THE ROLE OF GALANIN AND NEUROPEPTIDE Y IN A RAT MODEL OF NEUROPATHIC PAIN

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Declaration of Authorship

I hereby declare that the composition of this Thesis and the work presented in it are entirely my own work, with the exception of the initial setting up of the neuropathic model, including the behavioural assessment of this, and the electrophysiology experiments, which were carried out in collaboration with Margo Mark. This thesis has not been submitted for the purpose of obtaining any degree or qualification from any other academic institution.

February, 1998 **Lesley A. Colvin**

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Contents

Abstract

Galanin and neuropeptide Y (NPY) are not normally produced to any significant extent by primary afferent neurones. After peripheral nerve injury however, there is a marked increase in their synthesis - galanin mainly in small to medium sized neurones, and NPY mainly in large neurones. There is evidence of transport of these newly produced peptides to the dorsal horn, but it is not known what stimuli result in their release in the spinal cord. The antibody microprobe technique was therefore used to study the factors that might result in release of these peptides. The model that has been studied involved placement of four loose ligatures around one sciatic nerve in the rat, reliably resulting in the development of behavioural evidence of neuropathic pain.

In addition to observing for the characteristic signs associated with this model, the development of neuropathic pain was assessed quantitatively by the paw withdrawal response to von Frey hairs and pin prick. Thus, all the animals used in the study of peptide release or functional changes in the spinal cord, 10 to 14 days later, did indeed have evidence of neuropathic pain.

A general assessment of altered spinal cord responses, found significant changes in the neuropathic rats. There was found to be a bilateral increase in the cord dorsum potentials evoked by A fibre stimulation of the injured nerve, as compared tot he normal nerve, despite a decrease in the afferent volley from the injured nerve. As the synthesis of both galanin and NPY is stimulated by peripheral nerve injury, alterations in their spinal release was studied, including the effects of A fibre stimulation on this central release.

Microprobes bearing immobilised antibodies to galanin were inserted into the spinal cord of neuropathic rats. An increased area of immunoreactive (ir)-galanin release was detected in the superficial dorsal horn ipsilateral to nerve injury, as compared to that normally found. This occurred in the absence of any active peripheral stimulation, in an area of the dorsal horn where the small primary afferent fibres that have started to synthesise galanin terminate. Ir-galanin release was not increased by electrical stimulation of the injured nerve at a strength sufficient to activate A fibres,

but there was an increase in release as C fibres were stimulated. Conduction block proximal to the dorsal root ganglia did not reduce the increased basal ir-galanin release found ipsilateral to the nerve injury. An increase in ir-galanin release was seen, however, on the contralateral side of the cord, after all neuronal input had been blocked from the injured nerve.

Previous studies in this laboratory have found that, on the side ipsilateral to nerve injury, ir-NPY was released spontaneously in the dorsal horn and this release was increased by electrical stimulation of the injured nerve sufficient to activate only A fibres. The large diameter primary afferent neurones that synthesise NPY after nerve injury terminate in the deep dorsal horn, where ir-NPY was released, making it probable that these fibres are the source of this ir-NPY. Using antibody coated microprobes, the present studies found that the spontaneous release of ir-NPY was not altered by conduction block of the injured nerve proximal to the dorsal root ganglia. It appears therefore, that release of NPY in the spinal cord from large diameter primary afferent neurones does not require any peripheral neuronal impulses.

The large diameter neurones that start to produce NPY express the neurotrophin-3 (NT-3) receptor, trkC. It has also been shown that exogenous NT-3 can attenuate some of the increases in spinal levels of NPY, after bilateral axotomy. The effect of exogenous NT-3 on NPY release was therefore studied using a chronic infusion of NT-3 delivered to the nerve injury site and antibody microprobes to measure spontaneous NPY release. It was found that NT-3 infusion had no effect on spontaneous NPY release, but a minor alleviation of mechanical hypersensitivity was noted 10 to 14 days after nerve injury, in those rats receiving exogenous NT-3.

These studies have determined some of the stimuli responsible for spinal release of galanin and NPY after peripheral nerve injury, and excluded others. Further work is required to correlate these results with functional effects and possible role in the modulation of nociceptive transmission.

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Abbreviations

APTES aminopropyltriethoxysilane

BDNF brain derived neurotrophic factor

BSA bovine serum albumin

cAMP cyclic adenosine monophosphate

CCK Cholecystokinin

CB-HRP Choleragenoid beta- horse radish peroxidase

CCI Chronic Constriction Injury

Ci Curie

cGMP cyclic guaninine monophosphate

CGRP Calcitonin gene-related peptide

CNTF Ciliary neurotrophic factor

c.p.m. counts per minute

CRPS Complex Regional Pain Syndrome

EM electron microscope

epsp excitatory postsynaptic potential

GABA Gamma amino butyric acid

GalR1 Galanin receptor type 1

GalR2 Galanin receptor type 2

GAP-43 Growth associated protein-43

GTP guanine triphosphate

G-protein guanyl nucleotide binding protein

5-HT 5-hydoxytryptamine

IASP International Association for the Study of Pain

icv intracerebroventricular

iml interomediolateral

IP1 inositol monophosphate

ipsp inhibitory postsynaptic potential

ir immunoreactive / immunoreactivity

i.t. intrathecal

K^+ potassium

LDCVs Large dense core vesicles

LIF Leukaemia inhibitory factor

mRNA messenger ribonucleic acid

nmol nanomoles

NA noradrenaline

NGF Nerve growth factor

NMDA n-methyl-d-aspartate

NT-3 Neurotrophin-3

NPY Neuropeptide Y

PNL Partial Nerve Ligation

PP Pancreatic polypeptide

PYY Peptide YY

PBS-A phosphate buffered saline with 0.1% sodium azide

PACAP Pituitary adenylate cyclase activating polypeptide

PYX1 Ac-[3-(2,6-dichlorobenzyl) Tyr27, D-Thr32]] NPY(27-36)amide

PYX2 Ac-[3-(2,6-dichlorobenzyl) Tyr27,36, D-Thr32]] NPY(27-36)

s.e.m. standard error of the mean

SMP Sympathetically maintained pain

SNL Spinal Nerve Ligation

SP Substance P

SSV small synaptic vesicle

$t_{1/2}$ Half life

VIP Vasoactive intestinal peptide

Chapter 1. General introduction

1.1 Clinical Aspects of Neuropathic Pain

There is a significant difference between the normal 'physiological' pain that occurs as a response to acute tissue injury, which serves a protective function, and the pathological process that results in chronic pain problems, which apparently serve no useful function [466]. This type of pain is destructive in that many of the patients will have suffered for some considerable time, with major implications for themselves, their family and eventually for society. The exact incidence of neuropathic pain is difficult to determine, but some 40% of patients referred to pain clinics will have at least a component of their pain related to neural factors.

The definition of neuropathic pain has been varied, but relates to a group of chronic pain syndromes in which there is a neural component to the pain experienced. This is a heterogeneous group, making it difficult to study clinically. An attempt has been made recently, by the International Association for the Study of Pain, to try and classify these syndromes into two groups: Complex Region Pain Syndrome types I and II [390], to try to improve diagnosis of the syndromes, and evaluation of new treatments. This classification is based on signs and symptoms, as it is not yet possible to accurately diagnose these pain syndromes on the basis of pathological changes. The features of the classification are outlined in Table 1.

Empirical findings have implicated the sympathetic nervous system in the development of some forms of neuropathic pain. This has been reviewed succinctly by Bennett, 1991 [30]. There are often signs of abnormal sympathetic activity, such as abnormal skin temperature and trophic skin changes and sympathectomy often relieves the painful syndrome. Indeed, the term Reflex Sympathetic Dystrophy was commonly used as a diagnostic label, until it was replaced by Complex Regional Pain Syndrome Type I [390]. Evidence for changes in the sympathetic nervous system after peripheral nerve injury are increasing. There are several reviews [88,181,265] on the interactions of the sympathetic and sensory nervous system that might produce chronic pain. Possible explanations for the exacerbation of pain by the sympathetic nervous system include: ephaptic transmission, the influence of adrenergic receptors on sensory neurones

associated with increased ectopic discharges, indirect coupling of sympathetic and sensory neurones, sensitisation of nociceptive afferents and sensitisation of dorsal horn neurones.

Complex Regional Pain Syndrome Type II was previously known as causalgia, and was first documented after the American civil war [285]. It is a chronic pain syndrome that may develop soon after trauma to a peripheral nerve. There is variable involvement of the sympathetic nervous system, secondary hyperalgesia and mirror image pain [43,274]. The exact definition has been debated, probably reflecting the heterogeneous nature of the response to peripheral nerve injury [364].

The term neuropathic pain is a more general term, which includes causalgia, but also refers to a much wider variety of mono- and poly-neuropathies. Neuropathic pain may be due to many factors, and often the aetiology is not known. Possible causes include nerve injury due to trauma, whether accidental or secondary to surgery, ischaemia, infection, inflammation or compression (e.g. haematoma, malignancy), and secondary to specific disease processes or systemic disorders, such as diabetes, rheumatoid arthritis, myeloma or alcohol abuse [361]. The natural history of the condition is affected by the underlying aetiology, but the duration of the condition can range from months to years or even decades, and is often refractory to all currently available forms of therapy. Often patients have been treated by a number of specialists, without a coherent overview of treatment aims. Prior to referral to a pain clinic, they have usually already been subjected to a number of treatments, both medical, surgical and psychiatric, with varying degrees of success. The treatments themselves may exacerbate or at least complicate the original problem [127].

Perhaps one of the main reasons that there are so many treatments, that are often ineffective in the long-term, is that the underlying pathophysiology of the central response to peripheral nerve injury is still not well understood. This makes it almost impossible to formulate specifically designed treatment to alter or prevent the underlying neural changes that result in neuropathic pain.. The advent of animal models is greatly improving the understanding of the neural mechanisms underlying the neuropathic pain states [31]. As understanding of the mechanisms of neuropathic pain increase, a more logical approach can be used to developing rational treatment

strategies. Additionally, it may be possible to target pain management in the peri-operative period in order to prevent or minimise the incidence of chronic pain problems developing after surgery [133].

In this thesis the term " neuropathic pain" will be used to describe a peripheral mononeuropathy resulting in pain-related behaviour, as this is the loose definition used to describe the animal models.

Table 1. Classification of chronic pain syndromes

This shows the reclassification of reflex sympathetic dystrophy and causalgia into more strict categories. This was the result of a consensus conference of the IASP, as described by Stanton-Hicks et al, 1995 [390].

Classification of Chronic Pain Syndromes

<table>
<tr><th colspan="2">COMPLEX REGIONAL PAIN SYNDROME (CRPS)</th></tr>
<tr><td colspan="2">The diagnosis is excluded by the existence of conditions that would otherwise account for the degree of pain and dysfunction.

There is variable involvement of the autonomic nervous system</td></tr>
<tr><td colspan="2">Spontaneous pain or allodynia/ hyperalgesia not limited to territory of single nerve, with pain disproportionate to the inciting event. Evidence (or previous evidence) of oedema, skin blood flow, or abnormal sudomotor activity in the region of the pain since the event</td></tr>
<tr><td>Type I (Reflex Sympathetic Dystrophy)</td><td>Type II (Causalgia)</td></tr>
<tr><td>Develops after initiating noxious event, without definable nerve lesion</td><td>Develops after a nerve injury</td></tr>
<tr><td>Spontaneous pain or allodynia/ hyperalgesia not limited to territory of single nerve, with pain disproportionate to the inciting event</td><td>Spontaneous pain or allodynia/ hyperalgesia, not necessarily limited to territory of injured nerve.</td></tr>
</table>

1.2 Animal Models

One of the original animal models of peripheral nerve injury is that of complete nerve transection, or axotomy, with or without associated neuroma formation at the site of transection. This has provided much useful information about the central response to peripheral nerve injury. It is now likely that previously held ideas of a hard-wired central nervous system [309], responding to varying peripheral inputs are no longer tenable [465]. There is an accumulating body of evidence for significant plasticity in the central nervous system with biochemical, structural and functional changes occurring in response to events in the periphery [87]. However, a major disadvantage of the axotomy model is that it is difficult to correlate the changes found with the occurrence of pain. The degree of autotomy, where the animals gnaw at the denervated limb, has been used as an indicator of pain, with a scoring system for this to grade severity. There is debate as to whether or not this actually reflects painful sensation, or is due to the complete denervation of the limb and the animal ceasing to recognise it as its own [67,354]. As a result of this problem several animal models using a partial nerve injury have been developed. These models display altered behaviour and there are a variety of tests that have been used to test for the presence of mechanical, thermal or chemical allodynia and hyperalgesia. This allows some degree of correlation of central changes after nerve injury with the severity of pain-related behaviour and also the testing of interventions on the development of neuropathic pain syndromes. The three models that have been studied most extensively, mainly in the rat, are:

1. A chronic constriction injury of the sciatic nerve (Bennett and Xie, 1988 [33]).
2. Partial ligation of the sciatic nerve (Seltzer and Shir, 1990 [366]).
3. Spinal nerve ligation of L5 +/- L6 (Kim and Chung, 1992 [201]).

The general characteristics of these models and the changes associated with them are as follows:

1.2.1. Chronic Constriction Injury (CCI) [33]

This is the model that has been used for the studies outlined in chapters 3-5. A more detailed description of the production of the nerve injury is given in section 2.2.1. Briefly, four loose ligatures of 4/0 chromic gut were placed around the sciatic nerve at

mid-thigh level, under sodium pentobarbitone anaesthesia, resulting in the development of behavioural evidence of neuropathic pain.

General changes associated with the model [33]:

Characteristic changes in gait were seen, with motor behaviour consistent with pain. Pain-related behaviours reached a maximum at two weeks after surgery, and had effectively resolved at two to four months. Trophic changes of the skin and nails were also found, with some oedema of the affected paw. Skin temperature changes were initially very variable [32], with the injured hindpaw becoming more consistently colder as the injury developed. Neither autotomy nor mirror image pain were major features of the CCI model, nor was there any difference in behavioural signs seen between male and female rats. Variable effects of sympathectomy were seen on development or alleviation of the pain syndrome [311,336].

Evidence for evoked pain

1. Response to mechanical stimuli [12]: Using Randall and Selitto's method [345] of paw withdrawal to a graded mechanical pressure, Bennett's group did not find evidence of mechanical hypersensitivity [33]. Using a modification of this method , by measuring the vocalisation threshold to increasing pressure on the hindpaw, a decrease in threshold on the side of nerve injury was found from day 5, reaching a minimum at 2 weeks and resolving by 8 weeks [12]. A decrease in threshold was taken to indicate the presence of mechanical allodynia, as vocalisation was elicited at pressures that had not previously given a response. The disparate results obtained may reflect the effect of muscle weakness in the injured leg, and it was indeed noted by Bennett that there were several cases in which the hindpaw was not withdrawn, possibly reflecting an inability to move the leg. The presence of mechanical hyperalgesia was determined by an increased response to noxious paw pinch [12].
2. Response to thermal stimuli: The hindpaw on the side of nerve injury showed a decreased paw withdrawal latency and increased paw withdrawal duration, to radiant heat. Placement on a cold surface showed an increase in both paw withdrawal duration and frequency on the side of nerve injury. A role of the

saphenous nerve in these behavioural responses was excluded as complete transection of the saphenous nerve did not alter the hyperalgesic response [33]. Both hot and cold thermal hypersensitivity were found as measured by immersion in water at varying temperatures [12].

3. Response to chemical stimuli: Mustard oil applied to the skin evoked a much greater response after nerve injury, with a significantly prolonged increase in paw withdrawal duration [33].

1.2.2. Partial ligation of the sciatic nerve (PNL) [366].

This model was described by Seltzer and Shir in 1990 and involved tight ligation of one half to one third of the dorsal part of the sciatic nerve, just distal to the branching off of the nerve to biceps semitendinosis, near the greater trochanter.

General changes associated with the model:

Abnormal hind limb posture and grooming behaviour were noted, although no autotomy was seen. A marked feature was the bilateral effect on pain-related behaviour, possibly correlating with the clinically seen mirror image pain. Another feature is the role of the sympathetic nervous system, with sympathectomy alleviating or preventing the development of the syndrome [375].

Evidence for evoked pain [366]

1. 1. Response to mechanical stimuli: Bilateral mechanical allodynia was demonstrated using von Frey hairs, and mechanical hyperalgesia was demonstrated by a bilaterally exaggerated response to pin prick, especially on the side of nerve injury.
2. Response to thermal stimuli: Bilateral increases in response to noxious heat stimulation were reported, becoming ipsilateral to the injury as the stimulus strength increased. Cold stimulation elicited a lesser response bilaterally in the nerve injured animals compared to controls. There was no evidence therefore for cold allodynia in this model - in fact, the reverse seems to be true, with cold decreasing sensitivity [366].

1.2.3. Spinal nerve ligation (SNL) [201]

This model was first described by Kim and Chung in 1992 and involves tight ligation of L5 and L6 spinal nerves, or L5 spinal nerve alone.

General changes associated with the model

After nerve injury, there was abnormal gait, posture and grooming, with licking and biting of the paw. Although it has been shown that at least part of these abnormal behaviours are related to motor damage, a component is due to sensory abnormalities and may indicate spontaneous pain [305]. No evidence of autotomy was seen.
A role of the sympathetic nervous system in the prominent mechanical allodynia seen was suggested by the alleviation of allodynia by sympathectomy [203], or alpha-2 agonists such as clonidine [482].

Evidence for evoked pain [201]

1. 1. Response to mechanical stimuli: There was a marked increase in response to both low and high threshold stimulation with von Frey hairs on the side ipsilateral to nerve injury.
2. Response to thermal stimuli: Noxious radiant heat stimulation showed a marked decrease in latency on the side ipsilateral to nerve injury.

1.2.4 Overview of animal models

The main features of the models described are summarised in Table 2. Other models of partial nerve injury have been developed, such as sciatic cryoneurolysis [84], diabetes-induced neuropathy [353], or secondary to toxic levels of pyridoxine [158]. None of these other models have been as extensively investigated as those described here, and in some cases produce additional systemic problems, such as those associated with diabetes mellitus, as well as the neuropathy, making interpretation of findings more complex.
The Bennett and Xie model [33] has been used in the present studies because it has been so extensively studied, and thus allows integration of these results with those from other investigators to help improve understanding of the mechanisms of neuropathic pain. It is safe and simple to set up, and reliably results in the development of

behavioural signs of neuropathic pain. There is a low incidence of autotomy, wound infection and other morbidity. This allows study of a chronic pain syndrome using minimum number of rats, with minimum suffering (both severity and duration), thus following Home Office guidelines to try and reduce animal usage. Although much work can be done *in vitro, in vivo* studies are still necessary to understand the dynamics of neuropathic pain syndromes.

Table 2. Comparison of animal models of neuropathic pain

The main features of each model, and the advantages and disadvantages of each are summarised. This has been reviewed by Bennett [31], and more recently by Kim et al [200]. CCI= Chronic constriction injury; PNL= Partial nerve ligation; SNL= Spinal nerve ligation; + (presence) or - (absence) of sign.

	Axotomy	CCI [33]	PNL [366]	SNL [201]
Spontaneous pain	? + (assessed by autotomy score)	+ (marked)	+	+
Mechanical hypersensitivity	-	+	+	+
Thermal hypersensitivity heat	-	+	+	+
Thermal hypersensitivity - cold	-	+	+	-
Time course	-	2-4 months	up to 7 months	up to 5 months
Bilateral effect	-	-	+ (marked)	-
Sympathetic involvement		+	+	+(marked)
Advantages	• non-variable lesion	• behavioural signs correlate well with clinical syndrome • simple to set up with low morbidity • high % develop neuropathic pain	• all diameters of primary afferents affected • simple to set up • variable % develop behavioural signs of pain • good for study of mirror image pain	• separation of injured and intact spinal segments • non-variable lesion • good for study of involvement of sympathetic nervous system
Disadvantages	• not possible to correlate pain-related behaviour with central changes • significant self-mutilation	• variable lesion, with significant inter operator variation. • mainly large diameter primary afferents affected	• variable lesion • behavioural response variable depending on exact site of ligation	• relatively invasive surgery to produce model, with higher morbidity than other models

1.2.5 Aetiology of neuropathic pain in the CCI model

The chronic constriction injury model of Bennett and Xie [33] results in development of pain behaviours that have much in common with the neuropathic pain syndromes that are seen clinically. It is unclear however, what specific aspects of the nerve injury underlie the development of the pain syndrome seen in the rat. Several causes have been postulated:

Physical causes

At least part of the underlying mechanism is due to physical constriction of the nerve, as use of variable diameter polyethylene cuffs instead of sutures to produce the injury resulted in neuropathic behaviour only with small diameter cuffs [295]. Physical compression of the nerve, may result in the extensive endoneurial oedema that is found, leading subsequent fibre damage with degeneration and pain-related behaviours [23,297]. Although at the time of ligature placement, the endoneurial blood flow does not appear to be disrupted [33], there is evidence of decreases in local blood flow at the nerve injury site at a time when thermal hyperalgesia is apparent [304]. Thus disturbances in the microvasculature around the nerve may also play a role in the changes that occur.

Chemical causes

The type of suture material itself, rather than the physical constriction of the nerve might be important. It has been proposed that a chemical constituent of the chromic gut is important, as a variety of suture materials laid alongside the nerve had different effects on behaviour. Thus, chromic gut, but not other materials, either loosely around the nerve, or laid alongside resulted in the development of guarding behaviour and thermal hyperalgesia [188,261], and changes in neuropeptide levels in the dorsal horn [473]. A constituent in chromic gut may alter the chemical milieu of the nerve in such a way as to result in the development of pain. Either chromic salts or pyrogallol in the sutures [66,261] may have effects on neuronal function or a toxic action. Chromic gut initiates an inflammatory response, that could be important in the development of pain behaviour. The presence of a pro-inflammatory material may initiate an inflammatory

response with release of cytokines and activation of primary afferent neurones [385,430]. Increasing the inflammatory response by addition of Freund's complete adjuvant next to the suture material resulted in increased pain-related behaviour , providing further evidence for a role for peri-axonal inflammation in the development of neuropathic pain [66]. An additional factor might be the generation of an acidic pH, possibly secondary to induced inflammation, in the area immediately around the ligatures. Capsaicin-sensitive fibres can be activated by reductions in pH [136,137], and these fibres may be involved in the development of pain-related behaviour [271].

Finally, after nerve injury, the normal metabolic processes of the neurone are altered. In particular, there is a decrease in retrograde transport of substances such as nerve growth factor, and other neurotrophins. The alteration of the supply of neurotrophins to the primary afferent cell bodies in the dorsal root ganglia may well have major consequences for neuronal phenotype and function [93,176].

It seems likely that the development of neuropathic pain secondary to the placement of loose ligatures around the sciatic nerve is a combination of physical and chemical factors.

1.2.6 Morphological changes associated with the CCI model

There are marked morphological changes in fibre composition of the sciatic nerve after a chronic constriction injury, that develop with time after ligature placement. In the original description of the model [33], marked constrictions were found beneath each of the four ligatures. The epineurial vasculature was obstructed and swelling was evident around the lesion, particularly on the proximal side. From 3 days after the initial injury, a dense mass of connective tissue surrounded the nerve and the ligatures, anchoring it to the surrounding muscle. After two to four months, the connective tissue had been resorbed, although the ligated region was still thinner than the rest of the nerve.

At 10 to 14 days myelinated fibres had degenerated extensively, with almost no large myelinated fibres being found distal to the lesion, although smaller myelinated and unmyelinated fibres could still be seen. The large myelinated fibre loss appeared to be more uniform than that seen with the smaller myelinated fibres, which were variably affected according to which fascicle was studied. At ultrastructural level, the few

large fibres which remained were in an advanced state of degeneration. The effect on unmyelinated fibres was varied, with evidence of significant reductions in total number distal to injury, when compared to proximal. 1cm proximal to the ligatures, the morphology appeared to be normal, with no sign of degenerating fibres, thus indicating that most of the damaged axons had survived centrally, with a partial and differentiating deafferentation caused by the constriction injury. There was significant variability in amount of degeneration seen between nerves from different animals, indicating the variable nature of the lesion. These changes occurred at a time when the behavioural evidence of neuropathic pain was at a maximum [23,297].

Other studies have confirmed damage to all fibre classes distal to the injury site, with a particularly marked reduction in large myelinated fibres. Massive Wallerian degeneration was seen distally, especially in the outer portion of the transverse sections. Proliferating Schwann cells surrounded small myelinated and unmyelinated fibres. Neuroma formation was also seen, with disruption of the perineurium, at the site of nerve injury [53,317].

1.2.7 Correlation of behavioural responses with morphology

It has been suggested that the major changes in composition of the sciatic nerve after loose ligature placement may be related to the development of behavioural evidence of neuropathic pain. Alterations in all fibre types are found, mainly distal to the injury site, and the time course of these changes has been studied in parallel with behavioural changes. There is some discrepancy between the behavioural results obtained by the various groups, although the morphological changes seem less variable.

Thus, Guilbaud's group [134,154] found both hot and cold allodynia/ hyperalgesia and mechanical allodynia were maximal at week 2, as was evidence of degeneration of the injured nerve, with massive Wallerian degeneration of large myelinated fibres, Between weeks 10 -12 behavioural signs of neuropathic pain had disappeared, but less than half the fibres had returned to normal. Other groups [53,69] have found a similar correlation between the onset of heat hyperalgesia and fibre loss. The subsequent progression of fibre loss and behavioural changes did not seem to be related, with the behavioural signs resolving at 28-35 days after surgery, at a time when there were still major fibre abnormalities. Using acetyl cholinesterase transport as a monitor of axonal

regeneration, no correlation was found between transport rate and neuropathic pain behaviour [128].

Although there does not seem to be a strong correlation with the time course of morphological changes in the injured nerve and the neuropathic pain syndrome, it is likely that there is a link. It is, of course, difficult to assess the relative numbers of myelinated and unmyelinated fibres in a nerve where both degeneration and regeneration are occurring simultaneously. Fibres classified as unmyelinated fibres may in fact be regenerating, but demyelinated A fibres. Furthermore, there are few studies of electrophysiological properties of these injured fibres which might help to establish their origin, and help to relate function to anatomy.

1.2.8 Neuropeptide changes

Primary afferent neurones respond to peripheral nerve injury by major alterations in neuropeptide production (reviewed by Hokfelt, 1994 [164]). There are corresponding changes in peripheral and central transport of these substances, and subsequent changes in the spinal cord. There were striking alterations in neuropeptide synthesis in the cell bodies of the dorsal root ganglia and in neuropeptide levels in the dorsal horn of the spinal cord, both after complete nerve transection [425,492] and after partial chronic constriction injury of the nerve [308].

The synthesis of several peptides, normally produced by primary afferent neurones, was markedly decreased. Thus, substance P (SP), somatostatin and calcitonin gene-related peptide (CGRP) all decreased. In contrast, galanin, neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), cholecystokinin (CCK), dynorphin and pituitary adenylate cyclase activating polypeptide (PACAP) increased. The effects of nerve injury on galanin and neuropeptide Y are discussed in some detail in chapters 4 and 5, but the changes in other peptides are discussed briefly as follows.

Substance P (SP)

SP was found in about 30% of cells in the rat dorsal root ganglia, mainly of small or medium diameter. Transection of the sciatic nerve resulted in a decrease in SP mRNA in the dorsal root ganglia, being most pronounced at 10 to 14 days after the injury, and accompanied by a decrease in SP fibres in the spinal cord [183,316]. SP levels

decreased in the superficial dorsal horn of the spinal cord [20], and in some studies this decrease occurred bilaterally, peaking at day 14, and accompanied by decreased transport of SP from the dorsal root ganglia [425,426]. A similar marked decrease in SP production by primary afferent neurones was seen in the CCI model [308], with an ipsilateral decrease in dorsal horn levels after 7-28 days [51], becoming bilateral by 60 days [191,473]. Functional studies of SP indicate a mainly excitatory role in both rats and primates [98,474], with some evidence that it potentiated the effect of excitatory amino acids [286,307,383]. SP was released in the spinal cord in response to painful peripheral stimuli, and it has been postulated to play a role on the transmission of nociceptive information [108,112,456]. The major decrease in SP levels after nerve injury may reflect that it plays a lesser role in nociceptive processing in these states, although after axotomy, tachykinin antagonists still had an inhibitory effect on the nociceptive flexor reflex [250].

Somatostatin

Somatostatin was found in about 20% of rat dorsal root ganglia cells, of small to medium size [196,425], but its production by primary afferent neurones in dorsal root ganglia was markedly decreased after axotomy in the rat. A decrease from relatively high levels in the monkey dorsal root ganglia was also seen after axotomy [370,495]. The functional effects of somatostatin were mixed, with evidence of excitation in some circumstances [474]. However, it may also have an inhibitory role [159,346] in nociceptive pathways, as a longer acting analogue has had some limited success in reducing morphine requirements in cancer patients [288,335].

Calcitonin gene-related peptide (CGRP)

In the normal animal, CGRP was one of the most abundant neuropeptides in cells of the dorsal root ganglia, [186,425,426]. It co-existed with SP, somatostatin and galanin. In the dorsal horn it was found in large dense core vesicles (LDCVs) of primary afferent terminals, in laminae I and II, decreasing after axotomy, when it became localised mainly to small "dot-like" structures [493]. There are variable reports of the effect of nerve injury, with some evidence that axotomy had little or no effect on CGRP levels in the dorsal root ganglia, and a minor decrease in CGRP containing fibres in the

superficial dorsal horn [425]. There was however a significant decrease in CGRP-containing LDCVs in the dorsal root ganglia, as well as evidence for a marked decrease in its production by dorsal root ganglia cells after axotomy in the monkey [493,495]. Additionally, in the CCI model, the expression of CGRP mRNA in the dorsal root ganglia fell to approximately half of its normally abundant levels [308]. A more recent study using spinal nerve ligation, found an overall decrease in CGRP mRNA levels in the dorsal root ganglia, but a sub-population of medium to large sized neurones showed an increase. In the dorsal horn, little change was found in the superficial laminae, but an increase in CGRP-ir was found in deeper laminae [280]. There is considerable evidence that CGRP acts as an excitatory transmitter involved with nociception both in normal animals [79,359] and in inflammatory states [312]. In the CCI model, although a CGRP antagonist still increased mechanical and thermal thresholds, it was much less marked than that seen with uninjured rats [487].

Vasoactive intestinal peptide (VIP)

Very low levels of VIP were found in primary afferent neurones, with some VIP being found in intrinsic neurones of the spinal cord [130]. An ipsilateral increase in VIP levels in dorsal root ganglia was detectable after axotomy or partial nerve injury, mainly in small to medium sized neurones, often co-localised with galanin [308,493]. There was a corresponding increase in VIP-containing fibres in the central area of the superficial dorsal horn of, mainly in large dense core vesicles in laminae I and II [370,425,492]. VIP had a predominantly excitatory role, and its increased production by primary afferent neurones after nerve injury has led to the suggestion that it may take over the role of SP in the nerve injury state [456,457].

Cholecystokinin (CCK)

CCK has been found in a few dorsal root ganglia cells, of mainly small to medium sized in uninjured rats [186,425]. In the spinal cord, most of the CCK present was thought to arise from local dorsal horn neurones, or descending tracts [497]. Initial studies gave very variable results of the effect of axotomy on CCK levels, often showing a decrease. This was subsequently found to be due to technical problems with the antibody used and better controlled studies have found an increase in CCK mRNA in

primary afferent neurones after peripheral nerve injury [475]. Functionally, CCK inhibited the action of morphine, possibly explaining the reduced efficacy of opioids in neuropathic pain states [13,314,475]. CCK also facilitated the nociceptive flexor reflex, whereas CCK-B antagonists inhibit this reflex [457].

Dynorphin

Dynorphin, a kappa opioid agonist, was found in the dorsal horn of the rat, particularly in laminae I, II and V of normal rats [283]. In the CCI model, there was an ipsilateral increase in dynorphin, peaking at day 10. This increase was seen in laminae I-II initially, then at later time periods an increase was seen predominantly in deeper laminae VI-VII [189]. In another model of unilateral partial nerve injury, sciatic cryoneurolysis, a bilateral increase in dynorphin levels was found in laminae II-III of the dorsal horn, the magnitude of this increase correlating with the severity of autotomy [429]. The functional role of dynorphin after nerve injury is unclear [174], but it may inhibit any analgesic actions of morphine [315], and this may be mediated via an interaction with a non-opioid receptor, rather than the kappa receptor [428]. There is also some evidence that dynorphin may have an inhibitory role, both in inflammation [393] and after nerve injury [422].

Pituitary adenylate cyclase activating polypeptide (PACAP)

PACAP, a peptide with a structure similar to VIP, has been found in abundance in nerve fibres in the superficial dorsal horn of the rat, with some extension into deeper layers. Further areas of PACAP were present in the lateral funiculus, and into the interomediolateral cell column, but there was no PACAP-immunoreactivity in the ventral horn. Many dorsal root ganglia neurones also contained PACAP, with the densest staining being in small sized cells [113,296]. After sciatic nerve transection, an ipsilateral increase in PACAP mRNA levels were found in dorsal root ganglia, with an increased population of neurones now expressing PACAP. However, PACAP-containing fibres in the superficial dorsal horn may either increase [489] or decrease [499]. Increases in PACAP mRNA were found from as early as 15 hours after axotomy, peaking at day 3 and declining after day 10. These changes occurred earlier than those seen with other neuropeptides after nerve injury [489]. PACAP may have an excitatory

effect on transmission of primary afferent information, as it has been shown that topical application of PACAP increased cell firing in the dorsal horn [92].

1.2.9 Degenerative changes in the dorsal horn

Normally, the central terminals of primary afferent neurones distribute to distinct and specific laminae in the spinal dorsal horn [49,461]. However, after peripheral nerve injury, primary afferent fibres are damaged. There is evidence for effects on the central organisation of primary afferent terminations [240]. Retrograde transport of the C fibre specific lectin BSI-B4, from the injury site, was impaired, as from 4 days after sciatic axotomy, it was only transported as far as the cell bodies in the dorsal root ganglia, and not into the normal site of termination in lamina II [206].

There may also be transsynaptic damage to local and descending systems in the spinal cord [306], with major changes in the dorsal horn somatotopic map [400]. The initial injury barrage from the injured nerve may be excitotoxic, damaging cells in the spinal cord. In the CCI model, there was an increased incidence of hyperchromatic and pyknotic cells, likely to be degenerating neurones, found bilaterally from laminae I-IV, especially in medial laminae I-II. Transsynaptic degeneration after chronic constriction injury was increased in the superficial dorsal horn both by strychnine and bicuculline (glycine and gamma amino butyric acid (GABA) receptor antagonists respectively) [398,399]. The appearance of these cells led to the suggestion that inhibitory cells were preferentially damaged in the spinal cord. There may also be a link between the damage caused by the initial injury discharge and the induction of neuropathic pain., as preventing the injury discharge decreased pain behaviour and augmenting it increased neuropathic pain after partial nerve ligation [365] or in the CCI model [484]. The time course of behavioural changes in the CCI model also correlated with the time course of reductions in GABA levels in the superficial dorsal horn [175].

Descending and ascending influences to and from the brainstem to the spinal cord were also affected. There was an increase in serotonin and noradrenaline on both sides of the dorsal horn 7 days after a unilateral chronic constriction injury, thought to originate from descending control systems [360]. The responses of neurones in the spinothalamic tract to mechanical and thermal stimulation were altered in primates with either a chronic constriction injury of one sciatic nerve [333], or tight spinal nerve

ligation [332]. There was much higher background activity, with increased after-discharge in response to stimulation. Alterations in the number of inhibitory neurones, the effects of descending and ascending systems and the pattern of neurotransmitter co-existence may lead to impaired inhibitory function and central hyperexcitability after peripheral nerve injury.

1.2.10 Regenerative changes in the dorsal horn

As well as degenerative changes occurring in the dorsal horn after nerve injury, there are also regenerative changes occurring in parallel [50]. There is much new work on central axonal regeneration, some of which has been reviewed by Brosamle [48].The effects of these changes on nociceptive transmission and their role in the development of neuropathic pain is not yet understood.

Coggeshall has reviewed the evidence for a central regenerative response to peripheral nerve injury in 1994 [68]. The different types of sprouting that can occur are outlined:

1. Collateral sprouting is when central processes of undamaged or unconditioned dorsal root cells sprout into a neighbouring denervated area of cord [96,376].
2. Reactive collateral sprouting is when the central processes of conditioned dorsal root ganglion cells sprout into a neighbouring denervated region [462].
3. Reactive sprouting is when central processes of conditioned dorsal root ganglion cells sprout into different laminae in the cord. This type of sprouting is confined to the somatotopic boundaries of the injured nerve [376].

It was noted by Woolf and co-workers that after axotomy, there was an increase in production of a membrane phosphoprotein, Growth-Associated Protein GAP-43, by primary afferent neurones in the dorsal root ganglia [467]. GAP-43 is associated with neuronal development and is presumed to influence synapse formation and neurite elongation [35,63]. In transgenic mice overexpressing GAP-43 new synapses form spontaneously and there is significantly increased sprouting after injury. GAP-43 seems to be important in transducing intra- and extra-cellular signals to regulate organisation of the cytoskeleton (reviewed by Benowitz and Routtenberg,1997 [35]). GAP-43 appeared 3 days after axotomy in primary afferent cell bodies and 4 days after axotomy in the spinal cord, reaching a maximum at 14-21 days, in the substantia

gelatinosa ipsilateral to injury. The majority of labelled structures were unmyelinated axons, although a few myelinated elements were found, and in most cases GAP-43 seemed to be associated with growth cone-like structures. No labelling was found if the dorsal roots had been cut, indicating that the GAP-43 is likely to be of primary afferent origin [71]. Further work has confirmed the primary afferent origin of GAP-43 in the superficial dorsal horn, where it seems to be found mainly in unmyelinated fibres, even those showing signs of degeneration. Virtually none is found in A fibres sprouting into the superficial dorsal horn [97].

Further work on regeneration the dorsal horn, found evidence of extension of the site of termination of A-beta fibres into lamina II, an area where small fibres normally terminate [468]. Using Choleragenoid beta- horse radish peroxidase (CB-HRP) as a marker for large myelinated fibres, injected at the nerve injury site, detectable levels were found in lamina II by one week, reaching a maximum at 2 weeks after axotomy. The labelling in the dorsal horn was restricted mainly to axons and terminals of primary afferent fibres, with none in neuronal or glial cell bodies. The structure of the labelled terminals was compatible with A fibres, and similar throughout laminae I-IV, with a much higher proportion of undeveloped synapses labelled after nerve injury than in naive rats. GAP-43 appeared in almost the same area of the dorsal horn as CB-HRP after axotomy. This was seen after both nerve transection and nerve crush [469]. Thus, in approximately the same area of the superficial dorsal horn, there were increases in GAP-43 from unmyelinated fibres and new terminations from myelinated fibres sprouting from the deep dorsal horn to this more superficial area. The functional significance is not clear, but if these anatomical changes are relevant to the neuropathic syndrome, then there should be some correlation between behavioural changes and anatomical changes.

After a chronic constriction injury, when signs of thermal hyperalgesia were present, there was a substantial increase in GAP-43, in ipsilateral laminae I and II of the dorsal horn, similar to that seen after axotomy. Dorsal rhizotomy markedly reduced the GAP-43 response seen, indicating that it was mainly of primary afferent origin. [50,51]. Evidence of A fibre sprouting was also found in rats subjected to tight ligation of L5/6 spinal nerve roots, associated with a decrease in cell numbers in dorsal root ganglia

cells [232,233]. After sciatic nerve transection or crush, there was no apparent decrease in myelinated fibres in the dorsal roots, with these becoming the predominant type after nerve injury as unmyelinated axons were decreased by about 50% [70].

1.2.11 Stimulus for central regeneration

The nature of the signals controlling neuronal regeneration and GAP-43 expression are not clear. A role for a peripherally derived control factor seems likely as GAP-43 mRNA levels increased in the lumbar dorsal root ganglia after peripheral axotomy but not central dorsal root section [63]. Injury-induced C fibre degeneration may stimulate A fibre sprouting, as capsaicin treatment results in the same sprouting response into lamina II, as that seen after axotomy [255]. There are at least two possible signals underlying regeneration:

1. A peripherally-derived signal produced after nerve injury that is transported centrally after nerve injury may act as a stimulus to induce reorganisation in the dorsal horn [344].
2. Peripheral nerve injury may decrease or stop central transport of a peripherally derived growth factors necessary for maintaining normal neuronal phenotype of primary afferents and/or dorsal horn neurones.

Transgenic mice, over-expressing NGF, were found to have several ectopic sites of SP-ir and CGRP-ir in the white matter of the spinal cord, that were abolished by capsaicin treatment of adult mice [253]. Further evidence for a role of NGF is provided by a study delivering intrathecal neurotrophins to rats that had undergone sciatic axotomy. In the control group, evidence of A fibre sprouting and decreased CGRP-ir was found 2 weeks after axotomy. However, in those animals that had intrathecal NGF infusions, these changes were virtually abolished. Neither BDNF nor NT-3 were effective in preventing the changes [29]. NGF does not appear to be needed for the early expression of GAP-43, which is likely to be under the control of some other neurotrophin. In an *in vitro* culture model, using adult dorsal root ganglia cells, NGF did not regulate the neurite outgrowth that was found, especially after axotomy [116,173].

1.2.12 The sympathetic nervous system and behaviour

There is evidence for a link between the sympathetic nervous system and pain-related behaviour after nerve injury. Interventions to diminish sympathetic input alleviate some of the signs of neuropathic pain seen after a chronic constriction injury. Thus, either a chemical sympathectomy, with guanethidine [311,336], or a surgical sympathectomy [86], before or after the nerve injury, have been reported to reduce the subsequent hypersensitivity to thermal stimuli with inconsistent effects on mechanical hypersensitivity. Additionally, pre-treatment with an alpha-2 agonist, clonidine, delayed the development of thermal hyperalgesia, although the severity eventually reached was not altered [483].

