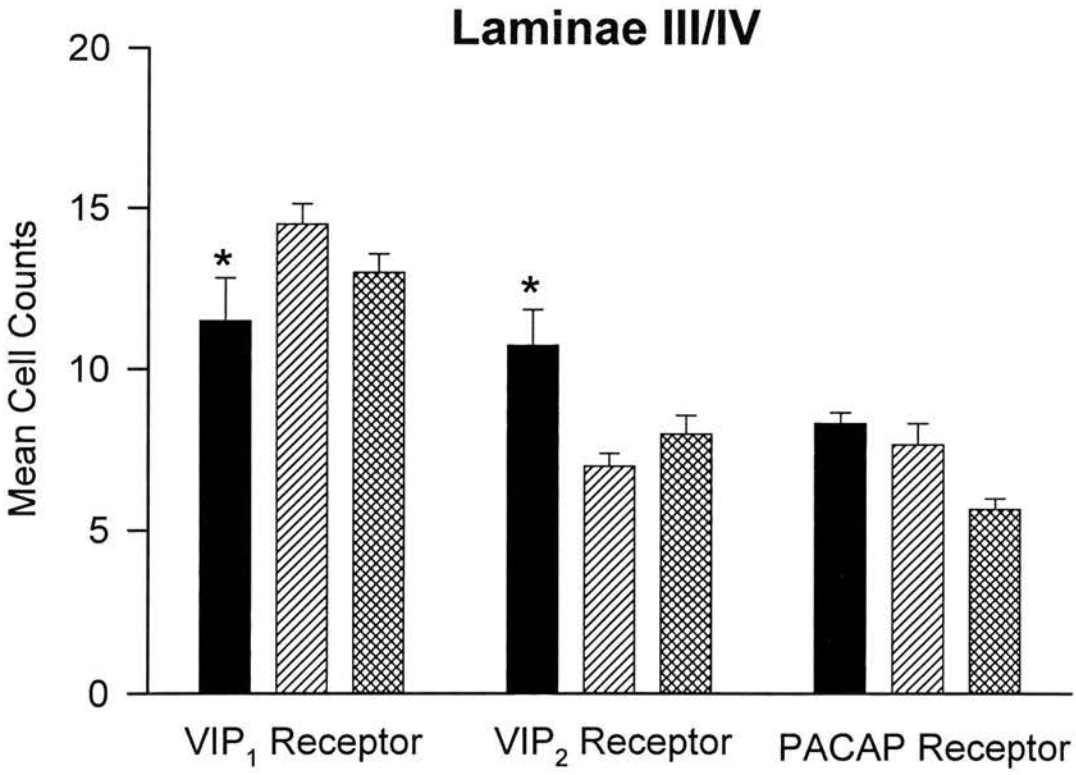
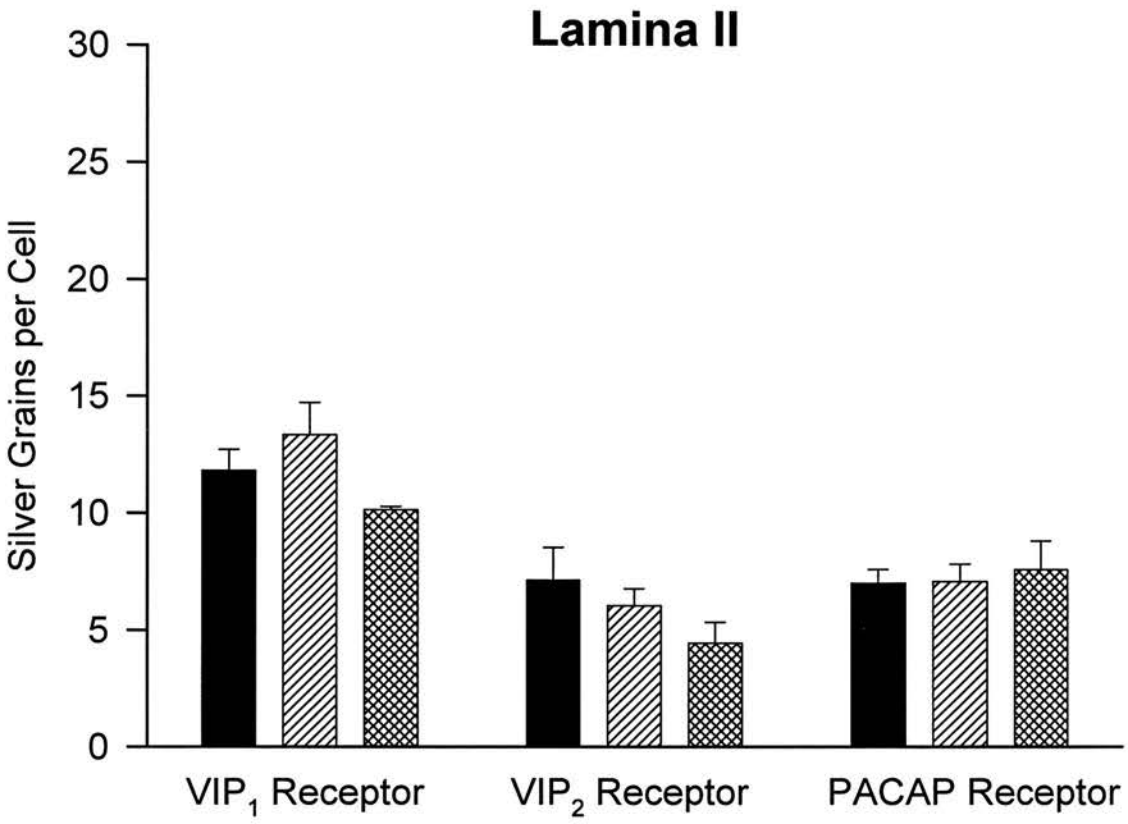

Laminae III/IV
Mean Cell Counts
20
15
10
5
0
*
*
VIP1 Receptor
VIP2 Receptor
PACAP Receptor

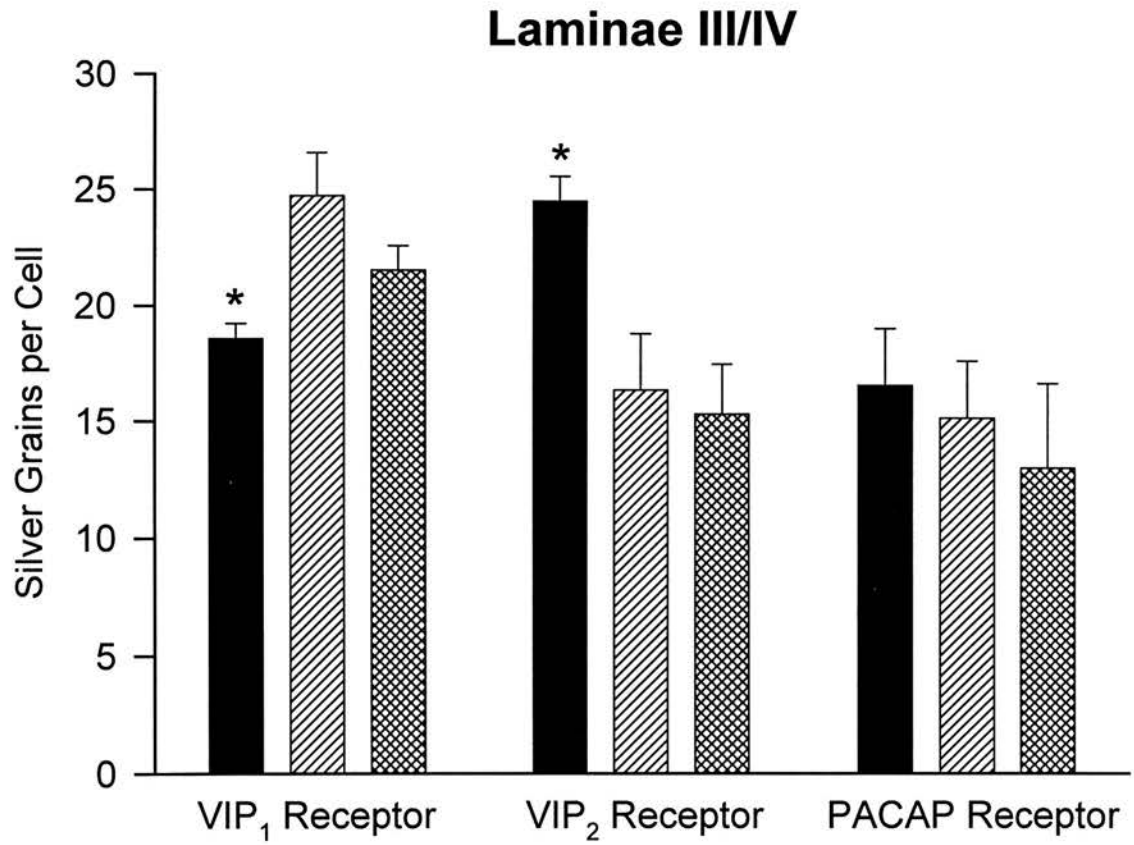
Figure 7.6

Average Silver Grain Density of Positively-Expressing Dorsal Horn Neurones for VIP_1, VIP_2 and PACAP Receptor mRNA

Summary histogram showing the average silver grain density for dorsal horn neurones positively-expressing VIP_1, VIP_2 or PACAP receptor mRNA in lamina II and laminae III/IV. Values ipsilateral to the nerve injury (■) are shown with respect to contralateral (▨) and control (⊠) values.

The average silver grain density per expressing cell, indicative of the expression of receptor mRNA, was relatively unchanged for all three receptors in Lamina II, in normal compared to neuropathic spinal cord. In laminae III/IV however, the density of expression of VIP_1 receptor mRNA was seen to markedly decrease ipsilateral to nerve injury compared to contralateral values, while that of VIP_2 receptor mRNA significantly increased (two-tailed paired student *t*-test: * $P \leq 0.05$). There were no significant differences between contralateral and control values for any of the three receptors (two-tailed unpaired student *t*-test: $P \leq 0.05$).


Lamina II
Silver Grains per Cell
30
25
20
15
10
5
0
VIP1 Receptor
VIP2 Receptor
PACAP Receptor


Laminae III/IV
Silver Grains per Cell
30
25
20
15
10
5
0
*
*
VIP1 Receptor
VIP2 Receptor
PACAP Receptor

7.4 DISCUSSION

These results provide new insight into the distribution of the VIP_1, VIP_2 and PACAP receptors within rat spinal dorsal horn, as well as revealing, for the first time, changes in expression of these receptor subtypes following an experimental peripheral mononeuropathy.

It has previously been shown that there is a distinct distribution of the VIP_1, VIP_2 and PACAP receptors in both the peripheral and central nervous systems of the rat (Harmar and Lutz, 1994; Lutz et al. 1993; Shioda et al. 1997; Usdin et al. 1994; Vertongen et al. 1997). ISHH studies have revealed that VIP_1 and VIP_2 receptor mRNA shows differential expression within the brain (Harmar and Lutz, 1994; Lutz et al. 1993; Usdin et al. 1994), with just some areas of overlap. More recently, the production of high affinity agonists with marked selectivity for the VIP_1 and VIP_2 receptors (Gourlet et al. 1997a; 1997b) has allowed autoradiographic mapping of the VIP receptor proteins to be carried out, using iodinated forms of these molecules (Vertongen et al. 1997). These studies have confirmed the differential expression of VIP_1 and VIP_2 receptors within the rat brain. The VIP_2 receptor was shown to be expressed in a number of areas where VIP_1 receptor expression was absent, or present at very low levels, including the thalamic nuclei, suprachiasmatic nucleus and the periventricular nucleus. In contrast, the VIP_1 receptor was the predominant receptor expressed in the dentate gyrus and supraoptic nucleus (Vertongen et al. 1997). As with previous findings, expression of both receptors was found to overlap in some areas of the brain, including the cerebral cortex, claustrum, the nucleus accumbens and the choroid plexus (Vertongen et al. 1997).

This differential expression is also observed in the periphery, as the VIP_1 receptor has been shown to be expressed in a number of peripheral tissues including the liver, lung, small intestine and the thymus (Harmar and Lutz, 1994; Usdin et al. 1994), while the VIP_2 receptor was found in a number of areas where VIP acts but where VIP_1 receptor mRNA is absent, or is present at very low levels, including the stomach and testes (Usdin et al. 1994). The combined VIP_1/VIP_2 receptor mRNA distribution, is consistent with the overall distribution of VIP-binding sites, as shown by autoradiography (Besson et al. 1986; Staun-Olsen et al. 1985), and so it is clear that the actions of VIP within the central and peripheral nervous systems may be

mediated by either of these receptor subtypes, and may reflect different functional roles for VIP within these different areas.

The expression of mRNA for the PACAP receptor shows some degree of overlap with that of the VIP_1/VIP_2 receptors. It is abundantly expressed in the brain where it shows some similarities to the distribution of the VIP_2 receptor, notably in the olfactory bulb, thalamus, hippocampus, pituitary and the hypothalamus (Cauvin et al. 1991; Masuo et al. 1992; 1993; Shioda et al. 1997). However, there are some distinct differences, with the preferential expression of the PACAP receptor within the hypothalamus, cerebellum, pons and medulla oblongata (Sheward et al. 1995). In addition, there is very little PACAP receptor mRNA expression in the peripheral tissues (Arimura and Shioda, 1995; Hashimoto et al. 1993), unlike VIP_1 and VIP_2 receptor mRNA. This distinct distribution of VIP/PACAP receptors may reflect different functions for each of the three receptor subtypes. As the distribution of these receptors within the spinal dorsal horn has not been previously investigated in any great detail, the aim of this study was to determine any differences in the expression of mRNA for the VIP_1, VIP_2 and PACAP receptors within normal rat spinal cord, and to try and ascertain whether the levels of expression change following a peripheral nerve injury.

Indeed, mRNA for VIP_1, VIP_2 and PACAP receptors appeared to show differing degrees of expression within the dorsal horn of normal animals. Expression of mRNA for all three receptors was found, to some degree, throughout the lumbar segments of the spinal cord studied, particularly in the dorsal horn. The small diameter neurones in lamina II showed a predominant expression of VIP_1 receptor mRNA expression, while the number of cells expressing either VIP_2 or PACAP receptors were relatively low in this lamina. This level of receptor expression appeared to be relatively unchanged following nerve injury.

In contrast, this study has demonstrated that CCI of the rat sciatic nerve leads to marked changes in the expression of VIP/PACAP receptor subtypes in the deeper ipsilateral dorsal horn. The most marked change in receptor expression was seen in the lateral edges of laminae III/IV, where the levels of VIP_1 receptor mRNA significantly decreased, ipsislateral to nerve injury, while the expression of VIP_2 receptor mRNA significantly increased. As discussed previously, it has been shown

that the mRNAs encoding the VIP_1 and VIP_2 receptors have a complementary distribution within the rat brain (Usdin et al. 1994; Vertongen et al. 1997), and so it was interesting to see that the levels of VIP_2 receptor expression appeared to increase in the same areas where VIP_1 receptor levels dropped. If these receptors do have different functional properties, this change-over may reflect the need for different receptor functions following the central changes brought about by neuropathy.

The ISHH results observed here as regards the expression of PACAP receptor mRNA were somewhat surprising. Five oligonucleotide probes were used in this investigation to try and increase the sensitivity of the assay, as mRNA for the PACAP receptor could not be detected in the spinal cord using one or two probes alone. All five probes were highly specific for regions of the DNA sequence for the PACAP receptor as produced by Hoysoya et al. (1993). Although a number of different subtypes of the PACAP receptor can be generated by alternative splicing (Spengler et al. 1993), the probe sequences used in the present study were common to mRNAs for all the variants and so should have bound to any mRNA for subtypes of the PACAP receptor present. At least 6 isoforms of the PACAP receptor have been described (Spengler et al. 1993) which differ in the amino acid sequence within the 3rd intracellular loop, an area which is considered to be crucial for interactions with G-proteins (Spengler et al. 1993). Expression of these different receptor subtypes in transfected cells, has been shown to be associated with different patterns of stimulation of adenylate cyclase and PLC, and PCR-amplified products of different regions of the brain, have revealed the differential prominence of these different splice variants of the PACAP receptor (Spengler et al. 1993) This highlights the possibility that PACAP could mediate a number of different physiological roles via different receptor variants.

From the results observed in the agonist study (Chapter 6) it is clear that PACAP-38 has the ability to activate approximately two-thirds of the dorsal horn neurones tested in both normal and neuropathic animals. In contrast, the present data suggest that the expression of mRNA for the PACAP receptor is relatively low in the rat spinal cord, in comparison to the VIP_1 and VIP_2 receptors. This may be due to the fact that the ISHH conditions were not at their optimum for hybridisation of the PACAP probes, but all of the probes used (for PACAP, VIP_1 and VIP_2) were of

similar base lengths (45-48mer) and had similar G/C contents (50-60%) and so the optimum conditions for all probes should be in a similar range. In addition, positively-labelled cells were observed in certain areas of the brain known to express the PACAP receptor, including the hippocampus and thalamus. Thus it seems likely that expression of the PACAP receptor within the rat spinal cord is in fact very low, although it is possible that other unknown technical factors, including for example low message stability, could have contributed to these findings. As PACAP can also bind with a relatively high affinity to the VIP_1 and VIP_2 receptors (Hashimoto et al. 1993; Shivers et al. 1991), this would suggest that any effects that PACAP may have within the spinal cord are mediated via the VIP_1 and VIP_2 receptors, and so may reflect a different physiological role for PACAP within the spinal cord than in other areas of the central nervous system (notably the brain) where the PACAP receptor predominates.

The changes in expression of VIP_1 and VIP_2 receptor mRNAs, but not of that for the PACAP receptor, seen in this ISHH study, parallel those deduced from the agonist study in Chapter 6. From these results therefore, we can speculate that VIP_2 receptor protein expression is increased within the deeper laminae of the ipsilateral dorsal horn following CCI, while that of VIP_1 decreases. This differential change in receptor expression may reflect a change in function for VIP/PACAP within the spinal cord of neuropathic animals (consistent with evidence for elevated expression of VIP and PACAP peptides in DRG neurones) and highlights the potential importance of the VIP_2 receptor in particular in the development of neuropathic pain states. These results therefore implicate the VIP_2 receptor as a possible target for analgesics in neuropathy. In addition, the relatively low expression of mRNA for the PACAP receptor within the spinal cord, and the lack of change of expression for this receptor after nerve injury, may suggest that any functional role PACAP has within the dorsal horn is most likely to be mediated primarily via the VIP_1 receptor in normal conditions and the VIP_2 receptor following neuropathy.

CHAPTER 8 SUMMARY AND CONCLUSIONS

Neuropathic pain due to nerve injury, may occur as a result of peripheral tissue damage due to disease or trauma, or may be a direct result of nerve transection, crushing or constriction. The chronic pain states which develop include spontaneous pain, hyperalgesia and allodynia, and they may persist long after the initial injury has healed (Scadding, 1984). These abnormal pain states show a reduced sensitivity to classical opioid analgesics (Arner and Meyerson, 1988; Mao et al. 1995), and anaesthetic nerve blocks or surgical sympathectomies often provide only temporary relief (Loh and Nathan, 1978; Luo and Wiesenfeld-Hallin, 1995). However, treatments using anticonvulsant drugs such as carbamazepine (Fields et al. 1997; McQuay et al. 1995; Tanelian and Brose, 1991), or tricyclic antidepressants (McQuay et al. 1996) have shown more promising results. The mechanisms underlying the development of neuropathic pain are poorly understood, but inflammatory mediators released at the periphery (Tracey and Walker, 1995), central changes within the spinal cord (Woolf, 1983), including changes in receptor and protein expression (Hokfelt et al. 1994), and biochemical and pathological changes within the damaged nerve itself (Coggeshall et al. 1993; Sebert and Shooter, 1993) are all thought to contribute.

A number of neurochemical changes within dorsal root ganglion neurones and the spinal dorsal horn, have been well-documented following peripheral nerve injury (Hokfelt et al. 1994) including a marked up-regulation of the expression of VIP and PACAP. Previous evidence has suggested that these peptides may have neurotransmitter and/or neuromodulatory functions within the CNS (Jeftinija et al. 1982; Phillis et al. 1978; Narita et al. 1996; Salt and Hill, 1981; Xu and Weisenfeld-Hallin, 1996) and so this project combined electrophysiological and molecular biological techniques to gain further insight into these proposed roles.