Similar effects on behaviour were found in the partial nerve ligation model, after guanethidine, as there was a delay in development of thermal hyperalgesia and no effect on mechanical allodynia, although if given several months after the injury, both mechanical and thermal signs were alleviated [375]. In the spinal nerve ligation model, there is evidence for a major involvement of the sympathetic nervous system [200], since sympathectomy at the L5 level completely abolished the abnormal sensory signs, as well as sympathetic sprouting seen after nerve injury (see section 1.2.13) [65,203]. However, lesioning preganglionic fibres had no effect on the hypersensitivity seen, indicating that the postganglionic fibre contribution is independent of sympathetic outflow, and is likely to involve sensory-sympathetic interaction within the dorsal root ganglia [205].

Of the animal models studied, that of Bennett and Xie probably demonstrates the least sympathetic involvement [200]. The reason for this difference is not understood, but the distance of the nerve injury site from the dorsal root ganglia may be an important factor in the degree of sympathetic sprouting occurring after nerve injury [202].

After a chronic constriction injury of the sciatic nerve there were alterations in hindpaw temperature, with a significant difference in paw temperature appearing between the injured and uninjured paw. The pattern of this change developed over time, progressing from abnormally hot to abnormally cold over several weeks. There was a high degree of variability in temperature changes within animals, with significantly increased asymmetry and lability after nerve injury [220,436]. Surgical

sympathectomy significantly suppressed the temperature abnormalities seen in the first week, but subsequently had no effect [437].

1.2.13 Interaction between sympathetic and sensory nervous systems

Anatomical changes

As well as behavioural evidence of a link between the sympathetic and sensory systems, there is also anatomical evidence [65,264,343]. McLachlan et al, 1993 [264] found that about two weeks after nerve transection, sympathetic efferents sprouted into dorsal root ganglia. Normally noradrenergic axons form varicose plexuses around blood vessels in the dorsal root ganglia, near the perimeter, but not penetrating into the dorsal root ganglia. However, post-axotomy, noradrenergic fibres penetrated into the dorsal root ganglia, forming basket-like structures around large diameter primary afferent neurones. This sprouting occurred to a much lesser extent contralaterally. The sprouting fibres appeared to be derived from the perivascular plexuses, as the sympathetic fibres within the lesioned nerve itself degenerated after transection. Repetitive stimulation of the sympathetic outflow resulted in firing of myelinated primary afferent neurones, that was blocked by phentolamine, indicating the potential for functional coupling of these noradrenergic neurones with primary afferent fibres in the dorsal root ganglia.

Sprouting of sympathetic fibres after partial nerve injury occurred earlier than after axotomy [64,65,343], with sympathetic sprouting occurring at a time when neuropathic pain behaviours were developing. A possible reason for the earlier sprouting after partial, as opposed to complete, nerve injury is that the products of Wallerian degeneration may contain a stimulus for sympathetic sprouting. In a strain of mice with delayed Wallerian degeneration after nerve injury, both the behavioural signs of neuropathic pain [303] and the degree of sympathetic sprouting were delayed compared to normal mice [344].

Both NGF and LIF are produced after axotomy by non-neuronal cells [401], with evidence for neuronal retrograde transport of these compounds [412,420]. They may be involved in the induction of sympathetic sprouting after nerve injury, as transgenic

mice overexpressing NGF in the skin, showed novel sympathetic sprouting in trigeminal ganglia [80].

Functional changes

Electrophysiological studies have found evidence for changes in primary afferent cell firing in response to adrenaline or noradrenaline depending on fibre type. There was a marked increase in responsiveness of polymodal C fibres to sympathetic stimulation or injection of noradrenaline, after partial nerve transection, although the A-delta fibres did not show any change [44,358]. It is possible that the alterations in sympathetic innervation in the dorsal root ganglia after nerve injury may result in the generation of spontaneous activity in the injured nerve [230], occurring both at the nerve injury site [89] and the dorsal root ganglia [90]. In anaesthetised rats, after sciatic nerve transection, primary afferent fibres displayed spontaneous firing [275,441], partly originating from the dorsal root ganglia. Sympathetic stimulation altered the firing rate of the majority of neurones [276]. In the CCI model, topically applied noradrenaline increased the spontaneous activity of most C fibres and A delta fibres, but had little effect on the rate of discharge of A beta fibres [472].

Dorsal root ganglia in culture, devoid of vascular supply and sympathetic innervation still showed increased sensitivity to noradrenaline and evidence of spontaneous activity if there had been a prior nerve injury (CCI), unlike the dorsal root ganglia taken from preparations without a chronic nerve injury [488].

The changes seen after nerve injury may be partly due to receptor changes. In normal rats, noradrenaline effects were mediated by beta-adrenoceptors in the dorsal root ganglia, whereas axotomized rats showed an alpha-2 adrenoceptor mediated effect [1]. After spinal nerve ligation, there were alterations in the sub-types of alpha-2 adrenoceptors from that normally found [144] in dorsal root ganglia cells, that might be related to functional changes [62].

The involvement of the sympathetic nervous system in the development and maintenance of neuropathic pain is variable, both in animal models and clinically [40,219], which may explain the variable effects of sympathetic manipulations on the development and treatment of neuropathic pain.

1.3 Galanin

1.3.1 Synthesis and structure

Galanin is a C-terminally amidated 29 amino acid peptide first isolated in 1983 [410]. It was isolated from porcine intestine and has the following sequence: Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys-Tyr-Gly-Leu-Ala-NH2. It was called galanin based on its N terminal and C terminal amino acids (**G**lycine & **Alanin**e). Although the C terminal tetrapeptide is structurally similar to that of SP (Phe-Gly-Leu-Met) [410], it is unlikely that there is any overlap on receptor activation (see section 1.3.4). Galanin is found in a wide range of species, with strict conservation of its primary sequence, especially the first 15 residues [198].

Galanin was found to exist in multiple conformational states, consistent with the extended and folded backbone conformations of 2 alpha-helical regions separated by a flexible hinge, at Pro13 [216].

It is produced from a 123 amino acid preprohormone, preprogalanin, that includes a 60 amino acid long flanking peptide called Galanin Messenger Associated Peptide (GMAP), a signal peptide and galanin itself [26,27]. The importance of peripheral neurotrophic factors in the regulation of neuronal galanin synthesis is unknown although there is some evidence that the transport of substances from the periphery may be important. Nerve growth factor may regulate some of the peptides, but many of the neurones containing galanin do not have nerve growth factor receptors [196,426].

1.3.2 Metabolism

In vitro, galanin(1-29) has a half life of between 60 and 120 min, depending on the tissue studied. Thus with a hippocampal membrane preparation, it had a $t_{1/2}$ of 100 minutes [224], whereas with a spinal cord preparation, it had a $t_{1/2}$ of 60 min [26]. Galanin appeared to be a relatively stable peptide that is predominantly degraded by endopeptidases, that were partially inhibited by bacitracin 1%, and may include a phosphoramidon-sensitive zinc metalloprotease [26,224]. None of the galanin metabolites

appear to have any affinity at the galanin receptor. Although galanin binding sites do seem to be concentrated in areas where galanin is distributed (see section 1.3.5), there is a possibility of paracrine effects at areas distant from its site of release, because of its relatively long half life. There have been several N terminally truncated forms of the peptide, gal(7-29) and gal(9-29), isolated from the adrenal medulla that appear to have low agonist activity at the spinal cord galanin receptors [28]. They have a longer $t_{1/2}$ than galanin itself, and the lower affinity at the receptor would be predicted from the fact that the N terminus sequence of amino acids is the major determinant of receptor-ligand interaction (see section 1.3.3).

1.3.3 Galanin receptors

Pharmacological studies of binding sites

Early pharmacological studies of galanin binding in the lumbar spinal cord found a single population of high affinity binding sites. The N-terminal amino acids (1-15) were very well conserved between species, and these peptides were sufficient and necessary for receptor binding and agonist activity [22]. Gal(1-12) is the shortest fragment with agonist activity, and includes the four most important pharmacophores - Gly1; Trp2; Asn5; Tyr9 needed for receptor interaction [27]. Binding studies, using the newer chimeric peptide antagonists, have found high and low affinity binding sites in the cord - this heterogeneous binding may indicate the presence of more than one galanin receptor site or it may reflect pharmacokinetic variables between the peptides [479].

In the adult rat spinal cord, galanin binding sites showed dense labelling in laminae I-III, moderate labelling in laminae IV-V and around the central canal, with low to moderate levels in the ventral horn and the white matter. The distribution of galanin binding sites correlated well with the distribution of galanin-containing fibres in the normal rat. Galanin binding sites were increased bilaterally by neonatal capsaicin treatment and unilaterally by dorsal rhizotomy [192,193]. These specific galanin binding sites may be post-synaptic to primary afferents, although there is some evidence for presynaptic binding sites, as galanin had a greater inhibitory action on the plasma extravasation induced by C fibre stimulation than on the response to exogenously

applied SP. This may be due to actions at a presynaptic site on the stimulated fibres, or the greater effect on C fibre response may be due to the release of substances other than SP [477].

Galanin binding sites changed during development. In early postnatal stages, there was a high density of galanin binding in the superficial dorsal horn, with some labelling in the deeper dorsal horn and very low levels around the central canal and the ventral horn. Three to four weeks postnatally, there was a generalised decrease in galanin binding throughout the cord. The regional alterations in galanin binding during development may indicate a role for peptides in neuronal growth and organisation of the spinal cord [193].

Molecular studies of galanin receptors

In 1995, the first galanin receptor, GalR1, was cloned, showing high sequence similarity to somatostatin (~30%) and delta opioid receptors [334]. It was subsequently found to be widely distributed in the central nervous system, particularly in the superficial dorsal horn [244]. Using in situ hybridisation GalR1 mRNA was found to be present in 23% of lumbar dorsal root ganglia neurones, the majority of which also expressed CGRP mRNA, and were of small to medium size. Although galanin receptor mRNA was found in dorsal root ganglia cells, the actual expression of the receptor protein may be much less, as radiolabelled galanin showed very low levels of binding in the dorsal root ganglia [481].

The GalR1 belongs to the G-protein coupled family, with seven transmembrane domains and appears to inhibit forskolin-stimulated cAMP production [114].

The domains on the human GalR1, from a Bowes melanoma cell line, were studied to determine the amino acids required for galanin binding and receptor activation. At least three ligand-receptor interactions were needed - Trp2-His264, Tyr9-Phe282 and N terminus of galanin bound to Glu271 for receptor activation [198].

Until recently, although pharmacological studies had indicated more than one galanin receptor sub-type, only GalR1 had been cloned. However, a second galanin receptor, GalR2, was cloned from rat brain [125,443]. It contained seven transmembrane domains, sharing only 40% sequence similarity with GalR1, with the greatest difference being at the amino and carboxyl termini. There was some conservation of G-protein coupled

receptor features. The major pharmacological difference detected between GalR1 and GalR2 was that the galanin fragment Gal (2-29) had a much higher affinity for GalR2. Activation of GalR2, like GalR1, led to inhibition of forskolin-stimulated cAMP production. GalR2 had a much wider distribution outside the CNS than GalR1, with strong signals being detected in the kidney, testis, skeletal muscle and liver. GalR2 was 26 amino acids longer than GalR1, at the amino terminus. It showed a 10 fold lower affinity for human galanin, although the potency order for the chimeric peptides was the same: M35>M40>C7 [169]. Both GalR1 and GalR2 require the 2 amino terminal residues for high affinity binding.

Further study of galanin receptor distribution found GalR2 mRNA in almost 25 % of normal rat dorsal root ganglia cells, of mainly small size [372]. GalR2 mRNA was often co-localised with CGRP mRNA, and 20% of the GalR2-containing cells also contained GalR1 mRNA. Axotomy markedly decreased expression of GalR2 mRNA in dorsal root ganglia cells [372].

A third galanin receptor, GalR3 has now been cloned [444]. It showed approximately 50% sequence similarity with Gal R1 and 2, but differs from these two receptors in that its distribution is more limited, with none being found in the brain.

All the galanin receptors cloned to date appear to belong to the G-protein superfamily of receptors. A pertussis toxin-sensitive G protein coupled galanin receptor [83] reduces intracellular calcium, either by inhibiting adenylate cyclase, opening K^+ channels or closing N type calcium channels. No effect of galanin was found on basal levels of cAMP, cGMP or inositol monophosphate, but K^+-stimulated cGMP production was inhibited by galanin. This inhibition could either be a direct effect by inhibition of guanylate cyclase, or could be mediated via closure of voltage sensitive calcium channels [22].

1.3.4 Antagonists

There are some high affinity chimeric peptide antagonists for the spinal cord galanin receptor, as reviewed by Bartfai, 1992 [22]. These recognise 2 receptors in which the galanin receptor recognition site is in the N-terminal of galanin and the C-terminal binds to other receptors, such as substance P. The conformation is stabilised by an α-helical structure at the C-terminus. These antagonists include: galantide(M-15), which

is gal(1-15) pro-SP(5-11) amide; the more potent spantide derivative (gal(1-15)pro-spantide), and M-35 (gal(1-12) pro-bradykinin2-9 amide). There are several problems with these peptide antagonists, in that they are not very stable, and that there is a possibility of binding both to the galanin receptor and to the other peptide receptor from which the chimera was produced. More recently high affinity chimeric peptide antagonists have been developed that are oxidatively stable. There are several of these available: C7, M32, M38 and M40. The last two have nonsense C-terminals, thus avoiding problems with bi-receptor activation [479].

1.3.5 Distribution of galanin

Galanin is widely distributed in the central nervous system, and is found in both the brain and spinal cord. There does appear to be some variation between species, therefore care must be taken when making comparisons [56,115,214,357].

Brain

In the rat, galanin has been found mainly in the posterior pituitary and hypothalamus, with undetectable levels in the frontal, parietal and occipital cortices and the cerebellum. The area with the highest levels of galanin in cell bodies was the hypothalamus, with fibres spreading to the periventricular nucleus [56]. A high proportion of locus coeruleus neurones containing galanin (with many also containing tyrosine hydroxylase) had fibres projecting to the hypothalamus, cortex and spinal cord. In addition, some of these fibres contained NPY, again often co-localised with tyrosine hydroxylase positive neurones in the locus coeruleus [166]. In primate brain, galanin was even more widespread. It was found in the hippocampus, cortex, hypothalamus, thalamus, pituitary, in all the circumventricular organs, the periaqueductal grey and the locus coeruleus. In humans, galanin was found in cell bodies in the supraoptic nucleus and the locus coeruleus, whereas in monkeys, galanin was found only in fibres in these areas [214].

Spinal cord and primary afferent neurones

In the rat spinal cord, there was a rostrocaudal increase in galanin with the highest levels being found in the lumbar and sacral spinal cord. Galanin was found both in cell bodies and fibres, being most abundant in laminae I - II, X (around central canal), Lissauer's tract and the dorsolateral funiculus [452,497]. Galanin is found in intrinsic neurones of the spinal cord, as well as in descending systems [269]. Galanin was found in islet cells in the dorsal horn that are the most common type of interneurone in lamina II, and have rostrocaudally elongated axons. These interneurones received input directly from primary afferents, as well as making frequent contact with other interneurones, most of which did not contain galanin [497]. Galanin levels were decreased by neonatal capsaicin treatment, particularly in laminae I-II, suggesting some input from small primary afferent fibres [381]. Dorsal rhizotomy also decreased levels in the superficial dorsal horn, and allowed clearer visualisation of galanin-containing cell bodies in this area. Thoracic cord transection resulted in a small increase in galanin levels in the lumbar and sacral areas of the spinal cord. This could either be due to accumulation of galanin normally transported in ascending systems, or it could be an effect due to loss of the influence of descending systems [56].

There is some disagreement about the number of primary afferent fibres containing galanin in the normal rat, as some studies have found insignificant levels in the dorsal root ganglia cells [161,163,425] whereas others find galanin at higher levels in dorsal root ganglia [186]. High levels have been detected in small axons in dorsal roots, even higher than CGRP, with up to 28% of small, unmyelinated and fine myelinated axons at the L5/S1 dorsal root level containing galanin [208]. Low levels of galanin in primary afferent fibres were also found in cats, in small to medium-sized cells in the dorsal root ganglia, most of which also contained CGRP [11]. Galanin expression in adult rat sensory neurones was also low [480], and the central release of galanin was not affected by stimulation of primary afferent neurones [168,292]. Thus, on balance, it would seem that galanin is not produced by primary afferent neurones to any significant extent in the normal state.

In the primate spinal cord [214], dense collections of galanin have been found in the spinal descending tract of the trigeminal nucleus, continuing into the substantia gelatinosa, as well as in Lissauer's tract, the nucleus of the tractus solitarius, the

dorsal motor nucleus of the vagus nerve and anterior horn cells in the cord. There may, therefore, be an important functional role for galanin in the primate sensory system, and the similarity of distribution to that found in the rat would indicate that the rat is a suitable model for study of galanin.

Colocalisaton of galanin with other neurotransmitters

Galanin has been found to co-exist with several other neurotransmitters in the spinal cord, including peptides. In the dorsal horn, galanin ir fibres also contained substance P and CGRP in LDCVs probably of primary afferent terminals in laminae I-II. Galanin-immunoreactivity (ir) was also found occasionally with CCK or NPY [496], and in local dorsal horn neurones in LDCVs, coexisting mainly with enkephalin, but also NPY (lamina II) and SP (deeper dorsal horn) [11,497]. Galanin has been shown to coexist extensively with GABA, in laminae I-II of the dorsal horn, but rarely with glycine. The predominant cells in the superficial dorsal horn in which galanin and GABA coexisted were islet cells, though to be inhibitory interneurones with long rostrocaudal dendrites [379]. In the ventral part of the motor nucleus, galanin co-existed with 5-hydoxytryptamine (5-HT), probably as part of the descending bulbospinal system, as the galanin-ir fibres disappeared with spinal transection [382]. Although only a small number of galanin-containing neurones were found in the lumbar dorsal root ganglia, most of those neurones also contained CGRP, and often substance P [186]. In the normal state therefore, galanin coexists with both inhibitory and excitatory transmitters. The functional significance of this requires further study, but any intervention that alters the pattern of galanin co-existence with other neurotransmitters may have major functional effects, due to alterations in the balance of substances released from neurones.

Effects of peripheral nerve injury on galanin distribution in the spinal cord

Galanin is normally found in a few small diameter dorsal root ganglia cells, but within 24 hours after sciatic nerve transection, there was an increase in galanin mRNA and galanin-ir in small, medium and a few large sized cells in L4/5 dorsal root ganglia, with a corresponding increase in galanin-ir in the ipsilateral superficial dorsal horn [163,452]. Thus peripheral nerve injury stimulated galanin production by many sensory

neurones, mainly of small to medium size. There are differences between species, with a less marked, but bilateral increase in ir-galanin in the dorsal root ganglia and spinal cord of the guinea pig than is seen in the rat after axotomy [357]. In primates, galanin mRNA in the dorsal root ganglia and galanin in the superficial dorsal horn changed in a similar pattern to that seen in the rat after peripheral sciatic nerve transection, with ipsilateral increases in galanin in the dorsal root ganglia and superficial dorsal horn [495]. Different types of peripheral nerve injury, including the CCI model [298], all resulted in an increase in galanin-ir in rats, but a recent study has shown that partial sciatic nerve injury resulted in a greater increase in galanin-ir, than complete sciatic nerve transection. There was a greater increase in galanin-ir in medium and large cells in dorsal root ganglia, as well as small cells after partial nerve transection, with both injured and uninjured neurones being affected. After partial nerve injury, galanin-ir was increased more in the superficial dorsal horn, with galanin-ir fibres running into deeper levels of the dorsal horn than seen after axotomy [252]. The response of sympathetic neurones to nerve injury was also to increase galanin levels [211], but this seems to be localised to cell bodies of uninjured axons [369]. There is some evidence that the increase in galanin found in sympathetic neurones is mediated by LIF derived from non-neuronal cells [210].

The factors that result in changes in peptide synthesis and distribution after nerve injury are unclear. There may be a role for some of the neurotrophins in regulating peptide expression in adult rats after nerve injury. Intrathecal infusion of NGF prevents the decrease in CGRP and SP found after axotomy, but not that of somatostatin. The up-regulation of galanin, VIP, NPY, and CCK was attenuated by about 50% in the NGF-treated rats [424]. However, when Kashiba and co-workers [195] tried to determine the effects of retrogradely transported NGF on the regulation of galanin expression in the dorsal root ganglion, they found a lack of NGF receptors on dorsal root ganglia cells containing galanin, as measured by anti-NGF receptor antibody binding. This led them to conclude that loss of NGF was not a significant factor in the up-regulation of galanin seen after nerve injury, and that other peripherally-derived neurotrophic factors may be important.

Factors other than changes in neurotrophin supply may include the central effects of peripheral cell death on neuropeptide expression. In one study, where the primary afferent cell death was prevented, by means of impermeable silicone tubes, there were still alterations in peptide levels in the dorsal horn, with decreases in CGRP, SP and CCK and an increase in VIP, as is normally seen after peripheral nerve injury. The effect on galanin-ir was however more complex, with an apparent decrease in galanin-ir in the medial dorsal horn, but a more widespread distribution to the lateral superficial dorsal horn [207].

The pattern of peptide co-existence changes after nerve injury, which may have major functional implications. In the dorsal horn of primates, there were changes in levels and coexistence of galanin and CGRP after L7 spinal nerve ligation, with an increase in galanin and a decrease in CGRP in the ipsilateral dorsal horn. However, neither the number of galanin-ir synapses nor the number or diameters of the LDCVs in which galanin was stored in the primary afferent synapses had actually increased. Instead, the increase in galanin-ir was found to be due to a significant increase in galanin-ir positive glial cell bodies. A significant decrease in CGRP-containing synapses was found, resulting in less co-localisation of CGRP with galanin [52].

After sciatic nerve transection, large type dorsal root ganglia neurones, that normally have almost undetectable staining for NPY and galanin showed coexistence of both these peptides, and also some weak staining for CGRP [194]. There was also a change in co-localisation with the immediate-early gene, c-jun, as prior to nerve injury there was no detectable co-localisation, whereas 48 hours after peripheral sciatic nerve transection, about 35% of dorsal root ganglia cells displayed galanin-ir and c-jun-ir [161]. There may be a link between these events, or the changes in c-jun levels may be quite non-specific.

Although earlier studies found no effect on galanin binding in the spinal cord after sciatic transection [27,452], more recently, galanin binding sites were found to be altered by peripheral nerve injury, with a marked decrease in the ipsilateral superficial dorsal horn. [192]. Both carrageenan induced inflammation and axotomy resulted in a down-regulation of GAL-R1 mRNA in the dorsal root ganglia, that was most marked with axotomy [481].

The results of the studies of galanin distribution in the spinal cord and dorsal root ganglia are summarised in Table 3. There is some minor variation between studies on the distribution of galanin in the dorsal horn, and more marked disagreement about levels in the dorsal root ganglia. However, there seems to be general agreement that after peripheral nerve injury, there is a marked increase in galanin production by primary afferent neurones.

Table 3. Galanin distribution in spinal cord and dorsal root ganglia

This shows the results from a range of studies on galanin distribution in the central nervous system. Abbreviations used:

drg = dorsal root ganglia

iml = interomediolateral

NA = not applicable (usually not studied)

i.t. = intrathecal

Galanin distribution in spinal cord and dorsal root ganglia

Author	Species	Production	Quantification / Localisation	Effect of neuronal injury
[208] Klein,1990	rat	NA	28% myelinated (mainly small diameter) &27% unmyelinated axons +ve for galanin	NA
[214]Kordower,1992	monkey human	NA	Fibres in Lissauer's tract, laminae II-VI, X & iml cell column; cell bodies inn laminae VI, iml cell column & anterior horn	NA
[56]Ch'ng,1985	rat/pig	NA	Sacral and lumbar spinal cord -large amount Galanin-ir fibres in laminae I-II, Lissauer's tract, dorsolateral funiculus and around central canal, also in dorsal roots; Small cell bodies: lamina II, X; drg: few weakly stained small cells	Neonatal capsaicin: ↓ galanin-ir in fibres of dorsal lumbar cord; Thoracic spinal cord transection: ↑ galanin in sacral cord; dorsal rhizotomy - ↓ galanin-ir in ipsilateral laminae I-II
[425] Villar et al,1989	rat	L4/5 dorsal root ganglia cells showed very low levels of galanin mRNA	<5% drg neurones contained galanin-ir(mainly small size)	Axotomy: ~50% drg cells galanin-ir +ve (small-large size) ; 120-fold increase in galanin mRNA.; ipsilateral ↑ in laminae I-II; sciatic crush - similar ↑ in galanin-ir
[426] Villar et al,1991	rat	NA	Very weak staining for galanin-ir in small dorsal root ganglia cells and in fibres in dorsal roots	Axotomy - drg cells: ↑ galanin-ir (small-medium), also in dorsal roots & sciatic nerve; dorsal rhizotomy - stronger galanin-ir (small-medium drg cells), ↓ galanin-ir in superficial dorsal horn. Axotomy + dorsal root crush - galanin-ir accumulated on the side distal to the dorsal root crush
[452] Wiesenfeld-Hallin,1992	rat	Very low levels of galanin mRNA in drg	Galanin-ir strongly staining in superficial dorsal horn & very weak staining in drg	1. I.t. colchicine - ↑ galanin-ir in drg cells 2. Axotomy - ↑ galanin-ir & galanin mRNA

1.3.6 Galanin-functional effects

The role of galanin in the processing of somatosensory information in the normal rat is unclear as functional studies give variable results depending on the preparation and dose used, and the effect studied. This is summarised in Table 4.

The behavioural effects of galanin applied to the spinal cord are inconsistent. Low dose intrathecal (i.t.) galanin (0.03nmol) alone had no effect on the paw licking latency in the hot plate test, but it significantly potentiated the antinociceptive effects of morphine in rats [452,454]. As the dose increased (3nmol), i.t. galanin, alone, caused a modest increase in paw licking latency in response to a hot plate, indicating weak mechanical hypoalgesia [455]. Henry's group, 1988 [79] reported that galanin caused a dose-dependent(0.65 - 6.5 nmol) increase in withdrawal latency in the tail flick reflex, but at higher doses (6.5nmol) vocalisation to previously innocuous touch was observed, as tested by von Frey hairs. Wiesenfeld-Halllin's group found no evidence of hyperalgesia, although it should be noted that a smaller maximum dose was used than in Cridland and Henry's study (3 v 6.5 nmol). Using similar tests for thermal nociception in mice it was found that galanin had an analgesic action (0.3-3 nmol) [340]. In spinalized, decerebrate rats, further studies of the effect of galanin on the hamstring flexor reflex found a biphasic action, with brief facilitation at very low doses, with a predominantly inhibitory effect as the dose increased. Galanin strongly potentiated the inhibitory actions of morphine, antagonising SP-, CGRP- and sural nerve-induced facilitation of the flexor reflex [452,456]. In an *in vitro* preparation from neonatal rats, galanin had a concentration-dependent inhibitory action on monosynaptic and nociceptive reflexes [486]. Further evidence for inhibitory actions of galanin come from single cell studies in the dorsal horn, where galanin appeared to hyperpolarize membranes and decrease excitability [346].

Both galantide and M35, selective galanin antagonists given intrathecally, inhibited the analgesic effect of morphine on noxious thermal and mechanical stimuli. When given alone the antagonists had no effect on the response to these stimuli, and galanin itself only showed antinociception at higher doses (2.7 nmol) [348]. These antagonists also attenuated the analgesic actions of tramodol (acts at mu-opioid receptors, inhibits

noradrenaline re-uptake and has serotonergic effects), DAMGO (a selective mu-opioid receptor agonist, [D-Ala2, MePhe4, Gly(ol)5]enkephalin), clonidine (alpha-2 agonist) and desipramine (noradrenaline re-uptake inhibitor) in tests using mechanical or thermal noxious stimuli [367].

A role for galanin in the modulation of nociceptive information would certainly be compatible with its anatomical distribution, with high levels of galanin-ir and galanin binding sits in the superficial dorsal horn.

Effect of nerve injury

After sciatic nerve section, 11-24 days previously, the action of galanin on the flexor reflex changed. It no longer had an inhibitory effect on SP actions, but instead a new inhibitory effect on VIP-induced facilitation was found. It has been postulated that, after peripheral nerve injury, VIP may become the predominant excitatory peptide in place of SP [456,474]. The inhibitory effect on sural nerve-induced and CGRP-induced facilitation seen prior to nerve injury was even more pronounced [454,456,474]. Further studies also indicated an inhibitory role for galanin in the nerve-injured state, with potentiation of the analgesic actions of morphine [454] seen to a greater extent after axotomy [453]. In addition, the high affinity galanin antagonist, M35, dose-dependently potentiated the conditioning stimulus-induced facilitation of the flexor reflex in rats. This action was strongly enhanced in rats that had undergone sciatic nerve transection 10-18 days previously, suggesting greater tonic inhibition by galanin after axotomy [453]. M35 also significantly increased the degree of self-mutilation (assessed by autotomy) in rats with a sciatic nerve transection [423].

From these limited behavioural and electrophysiological studies of galanin, it is still unclear as to what its role may be in the normal rat. However, after a peripheral nerve injury, it appears to have a predominantly inhibitory action, associated with an increase in its production by primary afferent neurones. It may be therefore, that galanin takes on the role of an endogenous analgesic compound in the nerve injured state. Further studies of its functional effects, particularly in models of partial nerve injury are required.

Table 4. Functional effects of galanin

This table summarises the studies of behavioural or electrophysiological studies of the actions of galanin in the spinal cord. As discussed in the text, its actions may be dependent on the model studied or the test used.

Abbreviations:

C= chemical

CS= conditioning stimulus

i.t.= intrathecal

M= mechanical

T= thermal

Table 4. Functional effects of galanin

Author	Model +test	Inhibitory	Excitatory
Yanagisawa, 1986 [486]	*In vitro* isolated spinal cord /tail from neonatal rat + galanin	Yes (C)	
Cridland, 1988 [79]	Rat; Chronic i.t. catheter for galanin administration +behavioural testing	Yes (T	Yes (M) - high dose
Wiesenfeld-Hallin, 1990 [454]	1. Rat; Chronic i.t. catheter for galanin administration +behavioural testing 2. Decerebrate, spinalized - hamstring flexor reflex	Yes (T): potentiation of morphine	
Xu, 1990 [474]	Rat; Decerebrate, spinalized - hamstring flexor reflex; Sciatic nerve transection + i.t.galanin	Yes (action of other peptides)	
Kuaraishi, 1991 [217]	Rat; i.t. catheter - galanin antiserum or galanin + carrageenan to 1 hindpaw +behavioural testing		Yes (M- inflamed paw)
Randic, 1987 [346]	Rat; Single cell recording + ionophoresis in dorsal horn	Yes(hyperpolarization)	
Post, 1988 [340]	Mouse; i.t. galanin + behavioural testing	Yes (T)	
Wiesenfeld-Hallin, 1992 [453]	Rat; It galanin antagonist-flexor reflex; sciatic nerve transection	Yes (CS facilitation) ↑ after nerve injury	
Verge,1993 [423]	Rat; i.t. infusion of galanin antagonist after axotomy; degree of autotomy	Yes (antagonist ↑ autotomy)	
Wiesenfeld-Hallin, 1993 [455]	Rat; i.t. galanin effect on response to noxious thermal & mechanical stimuli	Yes (T>M)	
Reimann, 1995 [348]	Rat; i.t. galanin antagonists: behavioural response to thermal & mechanical stimuli	No effect alone Antagonists inhibited morphine action	
Selve,1996 [367]	Rat; i.t. galanin antagonists: drug actions on behavioural response to thermal & mechanical stimuli	Yes(↓ effect of analgesics)	

1.4 Neuropeptide Y

1.4.1 Synthesis and structure

Neuropeptide Y is a 36 amino acid peptide that was first isolated from extracts of pig brain in 1982 [408]. The N and C terminals of NPY are tyrosine and tyrosinamide respectively, with the C terminus being needed for activity at NPY receptors. It exhibits 70% sequence similarity with peptide YY (PYY) and about 50% with pancreatic polypeptide (PP) [204,236]. There is marked primary and tertiary structural similarity between these peptides and they have therefore been classified into the pancreatic polypeptide family. The primary and tertiary structure of NPY has been highly conserved through evolution [228]. Studies from X ray crystallography have determined the structure outlined in Figure 1.

NPY is formed from proteolytic processing of an approximately 97 amino acid long prepropeptide [228]. A signal peptide, consisting of a hydrophobic N terminal sequence helps to direct the protein into the rough endoplasmic reticulum, where the signal protein is cleaved and degraded. The signal peptide is cleaved after 28 residues, resulting in Pro-NPY [284]. The tertiary structure of the peptide is then formed in the endoplasmic reticulum, with folding of the protein and formation of disulphide bonds [139]. After transport to the Golgi complex [337], further modifications occur, with amidation at the carboxyl terminal [45], and packaging of NPY into LDCVs, before transport to the nerve terminal. The degree of transcriptional and post-transcriptional control of NPY production is variable, with different cell lines showing that either protein kinase C or adenylate cyclase activation can increase transcription of the NPY gene, or prolong the half-life of NPY mRNA by increasing its stability [234].

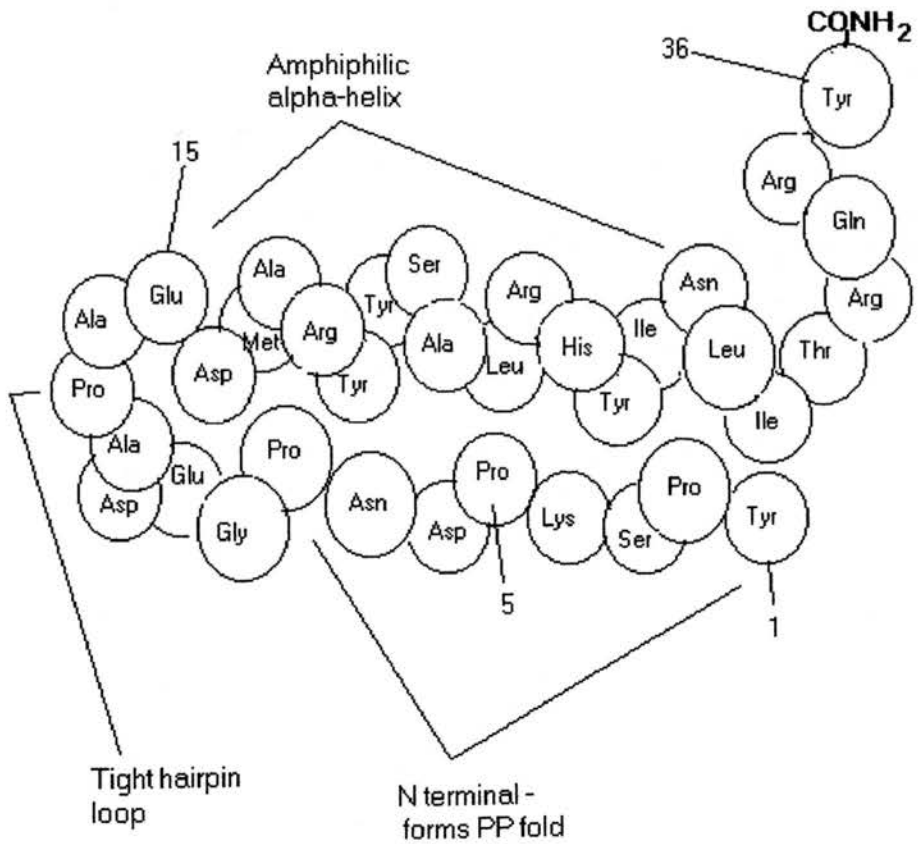
Figure 1. Structure of Neuropeptide Y and related peptides

A. The primary structure of neuropeptide Y and some related peptides in the pancreatic polypeptide family is shown in part. Abbreviations used:
NPY - Neuropeptide Y
PYY - Peptide YY
aPP - avian polypeptide
pPP - porcine polypeptide
bPP - bovine polypeptide
B. The tertiary structure of NPY as determined from X-ray crystallography studies. There is an N-terminal globular structure (the PP fold) consisting of a polyproline helix (1-8) followed by a tight hairpin loop formed by an anti-parallel amphiphilic α-helix (13-32) and a type I α-turn, ending in a short terminal tetrapeptide amide spatially close to the N terminal portion. The helices are held together by interdigitating hydrophobic side chains, with the C terminal projecting away from the loop at a 90° angle.

A. Primary amino acid sequence of some of the pancreatic polypeptide family

NPY	Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2
PYY	Tyr-Pro-Ala-Lys-Pro-Glu-Ala-Pro-Gly-Gu-Asp-Ala-Ser-Pro-Glu-Glu-Leu-Ser-Arg-Tyr-Tyr-Ala-Ser-Leu-Arg-His-Tyr-Leu-Asn-Leu-Val-Thr-Arg-Gly-Arg-Tyr-NH_2
aPP	Gly-Pro-Ser-Gln-Pro-THr-Tyr-Pro-Gly-Asp-Asp-Ala-Pro-Val-Glu-Asp-Leu-Ile-Arg-Phe-Tyr-Asp-Asn-Leu-Gln-Gln-Tyr-Leu-Asn-Val-Val-Thr-Arg-His-Arg-Tyr-NH_2
pPP	Ala-Pro-Leu-Glu-Pro-Val-Tyr-Pro-Gly-Asp-Asp-Ala-Thr-Rpo-Glu-Gln-Met-Ala-Gln-Tyr-Ala-Ala-Glu-Leu-Arg-Arg-Tyr-Ile-Asn-Met-Thr-Arg-Pro-Arg-Tyr-NH_2
bPP	Ala-Pro-Leu-Glu-Pro-Gly-Tyr-Pro-Gly-Asp-Asp-Ala-Thr-Pro-Glu-Gln-Met-Ala-Gln-Tyr-Ala-AlaGlu-Leu-Arg-Arg-Tyr-Ile-Asn-Met-Leu-Thr-Arg-Pro-Arg-Tyr- NH_2

A. Tertiary structure of NPY



1.4.2 Metabolism

NPY is resistant to degradation by non-specialised peptidases, that target a proline residue on the penultimate N-terminal position, and is protected against degradation by carboxypeptidases as a result of amidation of the C-terminus [272]. In the brain, astrocytes were almost always unable to catabolize NPY, whereas cultured neurones and microglia digested NPY through cleavage after Arg19, Arg25, Arg33 and Arg35. NPY was hydrolysed *in vitro* by neutral endopeptidase-24.11 (NEP) by cleaving at Tyr20-Tyr21 and Leu30-Ile31. *In vitro* studies have shown that the proline-preferring aminopeptidases, aminopeptidase-P (AP-P) and dipeptidyl peptidase IV (DPP-IV) processed NPY at its N terminus [272,273]. DPP-IV produced NPY(3-36) that did not have activity at the Y2 receptor on striatal synaptic membranes [245], but did in kidney epithelial cells [341]. NPY was resistant to the action of 2 other membrane aminopeptidases (N and W) and to the action of angiotensin converting enzyme [245,268]. The relative resistance of NPY to degradation may have functional implications. Thus if NPY persists for any significant length of time after its release, it may act by a form of volume transmission, at sites distant from release [132].

1.4.3 NPY Receptors

Pharmacological classification of NPY receptor subtypes has determined multiple subtypes, including Y-1, Y-1-like, Y-2 and Y-3 that share similarities with the PYY-PP family of receptors. More recent molecular studies correlate well with the pharmacological classification and have also found multiple NPY receptor subtypes. This has been reviewed most recently by Balasubramaniam, 1997 [14].

Pharmacological binding studies

Pharmacological study of NPY receptor subtypes was reviewed by Wan,1995 [442]. The Y2 receptor has good affinity for the whole NPY molecule and the C-terminal fragment , but the Y1 receptor requires the whole molecule [434]. Thus NPY(13-36) has activity at Y2 but not Y1 receptors. All the NPY receptor subtypes studied so far appear to act primarily by inhibition of adenylate cyclase via a G protein, although

subsequent intracellular events vary depending on the particular tissue and receptor type being studied [278,318]. Y1 receptor activation resulted in increased intracellular Ca^{2+} and neuronal excitation, whereas Y2 activation may have had the opposite effect [72]. Y1 sites were found particularly in the brain cortex and postjunctionally in some vascular beds in the rat, although in human brain, only the dentate gyrus of the hippocampus had any significant levels of Y1 receptors [179]. Y1 receptor mRNA has also been found in small cells in the rat dorsal root ganglia [491], and 5-20% of small to medium-sized dorsal root ganglia cells bound NPY [256]. The C terminal carboxyl-amide group seems to be essential for Y1 receptor recognition. The Y2 receptor was also activated by N-terminally truncated fragments, and was found mainly in the central nervous system.

A comparison of the distribution of Y1 and 2 receptors in the guinea pig and rat brain was carried out using radiolabelled subtype specific ligands. Major differences in distribution were found between the 2 species. High levels of Y1 were found in the rat thalamus, whereas there was very little Y1 binding found here in the guinea pig. Conversely, Y1 binding was low in the rat hippocampus, and high in the guinea pig. It seems likely therefore that Y1 and Y2 receptors perform different roles between the 2 species [135]. The human frontal cortex appeared to have a predominance of Y2 receptors, in contrast to low levels seen here in the rat [179].

The Y3 receptor was found in cardiac muscle and recognised NPY with a much higher affinity than PYY [142]. The main amino acid differences between PYY and NPY occurred in the 13-23 segment, which must be important for receptor binding, although the amidated C terminal was also needed [152]. A further type of NPY receptor mediated NPY-stimulated feeding , showing a broadly similar profile but with characteristic differences from the Y1 receptor, leading to its being called "Y1-like" [231]. It has also been suggested that NPY may interact with a subtype of sigma receptor in the rat brain, as it had very similar actions to specific sigma receptor agonists in potentiating the effect of NMDA in the hippocampus [290,291]. However, more recent studies indicated that the functional effect of NPY was mediated through a different receptor from sigma ligand [289].

The predominant receptor type on neurones seemed to be Y2. Pharmacological studies found that the presynaptic receptor in rat hippocampus shared identical properties to the presynaptic Y2 receptor found on sympathetic neurones [73]. NPY inhibited the release of noradrenaline in cultures of superior cervical ganglia cells, via a pertussis toxin-sensitive mechanism, decreasing the availability of intracellular calcium for the release process [323]. NPY was probably acting at a presynaptic site to inhibit sympathetic terminals by reducing calcium influx, possibly by selective inhibition of neuronal N-type calcium channels [414].