Although many different conditions can lead to the development of neuropathic pain, including diabetes and the herpes zoster virus (Scadding 1984), the most commonly used models of neuropathy usually involve peripheral nerve injury. In this study we utilised the Bennett and Xie chronic constriction injury model (1988), in which four chromic cat gut ligatures were tied loosely around the rat

sciatic nerve so as to barely constrict it. Consistent with previous studies (Attal et al. 1990; Bennett and Xie, 1988), the animals subsequently developed mechanical and thermal allodynia, as well as mechanical hyperalgesia, in their affected hind limb. These lowered thresholds to sensory stimuli showed maximal changes approximately 2 weeks after the initial nerve injury. At this time point, those animals showing strong signs of neuropathic pain (that is, a significantly reduced threshold to von Frey filaments, a prolonged paw elevation time when placed in a shallow cold water bath, and an exaggerated response to noxious pin-prick) were then used to investigate whether VIP/PACAP receptors are important regulators of the amplified sensory responses which had developed.

Selective agonists and antagonists were used to address the role of VIP and PACAP within the spinal dorsal horn, as mediated via the VIP_1, VIP_2 and PACAP receptors. Firstly, an electrophysiological study was carried out using novel selective antagonists for the three receptor subtypes. The effects of these receptor antagonists were investigated on the sustained neuronal activity of individual dorsal horn neurones, induced by sensory stimuli. These experiments were performed in normal, as well as neuropathic rats at approximately 2 weeks PO, and the results compared. Subsequently, the effects of ionophoretic application of novel selective agonists for the VIP_1, VIP_2 and PACAP receptors were examined on multireceptive dorsal horn neurones, in both normal and neuropathic animals. Finally, a complementary ISHH study was carried out to investigate the distribution of mRNA for the three receptor subtypes within the spinal dorsal horn, and to ascertain whether the expression of these receptors was altered in any way following an experimental peripheral mononeuropathy.

8.1 VIP_1/VIP_2/PACAP Receptor Involvement in the Transmission of Sensory Information

VIP_1, VIP_2 and PACAP receptors appear to play an important role in the transmission of sensory information within the spinal cord of both normal and neuropathic rats.

Despite the fact that VIP and PACAP are present in relatively low abundance in the spinal cord and DRG under normal circumstances (Noguchi et al. 1993;

Mulder et al. 1994), the present data provided clear evidence for the presence of functional VIP/PACAP receptors on multireceptive dorsal horn neurones in normal animals. The sustained sensory-induced activity of multireceptive dorsal horn neurones, was clearly mediated or promoted, at least in part, by the VIP_1, VIP_2 and PACAP receptors. Selective antagonists for the VIP_1 and PACAP receptors appeared to exert a generalised modulation of dorsal horn neurone responses, inhibiting both brush- and mustard oil-induced activity to similar extents. In contrast, the novel VIP_2 receptor antagonist was selectively antinociceptive (markedly inhibiting only the sustained C-fibre activity induced by topical application of the chemical irritant mustard oil) and thus suggesting a more restricted influence in the normal state.

In neuropathic animals a modulatory role for the three receptor subtypes was still evident, but the effects were clearly different from those seen in normal animals. The most obvious difference was the lack of effect of all three receptor antagonists on the brush-induced activity of dorsal horn neurones. The reduced threshold to von Frey filaments in CCI animals, indicative of mechanical allodynia, would imply that this stimulus would now be noxious in conscious animals. The development of mechanical allodynia therefore clearly has different contributing influences than those of thermal allodynia and mechanical hyperalgesia, which appear to be modulated, at least in part, by the VIP/PACAP receptors. In normal animals, VIP_1 and PACAP receptors appear to contribute to the brush responses, but this influence is apparently suppressed or outweighed in neuropathy. How this might be achieved is quite unknown, but the lack of Aβ fibre involvement in neuropathy is a likely candidate, as the CCI model of neuropathy causes a marked degeneration of these fibres within the sciatic nerve (Basbaum et al. 1991; Coggeshall et al. 1993). The neuropathy-induced increase in the expression of VIP and PACAP is predominantly in small to medium diameter DRG neurones corresponding to the small diameter Aδ and C fibres (Noguchi et al.1989; Zhang Q et al.1995) which would also suggest that VIP/PACAP are not likely candidates for direct mediators of the brush response in neuropathic animals unless new fibre types become involved. A gradual recovery of fibre composition within the sciatic nerve does occur, including both regeneration and sprouting (Coggeshall et al. 1993; Koerber et al. 1994; Ramer et al. 1997; Woolf

et al. 1995) and so it might be of interest to determine whether the inhibitory roles of the VIP_1 and PACAP receptors return in animals one or two months after the initial nerve injury, which would further implicate Aβ fibre involvement.

Whatever mechanisms underlie the changes that occur following nerve injury, an alteration in VIP/PACAP receptor function is clearly evident, as all three receptor subtypes showed selective antinociceptive actions in CCI animals. VIP_1 and PACAP receptor antagonists showed inhibitory effects on the cold-induced firing of dorsal horn neurones, implicating them in the development and/or maintenance of cold allodynia following nerve injury. On the other hand, exaggerated responses to noxious stimuli, indicative of hyperalgesia, may be mediated by all three receptor subtypes as antagonists for VIP_1, PACAP, and in particular, VIP_2 receptors all inhibited mustard oil-induced nociceptive activity in neuropathic animals. The clear differences in the effects of VIP_1/PACAP from VIP_2 receptor selective antagonists on the cold-induced neuronal activity, highlights the possibility of differential roles for each of the receptor subtypes in the regulation of different aspects of neuropathic pain.

These functional effects in neuropathy may be mediated by VIP and/or PACAP, as both peptides show a high affinity of binding to both VIP_1 and VIP_2 receptors (Christophe, 1993). Following peripheral axotomy or nerve ligation, the levels of both peptides increase dramatically in small diameter DRG neurones and nerve terminals within the superficial dorsal horn (Knyihar-Csillik et al. 1993; Noguchi et al. 1989; Shehab and Atkinson, 1986a; Zhang Q et al. 1995; Zhang et al. 1995), but with different time courses. There is a rapid upregulation of PACAP (within 1-2 days) which slowly reverses after 1-2 weeks (Zhang Q et al. 1995), while the levels of VIP are markedly increased within the first two weeks after nerve injury, and remain elevated for long periods of time (Nahin et al. 1994). This would be consistent with the possibility that PACAP may be an important regulator of the onset of neuropathy, while VIP may be more important in maintaining the prolonged pain states which subsequently develop.

Functional studies have already provided strong evidence of a regulatory role for VIP and PACAP at the spinal cord level, with both peptides showing facilitatory effects on the flexor reflex (Cridland and Henry, 1988; Narita et al. 1996;

Wiesenfeld-Hallin, 1987; Xu and Weisenfeld-Hallin, 1996), when applied intrathecally. This regulatory action appears to be particularly important in neuropathic animals, especially for VIP. It has been suggested that VIP takes over the role of SP as one of the main central neurotransmitters of nociceptive afferents following peripheral nerve section, as a VIP antagonist was shown to be highly effective in attenuating the electrically-induced flexor reflex in axotomised rats (Wiesenfeld-Hallin et al. 1990; Xu and Wiesenfeld-Hallin, 1991) while the SP antagonist Spantide II had no such effect, a complete reversal of the effects seen in normal animals.

So, VIP and PACAP both appear to play important regulatory roles in the transmission of sensory information at the spinal cord level. The fact that the VIP_1, VIP_2 and PACAP receptors are functionally active in normal animals, suggests that their roles may not simply be restricted to episodes of chronic pain. However, the selective antinociceptive nature of VIP_1, VIP_2 and PACAP receptors in neuropathic animals, makes them important new targets for the development of drugs to control currently intractable neuropathic pain states.

8.2 Altered Expression of VIP_1/VIP_2/PACAP Receptors Following Peripheral Nerve Injury and their Possible Function within the Rat Spinal Cord

The differential expression of the VIP_1, VIP_2 and PACAP receptors has been demonstrated on numerous occasions within the CNS (Harmar and Lutz, 1994; Lutz et al. 1993; Shioda et al. 1997; Usdin et al. 1994; Vertongen et al. 1997), and this was further confirmed here in both the agonist and *in situ* hybridisation studies performed within rat spinal cord.

Changes in the responsiveness of multireceptive dorsal horn neurones in normal and neuropathic animals, suggested that the expression of the VIP_2 receptor was increased following CCI of the rat sciatic nerve, while VIP_1 receptor expression appeared to decrease. Oligonucleotides complementary to specific areas in the mRNA of these two receptor subtypes also confirmed this. This dramatic increase in the expression of mRNA for the VIP_2 receptor may indicate a relatively greater role for this receptor in neuropathy compared to the normal state. Indeed, combined with

the antagonist results in the previous electrophysiological study, these data implicate the VIP_2 receptor as a prime target for the development of new analgesic agents.

In contrast with VIP_1 and VIP_2, the expression of PACAP receptor mRNA (and the appropriate agonist responses) appeared to be unaltered following neuropathy. It must be remembered however, that PACAP has the ability to act through all three receptor subtypes, as the VIP_1 and VIP_2 receptors show a high binding affinity for both VIP and PACAP (Christophe, 1993). So although these data suggest that there is no significant change in the expression of PACAP receptors, PACAP may still play an important role in the development of neuropathy via the VIP_1 and VIP_2 receptors.

Indeed, PACAP may even mediate its effect indirectly via NMDA receptor-mediated events, in addition to actions via the VIP_1 and VIP_2 receptors, as recent whole-cell patch-clamp recordings have revealed that PACAP-38 has the ability to exert modulatory effects on both cultured chick cortical neurones (Liu and Madsen, 1997) as well as sympathetic postganglionic neurones (SPN) (Wu and Dun, 1997). This potentiation of NMDA-receptor-mediated responses in SPN recordings is thought to be carried out via a cAMP-dependant mechanism (i.e. probably a VIP or PACAP receptor) (Wu and Dun, 1997), as prior incubation of the spinal cord slices with an adenylate cyclase inhibitor attenuated the facilitatory effects. With the cultured chick cortical neurones however, PACAP-38 appeared to have a direct modulatory effect on the NMDA receptor, which was independent of intracellular second messengers (such as cAMP, calcium and inositol phosphate), and is thought to be mediated instead, through the glycine coagonist site(s) (Liu and Madsen, 1997). Such a direct modulation of the NMDA receptor has previously been suggested for other peptides, including the opioids (Chen et al. 1995; Rusin and Randic, 1991) and the tachykinins, substance P and NKA (Rusin et al. 1992;1993). So there could potentially be two ways in which PACAP could contribute to the development of neuropathic pain states via its facilitatory effects at the NMDA receptor. The first is by direct chemical gating of the NMDA receptor to stimulate an influx of calcium ions, resulting in an increased responsiveness of the dorsal horn neurones to sensory inputs, and ultimately leading to the development of hyperalgesia and allodynia. The second is by triggering alterations in membrane excitability through interactions with

second messenger systems and protein kinases, which phosphorylate membrane-bound proteins (Nestler and Greengard, 1983). However, intracellular recording techniques are currently not advanced enough to distinguish between these two posiibilities. A large amount of evidence implicates NMDA receptor involvement in the development and maintenance of neuropathic pain states. Pre- and/or post-injury treatment with intrathecal MK-801 (a selective NMDA receptor antagonist) has been shown to significantly attenuate the development and/or onset of both mechanical and thermal hyperalgesia resulting from nerve injury (Kawamata and Omote, 1996; Mao et al. 1992a; 1992b; Smith et al. 1994). In addition, intrathecal MK-801, given just prior to axotomy, significantly suppresses autotomy in rats (Seltzer et al. 1991), suggesting that NMDA receptors may also be involved in the development of spontaneous pain following neuropathy. From these reports then, the long-lasting excitatory effects of PACAP-38 seen in the present studies, following ionophoretic application of the peptide on to multireceptive dorsal horn neurones, may in fact be NMDA receptor-mediated. This highlights a potential role for PACAP in the altered responsiveness of dorsal horn neurones to sensory stimuli and ultimately in the development and maintenance of the abnormal pain states which ensue neuropathy.

VIP on the other hand, only appears to have one mode of action, via the VIP_1 and VIP_2 receptors, which are both coupled only to adenylate cyclase (Ishihara et al. 1992). As mentioned previously, activation of these G-protein linked receptors triggers alterations in the cell membrane excitability through the activation of the cAMP pathway, which subsequently result in the phosphorylation of membrane-bound proteins (Nestler and Greengard, 1983). This increased metabolic activity can result in other intracellular changes however, including alterations in gene expression which may favour cell growth and differentiation, and/or cell survival.

Indeed, although the present study has concentrated on the potential neurotransmitter role of VIP and PACAP, activation of the VIP_1, VIP_2 and PACAP receptors can ultimately result in a number of biological and physiological changes both centrally and in the periphery. There is considerable data regarding the neurotrophic actions of VIP and PACAP. Both peptides have been shown to exhibit a number of distinct actions on the cell proliferation, survival and differentiation of well-characterised cell culture systems (Brenneman and Eiden, 1986; Gonzalez et al.

1997; Klimaschewski et al. 1995; Lioudyno et al. 1998; Pellegri et al. 1998; White and Mansfield, 1996). Some of these actions appear to be directly mediated by VIP and PACAP (Gonzalez et al. 1997; Klimaschewski et al. 1995; Lioudyno et al. 1998). However they also, and possibly more commonly, act indirectly by stimulating the release of trophic factors (including various cytokines and growth factors) from cells such as glial cells and lymphocytes (Pellegri et al. 1998; Tatsuno et al. 1996; Waschek, 1996; White and Mansfield, 1996). So it is clear then that VIP and PACAP have a multitude of biological actions which may directly contribute to the development of neuropathic pain states, or may even ultimately counteract them. These properties include neuronal protection (cell survival), enhancement of cell differentiation and proliferation, repair of damaged neurones through the indirect release of growth factors, as well as having the potential to enhance nerve fibre growth and regeneration (Brenneman and Eiden, 1986; Gonzalez et al. 1997; Klimaschewski et al. 1995; Lioudyno et al. 1998; Pellegri et al. 1998; Tatsuno et al. 1996; White and Mansfield, 1996).

In conclusion then, VIP and PACAP are extremely important mediators of neuropathy. The altered expression of the VIP/PACAP receptors, and the increased expression of the peptides themselves, may compensate functionally for the lack of classical neurotransmitters of nociceptive afferents (e.g. substance P) which are decreased in response to nerve injury, thus enabling VIP and PACAP to have direct regulatory roles in the transmission of sensory information within the spinal cord, and subsequently in the altered pain states which develop following nerve injury. In addition, they may also promote neuronal survival and fibre growth within the damaged nerve, as well as influencing spinal cord plasticity over the long-term, thereby contributing to the maintenance of chronic pain states.

8.3 Potential Therapeutic Value

This project provides new insight into the roles of VIP_1, VIP_2 and PACAP receptors within the central nervous system, and underlines the potential importance of VIP_1/VIP_2/PACAP receptor antagonists as new analgesic agents for use in currently intractable neuropathic pain states.

The profile of influence of these VIP/PACAP receptors is clearly very different from that of other modulators of sensory processing, such as the opioid and tachykinin peptides (Fleetwood-Walker et al. 1988;1993) as certain aspects of neuropathic pain appear to be less responsive to classical opioid analgesics (Arner and Meyerson, 1988; Mao et al. 1995). VIP_1, VIP_2 and PACAP receptors appear to play an important role in the regulation of nociceptive inputs of both normal and neuropathic rats. This may be a direct result of the cellular influences of VIP and/or PACAP acting at their receptors, or it could be that activation of these receptors may help to maintain a state which promotes responsiveness to nociceptive inputs. How this state of 'sensitisation' might be achieved is still unknown, and future pharmacological studies into the functions of VIP_1, VIP_2 and PACAP receptors may prove to be crucial in our understanding of the pain states which ensue nerve injury.

Overall, these results predict that VIP_2 receptor antagonists should serve as selective analgesics, strongly reducing polymodal C-fibre-mediated pain both in normal and neuropathic pain states. On the other hand, although the VIP_1 and PACAP receptor antagonists are rather non-selective inhibitors of sensory inputs in the normal state, they appear to have the useful property of attenuating cold allodynia as well as polymodal C-fibre responses in neuropathic animals, while preserving the low threshold mechanical responses.

In conclusion then, this study has highlighted potential regulatory roles for VIP and PACAP, via their selective VIP_1, VIP_2 and PACAP receptors, within the spinal cord of normal and neuropathic animals. This suggests that antagonists selective for these receptors, may be important new drugs for use in neuropathic pain. Further investigations to provide a better understanding of the function of VIP_1, VIP_2 and PACAP receptors, may therefore result in the development of new strategies for the treatment of chronic pain conditions.

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APPENDIX: PUBLICATIONS ARISING FROM RESEARCH

Some of the results presented in this Thesis have been published:-

1. Young MR, Fleetwood-Walker SM, Mitchell R, Dickinson T (1995) The involvement of metabotropic glutamate receptors and their intracellular signalling pathways in sustained nociceptive transmission in rat dorsal horn neurons. Neuropharmacology 34:1033-1041.

2. Dickinson T, Fleetwood-Walker SM (1996) The effects of VIP and PACAP receptor antagonists on sustained nociceptive and non-nociceptive inputs to rat dorsal horn neurones. [Abstract] IASP 8th World Congress on Pain: p134.

3. Dickinson T, Fleetwood-Walker SM, Mitchell R, Lutz EM (1997) Evidence for roles of vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) receptors in modulating the responses of rat dorsal horn neurons to sensory inputs. Neuropeptides 31:175-185.

4. Young MR, Fleetwood-Walker SM, Dickinson T, Blackburn-Munro G, Sparrow H, Birch PJ, Bountra C (1997) Behavioural and electrophysiological evidence supporting a role for group I metabotropic glutamate receptors in the mediation of nociceptive inputs to the rat spinal cord. Brain Research 777: 161-169.

5. Dickinson T, Robberecht P, Fleetwood-Walker SM (1998) The role of VIP/PACAP receptor subtypes in spinal somatosensory processing after chronic constriction injury to the sciatic nerve. [Abstract] 19th Annual Winter Neuropeptide Conference.

6. Dickinson T, Robberecht P, Fleetwood-Walker SM (1998) The role of VIP/PACAP receptor subtypes in spinal sensory processing following experimental mononeuropathy. [Abstract] Joint meeting of the European Neuropeptide Club and the Summer Neuropeptide Conference.

7. Dickinson T, Mitchell R, Robberecht P, Fleetwood-Walker SM (1998) The role of vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) receptor subtypes in spinal somatosensory processing in rats with an experimental peripheral mononeuropathy. Neuropharmacology (submitted).

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The Involvement of Metabotropic Glutamate Receptors and Their Intracellular Signalling Pathways in Sustained Nociceptive Transmission in Rat Dorsal Horn Neurons

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Summary—The excitatory responses of individual dorsal horn neurons to cutaneous brush, repeated application of the C-fibre-selective chemical algogen, mustard oil, or to ionophoretic (1*S*,3*R*)-ACPD [a metabotropic glutamate receptor (mGluR) agonist] were monitored by extracellular recording. We have previously shown that the responses of dorsal horn neurons to mustard oil are inhibited by several selective antagonists of mGluRs. Effects of ionophoresis of the mGluR antagonists (*R*,*S*)-CHPG and L-AP3 and a range of selective inhibitors of intracellular signalling pathways were examined on evoked responses here. The results suggest that protein kinase C, phospholipase A_2 and perhaps Ca^{2+}/calmodulin kinase II play a role in mediating the sustained elevated activity of dorsal horn neurons that is incrementally elicited by repeated application of mustard oil, but probably make little contribution to sustained brush-evoked activity. Concurrence in the sensitivity of mustard oil- and (1*S*,3*R*)-ACPD-evoked activity to (*R*,*S*)-CHPG, L-AP3 and to inhibitors of intracellular signalling pathways, suggests that mGluRs are an important origin of these intracellular signals required for sustained nociception.

Keywords—Metabotropic glutamate receptors, nociception, spinal cord, intracellular signalling, protein kinase C, phospholipase A_2.

he possible role of metabotropic glutamate receptors nGluRs) in spinal sensory processing has been ıvestigated relatively little. Their potential importance is ighlighted by evidence for their role in gating NMDA ceptor-dependent and -independent forms of the nsitization phenomenon in the hippocampus known as ng-term potentiation (LTP) (Bortolotto and ollingridge, 1993; Bortolotto *et al.*, 1994) and by the cilitation by mGluR agonist [(1*S*,3*R*)-ACPD] of MPA and NMDA responses in dorsal horn neurons leakman *et al.*, 1992; Cerne and Randic, 1992). We have eviously provided evidence for a mediatory role of GluRs in the sustained activation of dorsal horn urons elicited by repeated cutaneous application of the fibre selective chemical algogen, mustard oil (Young *al.*, 1994), which is known to bring about central nsitization of dorsal horn neurons to afferent inputs. ıe repeated cutaneous application of mustard oil elicits increasingly powerful and sustained activation of dorsal horn neurons (analogous to "wind-up") until a state of persistent elevated activity is reached (Munro *et al.*, 1993). This persistent mustard oil-evoked activity was inhibited by ionophoretic application of the selective mGluR antagonists L-(but not D-)-1-amino-3-phosphonopropanoic acid (L-AP3), (*R*,*S*)-4-carboxy-3-hydroxyphenylglycine (*R*,*S*-CHPG) and, more variably, by (*R*,*S*)-α-methyl-4-carboxyphenylglycine (*R*,*S*-α-MeCPG) (Young *et al.*, 1994). In behavioural analgesia experiments, intrathecally-applied (*S*)-CHPG, which is the enantiomer with activity at mGluRs, produces antinociceptive effects in thermal and mechanical paw withdrawal tests (Young *et al.*, 1995). In animals treated by unilateral intraplantar injection of carrageenan, these effects are amplified and an antinociceptive influence of L-AP3, but not D-AP3, is also apparent (Young *et al.*, 1995). Co-administration of AMPA and mGluR agonists is reported to bring about behavioural hyperalgesia in acute mechanical nociceptive tests (Meller *et al.*, 1993). The sensitization of dorsal horn neurons brought about by intra-articular inflammation is also inhibited by the mGluR antagonist L-AP3 (Neugebauer *et al.*, 1994).

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While these data provide compelling evidence that mGluRs play some role in spinal processing of nociceptive inputs (most likely in the sensitization of dorsal horn neurons to sustained high intensity C-fibre input), any evidence is lacking as to their intracellular mechanism of action. We have previously reported that neuronal activity evoked by repeated mustard oil application is profoundly inhibited by antagonists of protein kinase C (PKC) (Munro *et al.*, 1994a). This corresponds to the attenuation of behavioural nociceptive responses to subcutaneous formalin injection (and of their facilitation by glutamate) seen with PKC inhibitors (Coderre, 1992; Coderre and Yashpal, 1994). Since the intracellular signals leading to PKC activation are likely to also involve elevation of Ca^{2+} levels, it is possible that the Ca^{2+}-activated enzyme, Ca^{2+}/calmodulin-dependent protein kinase II [which has been implicated in LTP (Silva *et al.*, 1992)], may also play a role here. Additionally, evidence for the involvement of prostanoids in the nociceptive responses to formalin (Malmberg and Yaksh, 1992a,b; Chapman and Dickenson, 1992) and the facilitation of such behavioural responses by the prostanoid precursor arachidonic acid (Coderre and Yashpal, 1994), suggests that the arachidonic acid-generating enzyme, phospholipase A_2 (PLA_2) may play a role in sensitization. Further transduction mechanisms which could potentially play a role are cAMP-dependent protein kinase (PKA), since PKA activation can potentiate NMDA responses of dorsal horn neurons (Cerne *et al.*, 1993), and non-receptor tyrosine kinases, which can be activated by a number of phosphoinositide-hydrolysing, G protein-coupled receptors (Zachary *et al.*, 1991).

The present experiments utilized a range of selective inhibitors applied by ionophoresis, to assess the role of these various signal transduction pathways in neuronal responses to repeated application of mustard oil, to activation by the mGluR agonist (1*S*,3*R*)-1-aminocyclopentane-1,2-dicarboxylic acid [(1*S*,3*R*)-ACPD] and to light innocuous brushing of the cutaneous receptive field. Whilst these reagents were all chosen for their reported high degree of selectivity, it is not possible to say with certainty that their expected effects represent the actual mechanism of action in the present experiments. For this reason, pairs of reagents (with quite different structures, but with the common property of selectively blocking particular signal transduction pathways) were tested in each case.

METHODS

Experiments were carried out on 43 male Wistar rats (240–420 g). Following an initial halothane anaesthesia, animals were given intravenous α-chloralose (60 mg kg^{-1}) and urethane (1.2 g kg^{-1}). Supplementary doses of α-chloralose were given when required. Core body temperature was maintained at 37–38°C by means of a thermostatically controlled heat blanket. A light flow of O_2 (0.1 l min^{-1}) was passed over the end of a tracheal cannula to enrich the inspired air. Once the thoraco-lumbar spinal column was supported, using 3 pairs of swan-necked clamps, a laminectomy (L_1–L_4) was carried out. To provide stability, agar was injected under the most rostrally clamped vertebra, then over the whole area of the laminectomy. An agar core was removed to expose the recording region. The dura was then cut and a pool of 37°C liquid paraffin applied to the region.

Extracellular recordings were made from neurons in the deeper dorsal horn of spinal segments L_1–L_4, using the central barrel of 7-barrelled glass microelectrodes. The remaining barrels contained 1 M NaCl for automatic current balancing (Neurophore Ionophoresis System), 2% Pontamine Sky Blue in 0.5 M sodium acetate for histological marking of recording sites and combinations of the following drugs for ionophoresis: (1*S*,3*R*)-ACPD (Irving *et al.*, 1990), 10 mM aqueous, pH 8.0–8.5; (*R*,*S*)-and (*S*)-CHPG (Watkins and Collingridge, 1994), 10 mM aqueous, pH 8.0–8.5; L-AP3 (Schoepp *et al.*, 1990), 10 mM aqueous, pH 8.0–8.5; calmidazolium (Silver *et al.*, 1986), 50 μM in 0.5% dimethylformamide, pH 4.0–4.5; KN62 (Tokumitsu *et al.*, 1990), 50 μM in 0.5% dimethylformamide, pH 4.0–4.5; GF109203X (Toullec *et al.*, 1991), 100 μM in 0.5% dimethylformamide, pH 4.0–4.5; chelerythrine (Herbert *et al.*, 1990) 1 mM aqueous, pH 4.0–4.5; ONO-RS-082 (Banga *et al.* 1986), 100 μM in 0.5% dimethylformamide, pH 4.0–4.5 benzenesulphonamide 4 (BS4; Oinuma *et al.*, 1991), 50 μM in 0.5% dimethylformamide, pH 4.0–4.5; KT5720 (Kase *et al.*, 1987), 50 μM in 0.5% dimethylformamide pH 4.0–4.5; H89 (Chijiwa *et al.*, 1990), 100 μM in 0.5% dimethylformamide, pH 4.0–4.5; lavendustin A (Hsu *et al.*, 1991), 200 μM in 0.2% dimethylformamide, pH 4.0–4.5; and piceatannol (Geahlen and McLaughlin 1989), 300 μM in 0.3% dimethylformamide, pH 4.0–4.5 (1*S*,3*R*)-ACPD, (*R*,*S*)-, (*S*)-CHPG and L-AP3 were from Tocris Neuramin, calmidazolium was from Sigma piceatannol was from Boehringer Mannheim and all other compounds were from Calbiochem, except BS4 which was a gift from Mike Clark (Schering–Plough Research, Kenilworth, NJ, U.S.A.), and ONO-RS-082 which was a gift from Dr Tsuboshima (ONO Pharmaceuticals, Osaka, Japan). The signal transduction reagents were dissolved at the maximum concentration that could be achieved in an acceptable vehicle (0.5% dimethylformamide) that did not affect neuronal activity (see below). With certain reagents it was possible to use lower concentrations of the solvent in ionophoretic solutions.

Neuronal receptive fields were initially located by brushing of the ipsilateral hindlimb and then further examined with noxious heat (48°C, 10 sec). All neurons tested displayed a low basal firing rate of 0–1 Hz. The mustard oil experiments were carried out on a total of 2 multireceptive cells which responded to both brush and noxious heat. Repeated applications of mustard oil (Allyl isothiocyanate, Aldrich Chemical Company, 7.5% paraffin oil) were made to an area of about 3 cm^2 covering

the receptive field, and after 2–5 applications separated by 5 min intervals, a steady elevated firing rate (4–37 Hz) was maintained. The inhibitory effects of antagonists were calculated as the mean evoked activity through the 20 or 30 sec period encompassing greatest inhibition, compared to the mean evoked activity in the 20 or 30 sec period immediately prior to antagonist administration. Analysis was routinely over 20 sec, but was occasionally for the longer period of 30 sec if spurious variations occurred in the control sampling period, so that more truly representative values were obtained. The effects of antagonists, over a similar current range to that used on mustard oil-evoked activity, were also tested on responses of cells to a motorized brush applied continuously to the cutaneous receptive field and to ionophoretically-applied (1*S*,3*R*)-ACPD. The ionophoretic current ranges used were the lowest found to cause > 50% reduction of mustard oil-evoked activity without any detectable spike distortion; equivalent currents were then tested on brush- and (1*S*,3*R*)-ACPD-evoked responses. The statistical significance of drug-induced changes from control responses was assessed by the Wilcoxon test on the mean evoked raw firing rate in the sampling periods before and during drug administration. All analysed activity data are quoted as mean ± SEM values.

RESULTS

The majority of the neurons examined were multireceptive (i.e. responded to both innocuous brushing and noxious pinch or noxious heating to 48°C, of the cutaneous receptive field). As described previously (Munro *et al.*, 1993; Young *et al.*, 1994), the majority of these were vigorously excited by cutaneous administration of mustard oil, with several successive applications resulting in sustained elevated activity. The majority of Pontamine Sky Blue spots recovered from neuronal recording sites were within laminae III–V of the dorsal horn. Of the population of neurons recorded in the present study, approximately one-fifth (47 out of 244) were overtly excited by ionophoresis of (1*S*,3*R*)-ACPD (0–60 nA). Ionophoresis of 0.5% dimethylformamide-containing vehicle or saline at up to 80 nA, had no detectable or consistent effect on basal, mustard oil-, (1*S*,3*R*)-ACPD- or brush-evoked responses ($n = 5$). For each mGluR antagonist or signal transduction reagent, whenever recovery of responses was examined, full recovery was seen in most cases by 10 sec–15 min after ionophoresis was stopped.

Figure 1 extends the previously reported observations Young *et al.*, 1994; Munro *et al.*, 1994b) that mGluR antagonists inhibit mustard oil-evoked neuronal activation, by demonstrating that the potent mGluR antagonists (*R*,*S*)-CHPG and L-AP3 act selectively to inhibit mustard oil-evoked activity without affecting that evoked by innocuous brush. Mustard oil-evoked activity was significantly inhibited by (*R*,*S*)-CHPG [(−)15 to (−)55 nA] and L-AP3 [(−)15 to (−)50 nA] in 7 out of 8 cells (56 ± 9% inhibition, $P < 0.05$) and 11 out of 16 cells (68 ± 7% inhibition, $P < 0.05$), respectively (Wilcoxon test on raw data) (Fig. 1; Young *et al.*, 1994; Munro *et al.*, 1994b). In contrast, brush-evoked activity was never altered by more than 15% in 5 out of 5 cells by (*R*,*S*)-CHPG and 9 out of 9 cells tested with L-AP3 (not statistically significant). Furthermore, 12 out of 12 cells tested with (*S*)-CHPG [(−)15 to (−)55 nA] also showed negligible effects (<15% inhibition on brush-evoked activity (not statistically significant). Similar to their effect on mustard oil-evoked activity, (*R*,*S*)-CHPG and L-AP3 inhibited (1*S*,3*R*)-ACPD-driven activity in 5 out of 5 and 4 out of 6 cells, respectively (Fig. 1; Young *et al.*, 1994). The effect of (*R*,*S*)-CHPG on (1*S*,3*R*)-ACPD-evoked activity was a mean inhibition of 64 ± 6% (5/5 neurons) at currents of (−)15 to (−)40 nA ($P < 0.05$). When the percentage inhibition values of brush-, mustard oil- and (1*S*,3*R*)-ACPD-evoked activity caused by (*R*,*S*)-CHPG were compared by the non-parametric Mann–Whitney *U*-test, effects on each of the other responses were significantly greater than those on brush ($P < 0.05$). As a consequence of the lasting nature of activation caused by mustard oil, it was not possible to test drug effects in a side-by-side comparison of brush-, mustard oil- and (1*S*,3*R*)-ACPD-evoked responses on individual cells. Comparisons of a drug effect on brush/mustard oil, brush/(1*S*,3*R*)-ACPD or (1*S*,3*R*)-ACPD/mustard oil were however made in a number of cases. In individual examples of each combination, both (*R*,*S*)-CHPG and L-AP3 caused similar inhibition (by > 50%) of mustard oil/(1*S*,3*R*)-ACPD responses, but in examples of sequential brush/mustard oil and brush/(1*S*,3*R*)-ACPD tests the antagonists inhibited brush responses by <15%, if at all, despite causing > 50% reduction in mustard oil and (1*S*,3*R*)-ACPD responses.

When a range of selective inhibitors of signal transduction pathways was tested on activity evoked by brush, mustard oil or (1*S*,3*R*)-ACPD, there was general concurrence in the effects of each inhibitor on mustard oil- and (1*S*,3*R*)-ACPD-evoked responses. These responses were significantly inhibited by ionophoresis of each of the agents tested ($P < 0.05$, by Wilcoxon test, in each case; Table 1). However, in the case of certain inhibitors of Ca^{2+}/calmodulin kinase II, PKC and PLA_2, but not the other agents, there appeared to be a lesser effect on brush-evoked activity than on the other responses. The Ca^{2+}/calmodulin kinase II inhibitor, calmidazolium, the PKC inhibitor GF 109203X, and the PLA_2 inhibitor ONO-RS-082 had no significant effect on brush-evoked activity. The mean percentage inhibition of mustard oil- and (1*S*,3*R*)-ACPD-evoked responses by calmidazolium, ONO-RS-082 and a further PLA_2 inhibitor, BS4, was consistently at least 2-fold greater than their effect on brush responses (and in the case of GF109203X, many fold more). In the case of GF109203X, the percentage inhibition of mustard oil and of (1*S*,3*R*)-ACPD responses was significantly greater

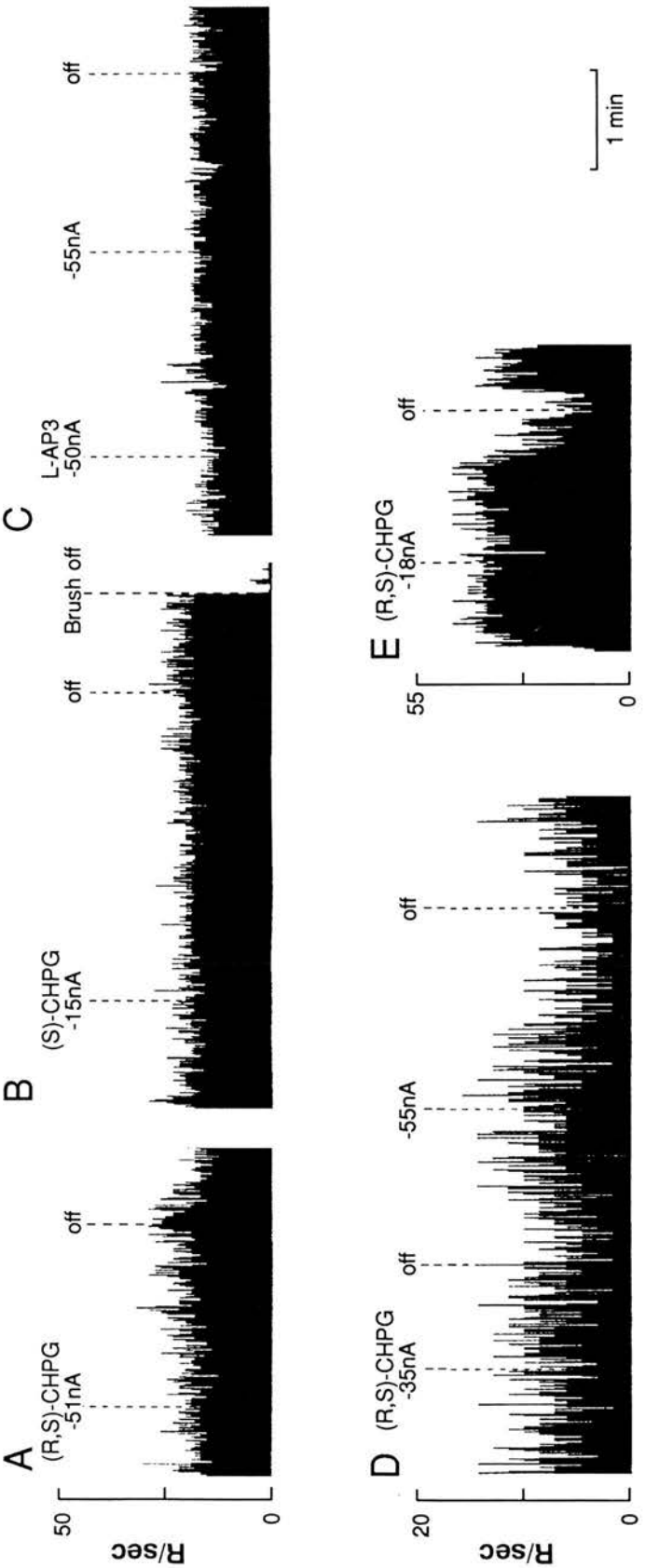

Fig. 1. Effects of ionophoretically-applied mGluR antagonists on evoked responses of dorsal horn neurons. Individual records of ongoing firing frequency are displayed as the action potentials per second (R/sec), integrated over 700 msec bins, plotted against time. (A–C) show the typical lack of any marked effect of mGluR antagonists on brush-evoked activity; (D, E) show inhibitory effects of (*R,S*)-CHPG on mustard oil- and (1*S*,3*R*)-ACPD-evoked activity (8 nA), respectively. Each trace is from a separate neuron and is entirely typical of the results obtained in the overall population sampled here.

than that of brush responses ($P < 0.05$, Mann–Whitney U-test). The effect of ONO-RS-082 on (1*S*,3*R*)-ACPD-evoked activity was also significantly greater than that on brush ($P < 0.05$), but no other such "between-response" comparisons revealed differences that were significant with the present samples. Figure 2 shows examples of the effect of the Ca^{2+}/calmodulin kinase II inhibitor KN62 and of the PLA_2 inhibitor BS4, illustrating concurrent inhibition of mustard oil- and (1*S*,3*R*)-ACPD-evoked activity with little or no effect on brush responses. In the overall populations, however, both KN62 and BS4 caused a relatively small, but significant, inhibition of brush as well as the other responses, with each drug showing no marked effect ($\leqslant 20\%$ change) in approximately 50% of the cells (as in the examples illustrated) (Fig. 2; Table 1). In the case of the PKA inhibitors, KT5720 and H89, and the tyrosine kinase inhibitors, lavendustin A and piceatannol, each reagent tested caused a significant inhibition of all types of evoked activity (Table 1). It was not possible to make direct sequential comparisons of a drug's effects on brush, mustard oil and (1*S*,3*R*)-ACPD responses of the same cell. However, comparisons between two types of evoked response were possible in a number of cases. For example, in the case of a single neuron tested with BS4, direct comparison of effects on brush/(1*S*,3*R*)-ACPD responses revealed selective inhibition of the latter, in accordance with the overall population of neurons investigated. Similarly, piceatannol caused inhibition of both brush and mustard oil responses when directly compared on a single cell, again typical of the overall population (Table 1). A total of 17 cells were tested sequentially with several drugs on either responses to brush (6 cells), mustard oil (6 cells) and (1*S*,3*R*)-ACPD (5 cells). In order to avoid any possible cumulative drug effects, this involved drugs considered to act on independent signal transduction pathways. Time for full recovery was always allowed between sequential tests on the same neuron (up to 15 min) and the results from these multiple tests were always typical of the overall population described in Table 1.