Both, pre and post junctional NPY receptors were found in the sympathetic nervous system with vasoconstriction mediated by a direct postjunctional effect and noradrenaline release suppressed by a prejunctional action [434]. There are species differences in the subtype of receptor found. Thus, rat and rabbit vas deferens appear to have two different prejunctional receptor subtypes - Y1 in the rabbit and Y2 in the rat. NPY and [Leu31,Pro34]NPY were potent agonists, whereas the C terminal fragments NPY(13-36) and NPY(18-36), both Y2 agonists, inhibited evoked twitches, but at differing doses in the rat and rabbit [94].

Molecular studies of NPY receptors

There have been several NPY receptors cloned, all with seven transmembrane domains, as occurs with members of the G protein family of receptors, confirming pharmacological studies. The cloned human Y1 receptor is coupled to both cAMP and calcium [262], and has been found in the colon, vascular smooth muscle cells, kidney, adrenals and heart. A human Y2 receptor has been cloned, showing about 30% sequence similarity with Y1 and also coupled to cAMP and calcium [227]. Although a bovine Y3 receptor was cloned, human and rat counterparts did not show any detectable interaction with NPY or related peptides, making it unlikely that this is a true NPY receptor subtype [152]. Further members of the PP family of receptors have been cloned with a putative Y4 receptor (or PP1 receptor) cloned in human, rat and mouse [249]. Most recently, a Y5 receptor has been cloned, with several variations, [362,447] and it is proposed that this might be the "Y1-like" receptor concerned with feeding [171,321].

In studies on a human neuroblastoma cell line expressing Y1 receptors, four monoclonal NPY antibodies, each directed against different epitopes on NPY, were used to examine receptor interactions. For NPY binding and subsequent inhibition of cAMP production by forskolin, sequences 11-24 and 32-36 were required. Those antibodies directed against 27-34 and 1-12 had little effect on NPY binding except at very high concentrations [149]. Also in a human neuroblastoma cell line, NPY and a cyclic analogue of NPY were both shown to inhibit calcium currents, via a pertussis toxin-sensitive mechanism. The inhibitory effects were additive with nifedipine, probably involved N-type calcium channels, and were even more marked with the cyclic NPY analogue with an agonist action [262].

The cloned Y2 receptor is also a 7-helix, G-protein coupled receptor that inhibited the activation of adenylate cyclase [138]. Y2 receptor mRNA was discretely localised in rat brain in a distribution consistent with the radioligand binding studies. In the brain, Y2 receptors were restricted to specific regions of the cortex, hippocampus, amygdala, thalamus, hypothalamus, mesencephalon and pons. The most intense labelling was in the CA3 region of the hippocampus, the arcuate nucleus of the hypothalamus and layer 3 of the piriform cortex, with less intense labelling in the trigeminal nucleus and the dorsal motor nucleus of the vagus [155].

The multiplicity of NPY receptor subtypes, both from molecular and pharmacological studies suggests diverse functional effects, many of which have been studied in the nervous, endocrine and cardiovascular systems. Additionally, it should be possible to develop drugs in such a way as to target specific subtypes of receptor, and thus gain specificity of action with minimal side effects [302,470].

1.4.4 NPY antagonists

A variety of NPY antagonists have been developed, and are discussed in detail in several recent reviews [14,247]. Many of the antagonists developed lack sufficient specificity to be of great use in pharmacological studies, and often show mixed agonist/ antagonist actions.

Peptide antagonists

Fragments of NPY have been synthesised that showed antagonist properties at one or more NPY receptor subtypes [450]. For example, NPY(18-36) acted as a competitive antagonist in rat cardiac ventricular membrane [15,282] and antagonised the potentiating effect of NPY on NMDA activity in rat hippocampus [291]. However, NPY(18-36) also acted as a partial agonist in some preparations with Y1 receptors and a full agonist in Y2 systems [117,277].

Other antagonists include PYX1 and PYX2, developed by Tatemoto [409], but again there is debate about their specificity [331]. These agents were based on the C-terminal end of the peptide, with at least the first 10 amino acids of the NPY structure being incorporated. Both PYX1 and PYX2 inhibited NPY-stimulated intracellular calcium release, in a human erythroleukaemia cell line, and displaced radiolabelled NPY from rat brain membrane [409]. There was also evidence that PYX2 can selectively antagonise NPY-induced feeding [231]. Unfortunately, these compounds were only effective at very high doses [25,117], but they have at least been useful in demonstrating that the C-terminal decapeptide moiety was suitable for the development of antagonists. More recent antagonists that used a d-Trp substitution at the C-terminal end, antagonised the feeding effects of NPY [16], but acted as agonists at the receptors involved in antinociception [47].

In order to characterize the many NPY receptor subtypes properly, it was necessary to develop multiple compounds [277]. A range of modified NPY fragments was therefore synthesised to produce Y1 selective compounds. Des-Asn29[d-Trp28,32]NPY(27-36) was found to have no agonist activity at Y1 receptors, and bound with higher affinity to Y1 than Y2 receptors. It antagonised NPY induced effects on cAMP and Ca^{2+} mobilisation [18]. Further non-selective analogues have been developed, with moderate antagonist properties differing between receptor subtypes [17,229]. 1229U91, a new peptide antagonist, was produced, with actions at postsynaptic vascular receptors in the rat. It was a cyclic, dimeric nonapeptide with no agonist activity, but acted as a competitive antagonist with NPY or [Leu13,Pro34]NPY (a Y1agonist) [235].

Nonpeptide antagonists

A number of nonpeptide antagonists have been used. Benextramine [58], an irreversible alpha adrenoceptor antagonist and HE90481 [279], a guanethidine based H1 and H2 histamine receptor antagonist were shown to have antagonist actions. Major disadvantages included lack of specificity, low affinity and irreversibility [117,433]. Benextramine had antagonist actions, but they were irreversible, limiting its usefulness, and there was some debate about its efficacy [331]. However, a series of benextramine analogues was produced, that had competitive antagonist activities at NPY Y1 and 2 receptors in rat brain and femoral artery, which may be more useful [58]. A substance with a similar molecular formula to a recently isolated substance P inhibitor (TAN-1612), BMS-192548, was found to be an antagonist at NPY receptors, [377]. Two further compounds may be useful, particularly as they are non-peptide agents - BIBP3226 [415,451] and SR120819A [368], that competitively and selectively antagonise Y1 receptor-mediated effects in some preparations.
Thus, recently developed antagonists for Y1 and Y2 receptor subtypes are more selective and specific, and the development of nonpeptide agents may be potentially useful in the treatment of a number of conditions [95,148,471], since these may be more likely to penetrate the blood brain barrier and may be degraded more slowly.

1.4.5 Distribution

NPY is one of the most abundant peptides found in mammalian central and peripheral nervous systems [6,7].

Brain

In the rat brain, NPY was extensively distributed in periventricular areas, forebrain, with widespread projections throughout the brain. NPY-ir was also found in all areas associated with olfaction in the rhinencephalon, as well as in the basal ganglia, thalamus, cortex (especially layer II/III and VI), hippocampus, amygdaloid and hypothalamus. It also coexisted with catecholamines in cells in the locus coeruleus and brainstem, projecting to the spinal cord [85]. Efferent projections of NPY-containing neurones from the locus coeruleus went mainly to the hypothalamus, but also to the spinal cord and cerebral cortex [166]. In rat, guinea-pig and cat, more than

90% of the NPY-ir found in the ventrolateral medulla was rostrally situated. There was significant overlap with those neurones containing tyrosine hydroxylase, and remarkable consistency between species [156]. Studies using 6-OHDA lesions of the rostral ventrolateral medulla confirmed that most of the catecholamine and NPY-containing terminals in the interomediolateral column originated in the rostral ventrolateral medulla. This is an area of the brain known to play a critical role in maintaining vasomotor tone and controlling vasomotor reflexes [418].

Spinal cord and primary afferents

In the rat spinal cord, NPY-ir was found in the substantia gelatinosa, the dorsolateral funiculus and the dorsal grey commisure in the lumbosacral cord. NPY-ir was also detected in fibres in an area of the cord concerned with autonomic function - around the interomediolateral preganglionic sympathetic neurones in thoracic cord and parasympathetic neurones in sacral cord [85]. Immunocytochemical techniques were used to study the ultrastructural arrangements of NPY-containing terminals in the dorsal horn of the cat. There was heavy staining in the superficial dorsal horn (laminae I-II) with axons and varicosities being stained. There was a much lower level in the deeper dorsal horn (laminae III-VI).The majority of NPY-ir was contained in axon terminals, most of which formed synaptic connections. Within these terminals NPY-ir was scattered throughout the cytoplasm as well as being associated with numerous LDCVs. NPY-containing terminals formed post synaptic connections of the axo-dendritic or axo-somatic type, although a few formed axo-axonic connections. Some NPY-containing boutons were presynaptic to unlabelled terminals in laminae II, having the appearance of primary afferent terminals. From the localisation of NPY, it may have both postsynaptic effect on local interneurones in the spinal cord as well as a presynaptic action on primary afferent fibres terminating in the superficial dorsal horn [99,100].

In the absence of any peripheral injury, NPY is not stored in the central terminations of primary afferents, but it is found mainly in intrinsic neurones of the dorsal horn [140], where it may coexist with galanin in a small number of intrinsic neurones in laminae II [497]. A proportion of GABAergic neurones in laminae I-III of the dorsal horn of the rat were found to contain NPY. In fact, all of the NPY containing neurones also

contained GABA, although very few showed any glycine or met-enkephalin staining [222,355].

NPY is not normally contained in primary afferent neurones in any significant amounts. NPY mRNA was virtually undetectable in normal dorsal root ganglia (<5%), as were signals for galanin and VIP. After capsaicin treatment, there was no change in NPY mRNA levels, but there was an increase to about 10% for galanin and VIP. This differential effect may be due to different subpopulations of neurones being affected by the capsaicin [197].

Sympathetic nervous system

Very high levels of NPY were found in sympathetic ganglia and in tissues with dense sympathetic innervation in guinea-pig, cat, pig and man [248]. In many of the cell bodies, NPY co-existed with tyrosine hydroxylase and dopamine-β-hydroxylase in large cytoplasmic granules [182]. These very high levels of NPY in the sympathetic nervous system suggest an important role in regulation of sympathetic tone [248]. NPY was also found in the majority of peripheral terminals of sympathetic neurones, mainly co-localised with tyrosine hydroxylase in LDCVs. NPY was released peripherally after stimulation of the sympathetic nerves [246]. In the dog spleen and vas deferens, it was found that the N-type calcium channel blocker, ω-conotoxin, reduced the release of both NA and NPY in an almost identical and dose dependent manner, whereas the L-type calcium channel blocker, nifedipine, had no effect. Additionally, if exhaustive stimulation was used, released NA was predominantly from small dense core vesicles, as the store of LDCVs became drastically depleted, and the amount of NPY released was significantly decreased [82]. From its distribution, it would seem likely that NPY does have a role to play in the sympathetic nervous system. The functional nature of this requires further study.

Effects of nerve injury on NPY in sensory and sympathetic neurones

After a peripheral nerve injury there are major changes in NPY levels in the dorsal horn, and also in its production by primary afferent neurones. There was no significant difference in the degree of NPY induction between different types of nerve injury, with axotomy, CCI and crush, inducing similar changes. There was an increase in

laminae III-V axons and varicosities and induction of NPY-ir in many large or medium diameter primary afferent neurones, with less than 2% having a diameter <25 μm [435]. Sciatic axotomy resulted in a marked increase in NPY levels in the dorsal horn, especially in laminae III and IV [492] and in the gracile nucleus, reaching a maximum at twenty-eight days. As simultaneous dorsal rhizotomy prevented the response in the spinal cord, it seems likely to be due to changes in primary afferent neurones [325].

In the CCI model, after twenty-eight days, there was a significant decrease in lectin IB_4 (used as a C fibre marker) in laminae I-II, with a corresponding decrease in SP levels. Although NPY showed no change in laminae I-II, there was a marked increase in laminae III-IV [298], that was still present even at 100- 120 days after injury [299]. There was a corresponding increase in NPY mRNA levels in the dorsal root ganglia from previously undetectable levels to high levels. This was seen in mainly medium to large sized dorsal root ganglia cells, such that 20-30% of all dorsal root ganglia neurones contained NPY mRNA. Capsaicin treatment caused only a small reduction in NPY mRNA levels (10%), suggesting that it is found mainly in myelinated fibres [194,316].

In the dorsal root ganglia of normal rats, NPY receptor mRNA was mainly in small type cells also containing SP and CGRP. After axotomy, the number of small neurones labelled increased, but labelling intensity decreased. There was also an up-regulation of NPY Y1 receptor mRNA in large neurones [164]. Despite this increase in NPY receptor mRNA, it may not be translated into functional receptors, as neither cuff-induced neuropathy (4 loose cuffs of polyethylene tubing) or axotomy altered NPY binding in the dorsal root ganglia. Pharmacological binding studies indicated that the predominant receptor type was Y2 [256].

In the normal situation, neuropeptides are rarely found in large neurones in the dorsal root ganglia. After axotomy, intense NPY-ir was seen in LDCVs budding off from the Golgi complex in large neurones. Some of these neurones had evidence of central transport and secretion of NPY, possibly by both regulated and constitutive pathways, although actual release of NPY was not studied [24,490].

In the trigeminal ganglia, changes similar to those seen in the dorsal root ganglia after axotomy were seen. Normally, about 5% of neurones were NPY mRNA positive and

19% were NPY receptor Y1 mRNA positive, mainly in small sized cells. After axotomy, 54% of mainly large sized cells were NPY mRNA positive [494]. Injury to branches of the mandibular nerve resulted in ~35% of cells (mainly of medium to large diameter) developing NPY-ir [438], with NPY-ir appearing in numerous thick nerve fibres, also containing the calcium-binding protein, calbindin [439,440]. After tooth extraction, NPY-ir also appeared in large to medium sized cells of the trigeminal ganglia [177].

The situation in sympathetic ganglia is unclear, as axotomy of the carotid nerves, close to their origin from the superior cervical ganglion decreased NPY-ir in cell bodies, but increased levels in nerve fibres. NPY mRNA decreased, although the numbers of cells containing it remained constant [215]. In LIF null mutant mice, there was a smaller decrease in NPY mRNA levels than in wild type mice, after axotomy. LIF mRNA expression was increased starting at 1 hour and peaking at 6 hours after axotomy. LIF may be one of the key regulators of NPY production in the sympathetic ganglia [402].

In the CCI model, NPY-ir decreased in vasoconstrictor efferents to the injured hindpaw, associated with a gradual loss of these sympathetic efferents [436]. Thus, nerve injury produces a loss of NPY in neurones which normally contain it, but de novo synthesis in a proportion of primary afferent neurones.

The stimulus for NPY production in primary afferent fibres after nerve injury is not known. However, as with galanin, peripherally derived neurotrophins may be important. Intrathecal NGF given for 7 days, 2 weeks after sciatic axotomy attenuated the expected increase in NPY by about 50%, as well as preventing a decrease in neurones expressing SP and CGRP, but not somatostatin, and attenuating the increase in VIP, CCK and galanin [424]. LIF seems to have less of a role in sensory ganglia than sympathetic ganglia, as LIF-deficient mice still showed an increase in NPY in primary afferent neurones after sciatic axotomy, although this was slightly less pronounced than that seen in the wild type mice [77]. Neurotrophin-3 (NT-3) may have a more important role in the control of NPY in primary afferent neurones, as after a bilateral axotomy, the transganglionic response of an increase in NPY in the gracile columns was prevented by exogenous NT-3 [326]. NT-3 seems to be important in development

and maintenance of the large type sensory neurones that start to produce NPY after injury [239,384], and lack of it after injury may be one of the stimuli resulting in NPY up-regulation. Both BDNF and NT3 have been shown to be of importance in the phenotypic maintenance of NPY-containing neurones in neonatal animals [54,310].

1.4.6 Functional effects of NPY

The ubiquitous nature of NPY indicates widespread effects in several systems. Thus it has actions in the nervous system, with effects on nociception, anxiety; memory and sympathetic tone [78,301]; the cardiovascular system, with actions on blood pressure, coronary perfusion and vasoconstriction [433]; and effects on the regulation of food intake and metabolism [268]. Most of this section will concentrate on the neuronal actions of NPY, particularly in the control of nociception.

NPY actions at the membrane and interactions with other neurotransmitters

The functional effect of NPY depends on the receptor activated. Several receptor types were found on sensory neurones in culture, and electrophysiological studies showed that receptor activation led to hyperpolarization [491] and a reduction in calcium currents [76,323]. Generally, activation of Y1 receptors results in an increase in Ca^{2+} availability, with subsequent neuronal excitation, whereas Y2 receptor activation may, instead, reduce Ca^{2+} availability and inhibit neuronal function [458]. However, Y1 receptors do not always result in neuronal excitation, as they have been shown to result in hyperpolarization of small primary afferent neurones [491]. There are many studies that provide further evidence for an inhibitory action of NPY. NPY inhibited or suppressed the response produced by electrical field stimulation of capsaicin-sensitive sensory neurones in guinea pig left atrium or bronchi via a prejunctional mechanism [141]. NPY inhibited calcium currents and modulated transmitter release by highly selective mechanisms. It inhibited both SP and acetylcholine release from sensory neurones in culture [72], and SP release into the substantia gelatinosa of the cat following stimulation of unmyelinated primary sensory neurones [110].

As well as a direct action on neurones, NPY may also modulate the effects of other neurotransmitters. NPY inhibited the release of several neurotransmitters in different systems. In rat hippocampal slices, NPY, PYY and NPY(13-36) (Y2 agonist) all

inhibited glutamate release, whereas the Y1 agonist ([Leu13,Pro34]NPY) had an insignificant effect [147]. However, *in vivo* studies, found that NPY potentiated the response of hippocampal neurones to NMDA [290] via a receptor subtype that was pharmacologically different from the already defined NPY receptors [291]. The potentiation was prevented by pre-treatment with pertussis toxin, indicating the involvement of a G-protein in mediation of this NPY effect [289]. NPY had effects on glutamate in areas other than the hippocampus, with inhibitory effects on both presynaptic glutaminergic neurones and presynaptic GABA-containing neurones in the suprachiasmatic nucleus [59,421]. NPY has also been shown to interact with both with catecholamine release [221] and metabolism [101].

Nervous system

NPY is likely to be involved in the modulation of nociceptive transmission in the spinal cord, a role that may be more important after nerve injury. When given centrally, it seemed to be mainly analgesic [172], but the exact effect may depend on the receptor subtype involved.

Intrathecal NPY had a dose-dependent biphasic effect on the spinal nociceptive flexor reflex in decerebrate, spinalized rats that developed with time. Low doses of NPY (10-100 ng) caused a brief facilitation, changing to inhibition at higher doses (1,10 μg), with intense and prolonged depression. This action was not reversed by naloxone or the α2-adrenoceptor antagonist, atipamezole. Sixteen days after axotomy, the facilitation by NPY was significantly enhanced, but the depressive effect was unchanged. However 31 39 days later, the inhibitory effect was more pronounced and seen at lower doses [476]. This effect may be related to changes in the types of NPY receptor expression after nerve injury. Behavioural studies confirmed the inhibitory effects of NPY, as it induced analgesia to thermal stimulation, and its C terminal fragments induced analgesia to mechanical stimulation [172].

Although there is evidence of a central analgesic action of NPY [172,476,478], in a study giving NPY intrathecally for 14 days, it has appeared to be hyperalgesic [448], with behavioural signs of hyperalgesia correlating with the peak of NPY levels in the spinal cord [298]. There may also be a peripheral effect, as subcutaneous NPY into the hindpaw resulted in hyperalgesia. This was mediated by a Y2 receptor, with Y1

agonists being analgesic [417]. Surgical sympathectomy also relieved the hyperalgesia and the effects of NPY, possibly indicating a role for NPY in the sympathetic nervous system. It is co-released with noradrenaline from sympathetic neurones, depending on the pattern of stimulation [82,416].

After peripheral nerve injury, a major increase in NPY production by the large diameter primary afferent cells [316] was associated with concurrent changes in NPY receptor distribution after nerve injury, such as decreased Y1 receptor mRNA in small dorsal root ganglia cells [491,498]. It has been suggested that the changes in NPY may be related to the development of spontaneous discharges in large myelinated fibres and the development of hyperalgesia, although the use of appropriate NPY antagonists would be useful to investigate this further [298,300]. In the long term, however, when the behavioural evidence of hyperalgesia had resolved, there was still an increase in NPY levels in laminae III-IV of the dorsal horn [299].

NPY was shown to have an antinociceptive action in the mouse writhing test [47], although in the hot plate test, NPY decreased the withdrawal threshold. In the formalin test, there was a decreased licking response in phase 1 with no effect on phase 2. As the dose was increased there ceased to be any effect of NPY in the tests studied [270].

Other actions of NPY

Apart from a possible role in the modulation of nociceptive transmission, NPY has been implicated in many homeostatic functions [302], including the control of blood pressure [151,433], stimulation of feeding [171,268,391] and effects on memory [129] and anxiety [46,118,157 302].

NPY has a role to play in the regulation of feeding. It simulated feeding, even in obese rats [171,268,391], and reversed the anorexic effects of some agents [386]. The effects on feeding seem to be mediated by the atypical Y1-like receptor (from pharmacological studies) that has recently been cloned, as the Y5 receptor [321]. NPY may have actions on energy expenditure, by effecting metabolic rate in brown adipose tissue. There is also evidence that NPY production is increased by the protein product of the *Ob* gene, resulting in an increase in calorie intake [392]. Interestingly, NPY does not seem to

be an essential component of regulation of feeding, as NPY knockout mice did not show any abnormality in weight gain or feeding behaviour [119].

Other possible functions of NPY include a role in the control of seizures. NPY knockout mice showed an increased propensity for developing seizures [119]. After kindling-induced epileptic seizures, there were long term increase in NPY, in mossy fibres of the hippocampus, that may be related to a generalised role in decreasing neuronal excitability [251]. NPY, acting via a Y5 type of receptor, strongly inhibited kainic acid induced seizures [463].

There is evidence that NPY can act as a growth factor itself, as it has been shown to increase DNA synthesis in vascular smooth muscle cells and increase smooth muscle cell proliferation [122,373]. In neuronal cultures, NPY in the presence of spinal cord explant induced significant neurite outgrowth, but not alone, indicating a possible indirect neurotrophic action [449].

1.5 Aims

The ultimate aim of the present studies was to contribute to the understanding of the pathophysiological changes occurring in response to a peripheral nerve injury, that result in the development of neuropathic pain. A major feature of the central response to nerve injury is the up-regulation of certain neuropeptides that are normally found at very low levels in primary afferent neurones. Galanin and neuropeptide Y are two of these peptides, and both display evidence of inhibitory actions in the spinal cord. It is important to correlate functional events with anatomical changes, and it was for this reason that the central release pattern of these peptides has been studied during experimental peripheral nerve injury. To do this a well validated animal model of neuropathic pain has been studied, and the stimuli that alter release of these peptides in the spinal cord have been determined *in vivo*.

Chapter 2. General Methodology

2.1 Antibody Microprobe Technique

2.1.1 Basic principles

The antibody microprobe technique has been used extensively to study neuropeptide release in the brain and spinal cord of both the cat [108,226,293,459,460] and rat [111,168,352]. This technique was described in detail by Duggan [104,107].

The basic principle of the technique is that a series of processes are used to immobilise a specific and sensitive antibody to the compound being studied, onto the whole of the outer surface of fine glass microprobes. This allows the relatively atraumatic study of the *in vivo* release patterns of neuropeptides of interest, as the tip diameter of each probe is only about 5-10μm. Microprobes are inserted into the spinal cord to a known depth and left *in situ* for a predetermined duration, to allow adequate binding of a proportion of endogenous peptide released into the extracellular space. The peptide binds to the antibodies coating the outer surface of the probe at localised sites of release. After a set length of time, the probes are removed from the cord, washed and incubated in radiolabelled peptide for 24 hours. This allows the radiolabelled peptide to bind to any unoccupied antibodies on the microprobe. A second wash is carried out then the tips of the probes placed next to X-ray film in order to produce an individual autoradiographic image for each probe. Deficits in binding of radiolabelled peptide represent areas where endogenously released peptide has bound. This is detected by an image analysis system, that measures variations in optical density along the length of the probe. A sorting programme can then be used to compare the means of defined groups of microprobes, and determine differences in release at specific sites within the spinal cord. The principles of this technique are outlined in Figure 2.

This technique therefore allows the study of the *in vivo* release pattern of particular neuropeptides, and the effect of peripheral stimulation or other manipulations of this release. In addition, release can be localised to specific areas in the spinal cord, which is not currently possible by any other technique. Techniques using surface perfusion do not localise the release of neuropeptides to specific areas within the spinal cord, and require a relative excess for peptide detection. Techniques using cannulae inserted

into the cord do give some degree of localisation, but the diameter of these cannulae is of the order of 300 - 500 μm, and thus cause considerable trauma and cell damage in the area of insertion [338,419].

The accuracy of spatial localisation depends on the accuracy of probe placement in the spinal cord and the resolution of the image analysis system. Maximum accuracy would be achieved by ejecting dye from each probe used at its insertion site in the cord. However, if several probes are used during the course of an experiment, it becomes difficult to relate the relevant dye spot to a specific probe. Accuracy of probe placement was improved by use of stepping motor micromanipulators, using an operating microscope to visualise insertion. Additionally, at the end of each experiment Pontamine Sky Blue was ejected from a microprobe inserted to a known depth and this was measured in post mortem cord sections. The image analysis system is capable of density measurements at 10 μm intervals, but comparisons between groups are carried out at 30 μm intervals, since a biological resolution of 10 μm is probably not possible [104,106].

The amount of neuropeptide that can be detected by this technique is very small. A complete inhibition of binding of ^{125}I SP over a length of 100 μm approximates to 3 x 10^{-8} moles of the tracer not being bound. The amount of endogenous SP producing such inhibition is likely to be of the same order of magnitude. More important is the concentration of endogenous ligand in the environment producing such inhibition [104]. With the majority of studies performed, *in vitro* studies assessing sensitivity demonstrate at least 50% inhibition of binding of the radiolabelled peptide by the corresponding unlabelled form of the peptide in a concentration of 10^{-7}M [103]. By relating the images of *in vitro* probes exposed to a known concentration of peptide, to *in vivo* images of probes inserted into the spinal cord it is possible to estimate concentrations at release sites [294]. The physiological relevance of this estimate is unclear, as it does not indicate peptide-receptor interaction or function.

It has been found that the duration for which each microprobe must be left in the spinal cord varies between peptides depending on variations in the antibody used. Substance P release can be detected by probes left in the spinal cord for 5 minutes during a specific peripheral stimulus [112]. Before beginning to study a particular peptide

using this technique, it is first necessary to determine the duration required for microprobes to stay *in situ*, for adequate binding for each peptide.

Additional problems occur with detection of release if the substance being studied is either inactivated very rapidly, or subject to rapid re-uptake into the cells. Both NPY and galanin are relatively resistant to degradation, and neuropeptides in general, have not been shown to taken up into cells after release [263]. However, as these peptides are not degraded rapidly there will be a degree of diffusion from site of release, with microprobes likely to show some spread outwith the actual site of release. Thus, although the peak of the detection area is likely to be in the zone of release, the spread around this may reflect diffusion of the peptide rather than other sites of release.

The detection of released peptide is referred to as "immunoreactive" (ir)-peptide release as the polyclonal antibody used binds to only one terminus of the peptide being studied. When using the microprobe method, interpretation of the significance of "ir-neuropeptide" is greatly aided by knowledge of the siting of the amino acid sequence to which the antibody binds, and whether degradation products also contain this sequence intact. Precursor molecules do not generally cause a problem as they do not tend to be released, but remain intracellular during processing of the peptide. If antibodies to both C and N terminus of the peptide are available, then this can be used to increase the likelihood that the substance being detected is in fact the peptide being studied. Substance P gives similar results with use of either C- or N-terminus directed antibodies [363]. However, with Bolton-Hunter labelled peptides, the binding of antibody to this portion of the molecule is hindered, thus making detection inaccurate.

Figure 2. Principles of the antibody microprobe technique

This is illustrated using a microprobe coated with galanin antibody as an example.

A. The antibody microprobe technique uses a series of treatments to the glass microprobes which are described in detail in the text. This results in immobilisation of galanin antibody (Y) on the outer surface of the microprobes. For clarity only one side of the microprobe is shown as having a coating of galanin antibody.

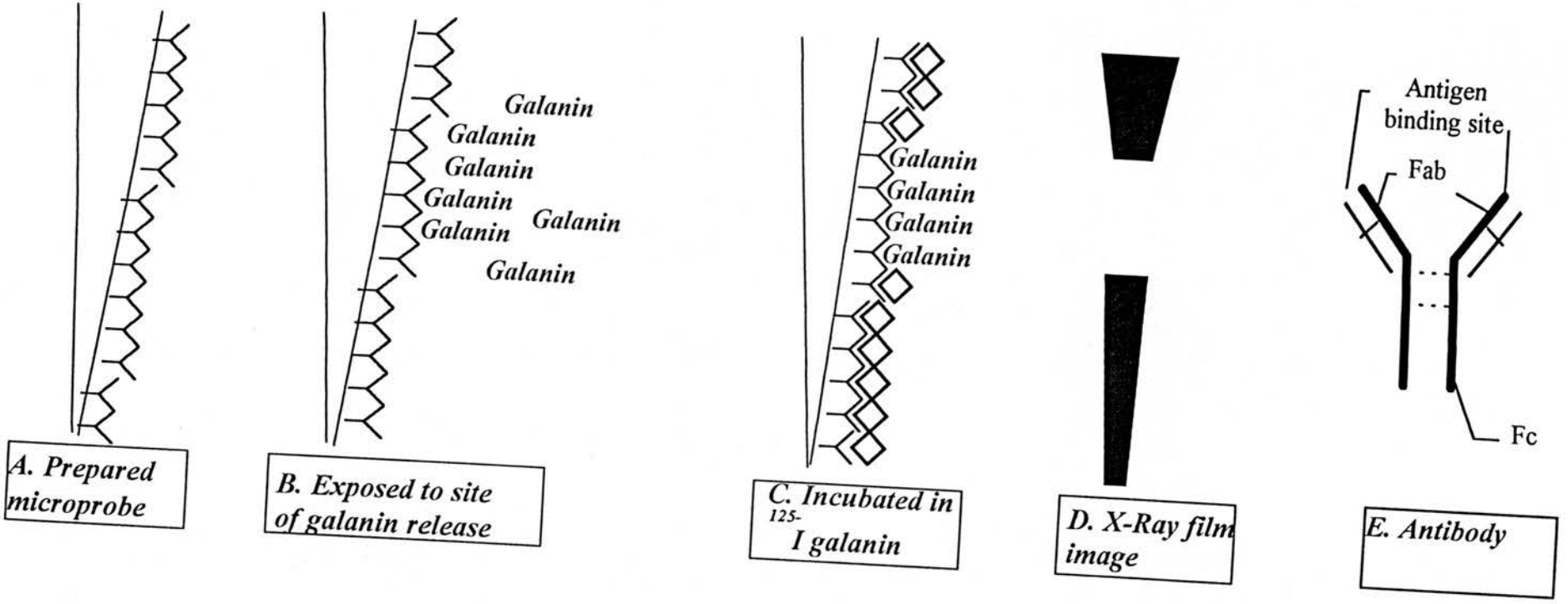
B. The coated microprobes are inserted into the spinal cord to a known depth and endogenous galanin binds to the specific and sensitive galanin antibody, at localised sites of release in the spinal cord.

C. The microprobes are then removed from the cord, washed and then incubated in radiolabelled ^{125}I galanin (◇) at 4°C for 24 hours. This allows binding of the radiolabelled galanin to any unoccupied antibody sites on the microprobes.

D. After washing, the microprobe tips are placed on card and an autoradiographic image of each microprobe is obtained by exposure for 8-10 days on X-ray monoemulsion film. Areas where endogenous galanin has bound are represented by deficits in binding of radiolabelled galanin, and thus areas of lower silver grain density. This can be analysed using an image analysis system and then related to the actual site of release within the spinal cord.

E. This is a diagram of the structure of the IgG antibody that is used. It consists of 4 peptide chains linked by disulphide bonds: two heavy and two light chains. The arms of the molecule - the Fab-fragments contain the antigen binding site, whereas the Fc fragment is the part that is immobilised onto the microprobe.

Figure 2. Principles of the Antibody Microprobe Technique



2.1.1 Chemicals

Antibodies

The particular antibodies used will be described in the appropriate chapter, but all were polyclonal antibodies raised in rabbit, against rat peptide sequences. As the antisera were polyclonal, other immunoglobulins of varying affinity and specificity were also likely to be contained in the freeze dried lyophilisate supplied by the manufacturer, and the degree of purification of this lyophilisate was unknown. The series of processes involved in antibody immobilisation serve to concentrate the specific and sensitive antibody of interest on the outer surface of the probe. All the antibody used for the NPY studies was obtained from Peninsula Laboratories, Inc, Belmont, Ca, USA. The galanin antisera were obtained from two sources - Peninsula Labs, as for NPY and Advanced Chemtech, Louisville, Kentucky, USA. The lyophilisate already contained buffering salts and bovine serum albumin, and was therefore dissolved directly in a small volume of Millipore filtered distilled water to the appropriate titre. This was then aliquoted out into Eppendorfs and frozen at -20 or -70 °C until used for microprobe coating. Once the antiserum was made up in solution it was never refrozen, but used straight after thawing, with any unused portion being discarded. The titre used for each antibody was kept constant during each series of experiments, and regular *in vitro* testing was carried out in parallel with the *in vivo* use of the antisera to ensure continued sensitivity and specificity at the titre used, during the time course of the experiments. One problem with using polyclonal serum was that the sensitivity of the antibody tended to vary between different lot numbers. These commercially produced antibodies varied to a slight extent in the antigen binding site resulting in a mixture of IgG molecules with high and low affinity for the inoculated peptide, as the response to antigenic peptide differed between animals. High affinity antibodies are needed for microprobes, since the labelled neuropeptide must not displace bound endogenous ligand to any significant extent. In those cases where the *in vitro* testing indicated a major change in sensitivity for any batch of antisera, this was discarded and not used for *in vivo* work.

Peptides

A range of peptides were used for *in vitro* testing of each antiserum to ensure specificity and test sensitivity. All peptides were obtained from Sigma Chemical Co, UK in freeze dried form, and reconstituted in phosphate buffered saline (PBS). All handling of peptides was done using silanised (Sigmacoted) containers, and the aliquots were stored at -20 or -70 °C until used for *in vitro* testing. Sigmacoting was used to prevent the peptide being adsorbed to the surfaces of any plastic material, which would reduce the accuracy of the *in vitro* testing. Any remaining peptide was discarded.

Radiolabelled compounds

Commercially prepared ^{125}I-labelled peptides were purchased in 10 μCi batches either from Amersham, UK or from Peninsula, Ca, USA, labelled with Bolton and Hunter's reagent. This reagent is [N-succinimidyl 3-(4-hydroxyphenyl) propionate] a molecule by which iodine can be coupled to the peptide via the phenol group. The molecule is linked to the peptide by its carboxyl group, either directly to free amino groups on lysyl or to the NH_2- terminal of the peptide [41]. Freeze dried ^{125}I-Bolton-Hunter peptides were dissolved in Millipore filtered distilled water with bacitracin. The bacitracin has been shown to improve the stability of the peptide in solution [225]. The resultant solutions were then divided into 2.5-10 μl aliquots, frozen and stored in a dedicated, lead-lined freezer at -20° C. For *in vitro* or *in vivo* work, the ligands were thawed then made up in a solution of PBS/azide containing either 0.5% bovine serum albumin (BSA) or 0.5% casein. These general binding agents served to reduce levels of non-specific binding to proteins, and non-specific binding to surfaces was decreased by Sigmacoting all surfaces in contact with the radioactivity. Previous work in the laboratory, and elsewhere [150] had shown that for NPY, 0.5% casein in the solution decreased levels of non-specific binding and improved the sensitivity of the assay to a greater extent than BSA. The final dilution was such that the concentration used gave a count of 2000 cpm/μl, using a gamma-counter. All radiolabelled materials were stored on ice until immediately before use. The half life of ^{125}I is approximately

60 days, therefore each batch of radiolabelled peptide was used for a maximum of 6 weeks.

2.1.3 Production of antibody microprobes

Preparation of glass microprobes

The microprobes were made from non-filamented, hollow borosilicate glass tubing with an outer diameter of 3 mm, inner diameter 1.62 mm (GC 300-10, Clark Electrochemical Instruments, UK). Although the glass was supplied pre-washed, it was important that the silane based coating needed for the antibody immobilisation was completely uniform, and for this reason all glass was washed as follows: Batches of approximately 50 glass tubes were incubated in xylene for a minimum of 24 hours, then washed in absolute alcohol for 30 minutes. This was followed by 2 further alcohol washes, then each batch was covered and left to dry in a clean oven at 200°C for two to three weeks before being used in the manufacturing process. Using a vertical microelectrode puller (PVS, University of Edinburgh) individual microprobes were pulled. Unlike microelectrodes, where it is desirable to have an abrupt taper in order to minimise electrical resistance a quite different shape is needed for this technique. An even, gradual taper was needed to produce a very fine probe tip in order to minimise trauma to the spinal cord during *in vivo* use, and allow breaking of the terminal 1cm without shattering. Additionally, it was essential that groups of microprobes that were to be compared to each other had a similar shape, otherwise differences in the physical shape would have prevented interpretation of differences between groups due to biological causes. For this reason a standard setting was used for the heat of the platinum coil and magnetic force exerted by the puller. To obtain the long thin taper it was necessary to melt a fairly large area of glass - thus a 1 cm length of platinum coil was used. Platinum has a high specific heat capacity and low resistance, thus allowing a high enough temperature to be reached. A diagram of the microprobe puller used and the microprobe shape produced is shown in Figure 3. After the probe had been pulled, several methods were used to ensure standardisation of shape. Visual inspection of every probe in comparison to a standard was used as an initial screen. From each batch of probes pulled a selection were chosen at random

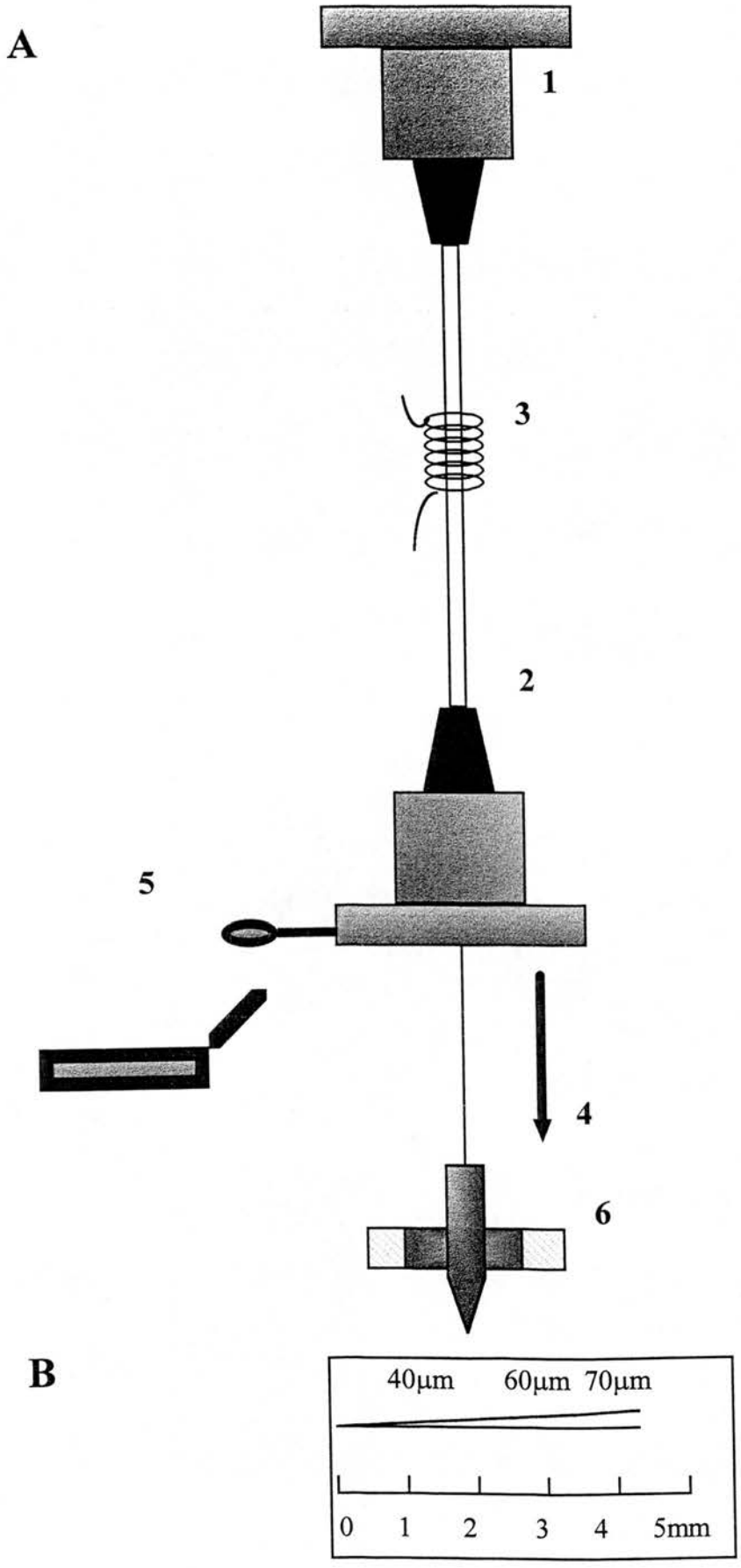
and analysed in the computerised image analysis system to ensure consistency of shape. Subsequently, the thick end of the probes were heat sealed in a gas flame, and the thin end of the probes were sealed by briefly touching the tip against a heated micro-coil (PVS, University of Edinburgh), using a micromanipulator (D10 Positioner, Research Instruments, UK) and binocular microscope to position the probe for heat sealing. At this time a further visual inspection was carried out on each probe, using a graticule with 10 μm divisions to measure probe diameter at the tip and at 2.25 mm from the tip, where it was approximately 40 μm.