DISCUSSION

The present experiments extend the evidence for a role of mGluRs in mediating the prolonged firing of dorsal horn neurons evoked by repeated application of mustard oil. Furthermore, they demonstrate modality specificity, in that neuronal activation caused by mustard oil, but not that due to continuous innocuous brushing, was inhibited by the mGluR antagonists (*R*,*S*)-CHPG and L-AP3 (Fig. 1). Administration of the single enantiomers

Table 1. Effects of ionophoretically-applied inhibitors of intracellular signalling pathways on the activity of dorsal horn neurons evoked by brush, mustard oil or (1*S*,3*R*)-ACPD

Principal target	Drug	Inhibition of evoked activity (% inhibition compared to mean pre-drug controls) Brush	Mustard oil	(1*S*,3*R*)-ACPD (0–10 nA)
Calmodulin, Ca^{2+}/calmodulin kinase	Calmidazolium (30–60 nA)	16 ± 10 NS $n = 8$ (25%)	42 ± 14* $n = 7$ (71%)	68 ± 16* $n = 5$ (80%)
	KN62 (30–70 nA)	42 ± 13* $n = 12$ (50%)	51 ± 19* $n = 5$ (80%)	83 ± 9* $n = 5$ (100%)
PKC	GF109203X (20–60 nA)	3 ± 3 NS $n = 5$ (20%)	53 ± 10* $n = 9$ (89%)	86 ± 12* $n = 5$ (100%)
	Chelerythrine (10–80 nA)	—	70 ± 10* $n = 8$ (100%)	84 ± 11* $n = 7$ (100%)
PLA_2	ONO-RS-082 (30–65 nA)	28 ± 13 NS $n = 9$ (44%)	70 ± 11* $n = 5$ (100%)	75 ± 9* $n = 8$ (100%)
	BS4 (30–60 nA)	37 ± 12* $n = 9$ (56%)	74 ± 11* $n = 5$ (100%)	86 ± 6* $n = 7$ (100%)
PKA	KT5720 (?0–60 nA)	59 ± 15* $n = 10$ (70%)	73 ± 13* $n = 7$ (86%)	87 ± 11* $n = 5$(80%)
	H89 (30–70 nA)	45 ± 15* $n = 10$ (50%)	66 ± 10* $n = 5$ (100%)	79 ± 13* $n = 6$ (100%)
Tyrosine kinases	Lavendustin A (30–70 nA)	60 ± 12* $n = 11$ (82%)	58 ± 10* $n = 8$ (88%)	69 ± 12* $n = 7$ (100%)
	Piceatannol (7–50 nA)	48 ± 15* $n = 13$ (61%)	87 ± 7* $n = 6$ (83%)	84 ± 9* $n = 6$ (100%)

The ranges of ionophoretic currents required for the effects reported, are indicated in parentheses below each drug. In the case of (1*S*,3*R*)-ACPD, higher currents ($\leqslant 60$ nA) were sometimes briefly used to initiate firing but, after the first few seconds, the elevated activity could always be maintained by $\leqslant 10$ nA.

The effects of the drugs on evoked responses are indicated as the mean ± SEM percentage inhibition compared to pre-drug control acitvity. In each case, the number of neurons contributing to the data is shown below and in parentheses, the proportion (percentage) of those neurons which individually gave a clear response of $> 20\%$ inhibition. The statistical significance of drug-induced changes in firing rate was assessed using the Wilcoxon test on mean raw firing rate data in action potentials per second (R/sec); comparing the 20 or 30 sec period immediately prior to drug administration with the 20 or 30 sec period centred on the greatest inhibitory effect of drug (*represents $P < 0.05$; NS represents not significantly different, $P > 0.05$).

The data for GF109203X and chelerythrine on brush- and mustard oil-evoked activity is taken from Munro *et al.* (1994a).

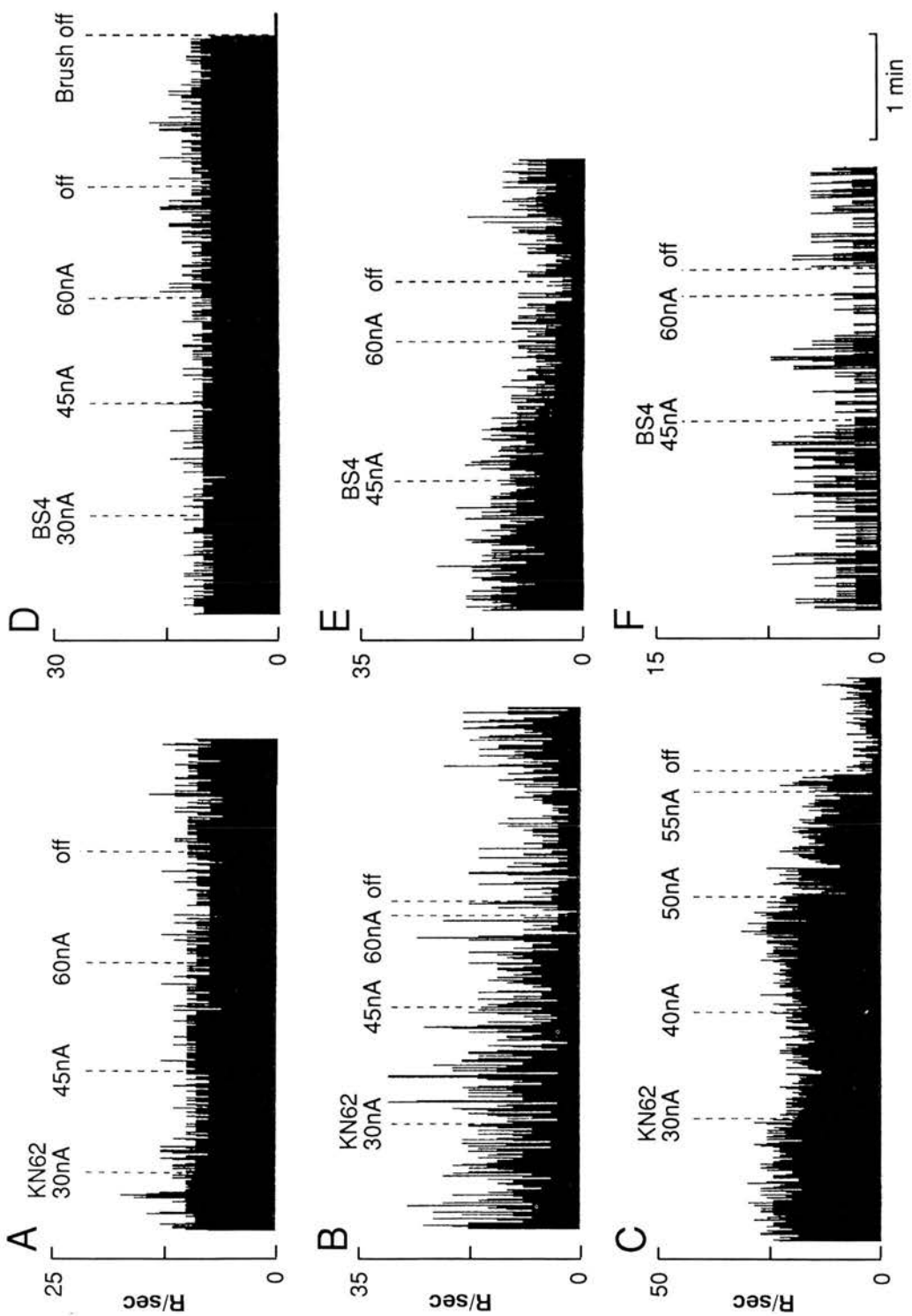

Fig. 2. Effects of ionophoretically-applied inhibitors of intracellular signalling pathways on evoked responses of dorsal horn neurons. Individual records of ongoing firing frequency are displayed as the action potentials per second (R/sec), integrated over 700 msec bins, plotted against time. (A–C) demonstrate typical effects of the Ca^{2+}/calmodulin kinase II inhibitor KN62 on brush-, mustard oil- and (1*S*,3*R*)-ACPD-evoked activity (1 nA), respectively; (D–F) show typical effects of the phospholipase A_2 inhibitor BS4 on brush, mustard oil- and (1*S*,3*R*)-ACPD-evoked activity (7 nA), respectively. The records are taken from separate neurons, but are entirely typical of the overall population in each case. In some cells, KN62 and BS4 attenuated not only mustard oil- and (1*S*,3*R*)-ACPD-evoked activity, but also brush responses.

(*S*)-CHPG and L-AP3, which display the greater activity as mGluR antagonists, failed to modify brush responses of the neurons. Interestingly, Eaton *et al.* (1993) have previously shown that (*S*)-CHPG affected nociceptive, but not non-nociceptive, responses of thalamic neurons. Neuronal activity evoked by the mGluR agonist (1*S*,3*R*)-ACPD was also significantly inhibited by (*R*,*S*)-CHPG or by L-AP3 (Fig. 1; Young *et al.*, 1994). α-MeCPG was not investigated in the present experiments due to the rather variable effects it exerts on dorsal horn neuron responses to mustard oil (Young *et al.*, 1994) and to dorsal root stimulation (Cao *et al.*, 1995). Although the mGluR antagonists inhibited mustard oil-evoked activity in 75% of the neurons tested here, (1*S*,3*R*)-ACPD only elicited neuronal firing in approximately 25% of cases. This apparent anomaly may be a consequence of the mustard oil-evoked synaptic input involving a number of other mediators beyond mGluRs; for example AMPA, neurokinin and perhaps NMDA receptors. Agonist stimulation of mGluRs alone, in the absence of other components, may be insufficient to regularly elicit overt neuronal firing in the recorded cell. Other explanations are of course possible.

The mGluR antagonist used here, (*R*,*S*)-CHPG, is a potent inhibitor of intracellular signalling responses elicited by the phosphoinositide-hydrolysing $mGlu_1$ receptor, but not by $mGlu_2$ or $mGlu_4$ receptors (Watkins and Collingridge, 1994). It is likely that the properties of $mGlu_5$ receptors correspond broadly to those of $mGlu_1$, and further subtypes to $mGlu_{2/4}$ receptors, which suggests that the receptor subtype involved in the mGlu antagonist effects may well be $mGlu_1$ or $mGlu_5$. Although the (*R*)-enantiomer of CHPG displays some NMDA receptor antagonism (Watkins and Collingridge, 1994) it can be inferred that the inhibitory effects of (*R*,*S*)-CHPG on mustard oil responses here are mainly due to mGluR blockade, since they are mimicked stereoselectively by L-AP3, but not D-AP3 (effective at mGluR and NMDA receptors respectively; Young *et al.*, 1994).

This evidence for mGluR involvement in a sensitized nociceptive response is consistent with evidence from other studies on the amplification of neuronal responses by intra-articular inflammation or carrageenan-induced (Neugebauer *et al.*, 1994; Young *et al.*, 1995). It is clear, however, that mGluRs are not the sole mediator of sensitization, since both NK_2 and NK_1 neurokinin receptors in the dorsal horn can also contribute to the sustained nociceptive activation and increased neuronal responsiveness (Xu *et al.*, 1991; Munro *et al.*, 1993; Thompson *et al.*, 1994). Interestingly, the $mGlu_1$ and $mGlu_5$ subtypes of mGluR, together with NK_1 and NK_2 receptors have the common property of signal transduction by means of phosphoinositide hydrolysis (Nakajima *et al.*, 1992; Watkins and Collingridge, 1994). Correspondingly, neuronal responses to mustard oil, but not brush, were significantly reduced by inhibitors of PKC and by calmidazolium, an antagonist of the Ca^{2+} binding protein calmodulin (thence Ca^{2+}/calmodulin kinase II) both classical mediators of phosphoinositide-hydrolysing receptors (Table 1). Responses to the mGluR agonist (1*S*,3*R*)-ACPD were also significantly inhibited by these reagents, consistent with the hypothesis that a phosphoinositide-hydrolysing mGluR may participate in mediating responses to mustard oil. Both Ca^{2+}/calmodulin kinase II and the Ca^{2+}/calmodulin-activated phosphatase calcineurin, have been implicated in long term changes in responsiveness in hippocampal neurons (Silva *et al.*, 1992; Lisman, 1989). It is not possible to draw any conclusion from the experiments here with KN62, since it significantly inhibited all types of activity tested in the overall population of cells and there is thus no assurance that its effects are other than non-specific.

The prominent blockade of mustard oil-evoked activity by PKC inhibitors (Table 1; Munro *et al.*, 1994a) is consistent with the translocation/activation of dorsal horn PKC observed in both inflammatory and loose-ligation neuropathy models of sustained nociception (Mao *et al.*, 1993; Munro *et al.*, 1994a). The sensitivity of hippocampal LTP to inhibition of PKC (Bliss and Collingridge, 1993) maintains the analogy between that phenomenon and the sensitization of dorsal horn neurons by sustained nociceptive input. Furthermore, PKC inhibitors attenuate the behavioural nociceptive responses elicited by subcutaneous formalin injection (Coderre, 1992; Coderre and Yashpal, 1994) and also attenuate the enhancement of such responses brought about by glutamate or substance P. Metabotropic glutamate, NK_1 and NK_2 receptor agonists can facilitate neuronal responses to NMDA (Cerne and Randic, 1992; Bleakman *et al.*, 1992; Rusin *et al.*, 1992) and, where tested (on the NK_1 agonist, substance P), these effects were reversed by a protein kinase inhibitor (Rusin *et al.*, 1992).

Not only PKC-activating phorbol esters (Gerber *et al.*, 1989) but also the PKA activator forskolin (Cerne *et al.*, 1993) can facilitate neuronal responsiveness in the dorsal horn, but the inhibitory effects of the PKA inhibitors KT5720 and H89 here are equivocal because all types of activity were inhibited and the possibility of non-specific actions cannot be excluded. No evidence was found in support of the hypothesis that the mGluR subtype here might be one acting by adenylate cyclase inhibition, in which case PKA inhibitors might have been expected to mimic rather than inhibit (1*S*,3*R*)-ACPD responses.

The two PLA_2 inhibitors tested, ONO-RS-082 and BS4, significantly inhibited both mustard oil and (1*S*,3*R*)-ACPD responses, whereas ONO-RS-082 had no significant effect on brush-evoked activity and BS4 showed at least twice the magnitude of effect on the other responses than on brush. These agents are reported to block low and high molecular weight species of PLA_2 respectively, and in each case both Ca^{2+} and PKC have been implicated in their activation cascades (Banga *et al.*, 1986; Oinuma *et al.*, 1991; Mayer and Marshall, 1993). The present results are consistent with observations in behavioural models implicating PKC and prostanoids in

the development of hyperalgesic states (Coderre, 1992; Malmberg and Yaksh, 1992a,b; Coderre and Yashpal, 1994).

Two selective inhibitors of tyrosine kinases (which could potentially disrupt the effects of either growth factor receptor tyrosine kinases or of receptor-independent tyrosine kinases operated downstream of phosphoinositide hydrolysis responses) were also tested. These agents caused generalized inhibition of all types of evoked activity and so no clear conclusions can be drawn about their actions in the context of the present experiments.

In summary, the present results suggest that PKC, PLA_2 and perhaps a calmodulin-dependent mechanism, such as Ca^{2+}/calmodulin kinase II, play a role in the mustard oil model of sustained and sensitized nociception in the dorsal horn and that an $mGlu_1$ or $mGlu_5$ receptor may be one of the synaptic mediators involved in triggering these signal transduction pathways.

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80 THE EFFECTS OF VIP AND PACAP RECEPTOR ANTAGONISTS ON SUSTAINED NOCICEPTIVE AND NON-NOCICEPTIVE INPUTS TO RAT DORSAL HORN NEURONES.

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Aim of Investigation: To investigate the role of Vasoactive Intestinal Polypeptide (VIP) and Pituitary Adenylate Cyclase Activating Peptide (PACAP) in the normal somatosensory processing of dorsal horn neurones.

Methods: Antagonists selective for VIP or PACAP receptors were ionophoresed locally onto functionally characterised dorsal horn neurones in the intact spinal cord of anaesthetised rats. Neurones were classified as being non-nociceptive if they responded to light brushing of the hairs of the cutaneous receptive field only, and multireceptive if they responded to brush, noxious pinch and/or noxious heat inputs. The activation of single dorsal horn neurones was induced by innocuous brushing of the hind limb or by peripheral application of the C-fibre-selective algogen, mustard oil.

Results: When applied near non-nociceptive neurones both VIP and PACAP antagonists showed negligible effects on sustained neuronal firing to a brush stimulus. However, upon ionophoresis near multireceptive neurones, [p-Chloro-D-Phe^{6}, Leu^{17}]-VIP showed 53 ± 6% inhibition (n=19) of responses induced by innocuous brush and 59 ± 8% inhibition (n=11) of mustard oil responses. Similarly PACAP (6-38) inhibited brush-evoked activity by 60 ± 9% (n=16) and mustard oil induced activity by 66± 9%, (n=9).

Conclusion: These results provide the first evidence that VIP and/or PACAP receptors expressed on dorsal horn neurones in normal conditions, may play an important role in mediating somatosensory processing in the spinal cord. Furthermore, they also suggest that VIP and/or PACAP act solely on cells which receive small diameter C and Aδ fibres.

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Evidence for roles of vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) receptors in modulating the responses of rat dorsal horn neurons to sensory inputs

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Summary The extracellularly recorded electrophysiological activity of single multireceptive dorsal horn neurons was markedly increased by ionophoretic administration of vasoactive intestinal polypeptide (VIP) or pituitary adenylate cyclase activating polypeptide (PACAP)-38. Some cells responded selectively to PACAP-38 (suggesting mediation by a PACAP receptor), whereas others responded to both VIP and PACAP-38 (suggesting a VIP_1 and/or VIP_2 receptor). Most non-nociceptive cells were unaffected by PACAP-38 and all were unaffected by VIP. The selectivity of VIP/PACAP receptor antagonists was established on cloned rat VIP_1, VIP_2 and PACAP receptors in vitro before their utilization to indicate the likely involvement of VIP_1, and possibly PACAP receptors, in VIP- and PACAP-38-mediated responses of dorsal horn neurons. The VIP/PACAP receptor antagonists inhibited responses of multireceptive cells to sustained innocuous (brush) and noxious (mustard oil) stimuli, with a selectivity suggesting the involvement of VIP_1 and PACAP receptors, although the participation by VIP_2 receptors cannot be excluded. These data implicate both VIP and PACAP in regulating the basal responsiveness of multireceptive dorsal horn neurons to sensory stimuli.

INTRODUCTION

The 28-amino acid peptide, vasoactive intestinal polypeptide (VIP)[1] is expressed widely in both the peripheral and central nervous systems (CNS) of a variety of species.[2] More recently, a related peptide pituitary adenylate cyclase-activating polypeptide (PACAP) was discovered[3] which shows 68% homology with VIP at its N-terminal.[4] PACAP is also widely expressed in many central and peripheral neurons.[5] VIP and the two alternative processing forms of PACAP (PACAP-38 and PACAP-27) are recognized by a family of three receptors: the PACAP receptor which displays a much greater affinity for the two forms of PACAP than for VIP, and the VIP_1 and VIP_2 receptors which display no marked selectivity for any one of the peptide ligands.[6–8] Immunohistochemical studies have revealed the presence of VIP-immunoreactive fibres in the superficial dorsal horn (especially at lower lumbar to sacral levels) which are depleted by dorsal rhizotomy and correspond to the presence of a modest number of small VIP-immunoreactive cell bodies in dorsal root ganglia.[9–14] Some VIP-immunoreactive cell bodies have also been observed in dorsal horn at all levels.[12,13,15,16] Similarly, PACAP-immunoreactive perikarya are present in dorsal root ganglia at all levels, and fibres are densely present in superficial dorsal horn.[17,18] Capsaicin treatment markedly depletes the number of PACAP-immunoreactive fibres in these tissues.[17] Messenger RNA for VIP is present at only low levels in lumbar dorsal root ganglia under normal

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conditions[19] whereas PACAP mRNA is normally present in about 10% of dorsal root ganglion cells.[20] PACAP mRNA is also present in spinal cord extracts.[5] Furthermore, release of VIP-like immunoreactivity occurs into spinal perfusates in response to sciatic nerve stimulation at Aδ- and C-fibre intensity.[21] Binding sites for VIP and PACAP ligands are correspondingly present in spinal cord,[22–25] and the mRNA for VIP_1,[6] VIP_2[26] and PACAP receptors[27] is also expressed in spinal cord, especially in dorsal horn.