After tip sealing, probes were placed into specially designed glass carrying buckets, holding up to 20 probes, with perforations in the base to allow drainage of any of the liquids used in the treatment process. Probes were washed in 50% nitric acid for 30 minutes, in order to promote the formation of silanol groups on the surface [446]. To remove the residual acid, probes were then washed in Millipore filtered distilled water for three times ten minute washes. Probes were dried for a minimum of four hours in a clean oven at 200°C. The aim of this process was to produce uniformly shaped microprobes with some degree of surface etching, on which the further reactions needed for good immobilisation of the antibody could occur.

Figure 3. Manufacture of glass microprobes for antibody coating

A. This diagram represents the basic principles of the probe puller used to produce the finely tapered microprobes required for *in vivo* work. The capillary glass is held firmly between the two holders (1 & 2), running through the platinum coil (3), at the midpoint of the length of the glass. It is important to ensure that the glass is exactly in the centre of the coil so that it is heated evenly. The coil is attached to a device allowing minor adjustments in position in relation to the capillary glass. The lower holder (2) is attached to a solid iron weight (4) by a strong metal cable. The heater to the coil is switched on and heats the coil, which in turn melts the glass within the area of the coil. The heated glass stretches and the lower holder gradually drops until a switch (5) is activated. This triggers a strong electromagnetic field (6), that rapidly pulls the weight and attached holder down to the base, drawing out the molten glass. This reaches a very gradual taper and separates at the tips to produce two microprobes. The taper can be varied by altering the heat of the coil, the distance to drop before the switch is activated and the strength of the force exerted by the electromagnetic field.

B. This shows the desired shape of a microprobe, with a long, fine taper. All probes are inspected under the microscope with a graticule to ensure the correct dimensions.


A
1
3
2
5
4
6
B
40µm
60µm
70µm
0
1
2
3
4
5mm

Siloxane coating process

The next step in preparing the microprobes for antibody immobilisation was to evenly coat the outer surface of each probe with a siloxane polymer bearing free amine groups. This process was carried out in sealed glass boiling tubes, each containing one bucket with approximately 20 probes. The mixture required for coating consisted of 30 ml of reagent grade toluene (Aldrich, UK), plus 10ml of gamma-aminopropyltriethoxysilane (APTES) (Aldrich, UK), plus 4-7 µl of distilled water. The amount of water was varied in order to control the amount of siloxane polymerisation at the surface of the probes and thus the thickness of coating. If a greater thickness was needed the amount of water was increased. The toluene solution had previously been treated with molecular sieves (150 g sieves/ 2.5 l toluene) for a minimum of 24 hours, in order to remove as much water from the mixture as possible. Water is critical in the coating process, as any excess results in siloxane polymerisation in the solution and not on the surface of the probes.

The buckets of glass microprobes were removed from the oven and lowered into the toluene / APTES mixture, in a fume cupboard. The stoppered tubes were then centrifuged (Mistral 2L, MSE Science Instruments, UK) at 6°C, 2000 rpm for 1 hour in order to increase the thickness of siloxane coating, by removal of small particles of debris or free water away from the probes, and increasing polymerisation on the probes rather than in the solution. After 24 hours at room temperature, the probes were removed from the boiling tubes and placed in an oven at 200°C for at least 24 hours. Heat curing of the probes in this way resulted in a stable, water-insoluble siloxane polymer coating on the microprobes' surface, and maximised the number of free amine groups available without steric hindrance [61].

After heat curing, each microprobe was examined under a binocular transmission light microscope at x125 magnification to assess the coating. A good coating appeared as a fine granular layer on the surface of the microprobe. This must evenly cover the probe for at distance of at least 4 mm from the tip. Any probes with patchy areas of coating or focal aggregations of polymer were discarded. Probes with too thin a coating were put through the coating process for a second time. Microprobes were stored

horizontally in specially designed boxes to protect from dust contamination and to protect the probe tips. After heat curing microprobes can be kept for at least six months prior to being coated with antiserum. Probes were sorted into those with heavy or medium coating and only those with similar density of coating used in any one experiment. As well as providing a chemical base for the next step in the process, the coating significantly increased the surface area on the probe eventually available for antibody immobilisation [108].

Antibody immobilisation to siloxane coated microprobes

This step of the process was carried out immediately before *in vivo* or *in vitro* use of the microprobes, as once the microprobes were coated in antibody they had a limited time during which they could be effectively used. This process took a minimum of 48 hours, therefore the preparation of peptides and radiolabelled compounds, and animals for *in vivo* studies had to be co-ordinated to prevent wastage of probes. This process was designed to immobilise the specific antibody of interest onto the outer surface of the probe. Despite this, when results from testing antibodies *in vitro* assays did not meet the criteria for specificity and sensitivity required for *in vivo* use, comparative assays using radioimmunoassay did not detect problems with the antibody. Although the process of antibody immobilisation may have interfered with the antibody affinity in some way, this discrepancy between radioimmunoassay and assays using microprobes with the same antiserum may be due to the fact that in RIA, tracer and endogenous ligand compete for the binding site at the same time. In the antibody microprobe technique, the antibody has to have a high affinity for the endogenous peptide, as the probes are sequentially incubated, first *in vivo*, with endogenous peptide, then *in vitro*, with labelled peptide.

The first stage of antibody immobilisation involved treatment of microprobes with glutaraldehyde. This was used as a cross-linking molecule, reacting covalently with the free amine groups of the siloxane polymer and the amine groups of protein A which is used subsequently. Buckets of 10-12 probes were immersed in a 2.5% glutaraldehyde solution (BDH Laboratory Suppliers, UK) for 30 minutes. This was followed by three 10 minute washes in distilled water.

Protein A (Sigma Chemicals) was then used, diluted in phosphate buffered saline containing 0.1% sodium azide (PBS-A) to a final concentration of 0.1 mg/ml. Protein A is a component of the cell wall of Staphylococcus aureus, and selectively binds to IgG fractions of the antisera. The protein A effectively excludes many unwanted proteins, and allows concentration of the specific antibody onto the outer surface of the probes. The Fc portion of immunoglobulins of the IgG class are bound by protein A whilst leaving the peptide binding sites free [143].

The tip of each microprobe was carefully inserted into a glass capillary filled with 5µl of diluted protein A, visualised under a dissecting microscope. Any probes with broken tips were discarded. The microprobes were then placed horizontally on a perspex rack and stored at 6°C for a minimum of 24 hours in a covered plastic tray containing PBS-A, to prevent drying out of the capillaries by evaporation.

The final stage in the preparation couples the antibody to the protein A now coating the microprobes. The protein A containing capillaries were removed from the tip of the microprobes and discarded. The microprobes were placed in glass tanks containing sodium borohydride (Sigma Chemicals, UK) 2.5% w/v in borate buffer for 5 minutes, during which time they were repeatedly moved in order to disperse hydrogen bubbles forming on their surface. This process reduced Schiff bases produced by aldehyde coupling to amino groups on protein A, and stabilised the binding of protein A. Batches of 10-12 microprobes were then put into buckets, and given three 10 minute washes in PBS-A solution. Glass capillaries (20µl, Camlab, UK) were then filled with antibody solution and the tip of each probe inserted as described for the protein A process. Microprobes underwent a minimum of a 24 hour incubation period in the antibody at 6°C.

2.1.4 *In vitro* testing of antibody coated microprobes

The end result of the series of processes just outlined was to produce fine glass microprobes with the antibody of interest immobilised onto the outer surfaces. Before being used to study the *in vivo* release pattern of neuropeptides, it was essential to check the manufacturer's data on antibody specificity and to determine the sensitivity

of each antibody after immobilisation. Additional checks were carried out to ensure consistent coating with antibody and to determine levels of non-specific binding. Each new batch of antibody was tested extensively prior to any *in vivo* use, and *in vitro* testing was carried out concurrently during *in vivo* experiments to ensure that the sensitivity and specificity of the assay was maintained.

For the *in vitro* tests, batches of 5-10 prepared microprobes were washed in PBS-A for 15 minutes. The subsequent treatment depended on what group each probe belonged to, but any incubations with other peptides, for cross reactivity studies, were carried out in 5 µl capillary tubes. All handling materials for peptides were Sigmacoted to prevent the peptides adhering to the glass or plastic surfaces. Microprobe tips were inserted into capillary tubes with the aid of a binocular microscope to minimise damage to probe tips.

The specificity of the immobilised antibody was checked by measuring the degree of inhibition of ^{125}I peptide binding by related peptides, using a gamma counter. A control group was used to check binding of the radiolabelled peptide to the antibody in the absence of any other peptides. This interaction depends on adequacy of antibody microprobe coating, binding affinity of antibody-peptide, and the correct binding of ^{125}I label to the peptide, as supplied by the manufacturer. Further groups of microprobes were incubated in 10^{-5} M solutions of a range of peptides for 30 minutes at 37°C then washed in ice cold PBS-Tween , followed by incubation in ^{125}I peptide for 24 hours at 6°C. After this, probes were washed in PBS-Tween for 15 minutes, the terminal 1 cm of the tips broken off and stuck to small pieces of card. Each piece of card was labelled according to the incubation process undergone by that probe, then placed in individual counting tubes for counting in the gamma counter. After counting, the cards were placed on X-ray monoemulsion film for 6-10 days to produce autoradiographic images for each probe. This was done as a check to ensure that there was uniform binding of the tracer to the surfaces of the microprobes.

The antibodies used were all C-terminus directed. The peptides used in cross reactivity studies are found in the dorsal horn, and included galanin, neuropeptide Y, VIP and substance P. Sensitivity tests were used to measure the degree of suppression of binding by a range of concentrations of the peptide being studied from 10^{-5} M to

10^{-9}M. The procedure followed was similar to that described for cross reactivity testing. An outline of a typical assay for galanin is given in Figure 3.

Every time a new batch of antibody was purchased, *in vitro* testing was carried out to ensure that:

1. Control counts were sufficiently high for that peptide to indicate adequate binding of labelled peptide to antibody. This varied depending on the peptide being studied, but was normally in the range of 1000 to 2000 c.p.m. for each control probe. Provided the assay met other criteria it was possible to use antisera-peptide combinations with low control counts, but the duration of exposure to develop autoradiographs that could be analysed was significantly increased. Unlike a conventional radioimmunoassay the antibody is exposed to the unlabelled peptide first, then incubated in the radiolabelled form A high affinity antibody is therefore required to ensure that bound unlabelled peptide remains bound to the antibody.
2. Incubation of probes in 10^{-5} M of the relevant non- radiolabelled peptide, prior to incubation with the radiolabelled form, gave at least 90% suppression of control counts, and 10^{-7} M gave approximately 50% suppression. Previous studies on tachykinin release have shown that this degree of suppression in *in vitro* assays was effective in detecting release in the spinal cord [112].
3. A range of concentrations of cold peptide, e.g. 10^{-5} M to 10^{-9}M, gave a corresponding range of suppression of control counts.
4. Levels of non-specific binding to the probes themselves (coated only with protein A) were less than 10%. A general binding agent such as BSA or casein was used to help reduce non-specific binding, and each stage was followed by washing in detergent (Tween 20 or 80), with the final wash for *in vivo* probes being carried out under suction.
5. If there was any doubt as to the adequacy of the coating procedure, antibody coated probes were incubated in ^{125}I $F(ab)_2$, which is raised against the Fc region of the IgG of the species in which the original antiserum was raised. Counts equal to or greater than those for control probes should be obtained, with autoradiographic images showing dense even binding along the length of the probe. This demonstrates that there is a good coat of antibody on the probe surface.

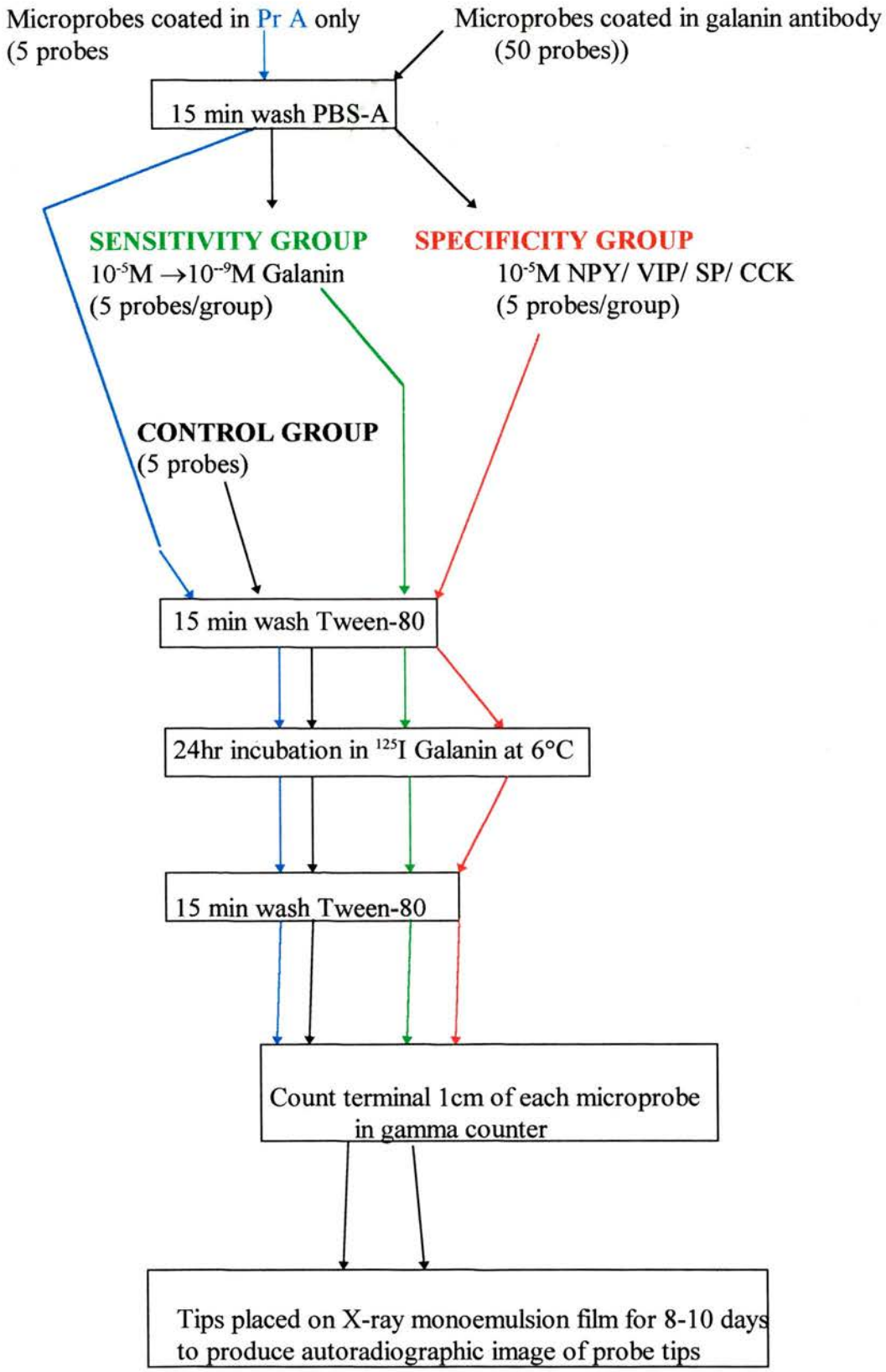
6. Cross- reactivity with other peptides was less than 10%, except for porcine forms of the peptide that would be expected to show higher cross reactivity.

Once these criteria had been met, the antibody could be used for *in vivo* work. During the course of a series of experiments (usually lasting about 6 weeks, as this was the useful life of the radiolabelled peptides), parallel *in vitro* testing was carried out each week to ensure that the assay continued to give acceptable results.

Figure 4. Typical *in vitro* assay for galanin

This diagram illustrates the procedure followed for an *in vitro* assay for a peptide, such as galanin, prior to *in vivo* use. The results obtained from such an assay allow confirmation of specificity from manufacturer's data, and give an indication of sensitivity under the conditions that will be used *in vivo*. The procedure is described in detail in section 2.1.4.

Figure 4. Typical *in vitro* assay for galanin



2.1.5 *In vivo* use of antibody coated microprobes

The process described in section 2.1.3 was followed to produce batches of approximately 30 antibody coated microprobes for each *in vivo* experiment. The experimental set-up used for the rat is described in detail in section 2.2.2.

On the day of experiment a fresh aliquot of radiolabelled peptide was made up in PBS-0.1% BSA or PBS-0.1% casein to result in a dilution of 2000 c.p.m./μl.

Shortly before use, microprobes were removed from the cold room, the antibody containing capillaries were discarded and the probes placed in buckets in PBS-A. The sealed, non-drawn out end of the probe was carefully removed using a glass saw. This allowed subsequent washing procedures to be carried out. Each probe tip was then examined under the binocular microscope at x125 magnification, with a calibrated graticule, and the tip size checked. If the tip was greater than 10 μm, indicating breakage during preparation, it was discarded. If the tip size was less than 10 μm, then it was gently broken back to this size against the metal coil previously used for heat sealing of the probes. Thus the tip of the probe was now open. This was why the final washing procedure was carried out under suction to remove any residual debris at the probe tip.

The peptide release experiments are described in section 2.2.2, but basically involved insertion of microprobes to a known depth into the spinal cord of the rat. This allowed binding of any endogenous peptide at localised release sites in the spinal cord. After washing, 24 hour incubation in radiolabelled peptide, and a final wash under suction, the tips of the probes were placed on card, next to X ray monoemulsion film (Kodak), with the emulsion side of the film in contact with the probes. The film was exposed to the probes for 6-10 days, depending on the intensity of image required and the peptide being used. After this time, the film was developed and an individual autoradiographic image was obtained for each microprobe.

2.1.6 Analysis of antibody microprobes [160]

A computerised image analysis system was used to analyse the autoradiographic images obtained from microprobes inserted into the spinal cord. *In vitro* microprobe images for each experiment were also analysed as controls. Binding of endogenously released peptide resulted in deficits in binding of ^{125}I-labelled peptide that were detected as a decrease in optical density by the analysis system. An simplified outline of the set-up used is shown in Figure 5.

For analysis, an autoradiographic image with a density well above the background silver grain density, but below the maximum density for the film, was required. If the image was too faint, then background subtraction could introduce errors. If the image was developed to the maximum density, then any variations in density from peptide binding would be obscured. The exposure length required varied between peptides, but for galanin, 8-10 days gave a good image, and for NPY, 6-8 days were sufficient. A clear image was achieved by having as close an apposition as possible of the probe tip onto the film, and ensuring no movement of the film against the card to which the tips were attached during the exposure period. If the tip was too far from the card, then an unfocused image was obtained, and if the film moved during exposure, then a double image was obtained. For each experiment it was usual to obtain two films of differing exposure times from each set of probes, so that if single probe images were unsatisfactory on one film, they could be used from the second film, thus allowing analysis of the majority of probes.

An Imaging Technology PC Vision Plus frame grabber board operating in a Data Control Systems 286e computer was used. The film was cut up into individual autoradiographic images, that were labelled and wiped clean with lens tissue before analysis. Single images were placed onto a microscope stage in a light proof box and illuminated from beneath through a narrow slit just wider than the image. The light source (2 X 25W microscope halogen bulbs) was mounted in a light box fitted with diffusion plates to give uniform distribution of light. A stabilised DC power supply

(Farnell) was used to power the light source and ensure an even and constant light supply.

A charged coupled device black and white camera (CCD, Panasonic) scanned the distal 5mm of each image, to give a video image for each microprobe tip, displayed on a colour monitor connected to the output of the image processing board. The automatic gain control function of the camera was disabled to allow use of the camera as a densitometer, by ensuring that the digitised video image was linear with respect to the autoradiographic density. The field of view of the camera produced a 512 x 512 pixel map, that was stored in the memory of the image board. Thus, each pixel corresponded to approximately 10 x 10 μm. The image board converted the video image to an optical density assigning an arbitrary grey scale from 0 to 255 to each location on the board, which was inverted by the processor for display, such that 0 = maximum light and 255 = complete blackness. If the light intensity from the light source was increased to greater than 255, linearity was lost from the greyscale. To avoid this, the light intensity was set at 1 when no film was present on the microscope stage, and the amount of light reaching the camera kept constant by varying the current of the light source.

Prior to analysis, incident light was subtracted using the average of two values of background density from unexposed areas on the film, and the focus adjusted such that a pre-calibrated scale bar equalled 1mm on the monitor screen. The first microprobe image, defined by a unique code, was then placed on the stage such that the probe was horizontal to the grid marked on the screen. Background subtractions were carried out by the computer, with the value of the background reading at each point being subtracted from the value for each pixel in the microprobe scan. The corrected image was displayed on the monitor in 16 false colours corresponding to 16 groups of grey scale values. Transverse integration of optical density were carried out to give a final resolution of 30 μm for each vertical column of pixels across the restricted part of the field containing the microprobe image [104]. For each microprobe image a plot of optical density (grey scale) against probe length was obtained. Areas where endogenous peptide had bound to the microprobe were shown by areas of low optical density.

Before comparing groups of probes it was necessary to correct for any variations in density simply due to variations in film exposure. This was done by choosing an area outwith the cord (thus avoiding the effects of endogenous ligand binding), and normalising all the probes to the chosen grey scale points over a length of 500 μm. Any images with obvious artefacts or damage were discarded from the analysis. Using a sorting program, it was possible to compare the means of defined groups of microprobes, at 30 μm intervals along the length of the probes. Mean image analyses were obtained from each group by plotting the mean grey scale value for each 30 μm interval along with the standard error of the mean, against the depth within the spinal cord. Pairs of mean image analyses representing different experimental manipulations, were compared statistically at 30 μm intervals, by calculating the difference between the two groups at each point. Each site on the probe was treated as being independent of other sites and thus comparisons between each site could be made. Statistical significance was assigned to each site using Student's unpaired t-test, and the *t* values thus obtained plotted against depth within the spinal cord. The t-test was used as the data at each point had previously been shown to be normally distributed, although analysis of variance could also have been used, with similar results. Both the difference between the two groups and the t values obtained were plotted against depth within the spinal cord.

Figure 5: Image analysis set-up

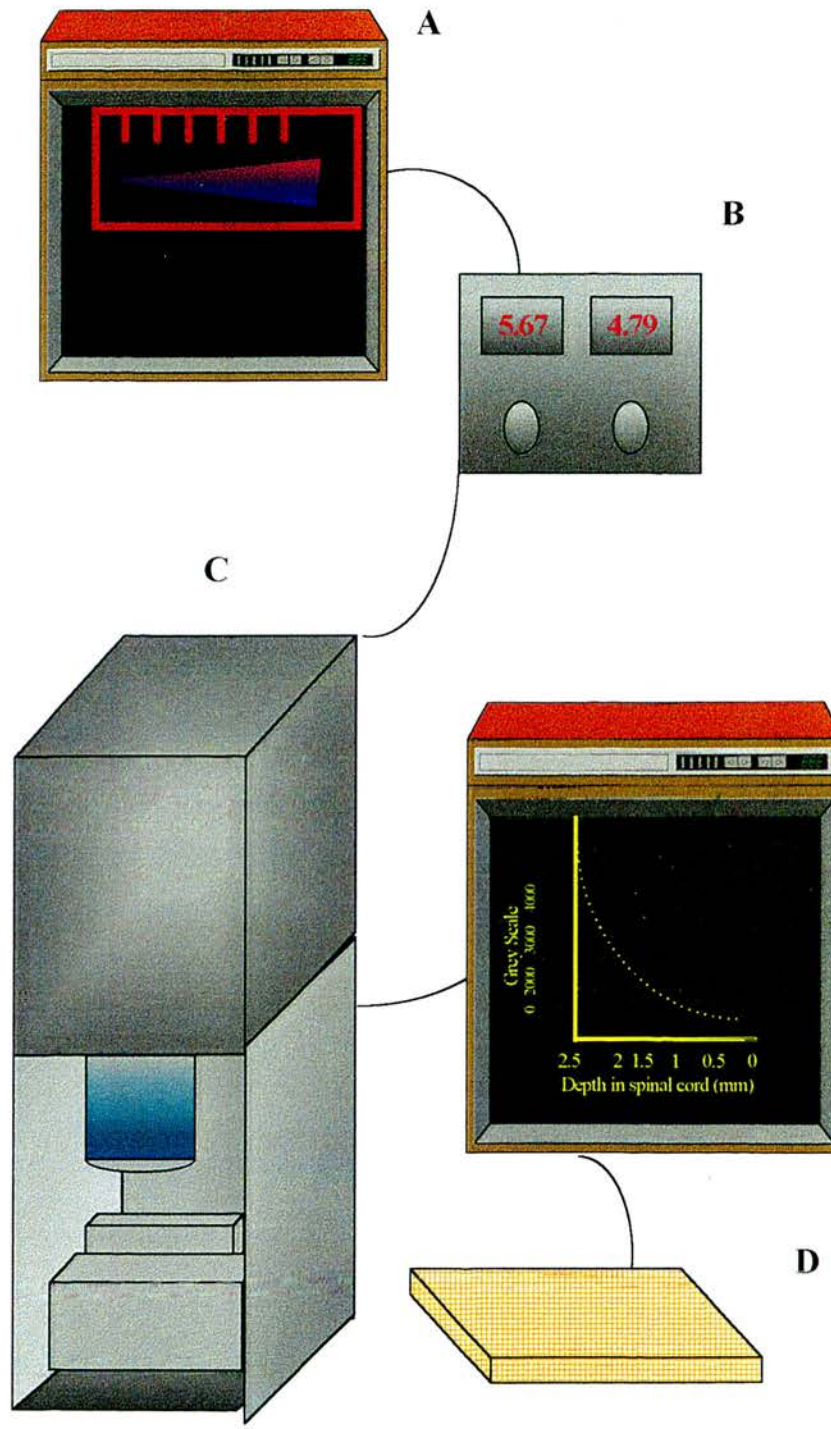
A. The colour monitor displayed the microprobe when it was aligned under the camera, and illuminated from below. The tip of the probe was seen at the left hand side of the screen, which was marked off in 1 mm intervals.

B. The light box was powered by a stabilised DC power supply to illuminate each image with a constant intensity of light.

C. The enclosed camera box was where the microprobe was placed, on top of the internal stage. The camera was then focused and the image appeared on monitor A. Probe position was adjusted so that the tip lay on the zero mm mark on the left hand side of the screen. The door of the box surrounding the camera arrangement was then closed to reduce levels of incident light and the analysis of optical density carried out along the length of the probe.

D. The computer monitor displayed the analysed image, The optical density was represented as a grey scale on the Y axis against the length of the probe on the X axis. Decreases in grey scale represented decreases in optical density and hence areas of endogenous peptide binding. The example shown here was typical of a control *in vitro* probe that has not been inserted into the cord and thus gave an indication of the underlying probe shape. The analysis programme could then sort groups of microprobes, that were plotted out as the mean (+/- s.e.m.) for each group on a plot similar to that illustrated.

Figure 5. Image analysis set-up



2.2 Animal Preparation

2.2.1 Preparation of Neuropathic Model

Male Wistar rats (200-320 g), Charles River Ltd, UK) were housed for at least two days before any handling in order to minimise the effects of stress secondary to transportation or change of environment. In those rats that were to be subjected to a nerve injury, behavioural testing was not carried out for the first two days after arrival in the animal unit.

A peripheral neuropathy was induced using the technique described by Bennett and Xie, 1988 [33]. Each rat was anaesthetised with 40-50 mg/kg of intraperitoneal sodium pentobarbitone, and maintained with supplemental halothane in oxygen, as required, supplied via a facemask. Full aseptic technique was used for this procedure, which was carried out in an operating theatre. Apart from the operation site, the rat was wrapped in insulating material to reduce heat loss. The skin area of the incision was shaved and thoroughly cleaned with a solution of Hibiscrubb in alcohol (Hospital Management & Supplies, UK). The sciatic nerve was exposed by an incision at mid thigh level and gently dissected free of surrounding connective tissue. The nerve was visualised under an operating microscope at x40 to ensure that four 4/0 chromic gut sutures were placed loosely around the sciatic nerve, proximal to its trifurcation. The ligatures were just tight enough not to slide freely on the nerve, but not as tight as to compromise epineurial blood flow. The tissue was closed in two layers with 4/0 Vicryl® (Ethicon, UK), to reduce tissue reaction to the suture material itself. A subcuticular stitch was used for the skin in order to try and minimise any gnawing at the wound or premature removal of stitches. The skin was then cleaned again with a solution of Hibiscrubb in alcohol. There were no cases of overt wound infection. Rats were allowed to recover in special cages with solid floors and no sawdust, under a heat lamp.

After the rats were fully awake, they were housed in solid floor cages in small groups of three to four, with free access to food and water, under controlled conditions of light and temperature. The guidelines for the care of experimental animals of the International Association for the Study of Pain were followed carefully [502]. Regular

monitoring of the rats to ensure that there was no evidence of excessive stress or pain was carried out daily in the post-operative period. If autotomy of greater than one proximal phalanx developed the rats were generally euthanased or used in an experiment that day. Regular behavioural testing was carried out to ensure that all the animals used in the release experiments did indeed have evidence of neuropathic pain. This is described in detail in section 3.1. At 10-14 days after ligature placement rats with behavioural evidence of neuropathic pain were used to study peptide release in the lumbar spinal cord, as described in Chapters 4 and 5.

2.2.2 Set up for peptide release experiments

Monitoring

Rats were anaesthetised with intraperitoneal urethane (1.25 g/kg) (Sigma Chemicals, UK). A 25% solution was used, and an induction dose of 0.5 - 0.7 ml/kg was given, followed by intermittent 0.25 ml increments as clinically indicated. Depth of anaesthesia was monitored by assessment of blood pressure, respiratory rate, absence of corneal blink reflex, movement of whiskers and hindpaw withdrawal reflex. The depth of anaesthesia was monitored regularly and further injections of urethane given as required. Vital signs were maintained in the physiological range. Continuous monitoring of blood pressure was commenced after the carotid artery was cannulated and connected to a pressure transducer, with an intermittent flush system using sterile Ringer's solution and 0.1% heparin. An external jugular vein was also cannulated to give intravenous access for warmed maintenance fluids or drugs. Several measures were taken to reduce heat loss and maintain body temperature between 36-38°C. Ambient temperature was increased by the use of additional heaters, and the rat was covered with insulating material. Body temperature was monitored continuously with a rectal probe, connected to a warming blanket. Any fluids given, including those for irrigation of the spinal cord were warmed to body temperature. Humidified supplemental oxygen was given via a T-piece inserted into the trachea, which aided unobstructed breathing, and allowed removal of excess secretions. If the rat deviated in any major way from normal physiological cardiorespiratory parameters, or body temperature dropped below 35°C, the experiment was terminated.

Surgical preparation

After intravenous and intra-arterial lines had been inserted, the injured sciatic nerve was exposed by an incision at mid-thigh level and gently dissected free of surrounding connective tissue. Once the rat was stabilised in the stereotaxic frame, a pool of warmed paraffin oil was formed around the nerve which was mounted on a platinum stimulating electrode, proximal to the nerve injury site.

With the rat placed in ventral recumbency, a midline skin incision was made from thoracic to sacral region of the spine, and the connective tissue and muscle layer from T11 to L5 dissected free of the vertebrae. The animal was supported and stabilised in a metal frame using bilaterally placed swan necked clamps below three pairs of transverse processes and ear bars from a separate neck support. The middle clamp was placed at the level of T13, as determined by the position of the floating rib, with the upper clamp one level rostrally and the lower clamp two levels caudally. This arrangement effectively immobilised the spinal cord, allowing accurate probe placement. Using an operating microscope, a laminectomy from T12 to L2 was performed to expose the lumbar spinal segments, L2 - L6. The cord was covered temporarily with cotton wool soaked in warmed sterile Ringers solution. Any bleeding points from the side of the laminectomy were packed with sterile haemostatic gauze (Spongel, Houde Laboratories). A pool was formed by suturing the four corners of the wound to bars on the corner of the frame, and filling with a thin layer of agar in Ringer's solution (4% w/v, Unipath Ltd, UK). A window was made in the solidified agar solution over the area of the cord under study, and the dura removed using sterile watchmaker's forceps. This pool was then irrigated with warmed sterile Ringer's solution at 37°C, this being removed by intermittent or continuous gentle suction, thus preventing drying out of the exposed cord, and minimising collection of any inflammatory exudates at sites of microprobe insertion.

The hindpaw with the dissected sciatic nerve was elevated and immobilised. The nerve was mounted on platinum stimulating electrodes as described above. The optimal sites for microprobe insertion in the spinal cord were determined by stimulation of the sciatic nerve at low intensity (1.5-2T, 1Hz, 0.1ms), and measuring the resultant cord

dorsum potential at several rostrocaudal sites, using a ball electrode. Microprobes were then inserted into the area where the largest field potential was measured.

Peptide release

Pairs of probes were inserted into the spinal cord, using two stepping micromanipulators (PVS, University of Edinburgh). Using the dorsal vein as an indicator of the centre of the cord, probes were inserted 500 μm from the midline, to a depth of 2.25 mm. This allowed study of peptide release throughout the dorsal and ventral horns in the rat. Previous studies in the laboratory have shown that for both NPY [258] and galanin [168], probes have to be left in the cord for 15 minutes to allow sufficient peptide - antibody binding to occur at release sites. To minimise the possibility of cord damage, probes were not inserted into exactly the same spot every time, but sites over the area measured were sampled during the course of the experiment. A maximum of ten probes was inserted into any one side of the cord for each rat. Damage to the spinal cord from excess probe insertion could result in extracellular release of enzymes from damaged cells, that might cause damage to the probes. By incubating probes in ^{125}I F(ab)$_2$ it was possible to demonstrate the integrity of the antibody coating on the probe surface. It was shown that there was no evidence of antibody damage using up to ten probes in each side of the cord. Probes were inserted into right and left sides of the cord alternately. After 15 minutes, probes were removed from the cord and washed in ice cold PBS-Tween-20 or -80 (depending on peptide) for 15 minutes. Each probe was then inserted into a 5μl Sigmacoted capillary tube containing the relevant ^{125}I peptide, then placed on a tray in a sealed container, containing PBS-A in the bottom to reduce evaporation, and prevent the probes drying out. The probes were incubated in the ^{125}I peptide for a minimum of 24 hours in the cold room at 4 °C. Next the probes were removed from the capillary tubes and washed in PBS-A Tween for 15 minutes under suction, in order to remove any tissue plugs or other debris from the tips. The terminal 1cm of each probe was then carefully broken from the rest of the probe and stuck to white cartridge paper using a small amount of white typing correction fluid on the proximal end. The card obtained from each experiment thus had approximately 26 probes that had been inserted into the cord, as well as approximately 6 *in vitro* probes for each

experiment. These were probes that had not been inserted into the cord, but simply incubated in radiolabelled peptide. In every other respect they were treated in exactly the same way as other probes used in the experiment. These probes provided an estimate of zero endogenous peptide binding, allowing an estimate of basal levels of neuropeptide in the spinal cord, in the absence of peripheral stimulation. They also gave an outline of the underlying basic shape of the probes, to allow comparison of the autoradiographic images. The card with the distal portions of the microprobes was placed in an X-ray film cassette with a sheet of monoemulsion film (Kodak NMC) for six to ten days to produce an individual autoradiographic image for each microprobe. To calibrate microprobe depth accurately within the cord, a microprobe previously filled with Pontamine Sky Blue was inserted into the cord to a known depth at the end of each experiment. Current was passed for 5 minutes to eject the dye as an anion from the probe tip. The rat was then euthanased with an overdose of 2.5 ml of sodium pentobarbitone (60 mg/ml) via the intravenous cannula. The spinal cord containing the dye spot was carefully removed and fixed in 25% formaldehyde. The exact position of the dye spot was measured in post mortem sections of the spinal cord, thus providing a direct measure of microprobe insertion, and a check on the accuracy of probe placement.

In those experiments where the effects of high intensity electrical stimulation of the injured nerve were being studied, neuromuscular blockade was necessary in order to remove the effects of excess muscle twitching on the stability of the spinal cord. As there is some evidence of involvement of the sympathetic nervous system in the development of neuropathic pain, it was desirable not to use any agent that might have either direct or indirect sympathomimetic effects. For this reason, vecuronium (1 mg/kg) was used as the neuromuscular agent of choice. This was given by repeated bolus injection, according to Home Office guidelines, as this allowed the assessment of depth of anaesthesia more effectively. A Harvard Animal Ventilator was used to ventilate at a rate of approximately 80 breaths / minute to maintain end tidal CO_2 (Datex Capnograph) at ~5%. This was monitored by sampling at the side arm of the T-piece in the trachea, and supplemental oxygen was administered via the ventilator.

Chapter 3. Effects of peripheral nerve injury on behaviour

3.1 *Introduction*

Since pain perception is subjective, there are obvious difficulties with the assessment of pain in animals. After a partial nerve injury, behavioural signs consistent with a chronic pain syndrome develop over time, as discussed in Chapter 1. There have been many tests developed to try and quantitatively assess the presence and degree of pain in such animals, with tests for mechanical, thermal and chemical allodynia and hyperalgesia. The degree of involvement of the different modalities varies depending on the type of nerve injury and the model being studied (see Table 2). Spontaneous pain, mechanical allodynia and hyperalgesia have been described after a chronic constriction injury of the sciatic nerve, and were studied here.

Spontaneous pain, arising in the absence of specific stimuli, has been assessed on the basis of changes in hindpaw position and movement. A modification of the scoring system used for the formalin test [102] has been adapted for use in neuropathic animals [12]. Thus a normal hindpaw position is rated at 0, to a grossly abnormal position, with the paw elevated and licked, scoring 3. A more precise description of paw posture has also been used [12] with up to 6 different positions, including ventroflexion of the toes and eversion of the hindpaw.

Mechanical allodynia is defined as a previously innocuous mechanical stimulus, such as light touch, being perceived as painful. In previous studies in this laboratory the Ugo-Basile Analgesymeter (Comerio-Varese, Italy) was used to test for mechanical allodynia. This device exerts a force that increases at a constant rate, via a dome-tipped plinth, to the dorsum of the rat hindpaw. It was originally developed by Randall and Selitto, whereby the threshold for paw withdrawal was used as a measurement for mechanical hyperalgesia [345], but has since been modified to use the threshold for vocalisation to increasing force as a measure of mechanical allodynia [199]. This avoids the problem of leg muscle weakness that can occur in nerve injured animals and affect paw withdrawal ability. It has been shown that training can improve the sensitivity of testing using an Analgesymeter [404]. However, in our hands, a high degree of variability

with individual animals was found with this device, despite training carried out prior to the nerve injury. There was also evidence of stress in the animals probably due to restraint, with hyperventilation, squealing and excess defaecation. For this reason, testing of mechanical allodynia in the studies described here did not use the Analgesymeter, but instead used a technique that could be carried out in unrestrained animals.

Von Frey hairs are a graded series of nylon monofilaments, which allow a quantitative assessment of sensitivity to touch, as the rat moves unrestrained on a cage floor. These were used to assess the degree of mechanical allodynia by establishing the lowest threshold to which the animal withdrew its hindpaw. The rats did not appear to be unduly stressed by this technique in contrast to the response seen with the Analgesymeter.

Table 5 Conversion values for von Frey hairs

This shows the values used to convert the markings on the handle of each von Frey hair to force in grams exerted by pressure on the plantar surface of the hindpaw. Theses values are derived from manufacturer's data (Stoelting, WoodDale, Ill, USA).

Table 5 Conversion values for von Frey hairs

Filament Marking	Hair diameter (mm)	Force (g)
4.08	0.229	1.202
4.17	0.254	1.479
4.31	0.305	2.041
4.56	0.356	3.630
4.74	0.381	5.495
4.93	0.406	8.511
5.07	0.432	11.749
5.18	0.483	15.136
5.46	0.559	28.84
5.88	0.711	75.85

Mechanical hyperalgesia is defined as an exaggerated response to a normally painful stimulus. Assessment of mechanical hyperalgesia is also possible by measuring the withdrawal response to pin prick or noxious pinch. The effects of a repeated noxious stimulus on response must be considered, as there is evidence that repeated noxious stimulation can lead to changes in the spinal cord and, sub-acutely, wind up of the response [2,464,465]. The paw withdrawal duration to pin prick was used in these studies as a quantitative assessment of mechanical hyperalgesia.

3.2 Methods

All rats that had undergone loose ligation of their sciatic nerve, as described in section 2.2.1, underwent a series of behavioural tests to ensure that study of the spinal cord changes in these animals was carried out only in rats with definite evidence of neuropathic pain. Prior to the nerve injury, pre-operative control values were established. The animals were observed for the three days immediately after nerve injury, to monitor full recovery from the residual effects of anaesthesia and surgery. From day three onwards until the day of the acute experiment at 10 - 14 days after ligature placement, a regular series of tests was carried out. All tests were performed on individual rats, with the other rats being studied screened from the test area.
The rat was placed on an inverted wire cage lid and allowed to acclimatise, unrestrained, while being observed for the characteristic changes seen in this model [12,33]. The rat tended to hold the injured leg off the cage floor, hopping, with the hindpaw everted and the toes ventroflexed. Although overt autotomy was rare, in the first few post-operative days it was not uncommon for one or two of the nails to be gnawed. If this progressed to damage to the phalanxes, the rat was either euthanased, using 180 mg/kg of i.p. sodium pentobarbital, or used in an experiment that day.
Once the rats had acclimatised, von Frey hairs (Stoelting, WoodDale, IL, USA) were used to assess the degree of mechanical allodynia. Starting with a very fine von Frey hair, inserted through holes in the cage floor, the plantar aspect of each paw was touched lightly up to five times on each paw or until the animal had exhibited a withdrawal response. If no withdrawal occurred the next hair up in the series was used in order to establish the lowest threshold at which paw withdrawal occurred. The von Frey filaments were labelled as Log 10 of (10 * Force in milligrams). The force

exerted by each hair is shown in Table 5. The highest value used was 5.88, as after this the filament was thick enough to lift the hindpaw from the cage floor. The exact force exerted does vary slightly depending on temperature, humidity and the exact shape of the cut off end. To minimise variations, the same set of hairs was used for all testing, which was always carried out in the same environment.

The duration of paw withdrawal to pin prick was used as a measure of mechanical hyperalgesia [406]. The plantar aspect of each hindpaw was touched lightly with a 16G hypodermic needle, sufficient to indent, but not pierce the skin. Normally a rapid and transient withdrawal response is elicited, which was estimated at approximately 0.5 sec. The withdrawal response that developed on the side of nerve ligation was much more prolonged, and often accompanied by licking of the paw or squeaking. The length of time for which each hindpaw was withdrawn above the cage floor was measured using a stopwatch. The stimulus was delivered only once to each hindpaw during any testing session, and the total number of testing sessions was limited. This was done to avoid any confounding factors that might arise from repeated noxious stimulation, such as sensitisation or tissue damage.