Functional studies have reported that ionophoretically applied VIP excites dorsal horn neurons and facilitates their sensory responses and similarly excites neurons of the trigeminal nucleus caudalis.[28,29] In addition, intrathecally applied VIP and PACAP-38 facilitate nociceptive flexor reflexes,[30–32] while intrathecal VIP decreases reaction latency in the thermal tail flick test.[33] In contrast, one study reported an attenuation of a nociceptive reflex with both VIP and PACAP-38,[34] while intrathecal administration of either PACAP-27 or -38 has been shown to reduce the instances of formalin-induced flinching behaviour.[35,36]

These data suggest that VIP, PACAP and their receptors may play an important regulatory role in the transmission of nociceptive information in the spinal cord of the rat. The present experiments were therefore carried out with several agonists and antagonists for VIP and PACAP receptors (having defined the specificity of these agents on receptors heterologously expressed from their cDNAs). Responses of functionally characterized classes of dorsal horn neurons to sustained innocuous and noxious stimuli were investigated in order to assess the specificity of VIP/PACAP influences with respect to neuronal cell type and sensory input.

MATERIALS AND METHODS

Electrophysiological techniques

Experiments were carried out on 45 male Wistar rats (296–394 g), anaesthetized with intravenous α-chloralose (60 mg/kg) and urethane (1.2 g/kg) following induction with halothane. Supplementary doses of α-chloralose were given as required and the core temperature was maintained at 37–38°C by means of a thermostatically controlled heated blanket. The trachea was then cannulated and oxygen (0.1 L/min) was passed over the end of the cannula to enrich the inspired air. The thoraco-lumbar spinal column was supported using three pairs of swan-necked clamps on alternate segments, and a laminectomy performed to expose segments L1–L4. Agar was injected under the most rostrally clamped vertebra and then over the whole area of the laminectomy to provide stability. A core of agar was then removed to expose the recording region, the dura carefully cut and a pool of 37°C liquid paraffin applied to the exposed area.

Extracellular recordings were made from single neurons located in dorsal horn laminae III–V, using the central barrel (4 M NaCl, pH 4.0–4.5) of 7-barrelled glass microelectrodes, with tip sizes 4.0–5.0 μm. One side barrel contained 1 M NaCl (pH 4.0–4.5) for automatic current balancing (Neurophore Ionophoresis System). A second side barrel contained 2% Pontamine Sky Blue (PSB) in 0.5 M sodium acetate for histological marking of recording sites. The remaining barrels contained the following compounds: VIP[37] (Sigma, UK) (0.25 mM aqueous, pH 4.5), PACAP-38[38] (0.25 mM aqueous, pH 4.5), [*p*-Cl-D-Phe^6,Leu^{17}]-VIP[39] (0.25 mM aqueous, pH 4.5), VIP(6–28)[40] (0.25 mM aqueous, pH 4.5), PACAP(6–38)[41] (0.25 mM aqueous, pH 4.5) or the vehicle control pH 4.5 H_2O. All peptides were from Bachem UK, unless otherwise stated. The peptides were ejected using positive currents of 30–70 nA and a retention current of −12 nA was applied to each barrel when not in use. The resistance of the side barrels was monitored regularly and electrodes with resistance values exceeding 45 MΩ were rejected.

Neuronal receptive fields were initially located by widespread manual brushing of the ipsilateral hind limb and then further examined with noxious pinch and heat (48°C, 10 s). The neurons were classified according to their sensory input with non-nociceptive neurons being those which responded only to light brushing of the hairs of the cutaneous receptive field, while those which responded to brush, noxious pinch, and/or noxious heat inputs were classed as being multireceptive. No nocispecific neurons were investigated in this experiment. Prior to ionophoresis of the solutions, or stimulation of the neuron by a sensory input, recording was started to determine the basal firing level. All neurons tested displayed a low basal firing rate of 0–1 Hz.

To demonstrate the presence of VIP-related receptors on dorsal horn neurons, either VIP or PACAP agonists were ionophoresed at increasing currents over a set period of time, initially starting at 30 nA and increasing to 45 and 60 nA, if necessary, after 90 s, until activation was achieved. To further investigate the receptor subtype involved, a number of neurons activated by both VIP and PACAP-38 were selected, and where possible, the firing was stabilized by decreasing the peptide current accordingly and then either the [*p*-Cl-D-Phe^6,Leu^{17}]-VIP or PACAP(6–38) antagonist was applied at 60 nA, for up to 3 min, to assess inhibition of the agonist-induced activity. If inhibition was present, ionophoresis of the peptide was stopped to look for recovery. The inhibitory effect of the antagonists was assessed as the mean evoked activ-

ity in the 30 s period encompassing the greatest inhibition, compared to the mean evoked activity in the 30 s period immediately prior to antagonist administration. The change in activity was expressed as a percentage of mean control activity ± the standard error of the mean (SEM). VIP was generally tested first, followed by a recovery period of at least 10 min to allow the firing to return to basal firing levels before PACAP-38 was tested.

To demonstrate the contribution of VIP-related receptors to sensory inputs, the effects of the VIP and PACAP antagonists were examined on sustained sensory inputs. Sustained innocuous inputs were set up by means of a rotating, motorized brush, producing a steady firing rate of 5–33 Hz, while a sustained noxious sensory input was produced by application of the C-fibre selective chemical algogen mustard oil (8% allyl isothiocyanate in paraffin oil). The mustard oil experiments were carried out on 26 multireceptive cells which responded to both brush and noxious heat. Repeated applications of mustard oil were made to an area of approximately 2 cm^2 covering the receptive field, and after 2–7 applications, separated by 5-min intervals, a steady elevated firing rate (6–45 Hz) was maintained. The antagonists were then applied during these sustained sensory inputs, initially starting at 30 nA and increasing to 45 or 60 nA, until clear effects were seen. The antagonists were usually ejected for 1.5–2 min in order to assess any maintained inhibition. If inhibition was present, ionophoresis of the peptide was stopped to look for recovery. In addition, current and vehicle controls were also applied to the brush- and mustard oil-induced activity. The data was analysed using the Mann-Whitney U-test and inhibitory values of $P \leq 0.05$ were considered as being significant.

In vitro characterization of antagonists

COS 7 cells were transfected (using DEAE dextran) with constructs in pcDNA1 encoding the rat VIP_1, VIP_2 and short-form PACAP receptors as previously described.[6,7,42] The cells were then plated into 24-well plates and used 72 h after transfection. After washing three times in MEM with 0.25% bovine serum albumin at 37°C, a further 0.5 mL aliquot of this medium was added with 0.5 mM isobutylmethylxanthine to block phosphodiesterase action. Following 15 min preincubation, the antagonists [*p*-Cl-D-Phe[6],Leu[17]]-VIP,[39] VIP(6–28)[40] or PACAP(6–38)[43] were added at concentrations through the range 10–10 000 nM. The agonist, VIP was then added, at a range of concentrations from 0.1 to 1000 nM and incubation continued for 20 min before the addition of an equal volume of ice-cold 0.2 M HCl and rapid freezing on solid carbon dioxide. After thawing, well contents were homogenized and 50 µL samples of the supernatant were assayed for cAMP using a double antibody immunoprecipitation radioimmunoassay based on rabbit primary antiserum RIB7.[44]

RESULTS

The effects of VIP and PACAP receptor agonists

These results were obtained from 22 neurons, 8 of which were classed as being non-nociceptive (excited by innocuous brush only) while the remaining 14 were multireceptive (activated by brush, pinch and/or noxious heat). All neurons displayed a low basal firing rate of 0–1 Hz, and current control tests had no detectable effect on any of the neurons tested. In all cases, the cells were tested separately with both VIP and PACAP-38, with currents ranging from 30 to 65 nA. The majority of cells were found to be within laminae III–V as determined by histological marking with Pontamine Sky Blue.

Both VIP and PACAP-38, when applied ionophoretically, were found to cause a marked excitation of multireceptive dorsal horn neurons. Only 3 of the 14 multireceptive cells tested were not activated by either VIP or PACAP-38. The remaining neurons showed variable effects, which may reflect a difference in receptor expression between dorsal horn neurons. Five out of the 11 cells activated responded to PACAP-38 alone (50–60 nA), while another five were activated by both VIP (40–60 nA) and PACAP-38 (30–65 nA). Figure 1(A) shows a typical example of the activation of an isolated dorsal horn neuron by VIP and PACAP-38. Only one neuron appeared to be activated by VIP alone. Excitation was observed as the initiation of firing of a previously quiescent cell or as an increase in basal firing, and had a latency of onset ranging from several seconds to 4 or 5 min following application of the agonist. Of the six neurons excited by VIP, two produced a marked and sustained increase in firing, two were seen to produce a sharp burst of high activity which then declined, while the remaining two displayed a modest increase in basal firing which declined to control levels when application of the peptide was terminated.

PACAP-38 appeared to be much more effective, demonstrating a much greater activation of the neurons tested. Only one out of the 10 cells excited showed a modest increase in basal firing, five of the remaining cells showed a sharp, sudden burst of firing which could not be maintained, while the remaining four neurons displayed a large increase in activity which often continued for several minutes after the application of PACAP-38 was terminated.

Of the eight non-nociceptive neurons tested here, none of them showed any apparent activation in response to VIP (30–60 nA), suggesting that VIP responsiveness may be restricted to neurons with nociceptive inputs. PACAP-38, however, demonstrated a marked acti-

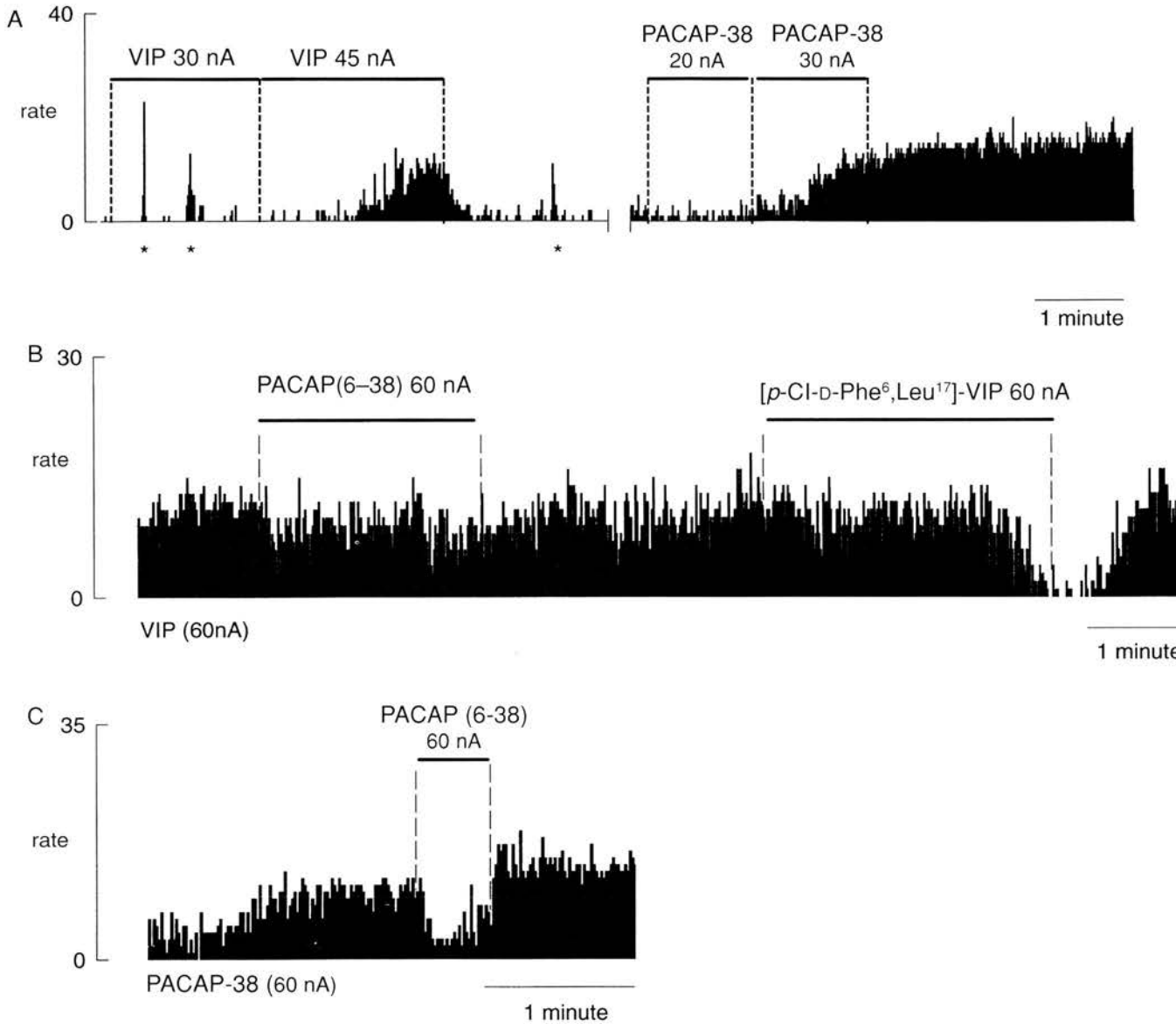

Fig. 1 Effects of ionophoretically applied VIP and PACAP-38 on multireceptive dorsal horn neurons. Individual recordings of ongoing firing frequency are displayed as the action potentials per second (rate, integrated over 1000 ms bins) plotted against time. (A) The excitation of a previously silent dorsal horn neuron by VIP, 45 nA (* indicates brief control challenges with innocuous brush), and the subsequent excitation of the same neuron by PACAP-38, 30 nA, 10 min after the termination of VIP. Note the sustained level of firing following application of PACAP-38, even after ionophoresis of this peptide was terminated; a phenomenon seen for a number of the neurones tested. (B) This trace shows the negligible effects of PACAP(6–38) (60 nA) and marked inhibitory effect of [*p*-Cl-D-Phe^6,Leu^{17}]-VIP (60 nA) antagonists respectively, on the sustained activity evoked by application of VIP (60 nA) in a neuron responding to both VIP and PACAP-38. (C) The effects of PACAP(6–38) (60 nA) on PACAP-38-evoked activity (60 nA), in a typical neuron activated by both PACAP-38 and VIP.

vation of three of the eight neurons. Only one of the responsive cells showed a sustained level of activity, the other two neurons showed a sudden burst of activity which could not be maintained. So it seems possible that some non-nociceptive dorsal horn neurons may express a receptor type enabling responsiveness to PACAP but not to VIP, i.e. a PACAP receptor.

Effects of VIP/PACAP receptor antagonists on sustained agonist-driven activity

In this study, a further six multireceptive neurons were selected for their ability to be clearly activated by both VIP and PACAP-38, in order to try and further ascertain the receptor subtypes expressed on dorsal horn neurons under normal conditions. VIP (45–60 nA) produced an

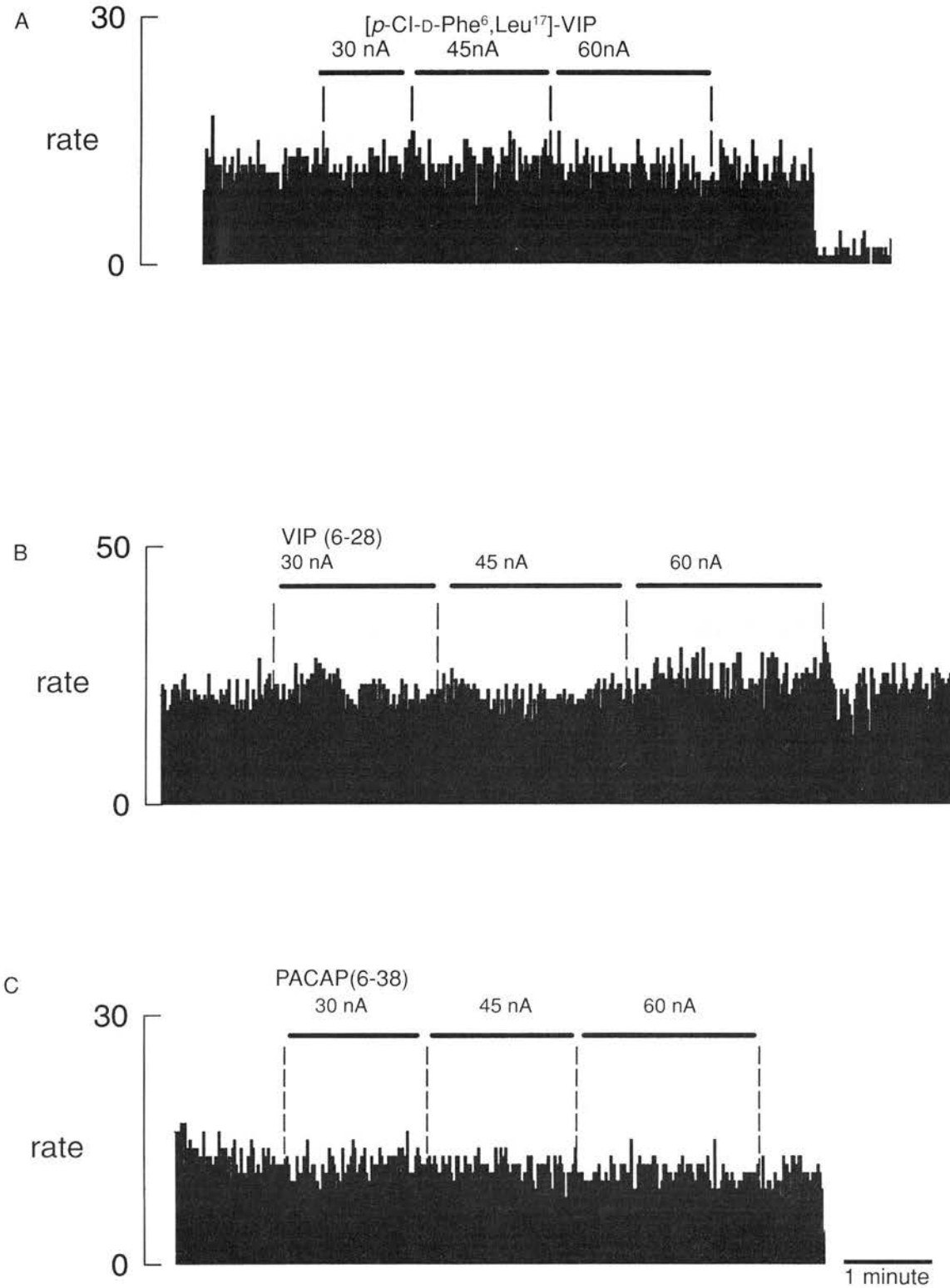

Fig. 2 Effects of ionophoretically applied VIP and PACAP receptor antagonists on sustained brush-evoked responses of non-nociceptive dorsal horn neurons. Individual records of sustained firing are displayed as the action potentials per second (rate, integrated over 1000 ms bins), plotted against time. (A), (B) and (C) show the generally observed lack of effect on the sustained firing of non-nociceptive dorsal horn neurons when [*p*-Cl-D-Phe^{6},Leu^{17}]-VIP, VIP(6–28) and PACAP(6–38) respectively, were applied to innocuous brush-evoked activity. Each trace is from a different neuron and is entirely typical of the results obtained in the overall population sampled here.