Statistical comparisons between right (injured) and left (uninjured) hindpaw withdrawal thresholds and durations were made using a Mann Whitney Rank Sum Test. Comparisons over time, from pre-operatively to 10- 14 days after nerve ligation were made using Kruskal-Wallis One Way Analysis of Variance on Ranks. $P<0.05$ was taken to indicate a statistically significant difference.

Histology

Some basic histology was carried out in addition to the behavioural testing, as a further confirmation, that in this laboratory, the histological changes expected to be associated with this model were seen. Thus 10 to 14 days after nerve injury, following the peptide release experiments and euthanasia of the animal, both the injured and uninjured sciatic nerves were dissected out and placed in formalin. An example of the gross specimen with the four loose ligatures around it is shown in Figure 6. The histology laboratory of the Dept of Preclinical Sciences sectioned the injured and uninjured nerves, and stained the sections for myelin with solochrome cyanin [330]. A typical example of this is shown in Figure 7.

Figure 6 Specimen of sciatic nerve excised after chronic constriction injury

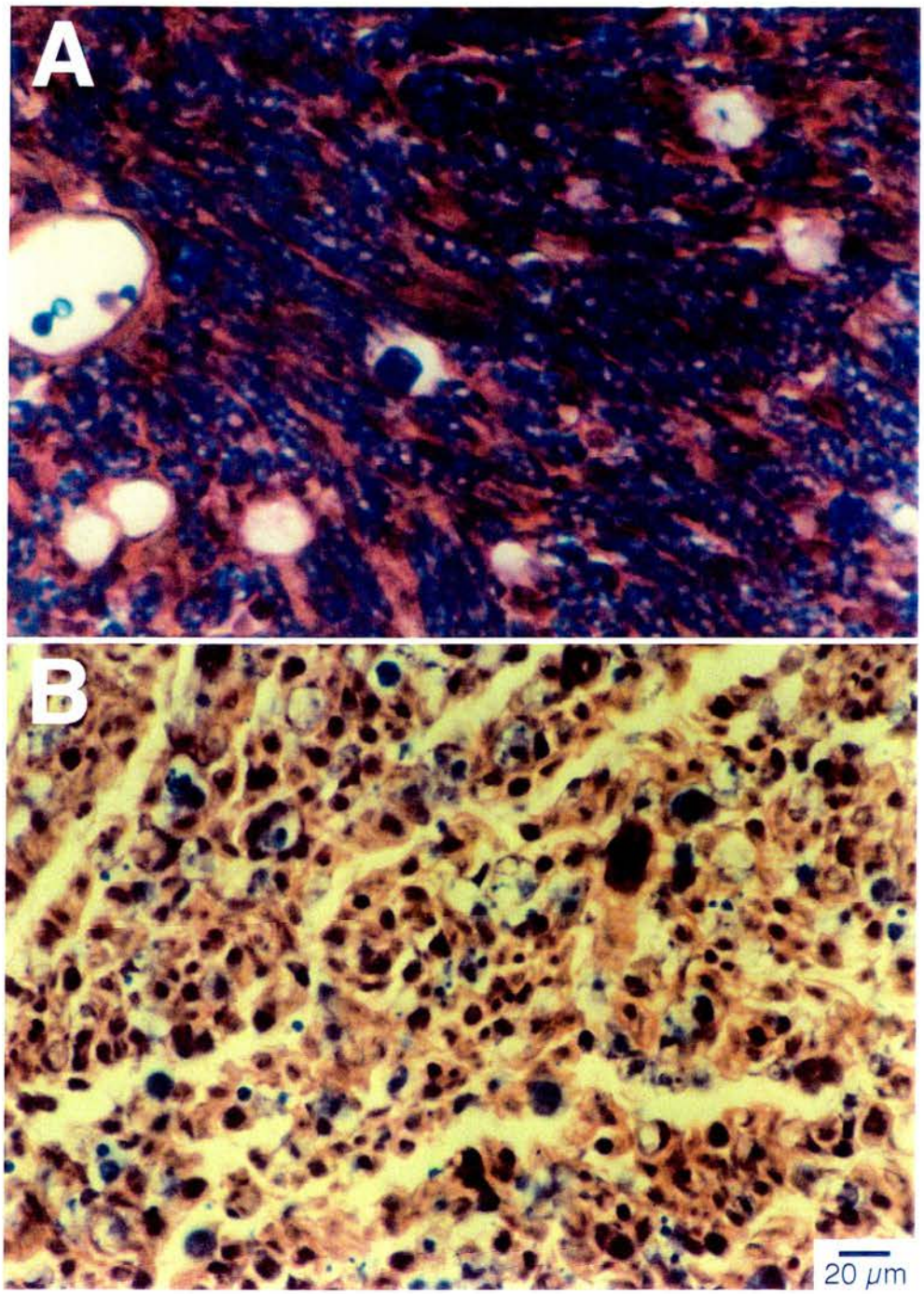
This shows a typical example of a sciatic nerve that was excised 12 days after four loose ligatures were placed around it, as described in section 2.2.1. There was extensive fibrosis around the site of the ligatures, and when these were removed there was evidence of constriction of the underlying nerve, as described by Bennett and Xie [33].


1mm

Figure 7. Sections from normal and ligated sciatic nerves

A. This shows a typical example of a transverse section taken from a sciatic nerve that had not been subjected to loose ligation. With solochrome cyanin, myelin shows up as a deep purple colour, that can been seen extensively throughout the section.

B. This is a section taken distal to the nerve injury site, 12 days after 4 loose ligatures were placed around the sciatic nerve. When compared to the section from normal nerve it is apparent that there is extensive loss of myelin. This corresponds well to the descriptions of changes occurring after this type of partial nerve injury [23,297], as described in section 1.2.6.


A
B
20 μm

3.2 Results

The characteristic changes in gait and hindpaw position were apparent in all animals from day three after ligature placement. A limp was evident and the paw held such that only the medial aspect touched the cage floor when walking. The foot was everted with the toes tightly ventroflexed, and there was evidence of abnormal grooming behaviour.

Von Frey hairs

The pre-operative control values of right and left hindpaws were virtually identical. However, from day 3 after loose ligation of the right sciatic nerve, a highly significant difference was detected between right and left hindpaws at each time point tested with a Mann Whitney Rank Sum test ($p<0.0001$). The values obtained at each time point are shown in Figure 8. Using Kruskal-Wallis One Way Analysis of Variance on Ranks, there was found to be a highly significant difference ($p<0.0001$) between the median pre-operative paw withdrawal threshold and each post operative time point tested for the right side (i.e. the side of nerve injury). The values for the left side remained relatively constant during the study period.

Pin Prick

The pre-operative control values between right and left hindpaws were essentially identical. At the time points tested in the post -operative period, there was a highly significant difference ($p<0.0001$) between right and left hindpaws as tested by a Mann Whitney Rank Sum test, and between pre-and post-operative values for the right hindpaw, as tested by Kruskal-Wallis One Way Analysis of Variance on Ranks. The mean values (+/-s.e.m.) are shown in Figure 9.

Figure 8 Response to von Frey hairs

A. The mean values +/- standard error of the mean (s.e.m.) for the paw withdrawal thresholds to von Frey hairs (grams) is shown for right and left hindpaws during the duration of the study period. The right side was the side of the loose ligation of the sciatic nerve, whereas the left side was unoperated. ** indicates where $p<0.0001$, for a difference between right and left sides, and from pre-operative control values.

B. The mean values (+/- s.e.m.) for paw withdrawal threshold to von Frey hairs (grams) are shown for each hindpaw from the pre-operative period until the day of experiment at 10-14 days after nerve injury.

A

Response to von Frey hairs - Paw withdrawal threshold (g) (n=48)		
No. of days after nerve injury	RIGHT: Mean +/- s.e.m.	LEFT: Mean +/- s.e.m
pre-op(control)	68.4 +/- 2.8	68.4 +/- 2.8
Day 3-4	5.5 +/- 0.4**	64.7 +/- 3.3
Day 6-8	3.0 +/- 0.3**	67.8 +/- 3.0
Day 10-14	2.3 +/- 0.1**	66.0 +/- 3.1

B

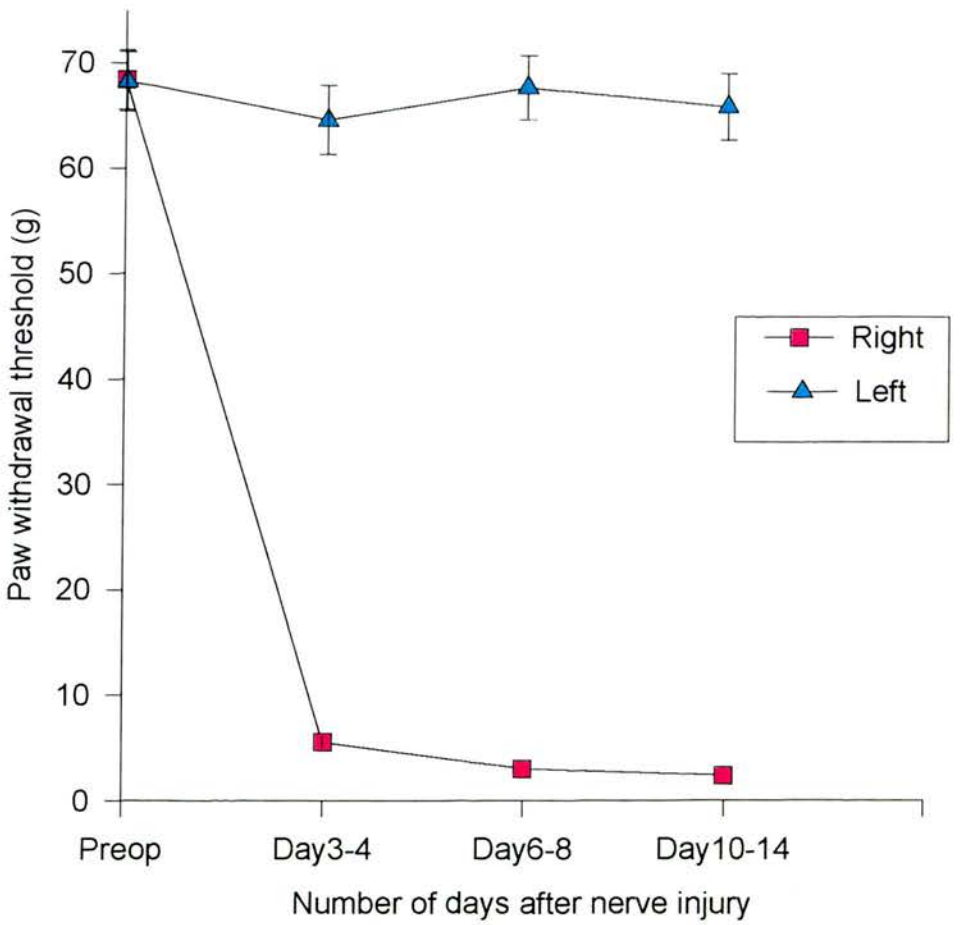


Figure 9. Response to pin prick

A. The mean values +/- standard error of the mean (s.e.m.) for the paw withdrawal duration to pin prick (s) are shown for right and left hindpaws during the study period. The right side was the side of the loose ligation of the sciatic nerve, whereas the left side was unoperated. ** indicates where $p<0.0001$, for a difference between right and left sides, and from pre-operative control values.

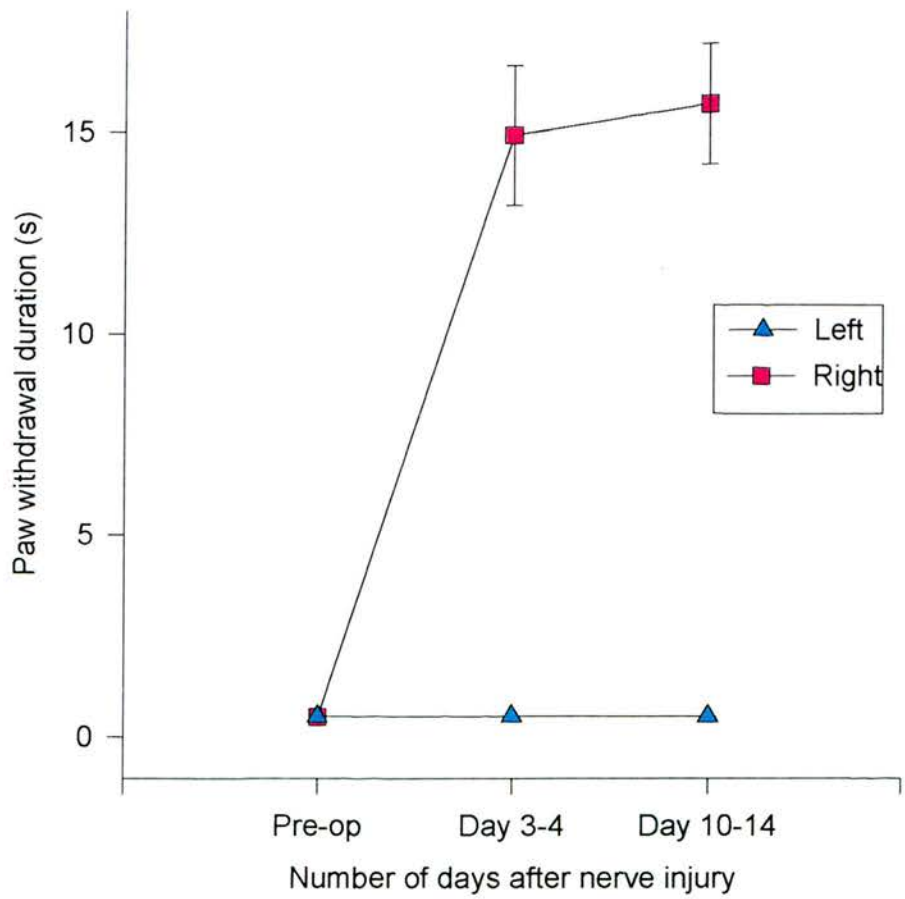
B. The mean values (+/-s.e.m.) for paw withdrawal duration to pin prick (s) are shown for each hindpaw from the pre-operative period until the day of experiment at 10-14 days after nerve injury.

Figure 9. Response to pin prick

A

Response to pin prick - Paw withdrawal duration (s) (n=48)		
No. of days after nerve injury	RIGHT: Mean +/- s.e.m.	LEFT: Mean +/- s.e.m
pre-op(control)	0.5 +/- 0	0.5 +/- 0
Day 3-4	14.9 +/- 1.7	0.5+/- 0
Day 10-14	15.7 +/- 1.5	0.5+/- 0

B



3.4 Discussion

A potential problem with behavioural testing is the effect of the testing itself - confounding factors such as training, acclimatisation and differing conditions for the test can all alter the sensitivity of the test. Those tests which are least invasive and cause least stress in the rats would therefore seem to have an advantage over other tests.

For tests measuring a withdrawal response the degree of motor nerve injury involved may alter the result. For example, in the chronic constriction injury model it is mainly large diameter fibres that are damaged, a significant proportion of which must be of motor origin. It is known that there is a reduction in weight gain in these animals; this could either be due to anorexia secondary to pain, or to the muscle wasting that can be seen on the side of nerve injury. There is also evidence that in the spinal nerve ligation model, both motor and sensory changes are involved in the abnormalities in hindpaw position, although at least some of the behavioural changes seen have a major sensory component, possibly related to pain [305].

Tests measuring a response to thermal stimuli may be affected by changes in blood flow and skin temperature, as well as the thermal properties of the surface on which the rat is placed. Thus as well as keeping ambient temperature constant during the period of behavioural testing, factors altering vasomotor tone must be minimised [165]. There is evidence that in the chronic constriction injury model there are unpredictable changes in skin temperature and vasomotor tone both on the side of the nerve injury and also on the contralateral hindpaw [32,218,436], thus making interpretation of thermal tests more difficult. Additionally, tissue damage from repeated noxious testing must be avoided, as the response in these circumstances does not reflect the initial nerve injury alone, but subsequent damage.

The interaction between spinal cord and higher centres may also alter the response seen. Noxious thermal stimulation of a hindpaw can decrease the response seen in the tail flick reflex. This seems to be mediated by a heterosegmental route, part of which is supraspinal, as it does not occur in spinalized rats [339]. Variations in degree of arousal can alter the baseline response, for example as occurs with stress-induced analgesia [40].

Another problem after nerve injury is the change in peripheral receptive fields that occurs, with the development of extra-territorial pain, thus making it harder to assess the nerve being tested. In the chronic constriction injury model, there is evidence of mechano-allodynia and hyperalgesia in the distribution of the saphenous (branch of the femoral nerve) as well as the sciatic nerve, that was abolished by acute sciatic nerve transection [406].

Despite all these factors, behavioural testing can be used to test for the development of signs of sensory disorders after nerve injury. Additionally, depending on the test used, quantitative assessment can be made, thus allowing comparison between animals as well as measuring the effects of specific interventions. The purpose of carrying out the tests described here was to ensure that the rats with loose ligation of one sciatic nerve did indeed develop behavioural evidence of neuropathic pain. These behavioural studies provided qualitative evidence of spontaneous pain and quantitative evidence of mechanical allodynia and hyperalgesia. Thus, conclusions drawn from further studies of peptide release in the spinal cord were based on rats with behavioural changes associated with pain-related behaviour. In addition, the well-described histological changes in the ligatured nerve were also present in those rats studied [23,297].

Chapter 4 - Studies of the spinal release of galanin

4.1 Introduction

Previous work from this laboratory found an extensive basal release of ir-galanin in the spinal cord of the normal rat [168]. Neither innocuous or noxious peripheral stimulation resulted in changes in spinal ir-galanin release [168,292]. The likely origin of this basal presence of ir-galanin was, therefore, either from intrinsic neurones of the spinal cord or of supraspinal origin, rather than from primary afferent fibres. This would correlate with results from immunohistochemical studies that have found significant amounts of galanin in the dorsal horn [56,214,497], but low levels in primary afferent neurones [161,163,425].

After sciatic nerve transection, galanin production increased in mainly small to medium sized primary afferent cells of the dorsal root ganglia [164,495], with an associated increase in galanin-ir in the superficial dorsal horn [493]. In the CCI model, a similar increase in galanin synthesis by primary afferent neurones was found [308], with a corresponding increase in galanin-ir in the ipsilateral dorsal horn [252]. The effect of peripheral nerve injury on the spinal release of ir-galanin under basal or evoked conditions, is not known.

The present experiments were therefore carried out to address directly the question of what changes occur in the central release of ir-galanin in the spinal cord of rats after a peripheral nerve injury.

Three series of experiments were carried out on spinal ir- galanin release:

1. Ir-galanin release was studied in the spinal cord of normal and neuropathic rats under basal conditions; to determine the effect of the nerve injury itself on ir-galanin release and allow comparison with the normal situation.
2. The effect of peripheral electrical stimulation of the injured nerve on the spinal release of ir-galanin was studied, in order to define the necessary stimuli required (if any) to alter spinal ir-galanin release.
3. Input from the injured nerve was blocked to determine the effect of altered input secondary to the nerve injury, on spontaneous ir-galanin release.

4.2 Materials and methods

4.2.1 Animal Preparation

General

The studies were carried out under Home Office Licence No PPL 60/1146, and the guidelines for the care of experimental animals set out by the International Association for the Study of Pain were followed carefully [502].

A total of 53 male Wistar rats (Charles River Ltd, UK), 240 g to 380 g were used in the ir-galanin release studies. Of these, 7 did not undergo a peripheral nerve injury, but were used as controls to determine the basal presence of ir-galanin in the spinal cord of normal rats, thus allowing comparison of basal levels of ir-galanin between normal and neuropathic rats.

A peripheral nerve injury was induced according to the model of Bennett and Xie [33], as described in section 2.2.1. Following this, all rats were monitored to ensure adequate wound healing, without infection, and underwent the behavioural tests outlined in chapter 3. All rats used in ir-galanin release studies had behavioural evidence of spontaneous pain and mechanical allodynia and hyperalgesia. For the ir-galanin release experiments, carried out at ten to fourteen days after ligature placement, the standard experimental set up outlined in section 2.2.2 was used. Microprobes were inserted into both sides of the cord and left in place for fifteen minutes to allow adequate binding of any extracellular galanin present in the cord, as described in section 2.1.5. Previous studies in the laboratory have shown this length of time to be optimal for binding of released ir-galanin [168]. Following removal from the spinal cord, microprobes were washed in cold phosphate-buffered saline containing 0.1% Tween 80 to reduce levels of non-specific binding and then incubated for 24 hours at 4 °C in a solution of radiolabelled ^{125}I galanin. After further washing in 0.1% Tween 80, the distal portions of microprobes were placed in an X-ray film cassette with a sheet of monoemulsion film (Kodak NMC) for eight to ten days to produce an individual autoradiographic image of each microprobe.

Stimulation experiments

In those experiments where the effects of peripheral electrical stimulation on ir-galanin release was being studied, a platinum stimulating electrode was used proximal to the ligatures. Before any stimulus had been delivered, six to eight antibody-coated microprobes were inserted into the spinal cord to a depth of 2.25 mm, as described in section 2.2.2, to confirm basal levels of galanin. This was followed by peripheral electrical stimulation in order to activate large myelinated fibres. Microprobes were inserted during stimulation and for the following two hours to measure any persistence of stimulus-released ir-galanin. This was followed by higher intensity stimulation to activate small myelinated and unmyelinated fibres as well. Again microprobes were inserted during and after stimulation for up to two hours. The stimulus parameters used were:

1. Stimulation of large myelinated fibres at 2 Hz, 3 x threshold, 0.1 ms pulse duration, for a period of 15 minutes;
2. Additional stimulation of small myelinated and unmyelinated fibres at 2 Hz, 50 x threshold, 0.1 ms pulse duration, for a period of 15 minutes.

In those animals where paralysis was required, during stimulation of C fibres, vecuronium (1 mg/kg) provided neuromuscular blockade and a Harvard Animal Ventilator was used to ventilate at a rate of approximately 80 breaths/minute. This minimised the effects of high intensity stimulation-induced muscle twitching on movements of the microprobes within the spinal cord.

The initial study on basal levels of ir-galanin, outlined in section 4.3.2, showed no effect of peripheral nerve injury on spontaneous ir-galanin release in the spinal cord contralateral to the side of injury [74]. Thus, microprobes inserted into the contralateral side of the cord could be used as controls for microprobes inserted ipsilateral to the nerve injury.

Blocking peripheral input

In order to investigate the effect of spontaneously firing injured primary afferent fibres on basal ir-galanin release, electrical input from the injured nerve was blocked in two

stages, using amethocaine gel 4% w/v (Smith & Nephew Healthcare Ltd, Hull, UK). Prior to application of amethocaine gel, six to eight microprobes were inserted under basal conditions to confirm previous results and give a definitive pre-intervention level for comparison. This was followed by placement of a piece of polyethylene tubing around the nerve, proximal to the site of the chronic constriction injury. This was cut to 1 cm in length and had a diameter of 5 mm, with a slit down one side to allow application around the nerve. Once the tubing was in place, ~0.1 ml amethocaine gel was injected into the tube in order to block neuronal conduction from the nerve injury site. The tube was used to try and minimise any effects from spread of the local anaesthetic and systemic absorption from the muscle vasculature around the sciatic nerve. This was left in place for a minimum of 30 minutes, before the adequacy of conduction block was checked, as described below. Once peripheral conduction was blocked, four pairs of antibody-coated microprobes were inserted into the spinal cord in sequence.

Blocking input from the dorsal root ganglia

The laminectomy was extended distally and laterally to expose the dorsal roots L3-S2. These were gently mobilised using a fine blunt probe, and a small piece (~0.5 x 1 cm) of black polythene inserted under the dorsal roots. This allowed the application of ~0.1 ml amethocaine gel around the dorsal roots without any of it contacting the spinal cord itself, so that the effect of peripheral, and not central, conduction block could be studied. In addition, sealant gel was used around the edges of the polythene to prevent leakage of the amethocaine gel pool from the dorsal roots onto the spinal cord. Continuous irrigation of the cord with warmed sterile Ringer's solution was suspended during this period to prevent washing the local anaesthetic onto the cord. Instead irrigation was carried out intermittently with warmed sterile Ringer's solution, and intermittent suction.

A minimum of 30 minutes was allowed after application of the amethocaine gel at both peripheral and proximal sites. A preliminary study had been carried out to determine the most effective type of local anaesthetic and the length of time necessary for adequate conduction block. Amethocaine gel was found to be superior to

solutions of 2% lignocaine, 0.25-0.75% bupivacaine and EMLA ® cream (Eutectic Mixture of Local Anaesthetic, Astra Pharm, UK), as it produced the quickest onset of block, and one that was sufficiently prolonged for the study duration.

Thus, prior to inserting any microprobes after conduction block, the adequacy of the block was checked. The injured nerve was stimulated peripherally at high intensity, distal to local anaesthetic, and the resultant activity evoked within the cord measured using a silver ball electrode on the cord surface as described in section 2.2.2. Provided no activity was detected, microprobes were then inserted into the cord to measure the effect of blocking conduction from the injured nerve on ir-galanin release. If activity was detected, more amethocaine gel was applied, and the preparation was left for a further 30 minutes to allow adequate diffusion of the local anaesthetic to its site of action.

By applying the amethocaine gel in two stages, it was possible to differentiate the relative contributions, if any, of spontaneous ectopic neuronal activity from either the nerve injury site or the dorsal root ganglia [190,407,441] to the increased spontaneous release of ir-galanin.

4.2.2 Antibody microprobe preparation [104,107]

Materials

Using the standard technique described in section 2.1 for antibody microprobe preparation, fine glass micropipettes, with a tip diameter of approximately 10μm, were coated with rabbit anti-rat galanin antiserum. Such coated microprobes can bind endogenous galanin, at localised sites of release, that is detected by subsequent inhibition of binding of exogenous radiolabelled ^{125}I galanin (Peninsula, CA).

Two suppliers were used for the galanin antiserum - Peninsula Laboratoreis, California, USA supplied the antiserum used in the study of basal and stimulated ir-galanin release, and Advanced Chemtech, Kentucky, USA supplied the galanin antiserum used in the study of ir-galanin release after conduction block from the injured nerve. This was because the original lot number supplied by Peninsula Laboratories was no longer available when the conduction block study was carried

out, and the new lot number supplied by them did not meet the requirements of the *in vitro* assays (as outlined in section 2.1.4), such that this batch had to be discarded. The galanin antiserum supplied by Advanced Chemtech did meet the requirements for *in vitro* assays.

In vitro tests

Prior to commencing *in vivo* work, *in vitro* assays were carried out to determine the specificity and sensitivity of the immobilised galanin antibody by measuring the degree of inhibition of ^{125}I galanin binding by related peptides or varying concentrations of unlabelled galanin. The procedure used for in vitro assays is outlined in section 2.1.4 and Figure 4. *In vitro* assays were carried out for each new batch of galanin antiserum, and continued in parallel with *in vivo* work to ensure no major deterioration in the assay. Sensitivity was determined by incubating microprobes in a range of unlabelled galanin peptide from 10^{-5}M to 10^{-8}M prior to incubation in ^{125}I galanin. Specificity values were supplied by the manufacturer's data, but these were checked by incubation of microprobes in 10^{-5}M to 10^{-6}M SP, VIP and NPY.

4.2.3 Analysis of Microprobes [160]

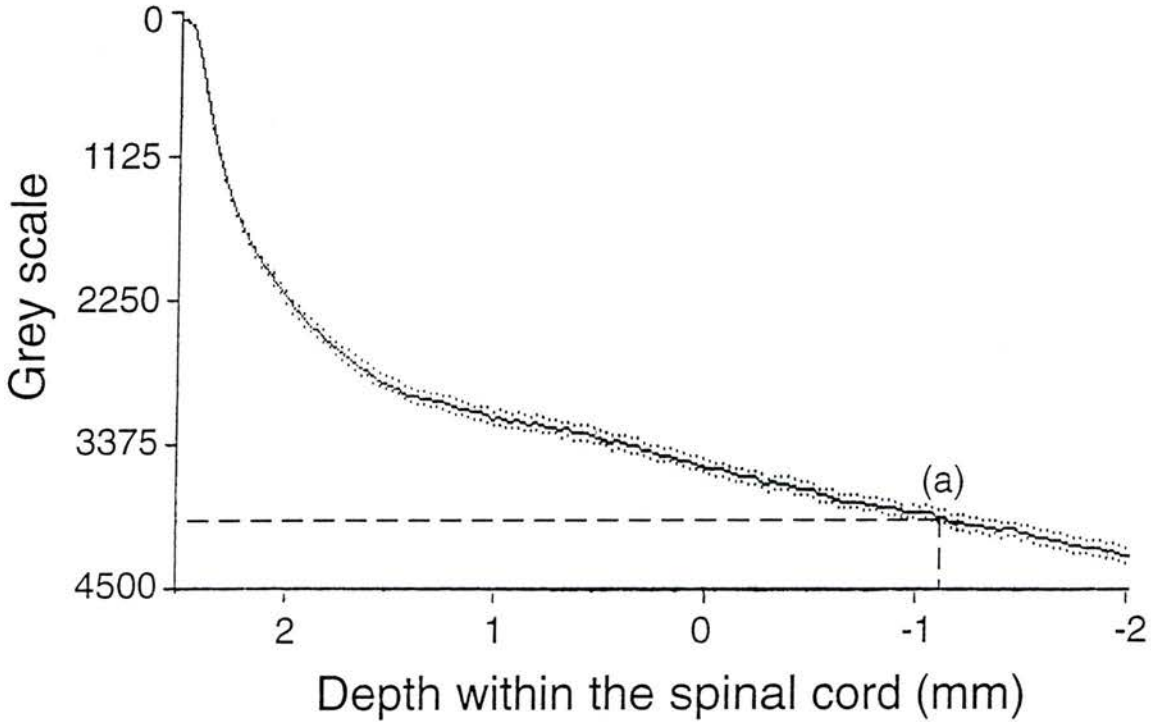
The principles of image analysis are described in section 2.1.6. Basically, a computerised image analysis system was used to analyse the autoradiographic images obtained from galanin antiserum-coated microprobes inserted into the spinal cord. *In vitro* microprobe images for each experiment were also analysed as controls. Binding of endogenous galanin resulted in deficits in binding of ^{125}I-labelled galanin that were detected as localised decreases in optical density by the analysis system. For each microprobe, a plot of grey scale (i.e. total optical density) against depth of the probe in the spinal cord was obtained. Thus areas where endogenous galanin had bound to the microprobe were shown by areas of low optical density and a low grey scale value.

A grey scale range of 50 points on the probe were chosen outwith the spinal cord as the site for normalisation of each series of experiments. This allowed comparison of probes that might otherwise have had minor variations in optical density due to

exposure time, rather than biological variation. An example of the plot used to determine the normalisation point is shown in Figure 10. The mean values for defined groups of microprobes, were plotted as the means (+/- s.e.m.) of the grey scale values at 30 μm intervals against the depth within the spinal cord. Statistical significance was assigned to each site using Student's unpaired t-test, and the t values thus obtained plotted against depth within the spinal cord. A p value of less than 0.05 was taken as showing a statistically significant difference between groups.

Figure 10. Mean image analysis of microprobes prior to normalisation

The mean image analysis (+/- s.e.m.) of all probes used to measure ir-galanin release under basal conditions (n=268) is plotted. In order to allow valid comparisons between groups of microprobes, is necessary that all probes are normalised to an identical optical density outwith the cord. If this were not done, a difference in optical density between groups of probes could be due to variations in the exposure of the X-ray film, rather than a true biological difference in ir-galanin release. As shown at (a), a normalisation point well outwith the cord was chosen. All probes were normalised to the optical density measured from point (a), and for 50 analysis points from here (i.e. 500 μm) along the probe length. All further analysis was done on normalised data.


0
1125
2250
3375
4500
Grey scale
(a)
2
1
0
-1
-2
Depth within the spinal cord (mm)

4.3 Results

4.3.1 *In vitro* testing

The galanin antibody used was C-terminus directed and showed minimal cross reactivity with other peptides found in the dorsal horn (according to manufacturer's data and confirmed by *in vitro* testing). Sensitivity tests found suppression of binding of radiolabelled galanin ranging from approximately 90% with 10^{-5} M galanin to approximately 50% suppression with 10^{-8}M galanin, indicating that the sensitivity of the galanin antibody was adequate to detect significant areas of galanin release in the spinal cord. Levels of non-specific binding of the radiolabelled galanin to the microprobes were assessed by measurement of the degree of binding to probes not coated in galanin antibody, but simply coated in protein A. The level of binding to such probes was in the region of 10% of control counts, thus suppression at a level greater than 10% can be assumed to de due to galanin-galanin antibody binding. To check the adequacy of the antibody coating of the microprobes, one group was incubated in ^{125} I Fab instead of ^{125} I galanin (see section 2.1.4). Both antibodies used showed evidence of good coating of the probes. The results of *in vitro* testing from the two batches of galanin antisera (one from Peninsula Labs and one from Advanced Chemtech) are shown in Tables 6 and 7. Although the control counts were higher with the Advanced Chemtech antiserum, the sensitivity and specificity of the two different galanin antisera used did not vary significantly.

Table 6. *In vitro* assay using galanin antiserum from Peninsula Labs.

This shows the results from microprobes used in *in vitro* assays to ensure adequate sensitivity and specificity of the galanin antibody coating on the microprobes. Low levels of non-specific binding are also demonstrated, and good antibody coating of the probes, as shown by the high counts obtained with those probes incubated in ^{125} I Fab.

Table 6. *In vitro* assay using galanin antiserum from Peninsula Labs.

Group (number of probes)	Mean corrected c.p.m. (+/-s.e.m.)	% suppression of control group
Control (109)	774 (+/-40)	
10^{-5} M galanin (76)	80 (+/-9)	90
10^{-6} M galanin (10)	155 (+/-26)	80
10^{-7} M galanin (49)	298 (+/-17)	62
10^{-8} M galanin (34)	348 (+/-46)	55
10^{-5} M SP (5)	1364 (+/-80)	+75
10^{-5} M VIP (17)	800 (+/-75)	+3
10^{-5} M NPY (17)	886 (+/-77)	+14
^{125} I Fab (8)	1041 (+/-73)	+35
Protein A (18)	81 (+/-19)	10 (non-specific binding, % of control)

Table 7. *In vitro* assay using galanin antiserum from Advanced Chemtech

This demonstrates similar *in vitro* assay to those outlined in Table 6, but using a galanin antiserum from a different manufacturer.

Table 7. *In vitro* assay using galanin antiserum from Advanced Chemtech

Group (number of probes)	Mean corrected c.p.m. (+/-s.e.m.)	% suppression of control group
Control (33)	1709 (+/-130)	
10^{-5} M galanin (32)	182 (+/-42)	90
10^{-6} M galanin (15)	396 (+/-37)	77
10^{-7} M galanin (9)	431 (+/-117)	75
10^{-8} M galanin (9)	900 (+/-130)	47
10^{-5} M SP (6)	1923 (+/-120)	+12
10^{-5} M VIP (9)	1777 (+/-331)	+4
10^{-5} M NPY (9)	1885(+/-305)	+10
^{125} I Fab (6)	1645(+/-104)	9
Protein A (8)	126 (+/-15)	7 (non-specific binding, % of control)

4.3.2 Basal ir-galanin release

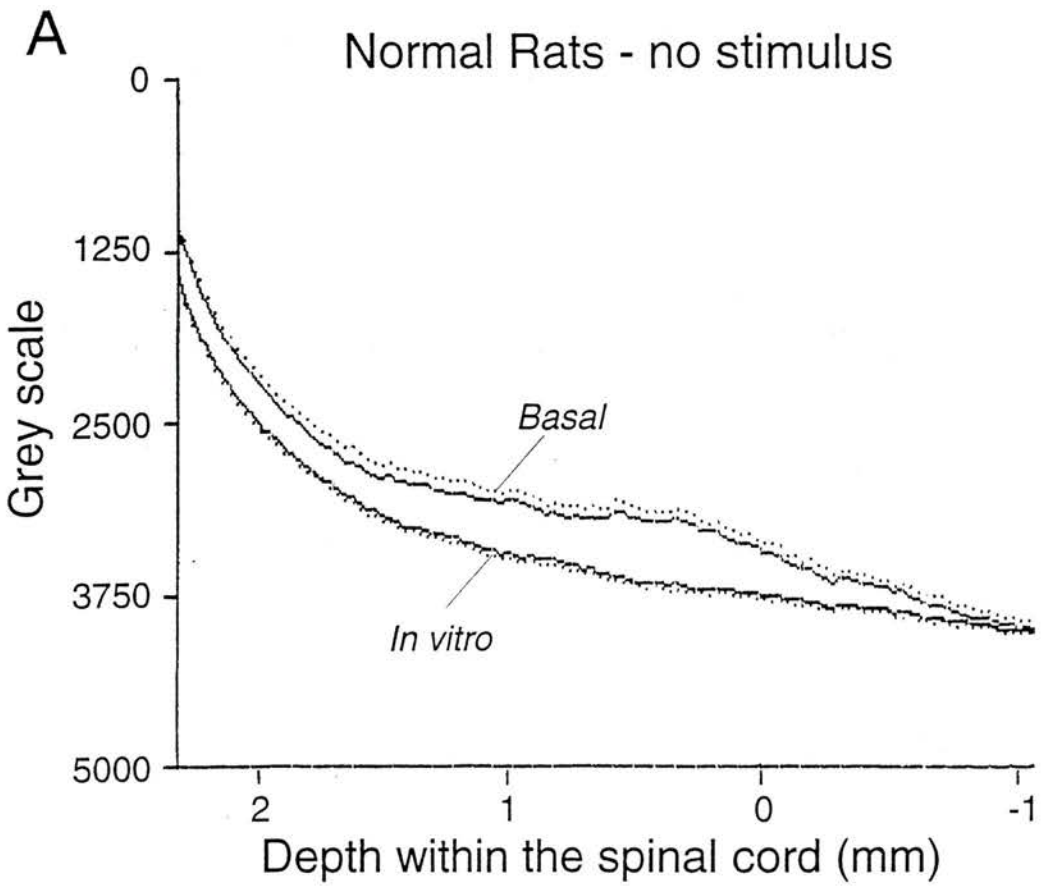
A total of 31 rats were used to determine the basal release of ir-galanin in normal (7) and neuropathic (24) rats. All rats used with a chronic constriction injury of the right sciatic nerve had behavioural evidence of spontaneous pain, mechanical allodynia and hyperalgesia on the side of nerve injury, as described in chapter 3. A total of 268 probes were inserted into the spinal cord, with a further 187 probes being used as *in vitro* controls. These *in vitro* microprobes had been prepared concurrently with the *in vivo* probes, but had not been inserted into the central nervous system. Instead, they were washed in 0.1% Tween 80, then incubated in ^{125} I galanin, thus representing zero endogenous galanin binding.

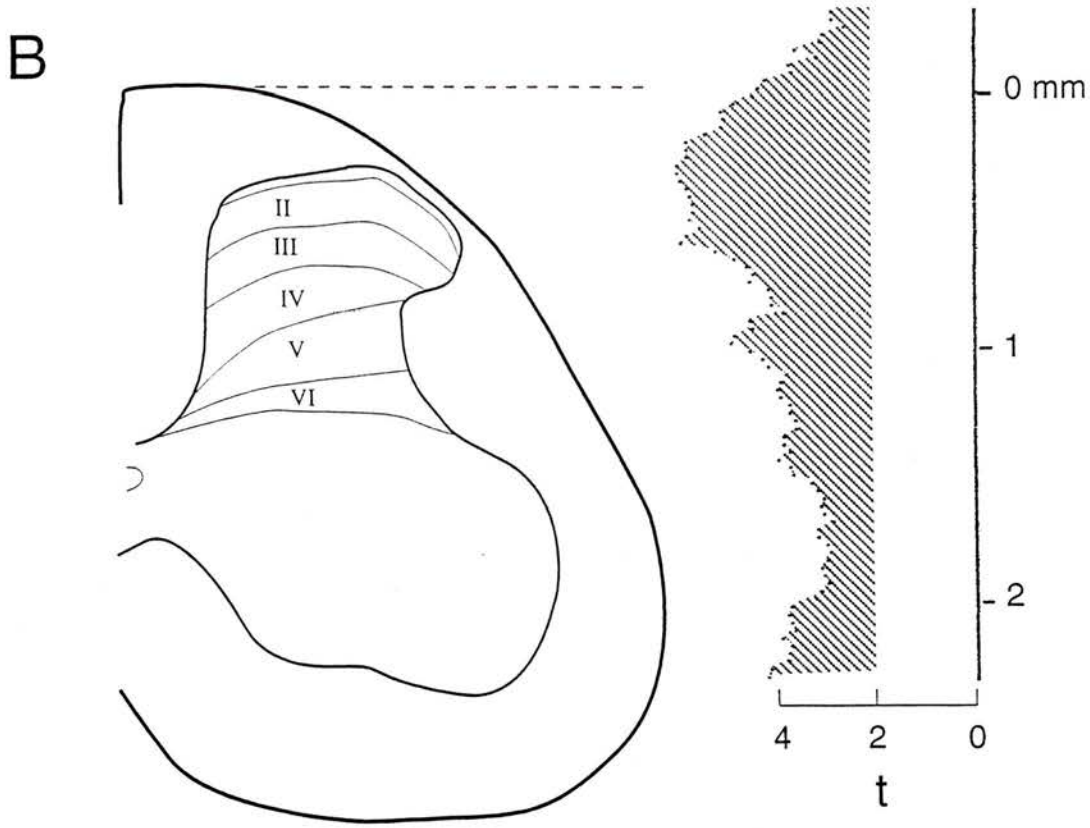
Microprobes were inserted into both sides of the spinal cord of normal rats in the absence of peripheral stimulation (n=57). Comparison of the mean image analyses of these two groups showed no difference in basal ir-galanin release between the right and left side of the spinal cord. Thus both sides of the spinal cord showed extensive basal ir-galanin release in the dorsal and ventral horns, when compared to *in vitro* microprobes (n=187). The area of ir-galanin release in the spinal cord of normal rats is shown in Figure 11. This confirms previous results in the rat from this laboratory [168] and studies from elsewhere in the cat [292], that have also shown no effect of peripheral stimulation on central ir-galanin release. There was no significant difference between the pattern of ir-galanin release on the right and left sides of the spinal cord in these normal rats.

Figure 11. Basal release of ir-galanin in the spinal cord of normal rats.

A. The mean image analysis of two groups of microprobes are plotted with respect to length: the *in vitro* microprobe group have not been inserted into the cord, but the equivalent length to microprobes used *in vivo* has been analysed to allow comparison. The optical density is represented by the grey scale (y axis), with any decrease in optical density (as shown by an upward deflection) indicating the binding of endogenously released ir- galanin to antibody-coated microprobes in the spinal cord. Each line is made up of the mean image analyses at 30 μm intervals along the length of the microprobe. The dotted line adjacent to the solid line for each group is the standard error of these means (s.e.m.). The two groups are the mean image analysis (-s.e.m.) of the *in vitro* microprobes (n=187) and the mean image analysis (+s.e.m.) of microprobes inserted into the spinal cord for 15 minutes without any concurrent peripheral stimulus (n=57). All microprobes were coated with galanin antiserum (Peninsula Labs, Ca, USA).

B. The right hand side of the diagram is a plot of the *t* statistics derived from comparison of the means at each analysis point in the mean image analyses shown in A. This is related to an outline of the spinal cord (modified from Molander, 1984 [287]) on the left hand side, to give an indication of the sites of ir-galanin release. The hatched area is where there are significant differences ($p<0.05$)between the two groups.


A
Normal Rats - no stimulus
0
1250
2500
3750
5000
Grey scale
Basal
In vitro
2
1
0
-1
Depth within the spinal cord (mm)


B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

In neuropathic rats, there was found to be no significant difference between spinal ir-galanin release contralateral to the nerve injury, compared to ir-galanin release in normal rats. The mean image analysis of microprobes (n=58) inserted into the spinal cord of normal rats and the mean image analysis of microprobes (n=50) inserted into the side of the spinal cord contralateral to the nerve injury, in neuropathic rats, were not significantly different. Thus, the initial extensive basal release of ir-galanin seen in the spinal cord of normal rats was unchanged in the spinal cord of neuropathic rats, contralateral to the peripheral nerve injury. This is shown in Figure 12.

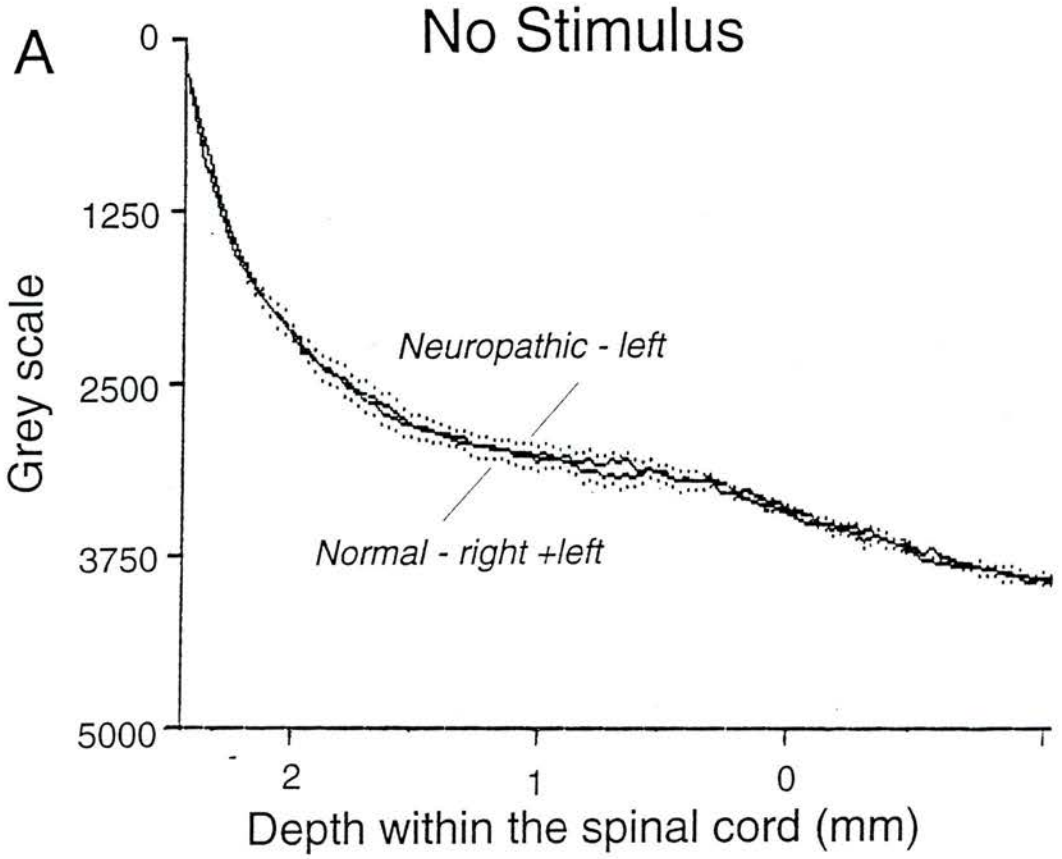
By contrast, an increase in spontaneous ir-galanin release was detected with a peak in the superficial dorsal horn ipsilateral to the side of nerve injury, both when compared to the release pattern found in normal rats, and to that seen in the side of the cord contralateral to the nerve injury (see Figure 13). This area of increased basal ir-galanin release is in the same area as the termination of the small to medium sized primary afferent fibres, that start synthesising galanin after nerve injury [164,308,316,495].

Figure 12. Basal ir-galanin release contralateral to a peripheral nerve lesion

A comparison was made between microprobes inserted into the spinal cord of normal rats to microprobes inserted into the spinal cord contralateral to the side of nerve injury in neuropathic rats, in the absence of any active peripheral stimulation.

A. The mean image analysis (- s.e.m.) of microprobes (n=58) inserted into the spinal cord of normal rats and the mean image analysis (+ s.e.m.) of microprobes (n=112) inserted into the left side of the spinal cord, contralateral to the nerve injury, for 15 minutes without any concurrent peripheral stimulus. The two groups are plotted with respect to depth of insertion into the spinal cord, with the cord surface being on the right hand side of the diagram.

B. The differences between the two groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord. At no point along the length of the probe was there any significant difference between the two groups.


A
No Stimulus
0
1250
2500
3750
5000
Grey scale
Neuropathic - left
Normal - right +left
2
1
0
Depth within the spinal cord (mm)

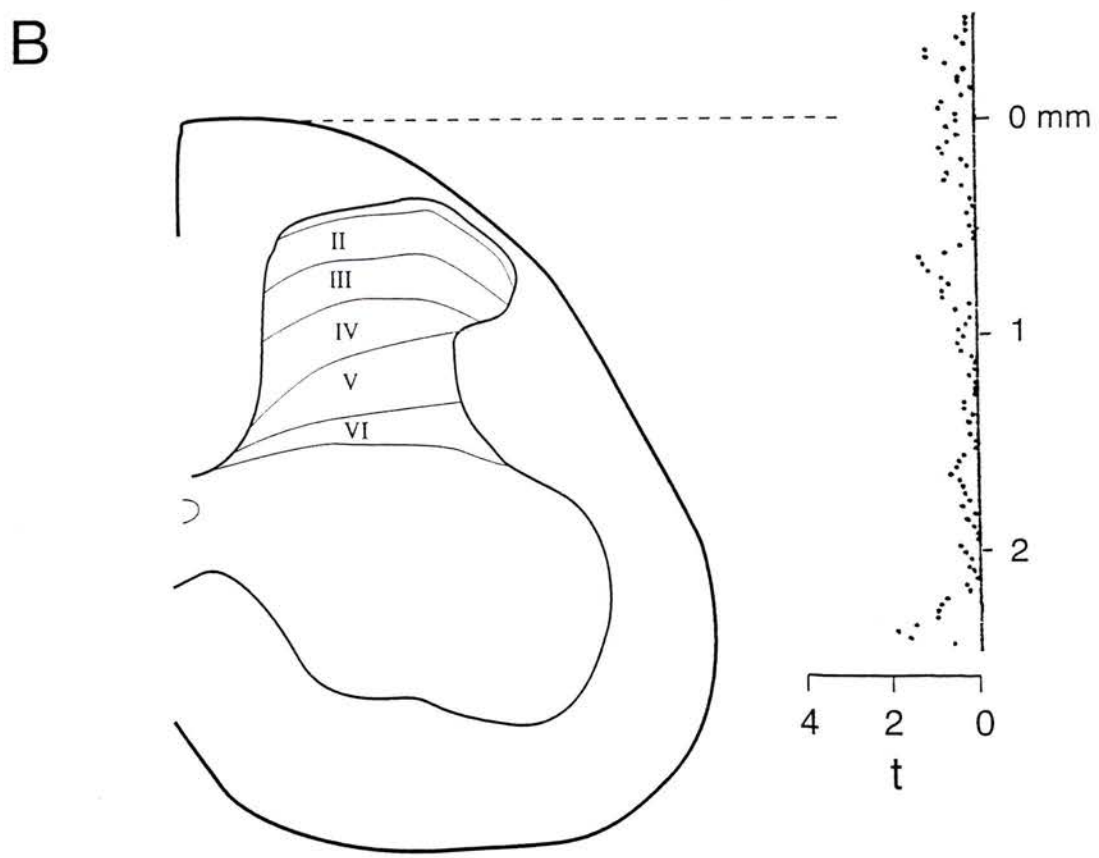
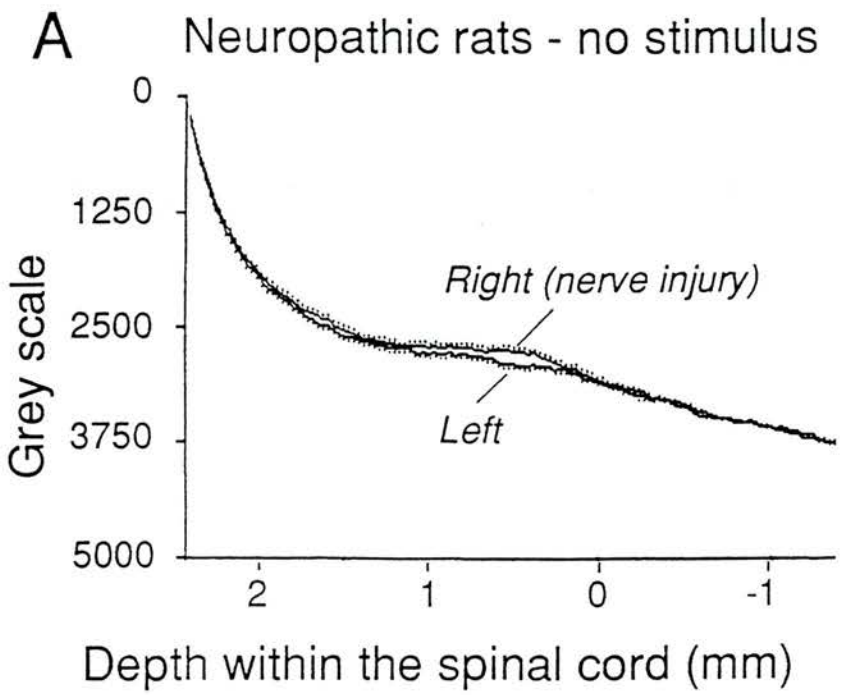

B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

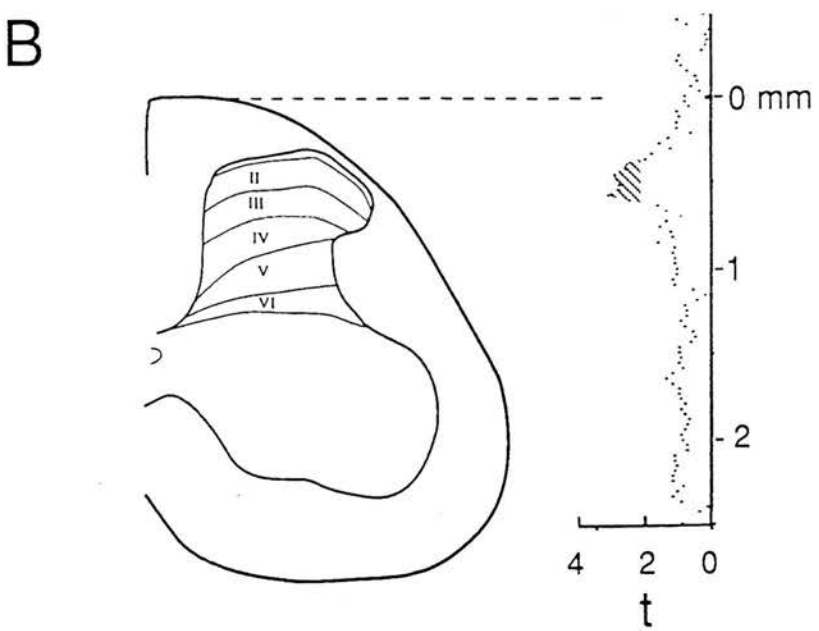
Figure 13. Basal ir-galanin release ipsilateral to a peripheral nerve lesion

A comparison was made between microprobes inserted into the ipsilateral and contralateral side of the spinal cord of neuropathic rats.

A. Microprobes were inserted into the spinal cord of neuropathic rats for 15 minutes without any concurrent peripheral stimulus. The plot shows the mean image analyses (+/-s.e.m.) of microprobes inserted into the ipsilateral (n=121) and contralateral (n=112) sides of the spinal cord relative to the nerve injury, which is on the right side. The microprobes inserted on the ipsilateral side are displaced above those inserted into the contralateral side, indicating a new zone of ir-galanin release under basal conditions.

B. The differences between the ipsilateral and the contralateral groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord. The hatched area shows where there is a significant increase ($p<0.05$) in spontaneous ir-galanin release in the superficial dorsal horn.


A
Neuropathic rats - no stimulus
0
1250
2500
3750
5000
Grey scale
Right (nerve injury)
Left
2
1
0
-1
Depth within the spinal cord (mm)


B
II
III
IV
V
VI
0 mm
-1
-2
4
2
0
t

4.3.3. Evoked ir-galanin release

A total of 12 neuropathic rats (240-350 g) were used to study the effects of peripheral stimulation on spinal release of ir-galanin. A total of 195 microprobes coated with galanin antiserum were inserted into the spinal cord, with a further 48 microprobes used as *in vitro* controls, so that basal ir-galanin release could be detected.

Basal ir-galanin release

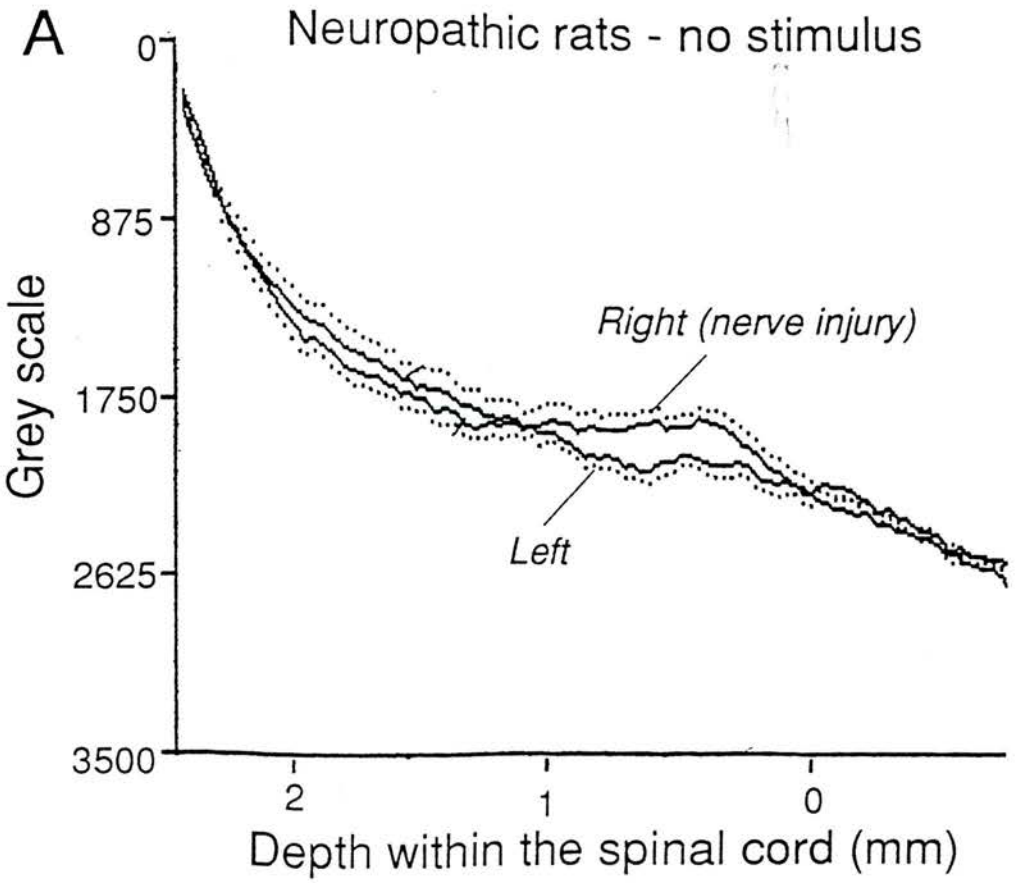
The mean image analyses of microprobes inserted into both sides of the spinal cord in the absence of any active peripheral stimulation were studied to confirm previous results and give a level for basal ir-galanin release with which to compare the effects of peripheral stimulation. The increased area of ir-galanin release, in the superficial dorsal horn, seen previously was again found, as shown in Figure 14.

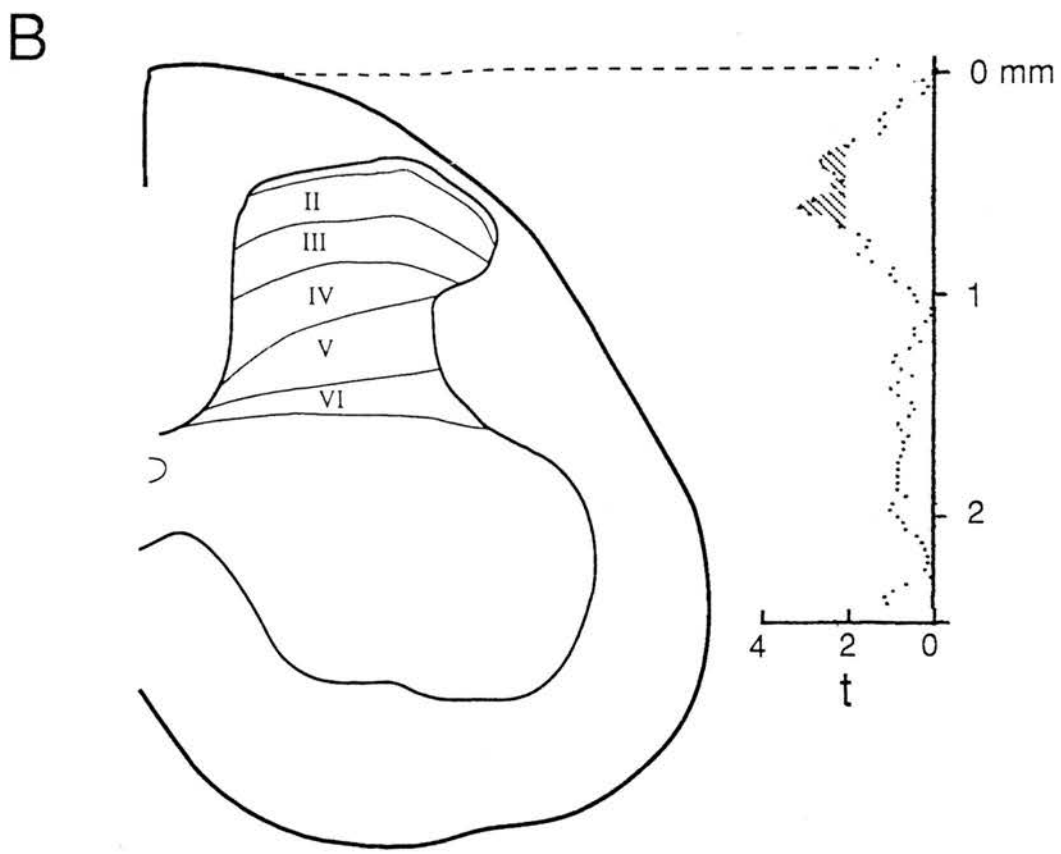
Figure 14. Effects of peripheral nerve injury on basal ir-galanin release.

This confirmed the previous finding (see Figure 13) of an increase area of spontaneous ir-galanin release in the superficial dorsal horn on the side of a peripheral nerve injury. The results are from microprobes inserted into the spinal cord of neuropathic rats prior to peripheral nerve stimulation. This was done to ensure that the same in crease in spontaneous ir-galanin release was found in those rats being studied in stimulation experiments.

A. The plot shows the mean image analyses (+/-s.e.m.) of microprobes inserted into the spinal cord, ipsilateral (n=18) and contralateral (n=17) to the nerve injury. The mean image analysis of microprobes inserted on the ipsilateral side is displaced above that from microprobes inserted into the contralateral side, indicating an increase in ir-galanin release under basal conditions.

B. The differences between the ipsilateral and the contralateral groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord. The hatched area shows where there was a significant increase ($p<0.05$) in spontaneous ir-galanin release in the superficial dorsal horn.


A
Neuropathic rats - no stimulus
0
875
1750
2625
3500
Grey scale
Right (nerve injury)
Left
2
1
0
Depth within the spinal cord (mm)


B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

Ir-galanin release evoked by A fibre stimulation:

Peripheral electrical stimulation sufficient to activate only large myelinated fibres in the injured nerve did not alter the release of ir-galanin in the spinal cord. Thus, the increased area of ir-galanin release detected ipsilateral to nerve injury was still present, but not increased by A fibre stimulation. The mean image analyses of microprobes inserted into the right side of the spinal cord (ipsilateral to the nerve injury) prior to (n=18) and during (n=8) 15 minutes of electrical stimulation at 3T, 2 Hz, 0.1 ms pulse duration, are shown in Figure 15. Thus, electrical stimulation of the injured nerve at a stimulus strength sufficient to activate mainly Aβ fibres did not alter the release pattern of ir-galanin in the spinal cord from that seen in the basal state.

No change in the pattern of ir-galanin release from that found under basal conditions was seen on the contralateral side of the cord during electrical stimulation of Aβ fibres in the injured nerve. This is shown in Figure 16. After electrical stimulation of the injured nerve had ceased, further microprobes inserted into both right (n=33) and left (n=32) sides of the spinal cord for the next two hours did not show any change in ir-galanin release from basal levels, indicating that there was no delayed effect of peripheral stimulation at low intensity, or change in ir-galanin release with time.

Figure 15. Effect of A fibre stimulation on ir-galanin release ipsilateral to peripheral nerve lesion

Microprobes were inserted into the spinal cord during 15 minutes of peripheral nerve stimulation sufficient to activate large myelinated fibres (2 Hz, 3 x threshold, 0.1 msec pulse duration). The stimulating electrode was placed proximal to the site of the chronic constriction injury.

A. The mean image analyses (+/- s.e.m.) of microprobes inserted into the side of the cord ipsilateral to nerve injury under basal conditions (n=18) and during peripheral stimulation sufficient to activate only large myelinated fibres (n=8).

B. The t statistics obtained from comparing these two groups at 30 μm intervals along the length of the microprobes are plotted alongside a diagram of the lumbar spinal cord. At no point was there any significant difference between the 2 groups.

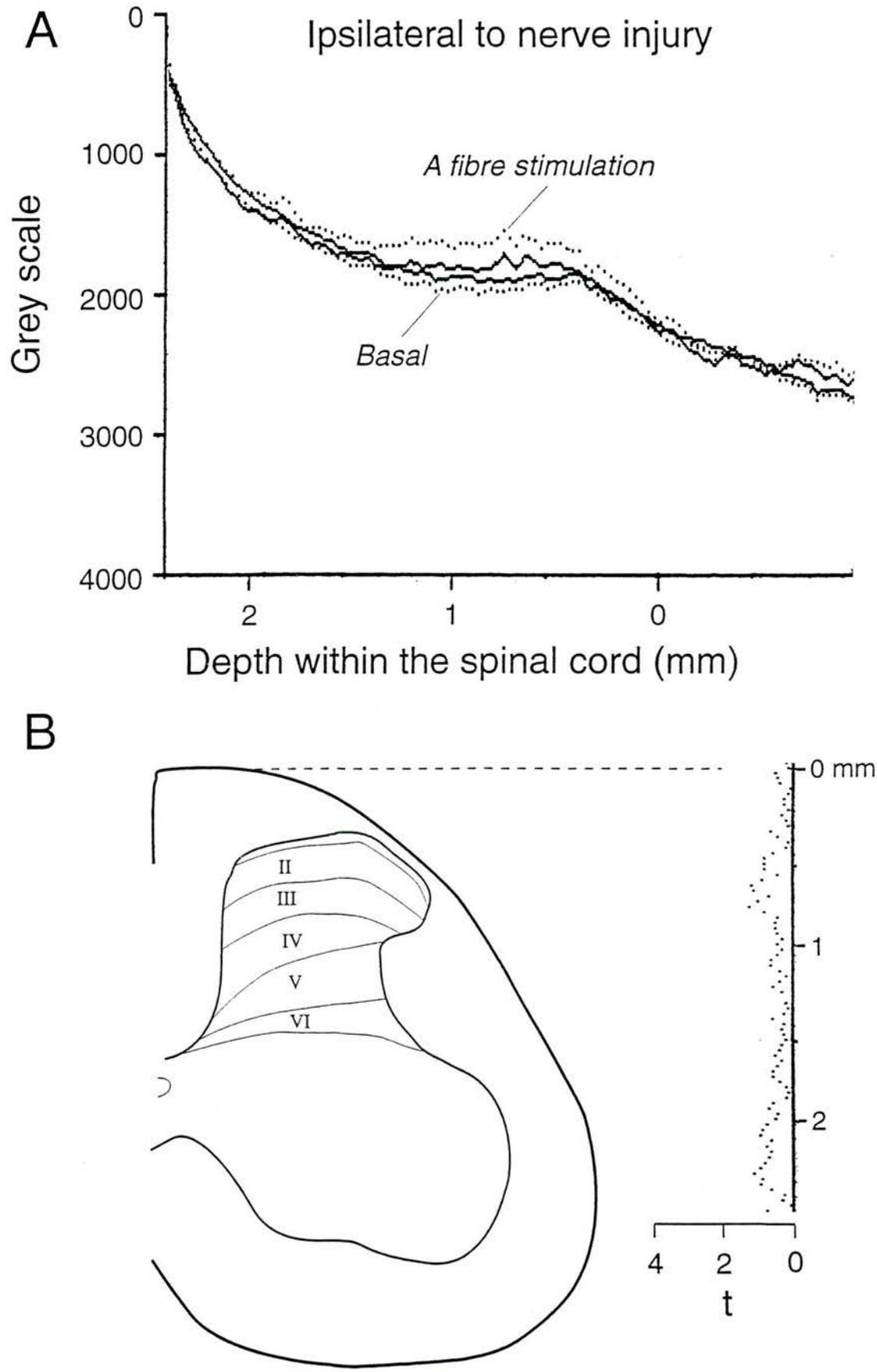
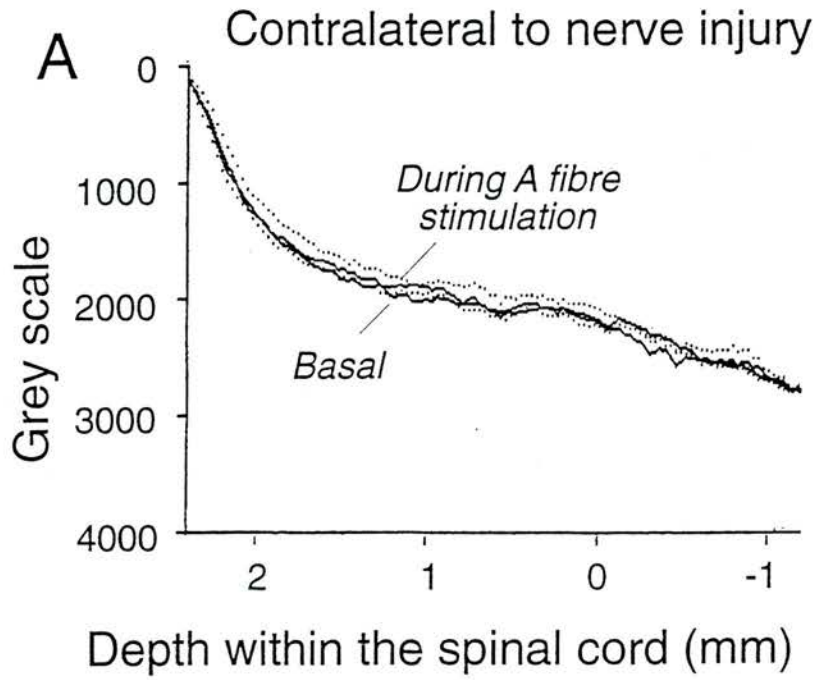

A
Ipsilateral to nerve injury
0
1000
2000
3000
4000
Grey scale
A fibre stimulation
Basal
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

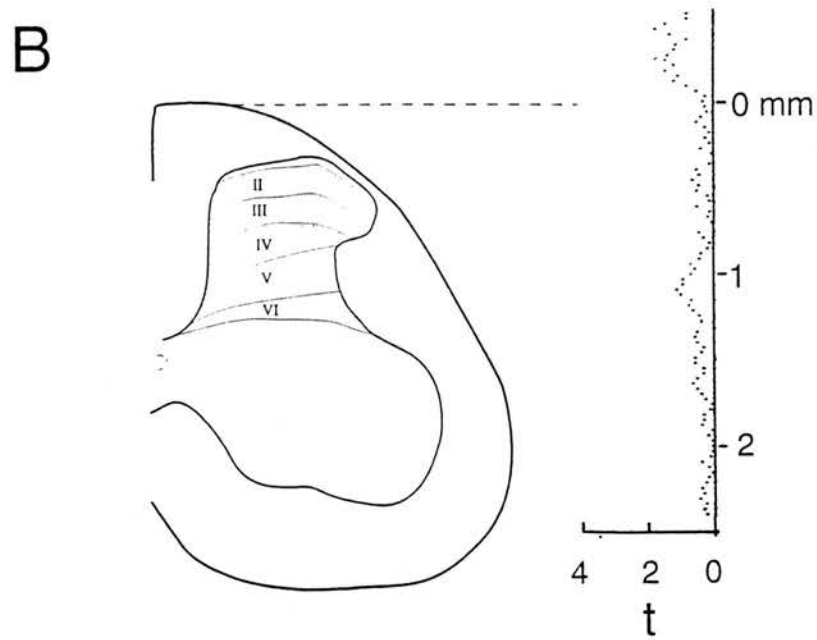
Figure 16. Effect of A fibre stimulation on ir-galanin release contralateral to peripheral nerve lesion

Using an electrode proximal to the nerve injury site, microprobes were inserted into the spinal cord during 15 minutes of peripheral nerve stimulation sufficient to activate large myelinated fibres (2 Hz, 3 x threshold, 0.1 msec pulse duration).

A. The mean image analyses (+/- s.e.m.) of microprobes inserted into the side of the cord contralateral to nerve injury under basal conditions (n=17) and during peripheral stimulation sufficient to activate only large myelinated fibres (n=10).

B. The t statistics obtained from comparing these two groups at 30 μm intervals along the length of the microprobes are plotted alongside a diagram of the lumbar spinal cord. At no point was there any significant difference between the 2 groups. This indicates that stimulation of the injured nerve at this strength did not have any effect on the release of ir-galanin on the side of the spinal cord contralateral to nerve injury.


A
Contralateral to nerve injury
0
1000
2000
3000
4000
Grey scale
During A fibre stimulation
Basal
2
1
0
-1
Depth within the spinal cord (mm)


B
II
III
IV
V
VI
0 mm
-1
-2
4
2
0
t

Ir-galanin release evoked by A plus C fibre stimulation

A minimum of two hours after A fibre stimulation, a further 15 minute electrical stimulus was delivered to the injured nerve. This was of a much higher intensity than used previously, sufficient to activate both myelinated and unmyelinated fibres (2 Hz, 50T, 0.1 ms). The mean image analyses of microprobes inserted into the spinal cord without any concurrent peripheral stimulus was compared with that of microprobes inserted during 15 minutes of high threshold electrical stimulation of the injured nerve as shown in Figures 17 and 18. Ir-galanin release was increased by this stimulus, on the side ipsilateral to nerve injury, with a peak in the superficial dorsal horn, with slightly deeper spread. This occurred in a similar area to the increased spontaneous ir-galanin release found in neuropathic rats as compared to normal rats. As this increase in ir-galanin release occurred in the area where the small primary afferent fibres terminate, it would be consistent with the hypothesis that the source of the new area of ir-galanin release in neuropathic rats was from small primary afferent fibres. High threshold peripheral nerve stimulation did not increase the basal pattern of ir-galanin release on the contralateral side of the spinal cord. Indeed, at one point on the analysis there was a significant decrease in ir-galanin release on the side contralateral to high intensity stimulation of the injured nerve.

Figure 17. Effect of A + C fibre stimulation on ir-galanin release ipsilateral to peripheral nerve lesion

Ir-galanin release was studied during electrical stimulation of the injured nerve at a stimulus strength sufficient to activate Aβ, Aδ and C fibres on (2 Hz, 50T, 0.1 ms) were studied. Microprobes were inserted into the side of the spinal cord ipsilateral to nerve injury for 15 minutes prior to and during peripheral electrical nerve stimulation.

A. The plot shows the mean image analyses (+/-s.e.m.) of microprobes inserted into the spinal cord before (n=18) and during (n=9) electrical stimulation of the injured nerve. At some points within the cord, the mean image analysis of microprobes inserted during stimulation is displaced above that of microprobes inserted before stimulation.

B. The differences between the basal and stimulus groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord. The hatched area shows where there is a significantly ($p<0.05$) increased zone of ir-galanin release evoked by high threshold stimulation sufficient to activate both myelinated and unmyelinated fibres.

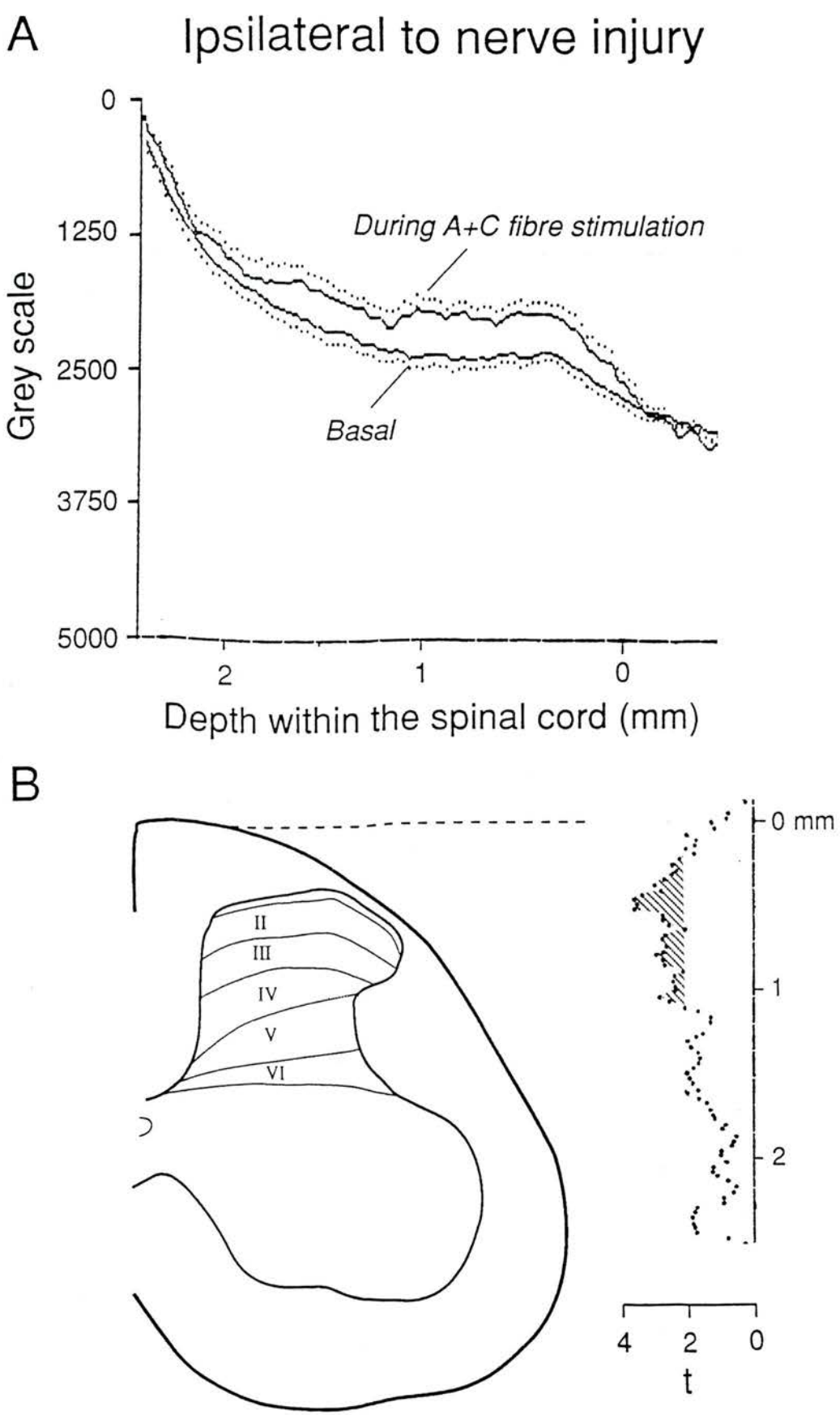
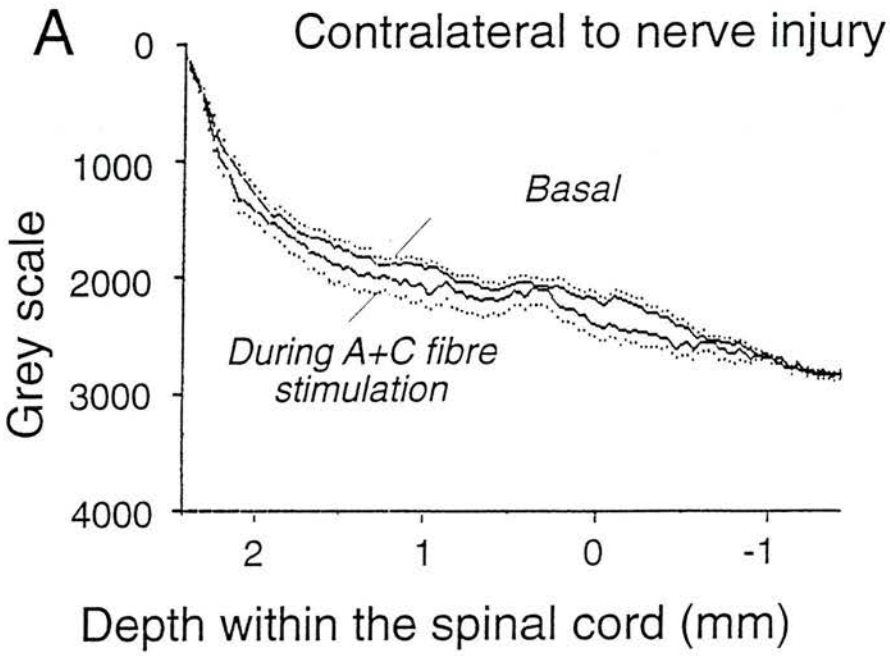

A
Ipsilateral to nerve injury
0
1250
2500
3750
5000
Grey scale
During A+C fibre stimulation
Basal
2
1
0
Depth within the spinal cord (mm)
B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

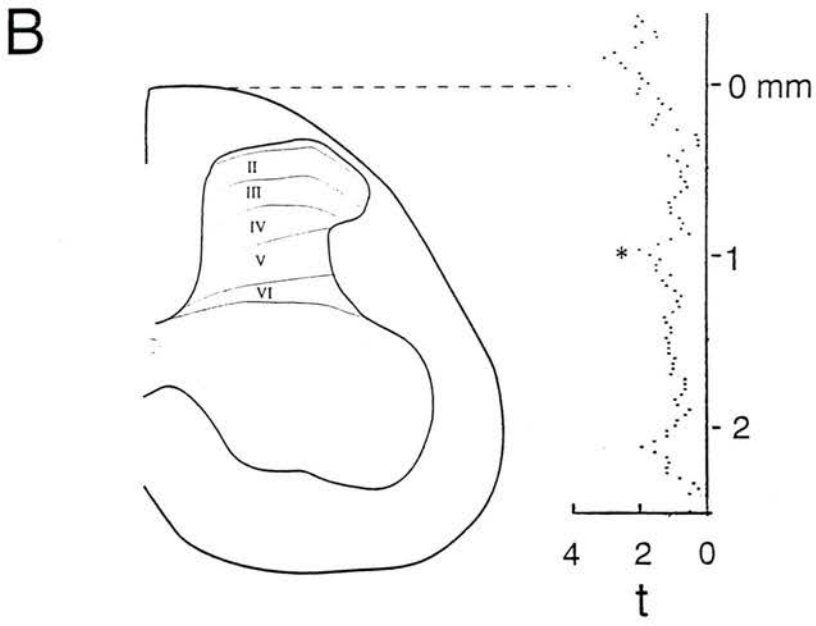
Figure 18. Effect of A + C fibre stimulation on ir-galanin release contralateral to peripheral nerve lesion

Microprobes were inserted into the side of the spinal cord contralateral to nerve injury for 15 minutes prior to and during peripheral electrical nerve stimulation at a stimulus strength sufficient to activate Aβ, Aδ and C fibres on ir-galanin release (2 Hz, 50T, 0.1 ms).

A. The plot shows the mean image analysis (+/-s.e.m.) of microprobes inserted into the spinal cord before (n=17) and during (n=10) electrical stimulation of the injured nerve. At some points within the cord, the mean image analysis of microprobes inserted during stimulation is displaced below that of microprobes inserted before stimulation.