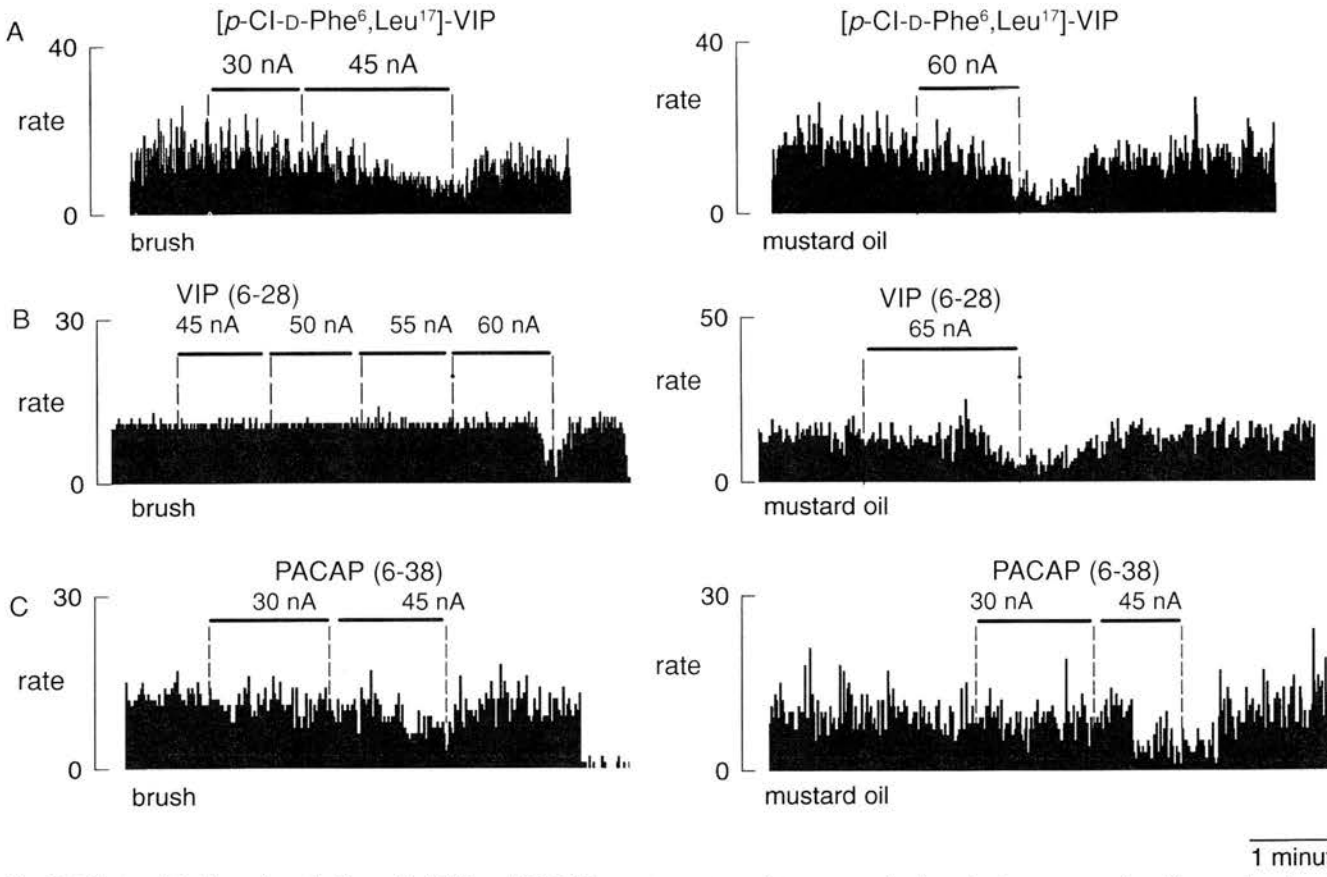

Fig. 3 Effects of the ionophoretically applied VIP and PACAP receptor antagonists on sustained evoked responses of multireceptive dorsal horn neurons. Individual records of sustained firing are displayed as the action potentials per second (rate, integrated over 1000 ms bins), plotted against time. (A) The marked inhibitory effects of [*p*-Cl-D-Phe^6,Leu^{17}]-VIP when applied to the sustained neuronal activity evoked by innocuous brush or noxious mustard oil application. Similarly (B) and (C) show clear antagonist effects of VIP(6–28) and PACAP(6–38) respectively on brush- and mustard oil-evoked activity. All examples show a marked inhibition of neuronal firing following application of the antagonist, with almost immediate recovery following termination of peptide application. In all three cases both the brush- and mustard oil-induced activities are evoked from the same multireceptive dorsal horn neurons, and the results are typical of those obtained in the population sampled.

average 9-fold increase in activity (4.2–22.3 Hz) when applied to multireceptive neurons ($n = 6$). [*p*-Cl-D-Phe^6,Leu^{17}]-VIP significantly inhibited this agonist driven activity ($P \leq 0.05$) showing a mean percentage inhibition of 42 ± 14%, while PACAP(6–38) exhibited little effect, 12 ± 9% inhibition (Fig. 1(B)).

Five of the six neurons tested produced agonist-driven activity which was sustained following application of PACAP-38 (30–60 nA), thereby allowing us to test the effects of antagonist application. The sustained levels of firing (10.3–31.8 Hz) were an approximate 17-fold increase from basal levels ($n = 5$). This time [*p*-Cl-D-Phe^6,Leu^{17}]-VIP showed negligible effects on activation by PACAP-38 with a mean percentage inhibition of 6 ± 4%. In contrast, PACAP(6–38) significantly inhibited neuronal activity by 29 ± 9% (Fig. 1(C)). Recovery was observed in all cases of inhibition, with the majority of neurons taking approximately 60 s to return to control firing levels.

Effects of VIP/PACAP receptor antagonists on sustained neuronal firing evoked by sensory inputs

The majority of neurons examined were within laminae III–V of the dorsal horn, as determined by histological marking with Pontamine Sky Blue. Eleven were classed as non-nociceptive neurons while 27 were classed as multireceptive. Only neurons with receptive fields on hairy skin were used, to facilitate classification and mustard oil application. Ionophoresis of vehicle (pH 4.5 H_2O) or NaCl current controls had no detectable effect

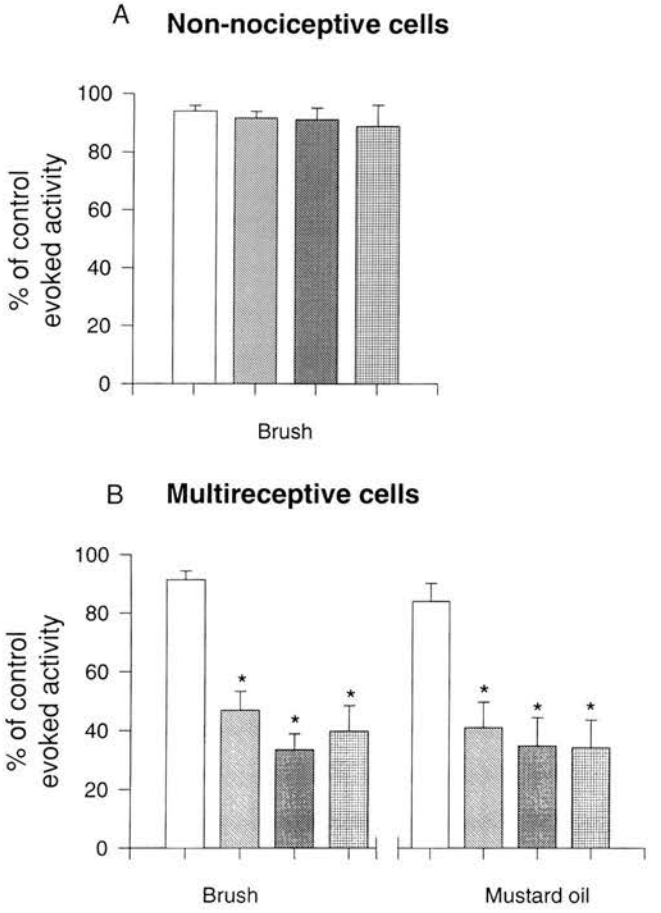

Fig. 4 Summary histogram showing the inhibition of stimulus-evoked neuronal firing rate of dorsal horn neurons caused by VIP/PACAP receptor antagonists in comparison with control vehicle application (pH 4.5 H_2O). Responses in the presence of □ vehicle control, ▒ [*p*-Cl-D-Phe^6,Leu^{17}]-VIP, ▓ VIP(6–28) and ▒ PACAP(6–38) are shown. All three antagonists showed negligible effects when applied to sustained neuronal activity of non-nociceptive neurons (A), while both innocuous brush- and noxious mustard oil-induced activity of multireceptive neurons were markedly decreased (* = $P \leq 0.05$) by application of any of the three antagonists (B).

on the brush or mustard oil-induced activity of those neurons tested.

In non-nociceptive neurons, all three antagonists showed negligible effects when applied to sustained neuronal firing induced by innocuous brush, with ejection currents of 30–60 nA. [*p*-Cl-D-Phe^6,Leu^{17}]-VIP showed a mean percentage inhibition of 8 ± 2% ($n = 14$), VIP(6–28) exhibited 10 ± 4% inhibition ($n = 7$), while PACAP(6–38) had a mean percentage inhibition of 11 ± 7% ($n = 6$) (Fig. 2). In multireceptive neurons, however, the VIP and PACAP antagonists significantly inhibited both brush- and mustard oil-induced responses (Fig. 3). [*p*-Cl-D-Phe^6,Leu^{17}]-VIP showed 53 ± 6% inhibition ($n = 19$) of sensory responses induced by innocuous brush and 59 ± 8% inhibition ($n = 11$) of those induced by mustard oil. The latency of inhibition varied slightly from cell to cell, but recovery was observed in all cases, with the activity of the majority of cells returning to control levels within about 90 s. Similarly, VIP(6–28) inhibited brush-evoked activity by 67 ± 6% ($n = 13$) and mustard oil-induced activity was inhibited by 71 ± 13% ($n = 7$). Again recovery was observed in all cases, from 2–3 s to 2 min. PACAP(6–38) also showed a marked effect on both types of response with 60 ± 9% inhibition ($n = 16$) of innocuous brush and 66 ± 9% inhibition ($n = 9$) of noxious mustard oil-induced activity. The activity of approximately half the cells tested with PACAP(6–38) returned to control firing levels within 5 s of terminating ionophoresis of the peptide, while the remaining neurons took anything from 1.5 to 8 min to recover. Figure 4 shows a summary histogram of the antagonist effects.

In vitro characterization of antagonists

VIP (0.1–1000 nM) caused concentration-dependent increases in cAMP generation by COS 7 cells expressing VIP_1, VIP_2 or PACAP receptors, with EC_{50} values of 3.3 ± 0.7, 0.9 ± 0.2 and 29 ± 6 nM respectively (means ± SEM from 8–12 separate determinations). Cells transfected with vector alone showed no response to VIP. Since the agents were not in all cases simple competitive antagonists, Schild analysis could not be completed and inhibition curves were constructed at a fixed submaximal agonist concentration of 5 nM. This concentration of VIP elicited cAMP generation of 12.2 ± 0.9-, 14.3 ± 1.3- and 8.5 ± 1.4-fold over basal levels at VIP_1, VIP_2 and PACAP receptors, respectively. Table 1 illustrates that [*p*-Cl-D-Phe^6,Leu^{17}]-VIP was a more potent inhibitor of cAMP generation at VIP_1 receptors (IC_{50} approximately 3 μM) than at either PACAP or VIP_2 receptors, with a margin of selectivity (estimated from inhibition curves) of 7- and 8-fold respectively. The potency of [*p*-Cl-D-Phe^6,Leu^{17}]-VIP was however quite low, even at VIP_1 receptors, and furthermore this compound alone acted as a weak partial agonist at VIP_1 and VIP_2, but not PACAP, receptors (to less than 20% of the VIP maximum at 10 μM). Additionally, VIP_1 receptor antagonism by this analogue was not purely competitive, with a clear reduction of maximal responses to VIP. Its greater effectiveness at VIP_1 than at VIP_2 receptors has been described previously.[45] VIP(6–28) was a moderately potent antagonist at PACAP receptors (IC_{50} approximately 2 μM), a weak antagonist at VIP_2 receptors

Table 1 Antagonist potencies on heterologously expressed VIP_1, VIP_2 and PACAP receptors

	IC_{50} value (μM) on cAMP generation elicited by 5 nM VIP		
Antagonist	VIP_1 receptor	VIP_2 receptor	PACAP receptor
[*p*-Cl-D-Phe^6,Leu^{17}]-VIP	3.39 ± 0.61	> 10 (29 ± 9% inhibition at 10 μM)	> 10 (36 ± 8% inhibition at 10 μM)
VIP(6–28)	> 10 (24 ± 4% inhibition at 10 μM)	8.87 ± 3.09	1.67 ± 0.38
PACAP(6–38)	>>3 (16 + 6% inhibition at 3 μM)	0.170 ± 0.028	0.014 ± 0.002

Each value is the mean ± SEM from 8–12 separate determinations.

(IC_{50} approximately 9 μM) and showed very little affinity for VIP_1 receptors. PACAP(6–38) was an extremely potent antagonist of PACAP receptors (IC_{50} of 14 nM) and unexpectedly also showed high potency at VIP_2 receptors (IC_{50} of 170 nM). In contrast, PACAP(6–38) demonstrated little or no interaction with VIP_1 receptors throughout the concentration range tested and thus displayed a selectivity sequence of PACAP > VIP_2, >> VIP_1 with selectivity ratios of approximately 12- and >>30-fold respectively.

DISCUSSION

These results further support previous evidence that VIP and its related peptides may play important physiological roles as neurotransmitters within the central nervous system, as well as providing us with new insights into the type of VIP/PACAP receptors functionally important in rat dorsal horn neurons, under normal conditions.

Despite the fact that VIP and PACAP are normally present in relatively low abundance,[19,20] the present results provide clear evidence for the presence of VIP/PACAP receptor subtypes on dorsal horn neurons under normal conditions. The agonist study carried out has provided the first direct evidence that PACAP is an effective excitant of spinal dorsal horn neurons, while reinforcing previous evidence of an excitatory role for VIP within the spinal cord. Although the two agonists used produced similar results, three main differences are apparent.

The first is that PACAP-38 appears to be more potent than VIP in terms of neuronal excitation, as it seems to have a much more marked excitatory effect, despite being used at the same concentration and similar ionophoretic currents. Approximately two-thirds of the neurons tested (10 out of 14) were activated by PACAP-38 compared to only 6 out of 14 with VIP. In the majority of cases PACAP-38 was also found to have a longer duration of action than VIP, lasting for at least 20 min in some cases. This long-term activation of a small proportion of some of the neurons, even after application of PACAP-38 had been terminated, would be consistent with the possibility that PACAP-38 may have induced some form of sensitization at the spinal cord level. The source of endogenous PACAP-38 which may be able to achieve this is likely to include afferent C-fibres, since it has been previously shown that the levels of PACAP in dorsal horn are markedly decreased by capsaicin treatment.[17] The relatively prolonged action of VIP and more especially PACAP-38, suggests a longer term regulatory role for these peptides, and the disparities between the two agonists could be due to a more prolonged effect of PACAP at the site of action, but may simply be due to differences in the enzymatic degradation processes for the two peptides.

The fact that some cells were activated by PACAP-38 and not by VIP (the second difference) suggests that a sub-population of cells may express PACAP receptors either instead of, or in addition to, VIP_1 or VIP_2 receptors under normal conditions. However, a number of other cells responded to both PACAP and VIP, suggesting a response mediated by either VIP_1 and/or VIP_2 receptor subtypes, where VIP and PACAP show similar potency. It is not clear whether PACAP and VIP receptors mediate dual regulation of individual cells. The efficiency of electrophoretic transport out of the electrodes may not necessarily be identical for VIP and PACAP-38, and so may bias our results. In the majority of cells tested, there was a relatively slow onset of effect and activation was often observed at fairly high currents (up to 60 nA), suggesting either quite low transport efficiency or diffusion to the site of action, so it is not possible to make a direct comparison. On balance, however, these results would suggest that PACAP may have a more widespread effect within the spinal cord than VIP.

The third difference involved the type of dorsal horn neuron activated. Responsiveness to VIP appeared to be highly selective for multireceptive neurons only, while PACAP-38 also excited a sub-population of the non-nociceptive neurons tested. Previous investigators have reported VIP to be equally effective in exciting both nociceptive and non-nociceptive trigeminal neurons,[29] but in the present study we found VIP to have no significant effect on any of the non-nociceptive dorsal horn neurons

tested. These results also correspond to the antagonist experiments carried out where none of the VIP/PACAP receptor antagonists used produced any significant effect on the brush-induced activity of non-nociceptive dorsal horn neurons. The agonist results suggest that whilst both VIP and PACAP cause a marked excitation of neurons within the spinal dorsal horn, there may be some differences in the distribution of their receptors and that they may each play a role (perhaps independently) in regulating sensory transmission at the spinal cord level.

The importance of VIP/PACAP-mediated transmission under normal conditions is highlighted in the antagonist study, where the sustained activity of multireceptive spinal cord neurons brought about by topical application of the C-fibre selective irritant mustard oil, innocuous brushing of the hindlimb or ionophoretic application of the agonists VIP or PACAP-38, is clearly regulated (or mediated), at least in part, by VIP/PACAP receptors. Blockade of these sensory responses shows a remarkable similarity between the effects of VIP and PACAP receptor antagonists. All three antagonists used showed a marked inhibition of the firing induced by either innocuous brush or noxious mustard oil application to the receptive fields of multireceptive neurons, while having no significant effect on the brush-induced activity of non-nociceptive dorsal horn neurons. This highlights the possibility that these peptides are largely restricted to neuronal pathways concerned with the regulation of C-fibre-mediated transmission, and the pharmacological profile suggests that both VIP and PACAP receptors may play a role. While a small population of non-nociceptive neurons may express PACAP receptors, the lack of effect of the PACAP receptor antagonist on the brush-induced responses of non-nociceptive neurons indicates that these receptors do not exert a significant functional influence under normal conditions.

Nociceptive afferents, including C-fibres, terminate in the superficial layers of the dorsal horn, and previous evidence has already demonstrated the fact that both VIP and PACAP are found in small diameter fibres,[18,22,46] inferred to be the Aδ– and C-fibres associated with polymodal and mechanical nociceptors. The majority of VIP- and PACAP-immunoreactive fibres are also present in laminae I and II of the rat spinal dorsal horn[17,18,21] with additional fibres being found around the central canal and in lamina VII. These histological findings initially provided the grounds for suggesting a role for these peptides in the control of normal sensory transmission, and the present antagonist data has now provided the first physiological evidence. The firing rate of the majority of cells tested was restored almost immediately the antagonist application was terminated. This suggests the possibility that the VIP/PACAP receptors are found on the recording neurons themselves and that VIP and PACAP are acting directly on the dorsal horn neurons. However, slow onset agonist responses could also be consistent with actions on cells which indirectly (but non-selectively with respect to input modality) regulate the recorded neurons. It has been shown previously that protein kinase A inhibitors such as H89 inhibit both brush and mustard oil responses of neurons[47] so it is possible that the VIP and PACAP receptors are acting here via cyclic AMP pathways. This is consistent with evidence for facilitation of dorsal horn neuron responses to excitatory amino acids following pre-incubation with the adenylate cyclase activator forskolin.[48]

Although totally specific agonists and antagonists are not yet available for VIP_1, VIP_2 and PACAP receptors, it is possible to use the relative selectivity of a number of compounds to make inferences about the type of receptor(s) which may be involved in physiological responses. PACAP receptors are activated with much greater potency by PACAP-38 than by VIP,[8,42] suggesting that a response elicited by PACAP-38 but not by VIP is likely to be mediated by a PACAP receptor. In contrast, VIP_1 and VIP_2 receptors are activated by VIP with a potency similar to or greater than that for PACAP-38,[6,7,45] suggesting that a response elicited by either VIP or PACAP-38 with similar ionophoretic currents is likely to be mediated by VIP_1 or VIP_2 receptors. Thus it appears that most multireceptive neurons are regulated by some form of VIP/PACAP receptor – with the predominant (or sole) effective form in about half of the population being VIP_1 or VIP_2 – while that in the remaining half appears to be the PACAP receptor. PACAP receptors may also be present on a minor subpopulation of non-nociceptive cells.

The receptor selectivity of the antagonists used allows further analysis of the subtypes of VIP/PACAP receptor likely to be involved in regulating sensory inputs. The cAMP production assay on individual receptor types expressed from their cDNAs characterizes the specificity of the antagonists used. The [*p*-Cl-D-Phe^6,Leu^{17}]-VIP antagonist appears to be selective for the VIP_1 receptor whilst showing only very low affinity for the VIP_2 or PACAP receptors. This is in agreement with a previous study which also provided evidence for selectivity of [*p*-Cl-D-Phe^6,Leu^{17}]-VIP for VIP_1 relative to VIP_2 receptors.[45] VIP(6–28), however, appeared to be moderately selective for PACAP receptors, with more than 4-fold lower affinity for VIP_2 receptors, and very little affinity for VIP_1 receptors. Although PACAP(6–38) is very highly selective for PACAP receptors, it also has a sufficiently high affinity for VIP_2 receptors to raise the possibility that it could be acting in the electrophysiological studies by VIP_2 rather than PACAP receptor blockade. PACAP(6–38) appeared to be essentially inactive at VIP_1 receptors through the concentration range tested.

Whilst agonist selectivity alone points strongly to the presence of PACAP receptors in cells responding to PACAP-38, but not VIP, the type of VIP receptor present in VIP-responsive cells can only be inferred from antagonist experiments. The blockade of VIP-evoked responses in such cells by [*p*-Cl-D-Phe^6,Leu^{17}]-VIP, but not by PACAP (6–38), suggests VIP_1 rather than VIP_2 receptor mediation because of their relative selectivity for VIP_1 and VIP_2 receptors, respectively. The blockade of PACAP-evoked activity by PACAP (6–38), but not [*p*-Cl-D-Phe^6,Leu^{17}]-VIP, is consistent with the additional presence of PACAP receptors, given that PACAP-38 will activate PACAP receptors at much lower concentrations than VIP_1 and VIP_2 receptors.

The issue of which of these receptors are functionally important in normal dorsal horn was addressed by applying antagonists during recordings of sensory responses. [*p*-Cl-D-Phe^6,Leu^{17}]-VIP, VIP(6–28) and PACAP(6–38) all inhibited both brush- and mustard oil-evoked responses of multireceptive cells, suggesting that VIP_1, PACAP (and possibly also VIP_2) receptors are involved normally in the regulation of dorsal horn neuron responsiveness. The origin of the endogenously released VIP/PACAP is not clear, with either local or afferent neurons being candidates. In summary, this is the first demonstration of an active role of VIP/PACAP receptor subtypes in regulating acute sensory inputs to dorsal horn neurons under normal conditions.

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Research report

Behavioural and electrophysiological evidence supporting a role for group I metabotropic glutamate receptors in the mediation of nociceptive inputs to the rat spinal cord

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BRAIN RESEARCH

Research report

Behavioural and electrophysiological evidence supporting a role for group I metabotropic glutamate receptors in the mediation of nociceptive inputs to the rat spinal cord

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Abstract

A combined study of behavioural and electrophysiological tests was carried out in order to assess the role of metabotropic glutamate receptors (mGluRs) in mediating sensory inputs to the spinal cord of the rat. In the behavioural study the responses of conscious animals, with or without carrageenan-induced inflammation, to noxious mechanical and thermal stimuli were observed both before and after the intrathecal administration of mGluR antagonists L(+)-2-amino-3-phosphonopropionic acid (L-AP3) and (*S*)-4-carboxy-3-hydroxyphenylglycine (CHPG). It was found that the mGluR antagonist (*S*)-CHPG was capable of increasing both mechanical threshold and thermal latency in both groups of animals, and L-AP3 did so in those with inflammation induced in their hindpaw. Following this study, the responses of single lamina III–V dorsal horn neurons to an innocuous Aβ fibre brush stimulus and a noxious C fibre (mustard oil) stimulus were extracellularly recorded and the effect of ionophoretically applied drugs was examined. Cyclothiazide (CTZ), a selective antagonist at mGluR$_1$, markedly reduced the activity evoked by mustard oil, but not that elicited by brushing of the receptive field. Activity induced in dorsal horn neurons by ionophoresing various mGluR subgroup agonists was examined. CTZ successfully inhibited the activity evoked by group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG). In comparison to the neurons which responded to the ionophoresis of DHPG, less were activated by the selective mGluR$_5$ agonist *trans*-azetidine dicarboxylic acid (t-ADA). Together these results indicate that group I mGlu receptors, in particular mGluR$_1$, play a crucial role in mediating nociception, particularly following a sustained noxious input. © 1997 Elsevier Science B.V.

Keywords: Pain; Metabotropic glutamate receptors; Spinal cord; Analgesia; Nociception

Introduction

There is a large body of evidence supporting a role for the excitatory amino acid glutamate in mediating sensory information and there are a number of receptors which may contribute to this role. These receptors have been divided into (i) the ionotropic group, non-*N*-methyl-D-aspartate (NMDA) and NMDA receptors, which contain intrinsic ion channels, and (ii) the metabotropic group, which are coupled to G proteins and several signal transduction pathways.

Despite the recent interest in the role of metabotropic glutamate receptors (mGluRs) in the central nervous system, their contribution to nociceptive processing in the spinal cord is still unclear. There are eight cloned mGluRs known at present [1,14,33,36,44], and these have been further divided into three subgroups, according to their pharmacological and signal transduction profiles: group I (mGluRs$_{1/5}$), group II (mGluRs$_{2/3}$) and group III (mGluRs$_{4/6/7/8}$) [1,34,37]. Until recently the pharmacological tools available could not distinguish adequately between the different receptors, however with the advent of newer, more specific drugs at the receptor subtypes it has been possible to begin to investigate the separate roles of these three mGluR subgroups. A number of studies have provided evidence that mGluRs are involved in selectively mediating noxious inputs to the spinal cord [35,51,52] as

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well as the thalamus [15,16], although the particular receptor(s) involved could not be fully elucidated from the compounds used. In a previous study we demonstrated how mustard oil-evoked responses could be blocked by mGluR I antagonists (*R,S*)-CHPG and L-AP3, while brush-induced activity remained unchanged [51,52].

The effect of mGluR compounds on activity at the NMDA receptor channel is of particular interest, as this receptor is important in nociceptive mechanisms. Several studies have demonstrated an interaction between the NMDA receptor and mGluRs. In electrophysiological experiments, both in vitro [5,8] and in vivo [6], NMDA-evoked responses are enhanced when mGluR agonists are co-applied. Also, in behavioural studies using the formalin model, the co-administration of NMDA with mGluR agonist ((±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*trans*-ACPD) results in a much greater increase in nociceptive behaviours than seen with either agonist given alone [11]. A more recent study utilising this model has confirmed that an increase in nociceptive behaviour is induced by intrathecally-administered mGluR agonists (*R,S*)-dihydroxyphenylglycine (DHPG; group I) or (1*S*,3*S*)-ACPD (non-selective mGluR), but not (2*S*,1′*R*,2′*R*,3′*R*)-2-(2′,3′-dicarboxy-cyclopropyl)-glycine (DCG-IV; group II) and that this can be reversed by the administration of NMDA antagonist D-2-amino-5-phosphonopentanoic acid (D-AP5) prior to testing [17]. It was in light of this evidence that we chose to investigate more closely the role of group I mGluRs.

We evaluated the role of these receptors in mediating noxious inputs in two ways: The first was a behavioural study in which we assessed the effect of intrathecally-delivered mGluR antagonists (which act preferentially at group I mGluRs) on the responses of conscious animals to noxious mechanical and thermal stimuli, both in normal and carrageenan-treated animals. The second was an electrophysiological study in which we observed the effect of ionophoresing $mGluR_1$ antagonist cyclothiazide (CTZ) during the activity elicited by (i) repeated topical applications of the C-fibre irritant mustard oil, and (ii) innocuous brushing of the receptive field. We also tested several mGluR agonists to assess their ability to induce excitation of dorsal horn neurons. In this way we aimed to determine the extent to which mGluRs are involved in mediating noxious inputs, particularly $mGluRs_{1/5}$.

2. Materials and methods

2.1. Behavioural testing

Animals were Glaxo-bred male and female Lister-hooded rats (30–70 g). All drugs, i.e. L(+)-2-amino-3-phosphonopropionic acid (L-AP3), D-AP3 ([38] 60 nmol) and (*S*)-carboxyhydroxyphenylglycine (CHPG) ([19] 48 nmol), were administered as a 10-μl injection in saline. The pH of each solution was adjusted to 7.2–7.4.

Animals received a single intrathecal (i.t.) injection by means of a 10 μl Hamilton microsyringe, as described by Hylden and Wilcox [23]. Young animals were used for this procedure since access to the intrathecal space was found to be much easier than in large adults. Baseline measurements of paw withdrawal threshold/latency to noxious mechanical/thermal stimulation were taken prior to drug administration. Following the i.t. injection of drug or vehicle, paw withdrawal reactions were tested at 1, 3 and 5 min. Paw withdrawal threshold to a noxious mechanical stimulus was measured using a Ugo Basile Analgesy-Meter and measurement of paw withdrawal latency to noxious heat (to the nearest 0.1 s) was measured with a Ugo Basile Tail Flick Unit. One group of animals had no treatment to their paws, whereas a second group were used to assess the effects of drugs in the inflamed state. In these animals inflammation was induced in one hindpaw by the administration of λ-carrageenan. Prior to the induction of this peripheral inflammation, baseline responses were recorded for each animal. A 100-μl unilateral injection of 2% λ-carrageenan was administered subcutaneously into the dorsal (hairy) surface of the right (ipsilateral) hindpaw under a brief halothane anaesthesia. After 3 h, paws were seen to be swollen and local oedema was apparent. Further reflex testing showed these animals to display decreased threshold responses, thus indicating hyperalgesia of the paw.

All withdrawal responses were calculated as a percentage of those determined before the administration of vehicle or drug. The data shown are mean ± S.E.M. values for groups of subjects; however, each individual animal had its own percentage change calculated. In separate groups of animals in which inflammation was induced a second set of baseline measurements were recorded 3 h after carrageenan administration, but prior to drug delivery. Drug induced effects were compared to those evoked by vehicle administration and statistical analysis was carried out using the Mann–Whitney *U*-test.

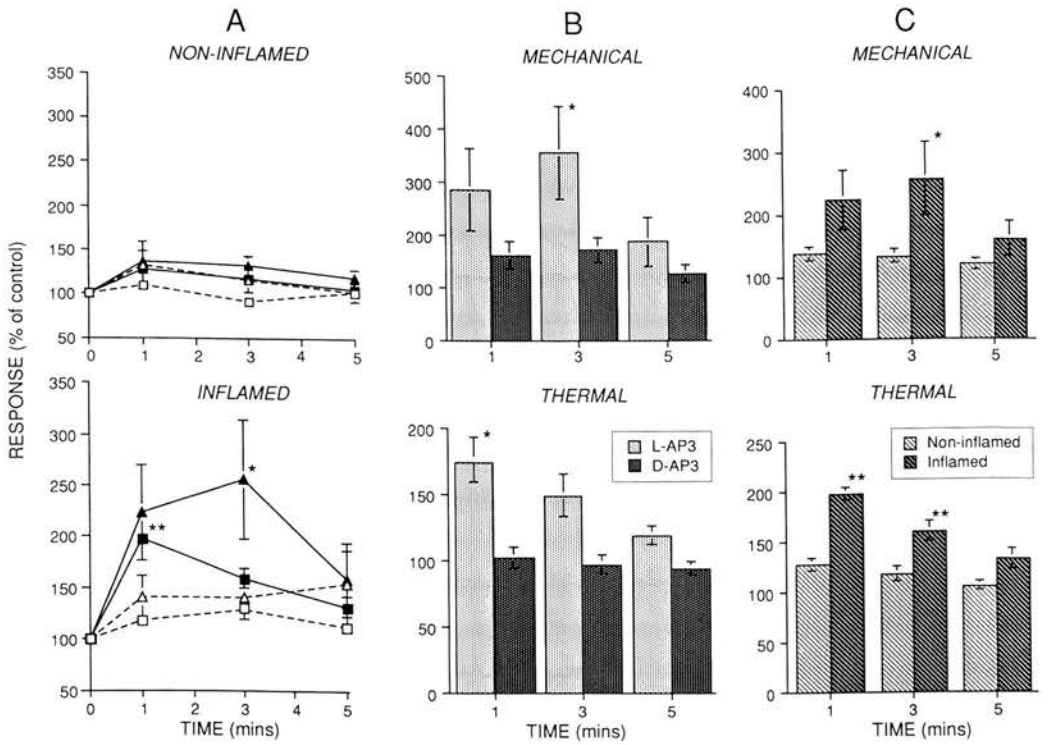
2.2. Electrophysiology

Experiments were carried out on 34 adult male Wistar rats (240–420 g). Under an initial halothane anaesthesia the jugular vein and trachea were cannulated. Animals were then given intravenous α-chloralose (60 mg kg^{-1}) and urethane (1.2 g kg^{-1}) with supplementary doses of α-chloralose (10 mg ml^{-1}) given throughout the experiment. Core body temperature was maintained at 37–38°C by means of a thermostatically-controlled heat blanket linked to a rectal probe. Animals breathed freely with a light flow of oxygen (0.1 l min^{-1}) passed over the tracheal cannula to enrich the inspired air. The animal was placed in a stereotaxic frame and the thoraco-lumbar spinal column was supported using three pairs of swan-neck clamps. A laminectomy (L1–L4) was performed and to provide stability agar was injected under the most rostral

ertebra and then over the exposed spinal cord. A small rea of this agar was removed to uncover the recording egion. Finally the dura was removed and liquid paraffin, 7°C, was then poured over the exposed spinal cord.

Extracellular recordings were made from single neurons n laminae III–V via the central barrel of a 7-barrelled lass microelectrode filled with 4 M NaCl (pH 4.0–4.5). he tip diameter ranged from 4–5 μm with DC resistances f 5–8 MΩ. The bandwidth of the recording amplifier was Hz–7 kHz. The following drugs were ionophoresed from ıe side barrels of the electrode: non-selective mGluR gonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic cid (ACPD; [24]), $mGluR_5$ agonist *trans*-azetidine-2,4-dicarboxylic acid (t-ADA; [28]), group II agonist (2*S*,3*S*,4*S*)-CCG/(2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine; L-CCG-I; [22]), group III agonist L(+)-2-amino-4-hosphonobutyric acid (L-AP4; [34]), α-amino-3-hydroxy-methyl-4-isoxazolepropionic acid (AMPA) and antago-sts L(+)-2-amino-3-phosphonopropionic acid (L-AP3; [38]), (*R,S*)-4-carboxy-3-hydroxyphenylglycine (CHPG; [47]), (*S*)-CHPG [4] and the selective group II antagonist (2*S*,1′*S*,2′*S*)-2-(2-carboxycyclopropyl)alanine (MCCG; [40]) were all 10 mM in distilled water, pH adjusted to 8.0–8.5 with sodium hydroxide; $mGluR_1$ antagonist cyclothiazide (CTZ; [41]), 0.1 mM in 0.5% dimethylformamide, pH 4.5; group I agonist 3,5-dihydroxyphenylglycine (DHPG; [39]), 10 mM aqueous, pH 4.5. All compounds were obtained from Tocris Neuramin. Retention currents of ±10 nA were employed to minimise drug leakage between tests. One of the remaining barrels contained 1 M NaCl (pH 4.0–4.5) for automatic current balancing, using a Neurophore BH2 Ionophoresis system, and current controls. A second side barrel contained Pontamine sky blue (PSB; 2% in 0.5 M sodium acetate) which was used to mark recording sites by ejecting for 12–14 min at 10 μA. The resistance of the side barrels was standardly measured and ranged from 25–30 MΩ.

The receptive fields of neurons were initially located



1. The effects of intrathecally-applied L-AP3 on paw withdrawal responses in normal and inflamed paws. Paw withdrawal responses (±S.E.M.) are wn as a percentage of the response immediately prior to drug administration (100%; that is the threshold and latency, respectively, in mechanical and mal stimulation experiments). In A, open symbols show the data from saline-treated rats, whereas filled symbols represent L-AP3-treatment; triangles responses to noxious mechanical stimulation and squares responses to noxious thermal stimulation. The data highlight the marked analgesic effect of P3 administration seen following the induction of inflammation by λ-carrageenan, but not in untreated animals. Although values in naive rats tended to ıigher with L-AP3 administration than in saline controls, this did not reach statistical significance in the present study. $^{**}P < 0.01$; $^{*}P < 0.05$, ificant differences with respect to saline (Mann–Whitney *U*-test). B shows a comparison of the effects of intrathecally-applied L-AP3 and its oisomer D-AP3 in animals which had been previously treated with carrageenan. The histogram demonstrates the analgesic effect of L-AP3 (lightly ed columns) in both mechanical and thermal tests, and how it was significantly different from the near-baseline effect observed with D-AP3 (heavily ed columns), thus implicating an mGluR-mediated effect. Data are shown as a percentage of pre-drug responses; $^{*}P < 0.05$ with respect to D-AP3 nn–Whitney *U*-test). C shows a comparison of the effect of L-AP3 on non-inflamed versus inflamed paws. There was a significant difference between ıal animals (lightly hatched columns) and carrageenan-treated animals (heavily hatched columns) following the intrathecal administration of L-AP3 (60 l). In both mechanical and thermal tests, L-AP3 was significantly analgesic only following inflammation, suggesting a more prominent role for uRs under these conditions. $^{**}P < 0.01$; $^{*}P < 0.05$ with respect to the untreated paw (Mann–Whitney *U*-test).

using a widespread manual brushing of the cutaneous surface of the hindlimb. Further characterisation was carried out using a noxious radiant heat stimulus (48°C, 10 s). All neurons exhibited low basal firing rates between 0 and 1 Hz. In the experiments using mustard oil (allyl isothiocyanate, Aldrich Chemical Co., 7.5% in paraffin oil); this irritant was repeatedly applied over an area of about 3 cm^2 and after 2–5 applications, separated by 5 min, steady elevated firing rates were observed (4–60 Hz). The inhibitory effects of antagonists were calculated as the mean evoked activity through a 20- or 30-s period encompassing the maximum observed inhibition, compared to the mean evoked activity in the 20–30-s period immediately preceding drug ionophoresis. The effects of the antagonists on motorised brush-, topically applied mustard oil- and agonist-evoked activity were compared over similar current ranges. Statistical analysis was carried out on raw data using the Wilcoxon test.

3. Results

3.1. Behavioural study

A total of 91 rats was used in this study, with each animal being randomly assigned to a test group. Nociceptive reflexes were assessed in these rats by their withdrawal reaction to a noxious (mechanical or thermal) stimulus applied to one hindpaw prior to and following intrathecal drug administration. The effects of mGluR antagonists were compared in normal rats and in those where a subcutaneous injection of λ-carrageenan had been given 3 h earlier, resulting in an inflammation of the paw. The mean changes in paw withdrawal responses per group of rats are given ($8 \leq n \leq 14$; mean percentage change ± S.E.M.) with statistical significance as determined using the Mann–Whitney *U*-test (see Fig. 1A,C and Fig. 2). All drug-induced effects were compared to those of saline vehicle. In a separate set of experiments, the effects of L-AP3 were directly compared with those brought about by its stereoisomer D-AP3, which is without effect at mGluRs, though it is a weak antagonist at the NMDA receptor [38] (see Fig. 1B).

In normal animals an intrathecal injection of L-AP3 (60 nmol) resulted in only minor changes in paw withdrawal responses to either mechanical or thermal stimulation (n = 14 and 8, respectively), which did not reach statistical significance in this study (see Fig. 1). However, when (*S*)-CHPG was applied (48 nmol; $n = 14$), both thermal latency and mechanical threshold were increased from pre-drug levels in control (as well as inflamed) animals and this was statistically significant at the 3-min time point from the effects observed using vehicle ($P < 0.05$; see Fig. 2). The difference between the effect of these compounds may well be due to the fact that (*S*)-CHPG is a far more potent antagonist at mGluRs than L-AP3 [37].

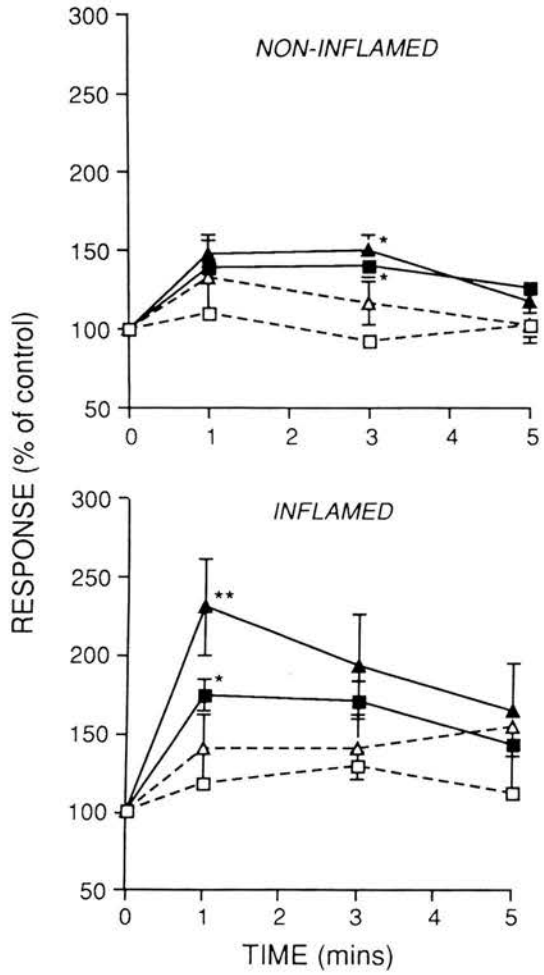


Fig. 2. Effect of intrathecally applied (*S*)-CHPG on paw withdrav responses in normal and inflamed paws. Paw withdrawal respon (±S.E.M.) are shown as a percentage of the response immediately pr to drug administration. Open symbols show the data from saline-trea rats, whereas filled symbols represent (*S*)-CHPG treatment; triangles responses to noxious mechanical stimulation and squares are to noxi thermal stimulation. In comparison to saline controls, this compou significantly raised responses in both tests, not only in carrageenan-trea animals but also in normal rats. ($^{**}P < 0.01$; $^{*}P < 0.05$; Mann–Wh ney *U*-test).