B. The differences between the basal and stimulus groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord. At no point did high threshold stimulation, sufficient to activate both myelinated and unmyelinated fibres, increase ir-galanin release on the contralateral side of the spinal cord. In fact, there is a suggestion that there is actually a decrease in ir-galanin release, this reaching significance only at the one point indicated *.


A
Contralateral to nerve injury
0
1000
2000
3000
4000
Grey scale
Basal
During A+C fibre stimulation
2
1
0
-1
Depth within the spinal cord (mm)


B
II
III
IV
V
VI
0 mm
1
2
*
4
2
0
t

Persistence of evoked ir-galanin release

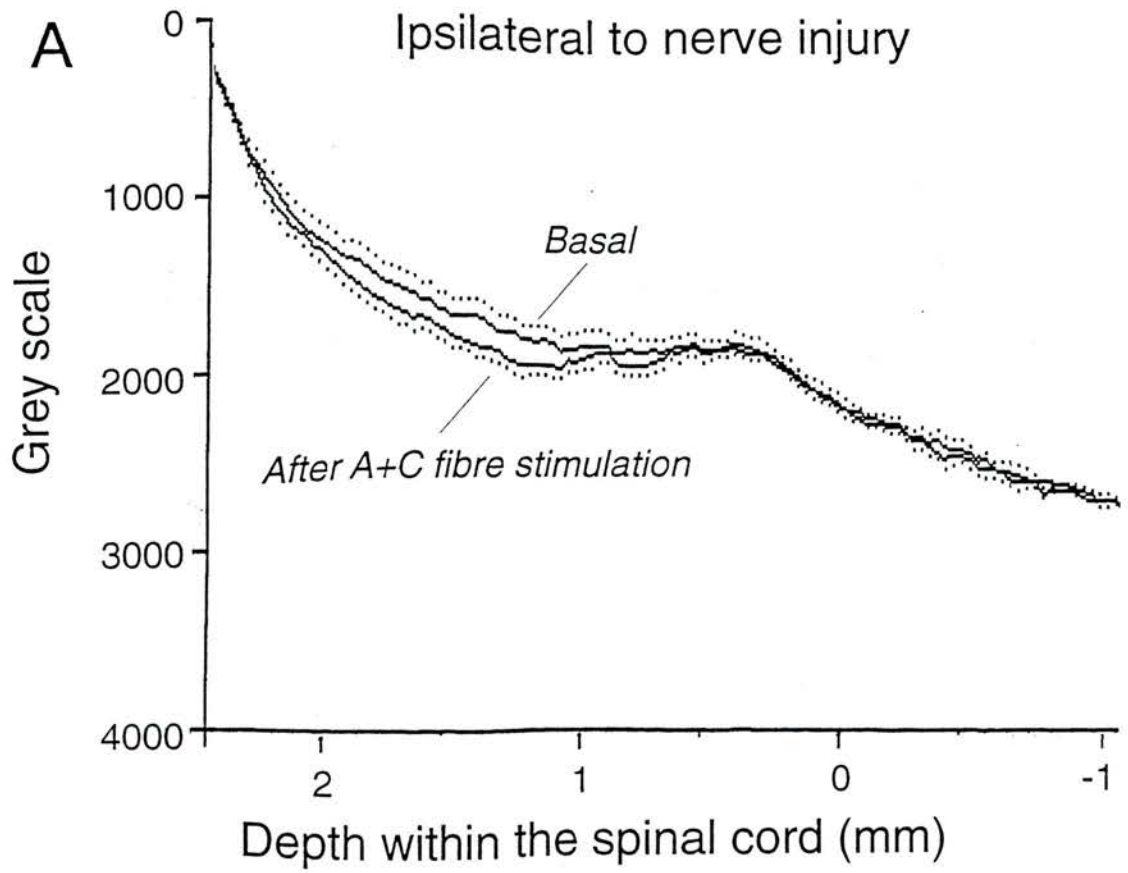
The mean image analysis of microprobes inserted into both sides of the spinal cord, at 15 minute intervals, for up to 2 hours after peripheral stimulation ceased, was compared to that of microprobes inserted under basal conditions. This is shown in Figures 19 and 20. During the time period studied there was no evidence of any persistence of the evoked ir-galanin ipsilateral to nerve injury. This remained the case even microprobes were split into groups of each individual 30 minute time period, from immediately after stimulation to 2 hours later. There was a persistent point of significantly decreased ir-galanin release contralateral to the nerve injury when the analysis was carried out. The temporal resolution of the microprobe technique is limited by the fact that microprobes have to be inserted for 15 minutes to allow adequate binding to the microprobes, thus changes in ir-galanin release during this time would not be detected. Thus, it is possible that the evoked ir-galanin could persist for some minutes. Despite this limitation, the present results with ir-galanin are different from other peptides studied using the same technique. Neurokinin A and neuropeptide Y, for example, have both been shown to persist and spread to sites distant from release, sometimes for hours after stimulated release [109,257].

Figure 19. Study of persistence of ir-galanin after evoked release from high threshold stimulation.

Microprobes were inserted into the ipsilateral side of the spinal cord to nerve injury after peripheral stimulation had ceased for up to two hours afterwards.

A. The mean image analysis (+/-s.e.m.) of microprobes inserted prior to (n=18) high threshold stimulation (2 Hz, 50T, 0.1 msec) and those inserted during the time period from 15 minutes to 2 hours after stimulation had ceased (n=31). The cumulative group of all post-stimulation probes is shown, but the result was the same when split into component groups (0-30 minutes, n=9; 31-60 minutes, n=10. 61-90 minutes, n=8, 91-120 minutes, n=4). There was no decrease in optical density of those probes inserted afterwards compared to those inserted under basal conditions.

B. The differences between the basal and post-stimulus groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord. There was no significant difference between these 2 groups, thus providing no evidence for long-term persistence of ir-galanin after its C fibre-evoked release.


A
Ipsilateral to nerve injury
Grey scale
0
1000
2000
3000
4000
Basal
After A+C fibre stimulation
2
1
0
-1
Depth within the spinal cord (mm)

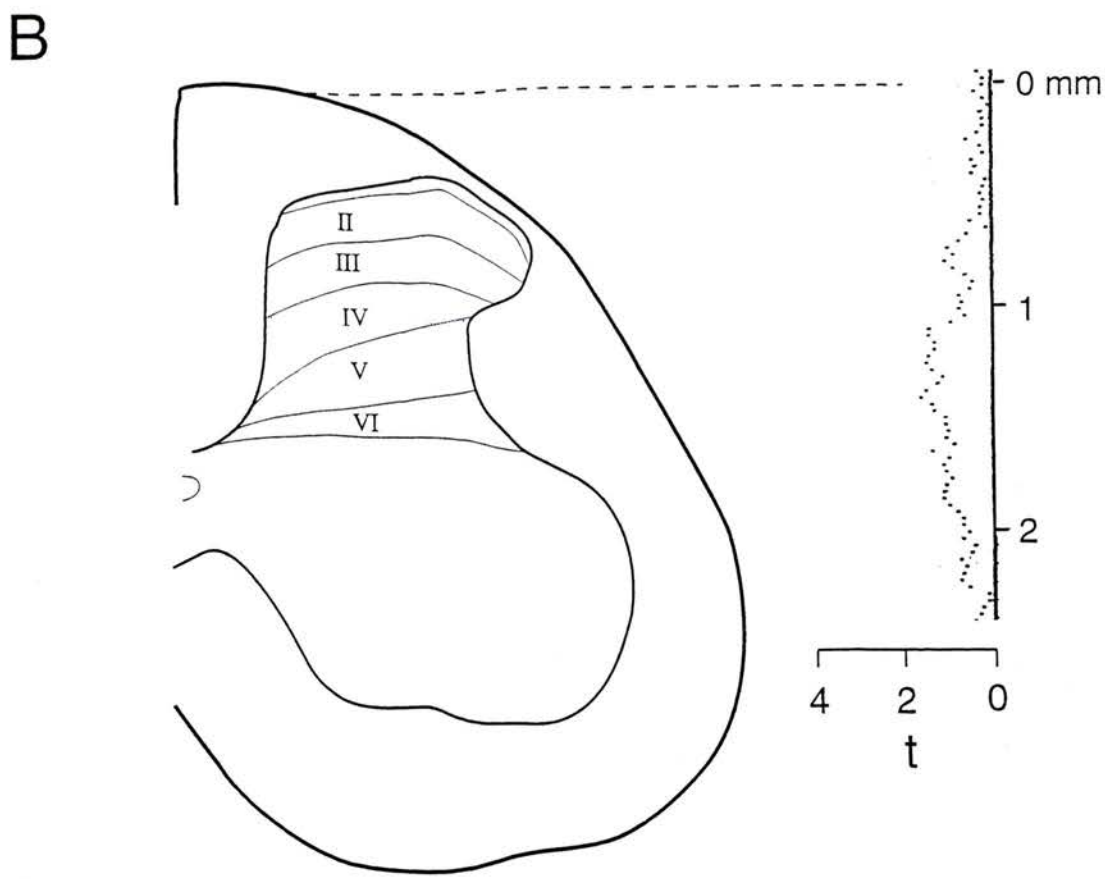
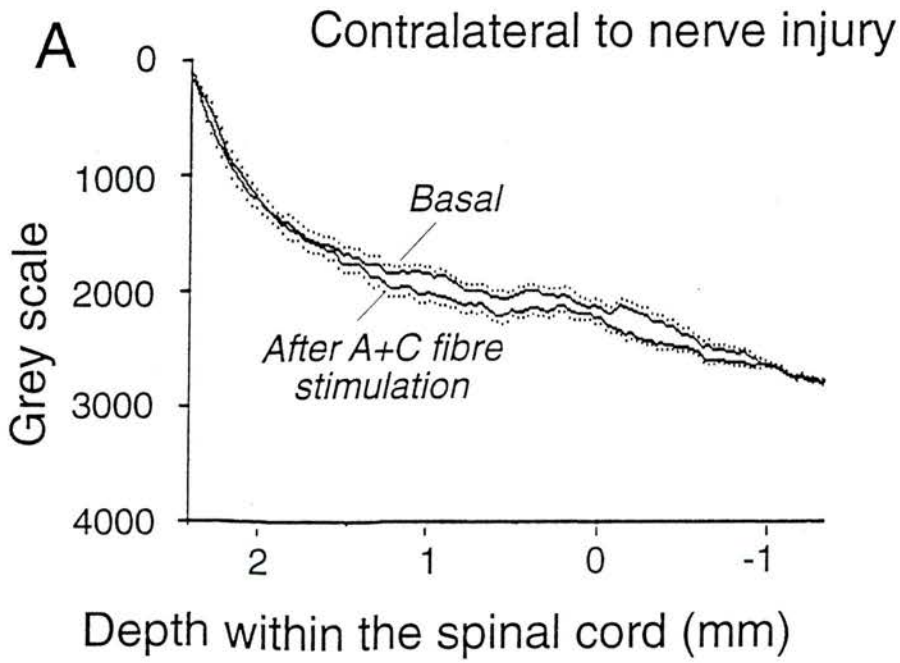

B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

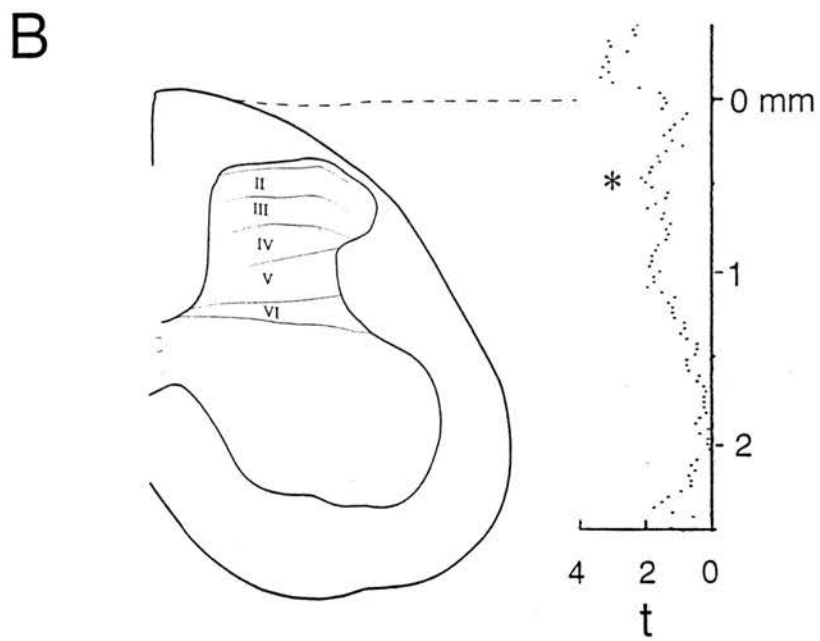
Figure 20. Changes in contralateral ir-galanin release after high threshold stimulation.

Microprobes were inserted into the contralateral side of the spinal cord to nerve injury after peripheral stimulation had ceased for up to two hours afterwards.

A. The mean image analysis (+/-s.e.m.) of microprobes inserted prior to (n=17) high threshold stimulation (2 Hz, 50T, 0.1 msec) and those inserted during the time period from 15 minutes to 2 hours after stimulation had ceased (n=26). There was a minor increase in optical density of those probes inserted afterwards compared to those inserted under basal conditions.

B. The differences between the basal and post-stimulus groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord. A significant decrease was detected in ir-galanin release contralateral to nerve injury, after high intensity stimulation of the injured nerve, as indicated by *.


A
Contralateral to nerve injury
0
1000
2000
3000
4000
Grey scale
Basal
After A+C fibre stimulation
2
1
0
-1
Depth within the spinal cord (mm)


B
II
III
IV
V
VI
0 mm
1
2
*
4
2
0
t

4.3.4 Effect of conduction block on ir-galanin release

A total of 12 neuropathic rats (245-350 g) was studied to determine the effect of blocking peripheral input from the injured nerve on spinal release of ir-galanin. A total of 261 galanin antibody coated microprobes were inserted into the spinal cord, with a further 60 *in vitro* probes and 14 probes incubated in cord perfusate. This was to check that probes inserted during intermittent rather than continuos irrigation of the spinal cord did not show any evidence of probe damage at the insertion site.

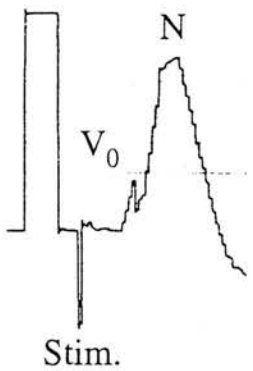
After basal probes had been inserted, and amethocaine gel placed around the nerve the adequacy of conduction block was assessed. Figure 21 shows a typical example of recording cord dorsum potentials from electrical stimulation of the injured nerve, before and after application of amethocaine gel proximal to the nerve injury site. Microprobes inserted into right (n=42) and left (n=30) sides of the spinal cord prior to any intervention confirmed the same increased peak of ir-galanin release occurring spontaneously in the superficial dorsal horn ipsilateral to nerve injury, as found previously (see Figures 13 and 14).

Figure 21. Cord dorsum potentials before and after application of amethocaine gel

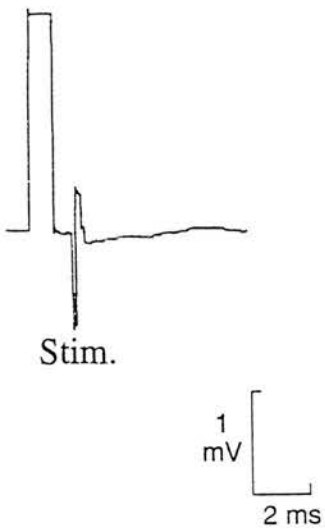
A. The injured nerve was stimulated at 2 T, 1Hz, 0.1 ms pulse width, and the cord dorsum potential measured on the cord surface as described in section 2.2.2. The expected field potential was obtained .

B. The injured nerve was stimulated at a much higher intensity (50 T, 1Hz,0.1 ms pulse width) 30 minutes after amethocaine gel was applied proximal to the nerve injury site. As can be seen from the averaged field potential, no activity was evoked in the spinal cord, confirming block of peripheral electrical input.

A Stimulation of injured nerve



B 30 mins after amethocaine gel



Effect of peripheral conduction block on ir-galanin release

Microprobes inserted after conduction block proximal to the nerve injury site, but distal to the dorsal root ganglia had no effect on the spinal release of ir-galanin on either the ipsilateral (n=38) or contralateral (n=37) side of the spinal cord relative to the nerve injury. The right to left difference between ir-galanin release was preserved after this intervention. This is shown in Figures 22 and 23.

A control group was used where the manipulations of the sciatic nerve required for placement of the plastic tubing around the nerve were carried out, but without application of the amethocaine gel. Microprobes inserted after manipulation of the nerve did not show any significant alteration in the release of ir-galanin in the ipsilateral (n=11) dorsal horn. As with the contralateral effects of high intensity stimulation, peripheral manipulation of the injured nerve resulted in a trend towards a decrease in contralateral ir-galanin release (n=11). This is shown in Figures 24.

Figure 22. Effect of blocking conduction proximal to nerve injury site on spinal ir-galanin release

Microprobes were inserted into both sides of the spinal cord after peripheral conduction block with amethocaine gel had been tested for effectiveness.

A. The mean image analysis (+/-s.e.m.) of microprobes inserted ipsilateral to (n=38) the site of conduction block, proximal to the chronic constriction injury of the sciatic nerve, and those microprobes inserted prior to this (n=42). There was no decrease in optical density of those probes inserted after conduction block compared to those inserted under basal conditions. The differences between the basal and post-conduction block groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord, at C1. There was no significant difference between these 2 groups, indicating no effect of peripheral conduction block on spontaneous ir-galanin release.

B. The mean image analysis (+/-s.e.m.) of microprobes inserted contralateral to (n=37) the site of conduction block, proximal to the chronic constriction injury of the sciatic nerve, and those microprobes inserted prior to this (n=30). There was no decrease in optical density of those probes inserted after conduction block compared to those inserted under basal conditions. The differences between the basal and post-conduction block groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord at C2. As there was no significant difference between these 2 groups, peripheral conduction block did not alter spontaneous ir-galanin release contralateral to the site of nerve injury.

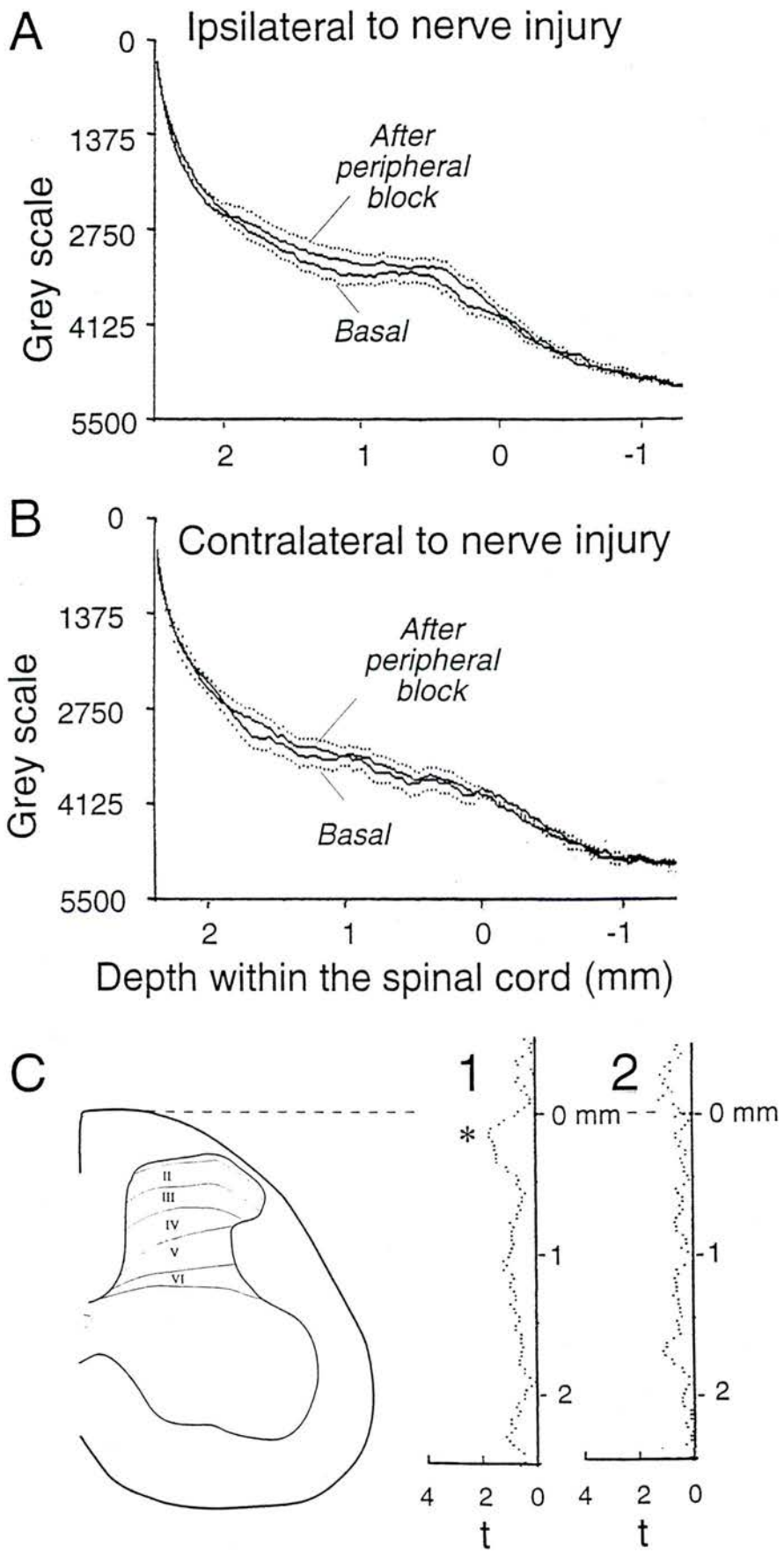
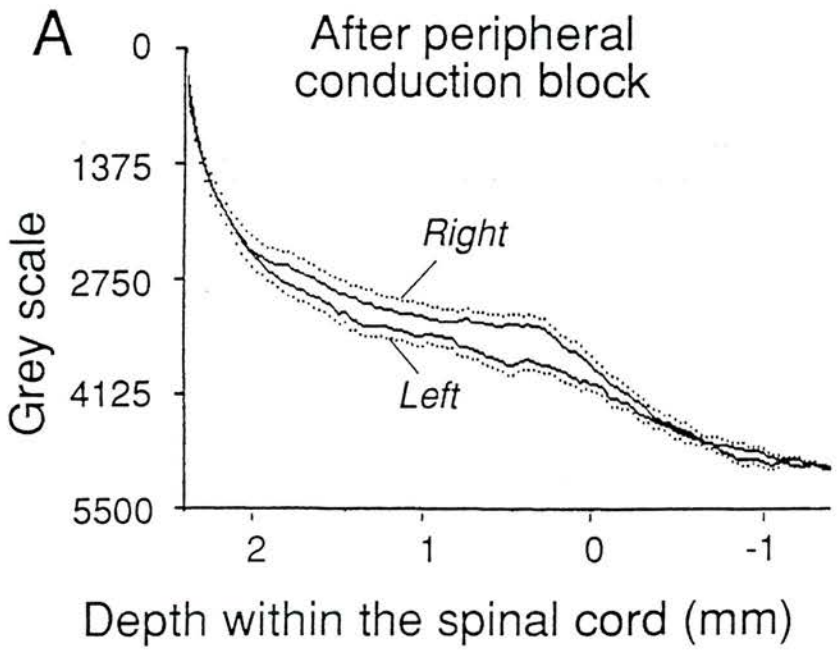

A
Ipsilateral to nerve injury
0
1375
2750
4125
5500
Grey scale
After peripheral block
Basal
2
1
0
-1
B
Contralateral to nerve injury
0
1375
2750
4125
5500
Grey scale
After peripheral block
Basal
2
1
0
-1
Depth within the spinal cord (mm)
C
II
III
IV
V
VI
1
2
*
0 mm
0 mm
1
1
2
2
4
2
0
4
2
0
t
t

Figure 23. Peripheral conduction block does not alter spontaneous ir-galanin release ipsilateral to nerve injury

A. This shows the mean image analyses of microprobes inserted ipsilateral (n=38) and contralateral (n=37) to the injured nerve, that has been previously blocked by amethocaine gel proximal to the injury site.

B. The difference between the two sides of the cord that has been demonstrated under basal conditions is preserved after peripheral conduction block, as demonstrated by the hatched area showing a significant ($p<0.05$) increase in ir-galanin release ipsilateral to the side of the blocked nerve.


A
After peripheral
conduction block
0
1375
2750
4125
5500
Grey scale
Right
Left
2
1
0
-1
Depth within the spinal cord (mm)

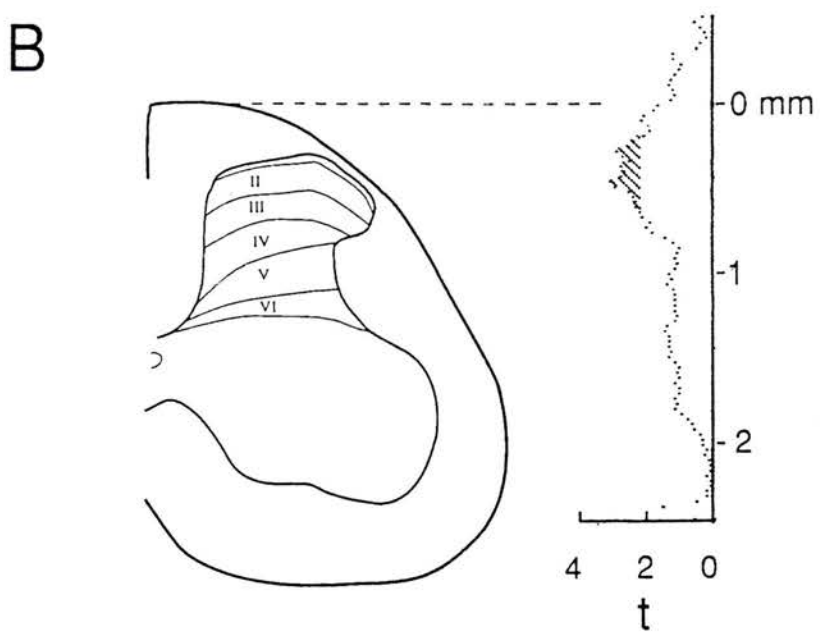
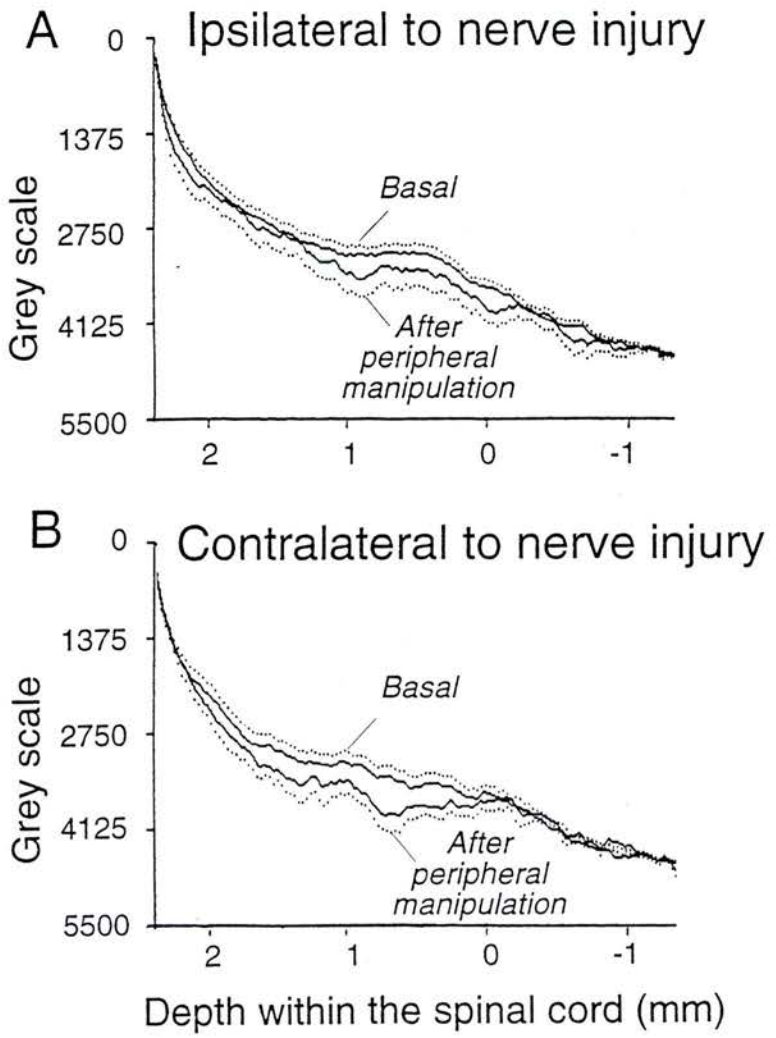

B
II
III
IV
V
VI
0 mm
-1
-2
4
2
0
t

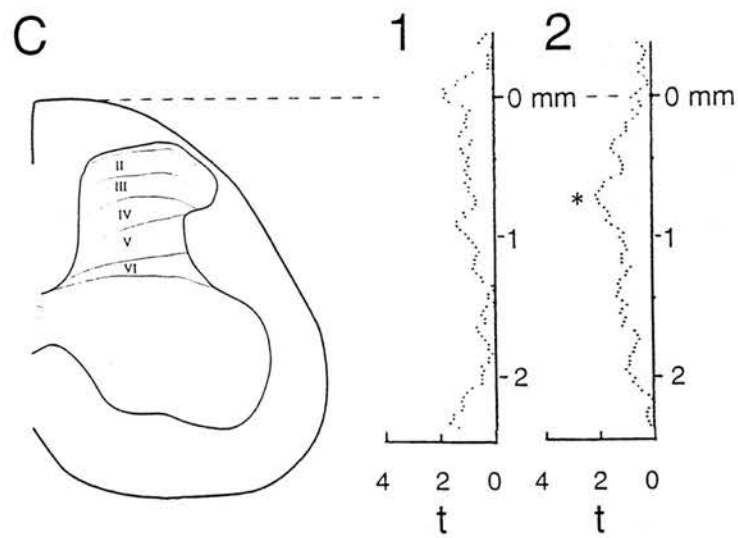
Figure 24. Effect of manipulation of injured nerve on spinal ir-galanin release

Microprobes were inserted into both sides of the spinal cord after peripheral manipulation of the injured nerve, without conduction block

A. The mean image analyses (+/-s.e.m.) of microprobes inserted ipsilateral to the injured nerve prior to (n=42) and after manipulation of the nerve (n=11). The differences between the basal and post-manipulation groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord at C1. There was no significant difference between these 2 groups, indicating no effect of peripheral manipulation on spontaneous ir-galanin release.

B. The mean image analyses (+/-s.e.m.) of microprobes inserted contralateral to the injured nerve prior to (n=30) and after manipulation of the nerve (n=11). The differences between the basal and post-manipulation groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord at C2. A trend towards a decrease in spontaneous ir-galanin release contralateral to the site of nerve injury after peripheral manipulation of the injured nerve was seen. Significant points are indicated *.


A
Ipsilateral to nerve injury
0
1375
2750
4125
5500
Grey scale
Basal
After peripheral manipulation
2
1
0
-1
B
Contralateral to nerve injury
0
1375
2750
4125
5500
Grey scale
Basal
After peripheral manipulation
2
1
0
-1
Depth within the spinal cord (mm)


C
1
2
II
III
IV
V
VI
0 mm
0 mm
1
2
1
2
*
4
2
0
4
2
0
t
t

Effect of proximal conduction block on ir-galanin release

After all electrical activity from the injured sciatic nerve had been effectively blocked by placement of amethocaine gel proximal to the dorsal root ganglia, further microprobes were inserted into the spinal cord. On the side of the cord ipsilateral to the nerve injury, there was a small area of increased ir-galanin release in the white matter of the spinal cord after proximal conduction block (n=35). However, the greatest effect of proximal conduction block was a marked, diffuse increase in ir-galanin release on the side of the cord contralateral to the nerve injury (n=34). The increase on the contralateral side of the cord was such that the previously observed difference between the two sides of the cord, with greater spontaneous ir-galanin release ipsilateral to the injured nerve was no longer apparent. This is shown in Figures 25 and 26.

The effects of manipulation of the dorsal roots, as described in section 4.2.1, but without application of local anaesthetic, on spinal ir-galanin release were also studied. This was to ensure that the changes seen in ir-galanin release were not simply due to the preparation required to place the amethocaine gel on the dorsal roots. Microprobes inserted ipsilateral to the nerve injury were compared before (n=42) and after(n=11) manipulation, and no significant difference was found in ir-galanin release. Microprobes inserted into the contralateral side of the cord, where the greatest change was detected after proximal conduction block, did not show any difference in ir-galanin release before (n=30) and after (n=12) manipulation of the dorsal roots.

In order to exclude the possibility of damage to the microprobes from exudate collecting on the spinal cord, when irrigation was intermittent, microprobes were incubated in 5 μl capillary tubes of this exudate, at 37°C for 15 minutes. Comparison of such microprobes (n=14) with the in vitro microprobe group (n=60) did not show any difference, thus excluding the possibility of enzymatic damage to the antibody coating. This is shown in Figure 27.

Figure 25. Effect of proximal conduction block on spinal ir-galanin release

A. The mean image analyses (+/-s.e.m.) of microprobes inserted ipsilateral to the nerve injury, before (n=42) and after (n=35) conduction block proximal to the dorsal root ganglia from the injured nerve. Those inserted after conduction block showed some increase in optical density compared to those inserted under basal conditions. The differences between the basal and post-conduction block groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord at C1. There was a significant difference ($p<0.05$) between these 2 groups, with a small area of increased ir-galanin just below the dorsal surface of the spinal cord.

B. The mean image analyses (+/-s.e.m.) of microprobes inserted contralateral to the nerve injury, before (n=30) and after (n=34) conduction block proximal to the dorsal root ganglia from the injured nerve. There was an increase in optical density of those probes inserted after conduction block compared to those inserted under basal conditions. The differences between the basal and post-conduction block groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord at C2. There was a large area throughout the spinal cord where ir-galanin release had increased significantly after complete block of conduction from the injured nerve.

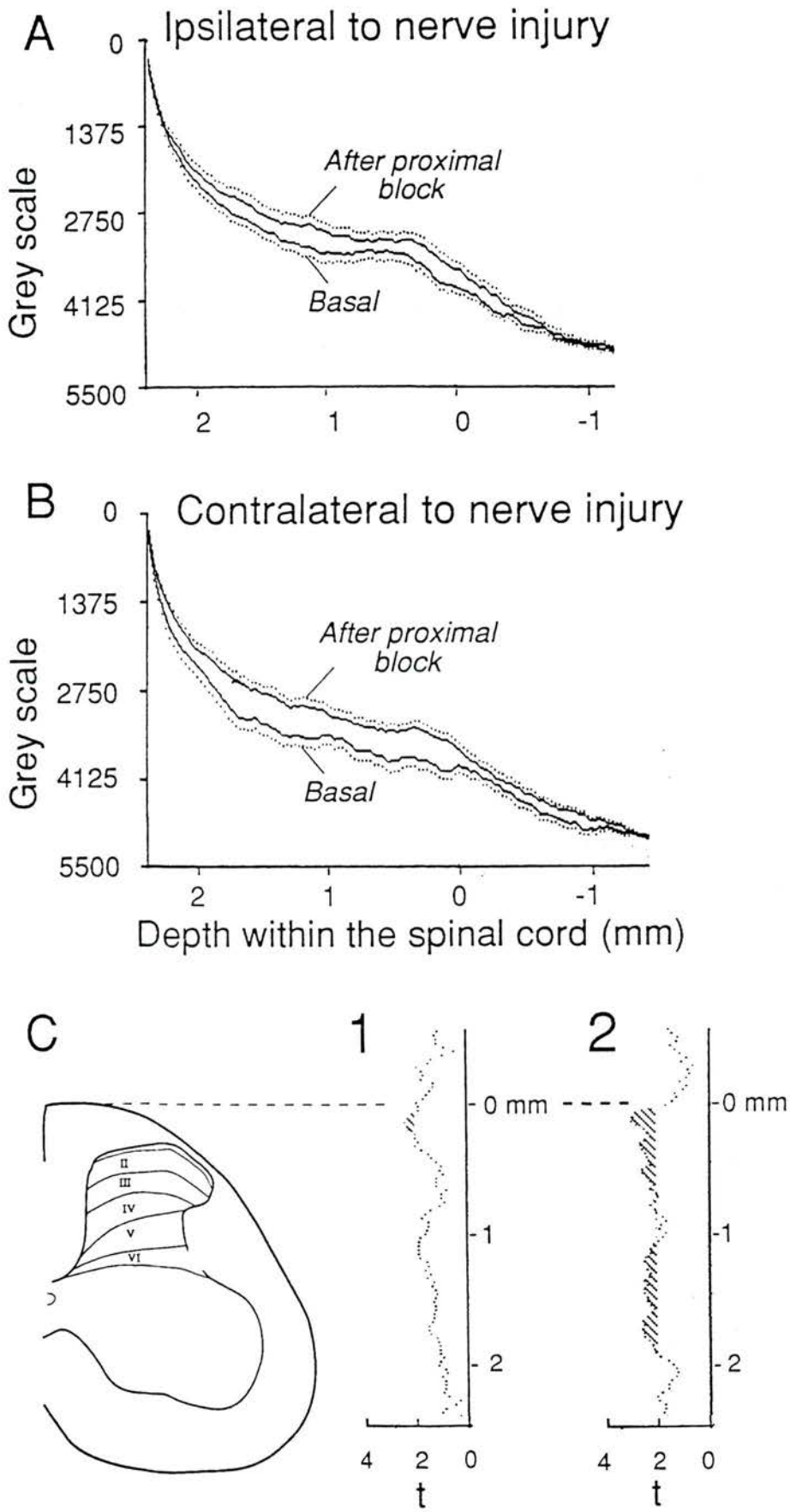
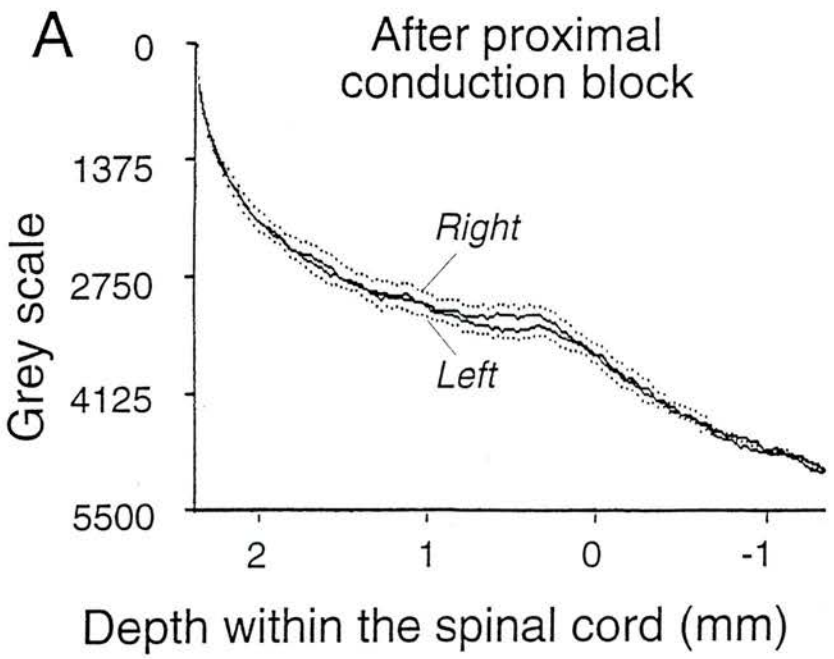

A
Ipsilateral to nerve injury
0
1375
2750
4125
5500
Grey scale
After proximal block
Basal
2
1
0
-1
B
Contralateral to nerve injury
0
1375
2750
4125
5500
Grey scale
After proximal block
Basal
2
1
0
-1
Depth within the spinal cord (mm)
C
1
2
II
III
IV
V
VI
0 mm
-1
-2
4
2
0
t
0 mm
-1
-2
4
2
0
t

Figure 26. Side to side comparison of ir-galanin release after proximal conduction block

A. This shows the mean image analyses of microprobes inserted ipsilateral (n=35) and contralateral (n=34) to the injured nerve, that has been previously blocked by amethocaine gel proximal to the dorsal root ganglia. The two groups are almost superimposed on each other, showing that blocking all neuronal input from the injured sciatic nerve results in eliminating the difference in spontaneous ir-galanin release between the two sides of the cord that occurs after nerve injury.

B. The difference between the two sides of the cord that has been demonstrated under basal conditions in neuropathic rats, is no longer present after proximal conduction block, as no significant increase in ir-galanin release can now be detected.