The results obtained from the animals which had inflammation induced using carrageenan were differ from those observed in the normal animals. (*S*)-CH ($n = 13$) now induced more profound effects with creases in thermal latency at the 1-min time point $+74 \pm 10\%$ ($P < 0.05$) and mechanical threshold $+130 \pm 31\%$ ($P < 0.01$; see Fig. 2). Interestingly, L-A was now also found to significantly increase the wi drawal responses (see Fig. 1A and C). The withdra threshold to noxious mechanical stimulation was increa by this compound ($n = 14$) to $+156 \pm 58\%$ ($P < 0.$ and thermal latency was increased by $+97 \pm 6\%$ (I 0.01). Neither of these analgesic effects were mimicked D-AP3 in inflamed animals (see Fig. 1B). (*R*)-CHPG not tested as it has antagonist properties at the NM

eceptor [4]. This effect of L-AP3 in the inflamed group vas significantly different from that observed in the normal group ($P < 0.05$ in mechanical testing and $P < 0.01$ on thermal tests; see Fig. 1C).

3.2. Electrophysiology

Most neurons tested were multireceptive, responding to both innocuous brushing of the receptive field as well as noxious heating to 48°C. Microdrive readings indicated that the neurons recorded from were in the deeper dorsal horn (laminae III–V), and this was confirmed with histological examination of PSB spots post-experimentally. Ionophoresis of Na^+ or Cl^- up to 80 nA had no detectable effects on any of the evoked activities discussed here.

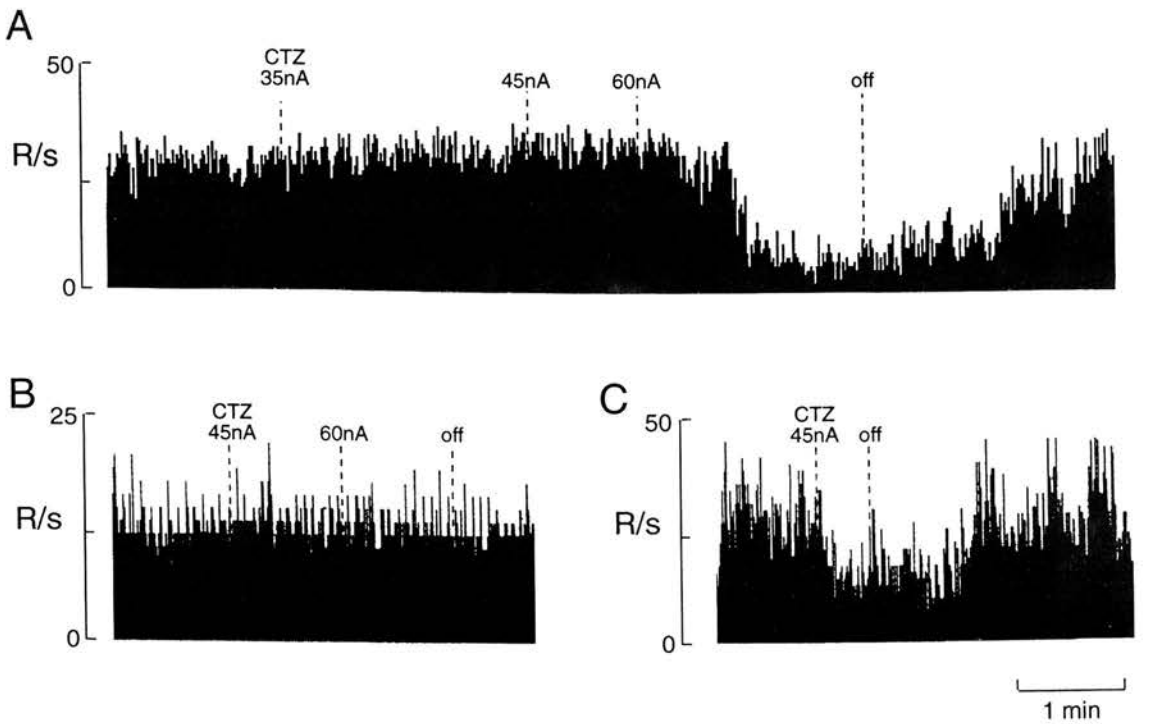
3.2.1. Agonist study

In a previous study we ionophoresed the non-selective mGluR agonist 1*S*,3*R*-ACPD and found that approximately one-fifth (47/244; 19%) of cells could be excited by this compound [52]. In the present experiments, we investigated a range of different mGluR agonists and observed their ability to evoke activity in single dorsal horn neurons. As well as the group I agonist 3,5-DHPG, we also used the $mGluR_5$-selective compound t-ADA, group II agonist L-CCG-I and group III agonist L-AP4.

Interestingly in the present experiments we found that ejection of the $mGluR_1$-selective agonist 3,5-DHPG (4–45 nA) could activate over 40% (55/137) of the neurons tested. Although this population was not identical to the one previously reported on using (1*S*,3*R*)-ACPD [52], it is likely that they shared broadly similar characteristics as the same search stratagem and depth range were used in both cases. These results therefore indicate that perhaps (1*S*,3*R*)-ACPD was activating some additional inhibitory systems, and the overall effect observed was one of lesser excitation. The activation of group II and III mGluRs by this compound would explain this, as they are negatively coupled to cAMP production. The other three agonists tested also activated a lower proportion of neurons: t-ADA induced activity in 6 of 31 cells (19%), L-CCG-I in 4 of 20 (20%) and L-AP4 only 1 of 19 units tested (5%). In the case of the latter two compounds, the lower proportion of responses may be due to the fact that they are linked to inhibitory cascades and the occasions on which we did see activation may indicate a polysynaptic event. In the case of t-ADA it is interesting that only a relatively small number of cells were activated, since it is clear from immunocytochemical experiments that $mGluR_5$ are present in the dorsal horn [46,47]. The clearly greater proportion of cells activated by 3,5-DHPG may result from its additional ability to activate $mGluR_1$.

3.2.2. CTZ study

Twelve of the 51 units which responded to ionophoresis of the $mGluR_{1/5}$ agonist 3,5-DHPG were tested further.



3. Ionophoresis of $mGluR_1$ antagonist cyclothiazide (CTZ) during evoked activity in single dorsal horn neurons. Traces show raw data as responses second, collected in 700-ms bins. The ejection of CTZ during co-ionophoresis of $mGluR_{1/5}$ agonist 3,5-DHPG resulted in a marked reduction of vity (A). Similarly, mustard oil-evoked C-fibre activity could be reduced when CTZ was ionophoresed (C), though the activity which resulted from cuous brushing of the hindlimb receptive field was unaffected (B).

When 3,5-DHPG was ejected (4–25 nA), the mean increase in background firing rate was 13 ± 2 Hz. This agonist-evoked activity was markedly reduced by the co-application of $mGluR_1$-selective antagonist CTZ (30–60 nA) in 12 out of 12 cases, with a mean inhibition of $72 \pm 7\%$ (see Fig. 3A). A further five cells were tested with repeated brief (10–20 s) applications of AMPA (20–30 nA). In all five cases, the brief afterdischarge, limited to 2–3 s, was more than doubled in the presence of CTZ (50–60 nA), as would be expected from its known attenuation of AMPA receptor desensitisation [7]. The results obtained when ionophoresing CTZ during evoked sensory inputs were very similar to those already published using L-AP3 and (*R,S*)-CHPG [51], in that in 9 out of 11 cases the noxious input elicited using the topical application of the C-fibre irritant mustard oil was markedly inhibited by CTZ (mean percent decrease for these 9 was $40 \pm 4\%$; $P < 0.05$ compared to pre-drug firing levels; see Fig. 3C). The lack of any noticeable inhibitory effect of L-AP3 and (*R,S*)-CHPG on innocuous brush-evoked activity [51] was also mirrored by ionophoretic application of CTZ ($n = 18$; see Fig. 3B). Therefore this $mGluR_1$ antagonist appears to be selectively blocking the transmission of nociceptive inputs evoked by the C-fibre irritant mustard oil, but not the low threshold brush-evoked activity.

In three out of three experiments, the 40–60% inhibition of mustard oil-evoked activity caused by (*S*)-CHPG (45–60 nA) was unaltered by co-application of MCCG (45–60 nA), thus providing no strong evidence to suggest a major role of group II mGlu receptors in the effects of (*S*)-CHPG here.

4. Discussion

The current study has demonstrated how antagonists which act preferentially at group I mGlu receptors are both analgesic in behavioural tests and selectively antinociceptive in electrophysiological recording experiments. Having found in a previous electrophysiological study that mGluR antagonists L-AP3 and (*R,S*)-CHPG attenuated the responses of single dorsal horn neurons to a sustained noxious input [51], we decided to assess the effect of administering these drugs intrathecally in conscious animals. The *S* isomer of CHPG had since become available and was used in the present experiments as it is the isomer with activity at mGluRs [4].

We evaluated the effect of applying these compounds in two models: firstly in normal animals and secondly in those which had inflammation induced peripherally with a subcutaneous injection of λ-carrageenan into one hindpaw. In the normal group we found that (*S*)-CHPG administration resulted in a decreased response to both noxious mechanical and thermal stimuli, thus implying that relative analgesia was obtained. This compound has been shown to act preferentially at group I mGluRs [37], so it would appear from our data that $mGluR_{1/5}$ are participating in the nociceptive reflexes under examination. The second antagonist used, L-AP3, is a weak antagonist at mGluRs [38]. This compound failed to significantly elevate mechanical threshold or thermal latency in the naive animals. Since (*S*)-CHPG was successful in causing analgesia, and both of these compounds are likely to act at the same receptors [37], the low potency of L-AP3 at mGluRs [37] is likely to be the reason for this lack of effect. Interestingly when we assessed the effects of the two antagonists in the carrageenan-treated animals we found the outcome was different to that in the normal group. Following inflammation L-AP3 was analgesic, in both mechanical and thermal tests, and (*S*)-CHPG was even more effective in elevating the responses than it had been in naive animals. There were no motor defects observed as a result of the administration of either mGluR antagonist. It therefore appears that $mGluR_{1/5}$ play a more profound role in an inflamed state. This is in agreement with the results of Neugebauer et al. [35], who found that L-AP3 inhibited the activity evoked in dorsal horn neurons following intra-articular inflammation, but not before and those of Fisher and Coderre [17] who showed attenuation of second phase, but not first phase formalin-induced nociceptive behaviour by (*S*)-CHPG. The increased mechanical threshold/thermal latency induced by L-AP3 in the inflamed state is highly likely to be due to mGluR blockade, as its stereoisomer D-AP3 did not elicit any effects discernible from saline controls. We chose not to assess the effects of the isomer of CHPG as this compound is an NMDA antagonist [4] and many studies have implicated NMDA receptor involvement in sustained rather than brief sensory input so effects of this reagent would be difficult to interpret. Although we cannot fully exclude the possibility that some of the effects of (*S*)-CHPG may be due to its agonist properties at group II mGluRs [20,49], pilot electrophysiological experiments provided no evidence that the selective group II antagonist MCCG could attenuate the reduction in mustard oil responses caused by (*S*)-CHPG. Clearly the current investigation provides evidence that the analgesic effects of L-AP3 and (*S*)-CHPG are very short-acting under the present conditions at least. This may be somewhat discouraging when considering the possibility of novel analgesic compounds. However, if a role for the receptors at which these drugs act can be elucidated, the identification of the target itself is a valuable tool for the further development of more longer-lasting analgesic compounds.

It would therefore appear that the role of mGlu (likely to be group I) in the spinal cord is far more profound following the induction of inflammation in the hindpaw. This reflects the somewhat similar observation made in our earlier electrophysiological experiments whereby mGluR antagonists markedly reduced the elevated C-fibre activity evoked when mustard oil was repeatedly applied to the cutaneous receptive field [51].

herefore returned to the electrophysiology paradigm in rder to further evaluate the specific mGluRs involved in his mediation of nociception.

Since our initial experiments, the compounds used (L-AP3, (*R,S*)- and (*S*)-CHPG) have been shown to act referentially at group I mGluRs [37]. In agreement with his profile we have shown that the excitatory effects of a elective group I mGluR agonist (3,5-DHPG) were much reater than those of non-selective group II or group III gonists. This is entirely consistent with the results of isher and Coderre [18], who showed that intrathecal jections of group I mGluR agonists elicited much greater ontaneous nociceptive behaviour than group II agonists nd that a group III agonist was without effect. In order to y and discern the role of $mGlu_1$ or $mGlu_5$ receptors, we herefore tested both a selective $mGluR_5$ agonist, t-ADA nd an agent described to selectively inhibit $mGluR_1$ com- ared to $mGluR_5$, CTZ. The proportion of cells activated y t-ADA was much less than that responding to 3,5-HPG, indicating that $mGluR_1$ and perhaps also $mGluR_5$ tes play a role in the activation of dorsal horn neurons. his is in agreement with a study in which it was reported at intrathecally administered 3,5-DHPG, but not t-ADA, icits spontaneous nociceptive behaviour [18]. These re- lts were also consistent with our observations using TZ. Although CTZ has been shown to have some facilita- ry effects at the AMPA receptor [7], we did not observe y increase in ongoing sensory responses to brush or ustard oil, despite evidence that AMPA receptors, partic- ate in both non-nociceptive and nociceptive pathways 3]. We were able, however, to demonstrate that CTZ used a marked prolongation of afterdischarges in five t of five cells challenged with brief ionophoretic pulses AMPA. This suggests that effects of CTZ on AMPA ceptor-mediated responses may be more apparent in brief ponses rather than the maintained stimuli used here to ess sensory inputs. When we ionophoresed CTZ during activity evoked in single dorsal horn neurons by a ht, innocuous brushing of the cutaneous receptive field re was no significant change, thus implying no major luence from facilitation of the AMPA receptor, as well no apparent involvement of $mGluR_1$. Conversely, if this npound was ejected whilst a cell was activated by eated topical application of mustard oil, a marked re- tion was observed. Although results with compounds ch as CTZ) that are active at multiple sites always uire interpretation with caution, these data provide evi- ce to suggest that $mGluR_1$ is selectively involved in diating a sustained C fibre input to dorsal horn neurons, not an innocuous Aβ fibre input. In further studies igned to address this hypothesis, it may be of interest to form similar studies in rats which have been neonatally ted with capsaicin. The mechanism by which mGlu ptors may contribute to sustained nociceptive inputs ains unresolved, although numerous downstream cellu- changes have been described [2,9,37,42,43]. Enhance- ment of NMDA receptor channel activity by mGluRs has been demonstrated, both in electrophysiological experiments in vitro [5,8] and in vivo [6], and more recently in behavioural studies [17]. Activity at group I mGluRs may result in initiation of a number of downstream signal transduction steps, including PKC activation [29], and we have previously demonstrated how inhibitors of PKC (as well as Ca^{2+}/calmodulin-dependent kinase II and phospholipase A_2) can block mGluR agonist-evoked activation of dorsal horn neurons [52]. PKC inhibitors can block LTP in the hippocampus [25–27], as well as the sustained activity in dorsal horn neurons elicited by the cutaneous application of C-fibre activator mustard oil, but not innocuous brushing of the receptive field [32]. Furthermore, [^{3}H]phorbol-12,13-dibutyrate ([^{3}H]PDBu) binding assays have shown how sustained noxious inputs result in the translocation of PKC to the membrane compartment in the spinal cord in this mustard-oil model [32], as well as in chronic constrictive injury (CCI, [30,31]) and in formalin-evoked hyperalgesia [50]. Such PKC activation has been demonstrated to correlate with behavioural hyperalgesia [30,31]. Also, the behavioural hyperalgesia brought about by CCI [21] or subcutaneous formalin, [12,50] is reduced if PKC activation is prevented. PKC-mediated enhancement of the voltage activation of NMDA receptors has been reported by several groups [3,10,45]. Furthermore, the spontaneous nociceptive behaviour and facilitation of formalin responses elicited by intrathecally-applied $mGluR_1$ agonist were inhibited by an NMDA antagonist [17,18]. Thus it seems possible that $mGluR_{1/5}$-elicited PKC activation may play a role in noxious, though not innocuous, inputs to the dorsal horn of the spinal cord by means of NMDA receptor phosphorylation and enhancement. We have also shown in the current investigation that 3,5-DHPG, which activates $mGluR_{1/5}$ is a relatively potent excitant of dorsal horn neurons, compared to agonists at group II and III mGluRs and that the selective $mGluR_5$ agonist t-ADA was much less effective. Since 3,5-DHPG-evoked activity (and mustard oil-evoked activity) could be reduced consistently by the co-ejection of CTZ, it appears that $mGluR_1$ may be activated in most of these cases of excitation.

In summary, our present data provide further evidence that mGluRs are crucial in processing high threshold sensory information, particularly following a sustained nociceptive input to the spinal cord. Furthermore, the evidence indicates that group I mGluRs, in particular $mGluR_1$, may be of particular importance in these events.

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ABSTRACT
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THE ROLE OF VIP/PACAP RECEPTOR SUBTYPES IN SPINAL SOMATOSENSORY PROCESSING AFTER CHRONIC CONSTRICTION INJURY TO THE SCIATIC NERVE.

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Peripheral nerve damage leads to a state of hyperalgesia and allodynia which involves important changes at the level of the spinal cord and is intractable to classical opioid analgesics. Since VIP and PACAP expression is increased in dorsal root ganglion cells following peripheral nerve injury, we have investigated the expression and influence of VIP_1, VIP_2 and PACAP receptors in dorsal horn in the chronic constriction injury (CCI) model of neuropathic pain. Selective antagonists of VIP_2 and, to a lesser extent VIP_1 and PACAP receptors, inhibited the sensitised responses of dorsal horn neurones to mustard oil, but not to brush. Neuropathy-induced responses to cold were attenuated by VIP_1 and PACAP, but not VIP_2, receptor antagonists. In CCI animals compared to controls, a selective VIP_2 receptor agonist excited twice as many cells to more than 5 fold greater extent. The number of cells excited by a selective VIP_1 receptor agonist decreased, whereas responses to PACAP-38 were unaltered.
In situ hybridisation histochemistry correspondingly showed that in particular laminae of the dorsal horn the expression of VIP_1 receptor mRNA was reduced, VIP_2 receptor mRNA was markedly increased and PACAP receptor mRNA was essentially unaltered as a result of CCI.
These data indicate that VIP/PACAP receptors may contribute importantly to neuropathic sensitisation and may represent novel targets for analgesics designed against the hyperalgesia and allodynia of neuropathic pain states.

ABSTRACT

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THE ROLE OF VIP/PACAP RECEPTOR SUBTYPES IN SPINAL SENSORY PROCESSING FOLLOWING EXPERIMENTAL MONONEURAPTHY

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Peripheral nerve damage often results in the development of chronic pain states, resistant to classical opioid analgesics. The expression of Vasoactive Intestinal Polypeptide (VIP) and Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is markedly up-regulated in small dorsal root ganglion neurones following a chronic constriction injury (CCI). We investigated the expression and influence of VIP_1, VIP_2 and PACAP receptor subtypes in the rat dorsal horn following CCI. Electrophysiological studies revealed that selective antagonists of VIP_1, VIP_2 and PACAP receptors inhibit mustard oil-induced activity, but not brush-induced activity, of dorsal horn neurones in CCI animals. Cold-induced neuronal activity was attenuated by VIP_1 and PACAP, but not VIP_2 receptor antagonists. Selective agonists for the three receptor subtypes revealed that the VIP_2 receptor agonist excited twice as many cells (to a 5 fold greater extent) in CCI compared to normal animals. The number of cells excited by the VIP_1 receptor agonist decreased whereas responses to PACAP-38 were unchanged. *In situ* hybridisation histochemistry revealed that CCI of the rat sciatic nerve resulted in a significant increase in the expression of VIP_2 receptor mRNA in laminae III/IV of the dorsal horn. In contrast, the expression of mRNA for VIP_1 receptors was seen to decrease while that for PACAP receptors was essentially unaltered. Therefore, VIP/PACAP receptors may be important factors in neuropathic pain states. The VIP_2 receptor appears to be particularly important in the transmission of nociceptive information, while VIP_1 and PACAP receptors may also be involved in certain aspects of allodynia.