A
After proximal
conduction block
0
1375
2750
4125
5500
Grey scale
Right
Left
2
1
0
-1
Depth within the spinal cord (mm)

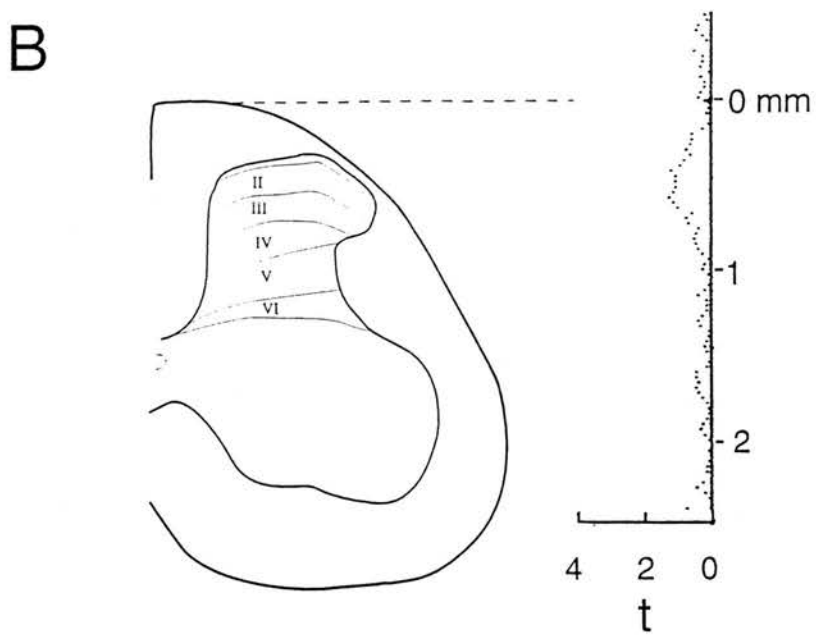
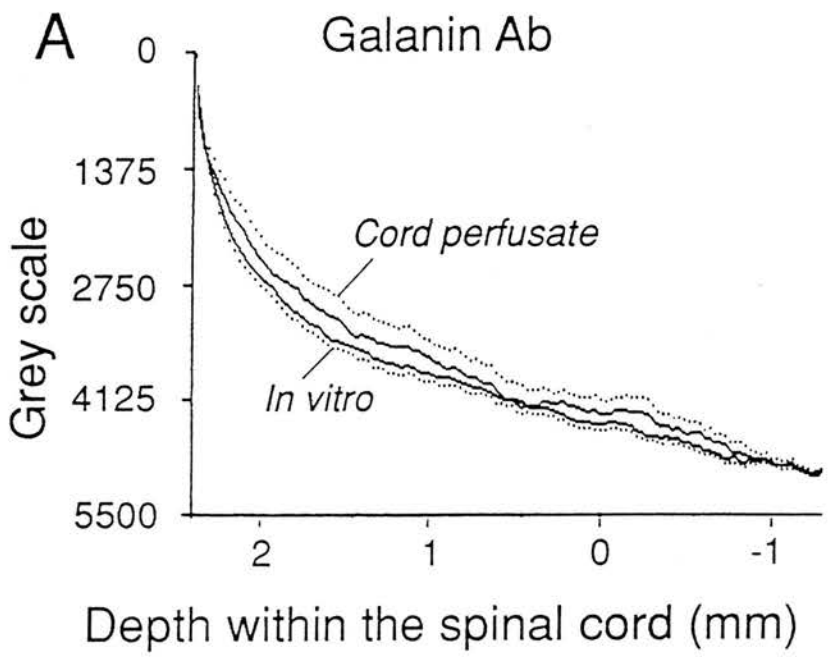
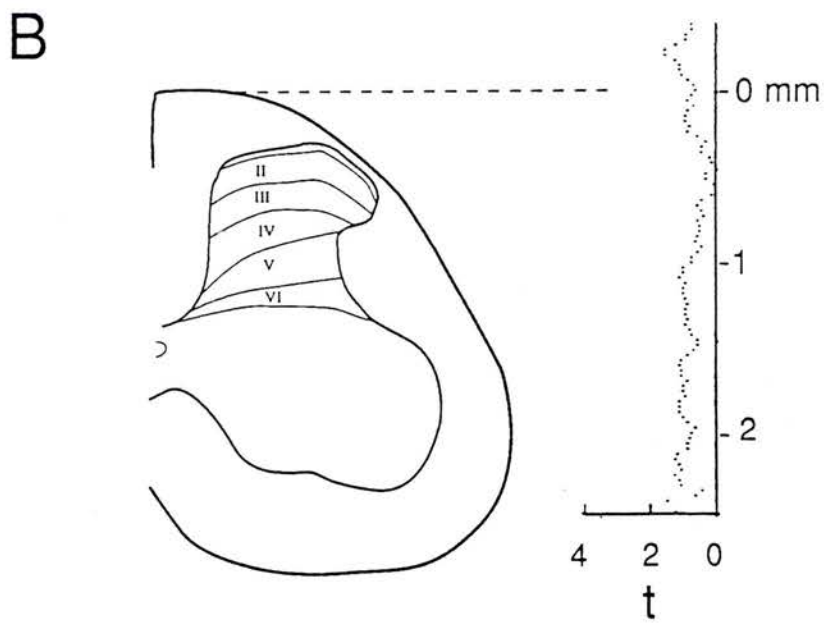

B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

Figure 27. Effect of cord perfusate on antibody coating of microprobes

A. This shows the mean image analysis of microprobes (n=14) incubated in 5 μl capillary tubes of the fluid on the cord surface, during intermittent irrigation, at 37°C for 15 minutes. The microprobes were then treated as for the other experimental probes. The second mean image analysis shows the *in vitro* group (n=60) of microprobes, to allow detection of any areas along the length of the probe that might be damaged.

B. The t statistics obtained from comparing *in vitro* probes with those incubated in cord perfusate shows that there is no difference between these two groups. This would help to confirm that decreases in optical density from in vivo probes is due to ir-galanin release and not probe damage.


A
Galanin Ab
0
1375
2750
4125
5500
Grey scale
Cord perfusate
In vitro
2
1
0
-1
Depth within the spinal cord (mm)


B
II
III
IV
V
VI
0 mm
-1
-2
4
2
0
t

4.4 Discussion

In summary, the findings from the study of ir-galanin release in the spinal cord are:

1. In neuropathic rats there was evidence of a new site of ir-galanin release occurring in the absence of any active peripheral stimulation, peaking in the superficial dorsal horn ipsilateral to the nerve injury. The pattern of ir-galanin release contralaterally was unchanged from that normally found.
2. This new site of ir-galanin release was increased, with a peak in the same area, by electrical stimulation of the damaged nerve only at stimulus strengths sufficient to activate small myelinated and unmyelinated primary afferent fibres, but not at lower stimulus strengths. The stimulus-evoked release of ir-galanin did not persist for any significant length of time after release.
3. Blocking all neuronal input from the injured nerve resulted in little change in ipsilateral ir-galanin release, but a significant increase in ir-galanin release on the contralateral side of the cord. This was such that the difference in ir-galanin release between the two sides of the spinal cord found consistently in neuropathic rats was no longer present. Both high intensity stimulation and peripheral manipulation of the injured nerve resulted in a significant decrease in contralaterally released ir-galanin at one point in the superficial dorsal horn.

4.4.1 Source of ir-galanin

It is important to consider the source of the increased ir-galanin release. Possible sources of release include primary afferent neurones, intrinsic neurones of the dorsal horn, or descending spinal tracts. Although it has been demonstrated both in this study and others [168], that there was extensive basal release of ir-galanin in normal rats, this was unlikely to be from primary afferent neurones, for several reasons. Firstly, there were very low levels of galanin mRNA in the dorsal root ganglia [11,425], and secondly, peripheral innocuous and noxious stimulation did not alter the basal ir-galanin release in the spinal cord of normal rats or cats [168,292]. The major difference in the neuropathic rats was that an increase in ir-galanin release was found in the dorsal horn ipsilateral to the nerve injury, and this was increased further by stimulation of

high threshold neurones (A-delta and C fibres). Other studies have shown dramatically increased galanin synthesis after nerve injury in the small to medium sized primary afferent cells that normally terminate in the superficial dorsal horn [308,316]. There is also immunohistochemical evidence that the increase in intra-axonal galanin levels in the dorsal horn after nerve injury arises from central transport from the cell bodies in the dorsal root ganglia [426,490].

The results from the current studies indicate that the increased spontaneous and evoked release of ir-galanin in the superficial dorsal horn of neuropathic rats is likely to arise from small primary afferent neurones. It is also likely that the significantly increased spontaneous release of ir-galanin found in the superficial dorsal horn of neuropathic rats arises from these primary afferent neurones.

The interpretation of theses studies is made more complicated by the fact that ir-galanin release is increased in an area of the spinal cord where several systems interact. Thus, the small sized primary afferent neurones that start to produce galanin terminate here, in an area where either intrinsic or descending neurones also release ir-galanin. Although it is highly probable that the increased ir-galanin release is indeed from primary afferent neurones, there may also be parallel effects of nerve injury on other galanin-containing neurones, of non-primary afferent origin, in this area of the spinal cord. The results of blocking peripheral conduction, with an increase in ir-galanin release on the contralateral side of the spinal cord would certainly seem to indicate the involvement of either intrinsic loops with bilateral neuronal connections in the spinal cord [180], or bilateral effects of supraspinal systems, both of which must be affected by ongoing neuronal input from the injured nerve. If this were the case, then inhibition of ir-galanin release contralaterally, either by high intensity stimulation of the injured nerve, or manipulation around the site of nerve injury could also occur by this mechanism. This would indicate that some input from the injured nerve results in inhibition of ir-galanin release from neurones within the dorsal horn. As this effect is seen on the contralateral side of the cord, it seems that input from a unilateral nerve injury has bilateral effects. This would certainly correlate with bilaterally altered responses previously found in response to electrical stimulation of the injured nerve [75].

4.4.2 Stimulus for ir-galanin release

If the increase in spontaneously released ir-galanin did arise from primary afferent neurones, one explanation for this is that abnormal activity, developing in the primary afferent neurones, provided a stimulus for release. There is an increasing amount of evidence that after nerve injury, primary afferent neurones behave abnormally, with the generation of spontaneous ectopic activity both at the nerve injury site [275,407,488], and at cell bodies in the dorsal root ganglia [190,472].

In the CCI model, it was found that the majority of cells showing evidence of spontaneous activity were of the A-beta type. Spontaneous activity in C fibres did not appear to increase significantly until about 10 days after nerve injury [187]. A high level of spontaneous activity was also found in medium sized neurones [396]. This high level of spontaneous activity from primary afferents is likely to contribute to changes in somatosensory processing that occur centrally in response to peripheral nerve injury. Tal and co-workers [407] did find that there was a correlation between the amount of spontaneous activity in myelinated primary afferents and the development of thermal hyperalgesia.

As these afferents are firing spontaneously, it is possible that the central release of neuropeptides such as galanin is due to these abnormal impulses.

Although the block of ectopic discharges was not measured directly in the experiments using local anaesthetic to block conduction, there is evidence of ectopic discharges being generated via TTX-sensitive sodium channels [328], that are sensitive to local anaesthetics at much lower doses than those required for conduction block [57,260]. It can be assumed therefore that by effectively blocking the measured cord dorsum potential resulting from stimulation of the injured nerve, all ectopic discharges were also blocked.

It is likely that the changes in ir-galanin release found after conduction block are due to effects in a mixed neuronal population - thus ir-galanin released from primary afferent activity ipsilateral to the nerve injury may have been eliminated, whereas the block of all peripheral input led to disinhibition of intrinsic neurones or supraspinal systems releasing ir-galanin. This would account for the contralateral increase in ir-galanin, with the increase in ipsilateral ir-galanin from intrinsic neurones being

balanced by a decrease from primary afferent neurones. Thus, as galanin is produced in mainly small to medium sized primary afferent neurones, it may be that spontaneous activity in these results in central ir-galanin release. However, the effects of spontaneously firing large diameter primary afferent neurones may also have an effect on spinal release of ir-galanin. This may, in part, explain the differential effects in ir-galanin release from primary afferent neurones and from intrinsic or supraspinal neurones.

4.4.3 Functional significance of galanin changes

The functional role of galanin after nerve injury is not known, but there are several possibilities:

1. Galanin may act as an endogenous analgesic substance. The functional significance of galanin is not clear, with evidence of both inhibitory [348,455] and excitatory [79,217] effects, including potentiation of the inhibitory effects of morphine [454]. Its presence in the superficial dorsal horn and its increased release here after nerve injury, implies that it may have an important role in processing of nociceptive information. After peripheral nerve injury, behavioural and electrophysiological studies show a greater inhibitory action of galanin. It may therefore be more important as an inhibitor of ongoing neuronal activity only after a peripheral nerve injury has occurred [423,453]. The lack of persistence of released ir-galanin for any significant length of time after its release, provides a further indication that it acts in or close to its area of release, i.e. in an area of the dorsal horn thought to be important in the processing of nociceptive inputs. Additionally, the increase in contralateral ir-galanin release after conduction block may indicate that one reason for the generation of spontaneous pain is that galanin release is inhibited, thus preventing its endogenous analgesic effect - it could be that if an inadequate amount of galanin is released, there is inadequate damping of abnormal incoming activity from the injured nerve. Thus, the release of ir-galanin from primary afferent fibres may be inadequate to inhibit transmission of abnormal nociceptive messages. As the spontaneously released ir-galanin is likely to be from small to medium sized primary afferent neurones, it may be that spontaneous firing from large primary

afferent neurones inhibits further ir-galanin release, thus it could be that pain develops because of an inadequate galanin response.

2. It must also be considered that galanin may in fact be involved in the development of the central sensitisation accompanying neuropathic pain. There is a correlation between the rate of ectopic discharge and the degree of spontaneous pain in both humans and animals [320,347], and it may be that ectopic impulse-evoked release of neuropeptides is related to the generation of spontaneous pain. Ir-galanin release occurred in an area where new synaptic contacts have been formed, and could amplify an ongoing neural input, resulting in pain. Electrophysiological studies of the dorsal horn in animal models of nerve injury have shown both an increase in spontaneous activity of dorsal horn neurones [223,388,389] and ascending systems [332,333], as well as bilaterally increased evoked activity [75]. However, this increase in activity has neither been related to galanin release nor to the generation of pain. More recent evidence has shown almost complete loss of GABA-containing cells in laminae I- III of the dorsal horn after nerve injury, with lesser decrease contralaterally [175]. Galanin has been shown to co-exist with GABA in the superficial dorsal horn in normal rats [379], a pattern of co-existence that must be altered after nerve injury. Alterations in the number of inhibitory neurones and the pattern of neurotransmitter co-existence may lead to impaired inhibitory function and central hyperexcitability after peripheral nerve injury.
3. Alternatively, galanin may be not be acting as a neurotransmitter at all, but may be involved in the reorganisational changes that occur in the dorsal horn after nerve injury [68]. An enhancement of central regeneration of primary afferent neurones has been found after axotomy and nerve crush [351]. Large myelinated fibres have been shown to sprout from deeper in the dorsal horn up to laminae I-II [232,468,469], with some evidence of synaptic contacts being formed in these superficial areas. A protein associated with regrowth, growth associated protein-43 (GAP-43), appears in the superficial dorsal horn after peripheral nerve injury with a distribution similar to that of small primary afferent terminals [51,71,467]. Thus, there is anatomical evidence of altered primary afferent connections in the same area as the increased release of ir-galanin has been found. There is some evidence that peptides can have

neurotrophic effects themselves. PACAP, VIP and NPY have been shown to induce neurite outgrowth [145,178], but galanin did not have any neurotrophic effect [212].

In conclusion, the findings of increased release of ir-galanin in the superficial dorsal horn on the side ipsilateral to a peripheral nerve injury suggest that it plays a greater role in spinal processing in this abnormal state. The spontaneous release of galanin, as well as increased release during stimulation of small diameter primary afferents, indicate a role in the processing of nociceptive information. The widespread effects of blocking conduction from the injured nerve on ir-galanin release also indicate an important functional role in the processing of information from the injured nerve. There is evidence for ongoing inhibition of ir-galanin release bilaterally in the neuropathic state, which may have widespread functional consequences. The neuronal systems involved are unlikely to be simple mono-synaptic connections, but to involve complex interactions between primary afferent neurones, local dorsal horn neurones and ascending and descending systems. However, galanin, or a galanin analogue, may provide a potential new treatment for neuropathic pain syndromes, perhaps by influencing an endogenous analgesic system similar to that found with opioid peptides. However, further studies are required to elucidate the precise functional role of galanin released into the spinal cord, both from primary afferent neurones and from other sources.

Chapter 5. Studies of the spinal release of ir-neuropeptide Y in neuropathic rats

5.1 Introduction

In normal rats, ir-NPY was released in the superficial dorsal horn, and peripheral electrical stimulation had no effect on this release. As NPY is not synthesised by primary afferent neurones in the normal situation [100,435], and spinalisation had no significant effect on release, then the ir-NPY release detected in the superficial dorsal horn was likely to be from intrinsic neurones [258]. Studies of neuropathic rats, in which I was involved, detected an area of spontaneously released ir-NPY deeper in the dorsal horn than normally found, on the side ipsilateral to nerve injury. This new area of ir-NPY release was increased by stimulation of large myelinated fibres and could still be detected for some time after its release [257]. As this new zone of ir-NPY release occurred in a deeper area from that where intrinsic neurones normally released ir-NPY, in the superficial dorsal horn, it is likely that this spontaneous release of ir-NPY was from large diameter primary afferent neurones [257], that have started to produce NPY after nerve injury [308,316,492].

As with galanin, one possible stimulus for spinal release of ir-NPY from primary afferent fibres could be spontaneously occurring ectopic discharges that develop at the nerve injury site and the dorsal root ganglia, particularly in myelinated fibres. It has been well documented that in the CCI model, spontaneous neuronal activity arises at ectopic sites - both at the nerve injury site [187,407] and also at the dorsal root ganglia from the injured nerve [396,472]. Initially, ectopic activity was found in the majority of A-β fibres, to a lesser extent in A-δ fibres and least in unmyelinated fibres. The pattern of ectopic activity developed with time, with myelinated fibres developing this activity at an earlier time point than was seen in unmyelinated fibres [187]. There was also evidence of abnormal ectopic activity spreading to influence non-injured primary afferent neurones, with cross-excitation occurring between both primary afferent neurones [8] and spontaneously firing sympathetic neurones [90] in the dorsal root ganglia.

The abnormal ectopic activity may involve voltage sensitive Na^+ channels [260] that are sensitive to local anaesthetics at much lower doses than those required for conduction block [57,91]. If NPY were released by this mechanism then block of ectopic discharges, as described in section 5.2.1, would eliminate the deep zone of ir-NPY released in response to spontaneous activity in primary afferent neurones.

Another factor that may be important in the changes in peptide synthesis and release after nerve injury, is interruption to the supply of peripherally derived growth factors. There is evidence that after nerve injury there is a decrease in production of NT-3 distally, both in neurones [239] and Schwann cells [131], as well as decreased retrograde transport from the periphery [158]. It may be therefore, that lack of NT-3 plays at least a partial role in the changes in peptide production of at least some of the primary sensory neurones. Lack of both NGF [424] and NT-3 [326] has been shown to influence the increase in NPY synthesis by primary afferent neurones after axotomy. Like the primary afferent neurones that start to produce NPY after nerve injury, the NT-3 responsive neurones are also large diameter primary afferent neurones. Thus, NT-3 deficiency may be an important factor in the changes seen in NPY production in these large diameter neurones after nerve injury. Studies of NT-3 knockout mice have found a marked decrease in sensory neurones in the dorsal root ganglia, particularly large diameter sensory neurones, with associated decreases in Ia muscle afferents, slowly adapting mechanoceptor and proprioceptive fibres [3,4,384]. In addition, it is mainly the large diameter primary sensory neurones that express the high affinity NT-3 receptor, the trkC receptor [239]. Mice lacking the trkC receptor show marked sensory abnormalities, and a similar loss of large myelinated fibres to that seen in the NT-3 knockout mice [209,378]. NT-3 itself has been shown to have antinociceptive effects [380], as well as attenuating behavioural and anatomical effects of a large fibre sensory neuropathy induced by pyridoxine [158]. Thus lack of NT-3 might result in the development of pain-related behaviour. There is also recent evidence that neurotrophins may actually alter neurotransmitter release, possibly by altering the levels of secretory proteins at the synapse [405], although the effect of NT-3 on neurotransmitter release has not yet been studied.

To investigate the effect of a peripheral nerve injury on spontaneous ir-NPY release further, two series of studies were carried out using the antibody-coated microprobe technique previously described.

1. All neuronal input from the injured nerve was blocked by local anaesthetic placed proximal to the dorsal root ganglia from the injured nerve.
2. Sub-cutaneously implanted osmotic pumps chronically infused NT-3 to the nerve injury site, from the time of nerve injury, for 10 to 14 days, when the effect on spontaneous ir-NPY release was studied. During the infusion period quantitative assessment of the degree of mechanical allodynia and hyperalgesia was studied.

5.2 Materials and methods

5.2.1. Animal preparation

Set up of neuropathic model and implanting osmotic pumps

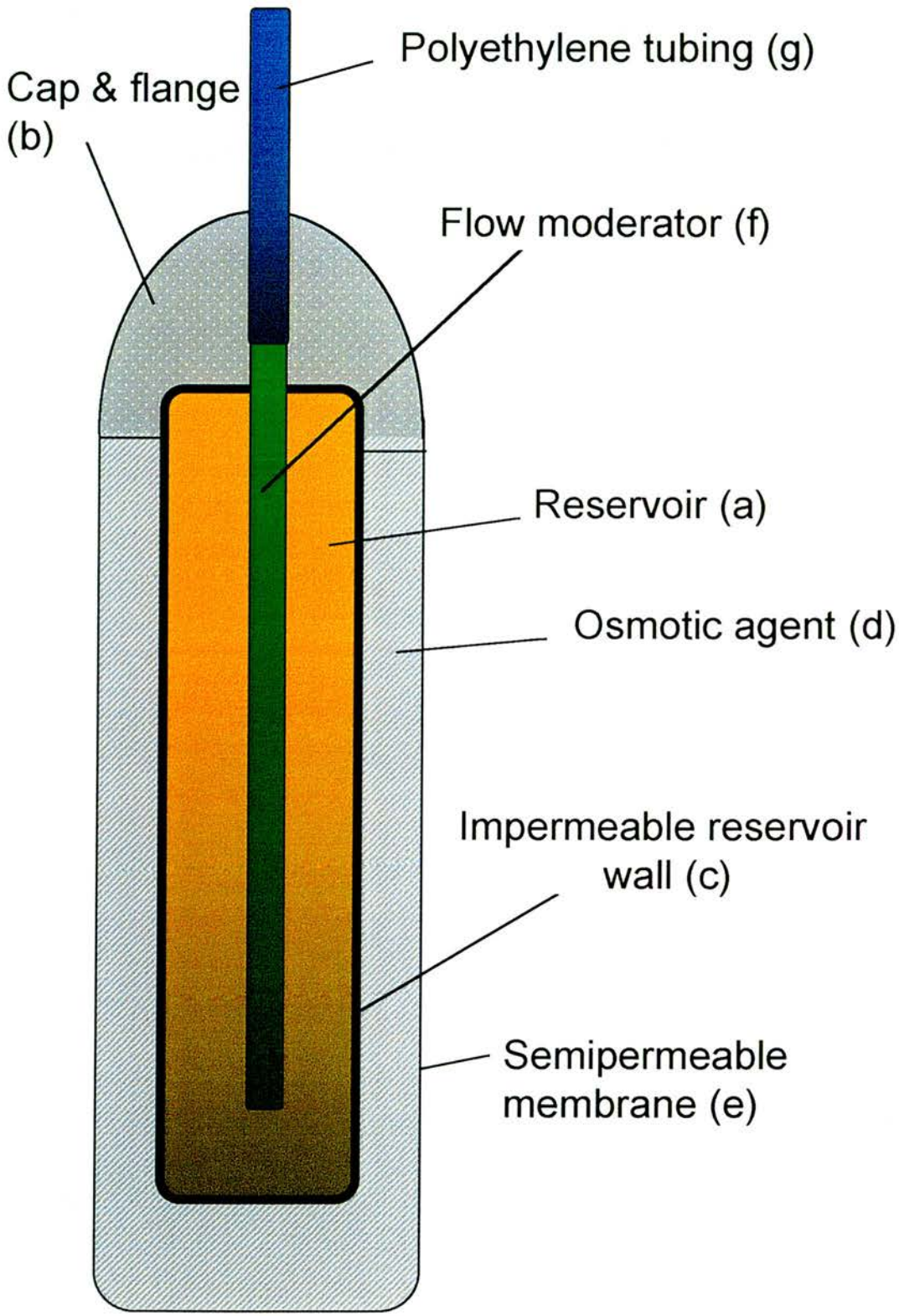
A total of 31 male Wistar rats were used (220 g to 360 g; Charles River, UK) in the studies of ir-NPY release. In all the rats used, the CCI model was set up as previously described in section 2.2.1, except for those rats in which the effects of NT-3 infusion were being studied. In those animals, at the time of loose ligation of the right sciatic nerve, Alzet® mini-osmotic pumps (Alza Scientific Products, Ca, USA) were also implanted sub-cutaneously into the lower abdomen. The pump used had a reservoir volume of 200 µl (model No 2002) and an infusion rate of 0.5 µl/hr. The pumps were pre-filled with the filling tube supplied, according to manufacturer's instructions, using a flow moderator attached to a 3 cm length of polyethylene tubing (PE-60). All handling of the pumps and materials used were carried out using strict aseptic technique to ensure that the pump and tubing were sterile, when implanted into the rat. A diagram of the pump and an outline of the principles behind its operation [411] are shown in Figure 28. After the pump was implanted the attached catheter was tunnelled sub-cutaneously to the site of nerve injury, at mid-thigh level. A larger diameter piece of tubing, approximately 1 cm in length, with a longitudinal split, was placed around the nerve and the ligatures. To prevent this slipping down the nerve this was anchored by a suture (4/0 Vicryl) to the end of one of the ligatures. The slit was held shut by further sutures, and the catheter itself sutured within the tubing. This allowed delivery of the NT-3 solution close to the nerve injury site, thus ensuring that it received the highest concentration of the solution. It did not prevent systemic absorption of NT-3, thus the possibility of systemic and possibly bilateral effects could not be excluded. For this reason, a control group of rats was used with 0.9% saline only in the implanted osmotic pumps. After implantation of the pumps, tissue layers were closed as described previously (section 2.2.1) and the rats recovered in the standard fashion.

From day 3 after ligature placement a regular series of behavioural tests were carried out as outlined in chapter 3. These were analysed using Mann Whitney Rank Sum tests to determine the effect of NT-3 infusion on the development of the behavioural syndrome. At ten to fourteen days after nerve injury ir-NPY release experiments were carried out.

Figure 28. Diagram of Alzet® osmotic pump

This outlines the principles of the osmotic pump. NT-3 or sterile 0.9% saline fills the drug reservoir (a), sealed with a cap and flange (b). This is surrounded by a compressible, impermeable reservoir wall (c). Outwith this is an osmotic agent - a high concentration of sodium chloride, contained within an osmotic sleeve (d), with a semi-permeable membrane (e) separating this from the implantation site. The difference in osmotic pressure between this compartment and the implantation site draws in water, compressing the drug reservoir and driving delivery of the test solution. The rate of flow is controlled partly by the flow moderator (f), and partly by the characteristics of the semi-permeable membrane. The flow moderator is tightly connected to the polyethylene catheter (g) that delivers the NT-3 or saline to the nerve injury site. Diagram modified from manufacturer's data.

Figure 30. Diagram of Alzet osmotic pump



Blocking neuronal input from the injured nerve

A total of 12 rats with behavioural evidence of spontaneous pain and mechanical allodynia and hyperalgesia were studied (see chapter 3). The standard experimental set up described in section 2.2.2 was used. Prior to conduction block, 6-8 NPY antibody-coated microprobes were inserted into the spinal cord to establish basal levels of spontaneous ir-NPY release. As for the studies of ir-galanin release, after proximal conduction block, the laminectomy was extended distally and laterally to allow gentle mobilisation of the dorsal roots from the sciatic nerve, and a small piece (~0.5 x1cm) of black polythene inserted under the dorsal roots. Approximately 0.1ml amethocaine gel was applied to the dorsal roots, on top of the polythene, and surrounded by sealant gel around the edges of the polythene to prevent leakage of the amethocaine gel pool onto the spinal cord. Intermittent irrigation of the cord with warmed sterile Ringer's solution was used to prevent washing the local anaesthetic onto the cord.

A minimum of 30 minutes was allowed after application of the amethocaine gel to the dorsal roots. As was described in section 4.2.1, adequacy of conduction block was checked by high intensity electrical stimulation proximal to the nerve injury site. Only after an adequate block had been established were further microprobes inserted into the spinal cord for up to two hours after conduction block had been established.

At the end of each experiment a pair of microprobes was incubated in 5µl capillary tubes of cord perfusate from the surface of the spinal cord, for 15 minutes at 37°C. These were then washed, as for *in vivo* probes and incubated in ^{125}I Fab, instead of ^{125}I NPY. For comparison, some of the *in vitro* probes from each experiment were also incubated in ^{125}I Fab. This allowed assessment of any damage to the NPY antibody coating on the probe surface.

Effect of NT-3 infusion on ir-NPY release

A total of 20 rats were used in these studies, of which 13 were implanted with NT-3-containing osmotic pumps at the time of nerve injury, and 7 were implanted with

0.9% saline-containing osmotic pumps. Prior to study of ir-NPY release, regular behavioural testing was carried out to determine whether or not NT-3 infusion had any effect on the development of neuropathic pain. Tests for mechanical allodynia and hyperalgesia were used, as outlined in section 3.2. These were carried out by a single observer who was blinded to the contents of the osmotic pump.

Ir-NPY release experiments were carried out at 10-14 days after nerve injury and pump placement. The standard experimental set up described in section 2.2.2 was used. Up to 24 microprobes coated with NPY antibody were inserted into the spinal cord under basal conditions, with the final pair of probes being incubated in ^{125}I Fab, to exclude probe damage secondary to enzyme release from ruptured cells. After incubation with ^{125}I NPY, washing and placing on X-ray monoemulsion film for 6-8 days, autoradiographic images were obtained for each microprobe.

Several methods were used to check correct functioning of the osmotic pumps, and delivery of the reservoir contents to the nerve injury site. At the time of set up for release experiments, correct placement of the pump and catheter were checked. At the end of each experiment, after the rat had been euthanased, the sites of pump insertion and catheter were examined to exclude signs of infection, the patency of the catheter checked, and the drug reservoir was examined to ensure that it was empty.

5.2.2. Antibody microprobe preparation

Materials

NPY antibody coated microprobes were prepared as described previously (section 2.1). Only one supplier of NPY antiserum was used (Peninsula, Ca, USA), and the same lot number was used for all the experiments described. The basic technique used is the same for both galanin and NPY antibody-coated microprobes, with a few differences. When reconstituting the ^{125}I NPY, 0.1% casein was used instead of BSA. This was because previous studies in this laboratory had found unacceptably high levels of non-specific binding with BSA, and much lower levels when casein was used. The washing solution used for both *in vitro* and *in vivo* probes was always 0.1%

Tween-20, as opposed to the Tween-80, that was used in the galanin experiments, for the same reason.

The NT-3 was kindly gifted by Regeneron, NY, USA (Dr RM Lindsay), and was reconstituted in sterile 0.9% saline to give a solution of 1 mg/ml. This dose was slightly higher than that used in the study of the effects of NT-3 in axotomy (800 μg/ml) [326]. The solution was aliquoted into sterile Eppendorfs and frozen at -70°C until used.

In vitro tests

Prior to commencing *in vivo* work, *in vitro* assays were carried out to determine the specificity and sensitivity of the immobilised NPY antibody by measuring the degree of inhibition of ^{125}I NPY binding by related peptides or varying concentrations of unlabelled NPY. The same basic procedure used for *in vitro* galanin assays (see section 2.1.4 & 4.2.2; Figure 4) was used for the NPY assays. *In vitro* assays were carried out for each new batch of NPY antiserum, and continued in parallel with *in vivo* work to ensure that there was no major deterioration in sensitivity or specificity. Sensitivity was determined by incubating microprobes in a range of unlabelled NPY from 10^{-5}M to 10^{-8}M prior to incubation in ^{125}I NPY. Specificity values were supplied by the manufacturer's data, but these were checked by incubation of microprobes in 10^{-5}M to 10^{-6}M SP, VIP, galanin, PYY and PP. Levels of non-specific binding were assessed by incubation of microprobes coated only with PrA in ^{125}I NPY, and the antibody coating was assessed by incubation in ^{125}I Fab.

5.2.3. Analysis of microprobes

The same image analysis set up as that used for the galanin experiments was used, and is described in detail in section 2.1.6. Basically, the computerised image analysis system analysed autoradiographic images of both *in vivo* and *in vitro* microprobes. The *in vitro* probes gave an estimate of probe shape and allowed estimation of basal levels of ir-NPY release in the spinal cord. Binding of endogenous NPY resulted in deficits in binding of ^{125}I NPY that were detected as localised decreases in optical density (grey scale) by the analysis system. A plot of grey scale against depth of the probe in the spinal cord was obtained for each microprobe.

All analysis was carried out on normalised data, as described in section 2.1.6. This allowed comparison of probes that might otherwise have had minor variations in optical density due to exposure time, rather than biological variation. The mean values for defined groups of microprobes, were plotted as the means (+/- s.e.m.) of the grey scale values at 30 μm intervals against the depth within the spinal cord. Statistical significance was assigned to each site using Student's unpaired t-test, and the t values thus obtained plotted against depth within the spinal cord. A p value of less than 0.05 was taken as showing a statistically significant difference between groups.

5.3 Results

5.3.1 *In vitro* testing

The NPY antibody used was C-terminus directed and showed minimal cross reactivity with other peptides found in the dorsal horn, according to manufacturer's data and confirmed by *in vitro* testing. Sensitivity tests found suppression of binding ranging from approximately 90% with 10^{-5} M NPY to approximately 25% suppression with 10^{-8}M galanin, indicating that the sensitivity of the NPY antibody was adequate to detect significant areas of ir-NPY release in the spinal cord. Levels of non-specific binding, as assessed by measurement of the degree of binding to probes coated in protein A, were of the order of 7%. Microprobes incubated in ^{125} I Fab gave counts within 8% of control counts, indicating good coating of NPY antibody on the probe surface. Cross-reactivity with other peptides found in the dorsal horn was negligible, although significant cross-reactivity was found with the related peptides PYY and PP. This is in agreement with data from the manufacturer. The results of *in vitro* testing of the NPY antiserum from Peninsula Labs is shown in Table 8.

Table 8. *In vitro* assay for NPY antiserum (Peninsula, Ca, USA)

This shows the results from microprobes used in *in vitro* assays to ensure adequate sensitivity and specificity of the NPY antibody coating on the microprobes. Low levels of non-specific binding are also demonstrated, and good antibody coating of the probes, as shown by the high counts obtained with those probes incubated in ^{125} I Fab.

Group (number of probes)	Mean corrected c.p.m. (+/- s.e.m.)	% suppression of control group
Control (50)	1738 (+/-860)	
10^{-5}M NPY (51)	166(+/-77)	90
10^{-6}M NPY (17)	667(+/-129)	62
10^{-7}M NPY (27)	1020(+/-93)	41
10^{-8}M NPY (8)	1312(+/-140)	25
10^{-5}M Galanin (8)	1674(+/-271)	4
10^{-5}M SP (5)	2457(+/-224)	+41
10^{-5}M VIP (21)	1646(+/-108)	5
10^{-5}M PP (4)	1067(+/-191)	39
10^{-5}M PYY (6)	631(+/-74)	64
Fab (11)	1596(+/-58)	8
Cord perfusate (12)	1620(+/-101)	7
PrA (33)	116(+/-9)	7 (non-specific binding, % of control)

5.3.2. Effect of conduction block on ir-NPY release

A total of 154 probes were inserted into the spinal cord of rats with behavioural evidence of neuropathic pain, and a further 56 probes were used as *in vitro* controls to provide an estimate of probe shape in the absence of bound endogenously released ir-NPY. An additional group of *in vitro* probes was incubated in ^{125}I Fab, to allow comparison with probes incubated in cord perfusate then in ^{125}I Fab. This was to exclude the possibility of antibody damage to the probes. The adequacy of the conduction block was confirmed in all cases using electrical stimulation and central measurement with a ball electrode, as described in section 2.2.2, and a typical example of the recordings before and after conduction block is shown in Figure 21.

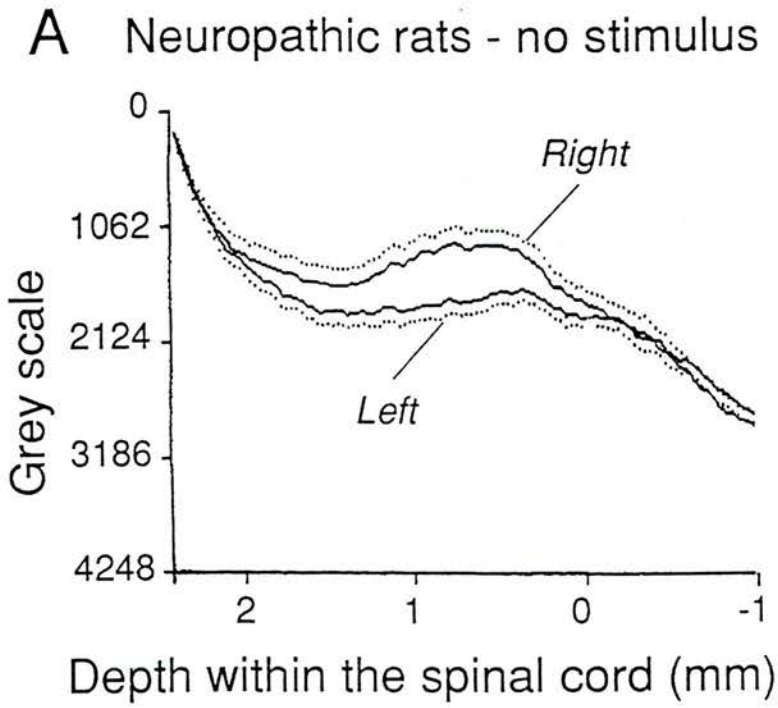
When *in vitro* microprobes (n=56) were compared to microprobes inserted into the contralateral (n=27) side of the cord to the nerve injury, a similar pattern of ir-NPY release was seen as is found in the spinal cord of normal rats [258]. Thus an area of ir-NPY release was found in the superficial dorsal horn, contralateral to nerve injury. As already discussed, this area of ir-NPY release is likely to be from intrinsic neurones rather than primary afferent neurones.

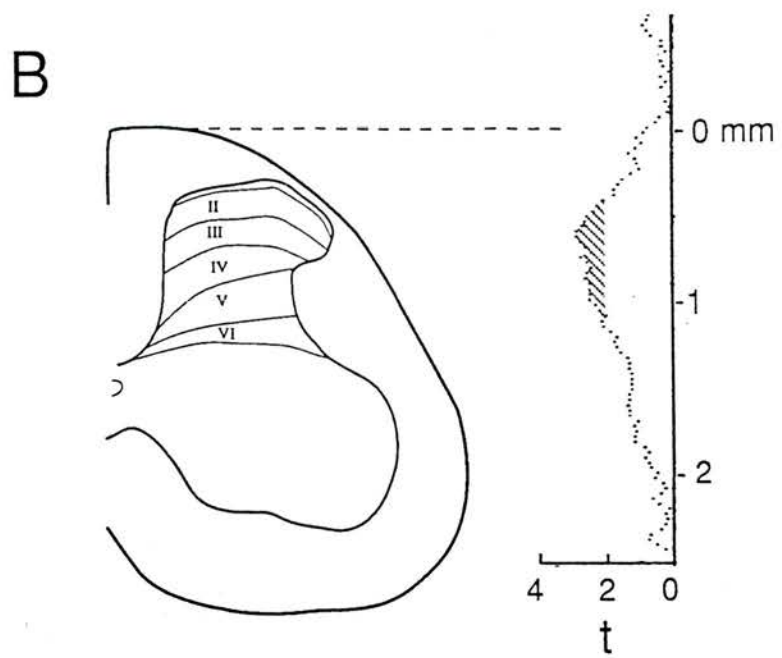
Microprobes inserted into the side of the cord ipsilateral to nerve injury (n=33) showed a new area of ir-NPY release in the deeper dorsal horn, when compared to probes inserted into the contralateral side (n=27). This is shown in Figure 29, and confirms previous work in which I was involved [257]. This new area of ir-NPY release is occurring in an area of the dorsal horn where ir-NPY is not normally produced [100,184] or released [258]. This is the same area where the large diameter primary afferent neurones, that start to synthesise NPY after nerve injury, terminate, and ir-NPY release is increased here by stimulation of these fibres [257]. It is highly likely therefore that this new area of spontaneously released ir-NPY is arising from primary afferent neurones.

Figure 29. Effect of peripheral nerve injury on spinal ir-NPY release

A. Microprobes were inserted into the spinal cord of neuropathic rats for 15 minutes without any concurrent peripheral stimulus. The plot shows the mean image analyses (+/-s.e.m.) of microprobes inserted into the ipsilateral (n=33) and contralateral (n=27) sides of the spinal cord relative to the nerve injury, which is on the right side. The microprobes inserted on the ipsilateral side are displaced above those inserted into the contralateral side, indicating a new zone of ir-NPY release under basal conditions.

B. The differences between the ipsilateral and the contralateral groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord. The hatched area shows where there was a significant increase ($p<0.05$) in spontaneous ir-NPY release in the deep dorsal horn.


A
Neuropathic rats - no stimulus
0
1062
2124
3186
4248
Grey scale
Right
Left
2
1
0
-1
Depth within the spinal cord (mm)


B
II
III
IV
V
VI
0 mm
-1
-2
4
2
0
t

After blocking input from the injured nerve proximal to the dorsal root ganglia, it was found that ir-NPY release ipsilateral to the nerve injury was not altered. Probes inserted after effective proximal conduction block (n=45) did not show any change in ir-NPY release when compared to those inserted under basal conditions (n=33). Thus complete block of neuronal input did not prevent the deeper area of ir-NPY release in the dorsal horn ipsilateral to the side of nerve injury. The mean image analysis of probes inserted into the spinal cord contralateral to nerve injury, before (n=27) and after (n=41) conduction block proximal to the dorsal root ganglia from the injured nerve, did not show any change in the pattern of ir-NPY release. Thus, unlike ir-galanin release, there was no evidence of contralateral effects from blocking neuronal input from the injured nerve. The effects of proximal conduction block on ir-NPY release are shown in Figures 30 and 31. As a control, the effect of dorsal root manipulation, without application of amethocaine gel was determined ipsilateral to the nerve injury site. There was no difference in ir-NPY release before (n=33) and after (n=8) manipulation of the dorsal roots.

To ensure that during the course of the experiment there had been no cell damage with release of enzymes into the cord perfusate, that might damage the NPY antibody coating of the probes, probes incubated in cord perfusate then ^{125}I Fab (n=23) were compared to *in vitro* probes (n=11) also incubated in ^{125}I Fab. There was no evidence of damage to the NPY antibody coating on the probe surface. This is shown in Figure 32.

Figure 30. Effect of proximal conduction block on spinal ir-NPY release in neuropathic rats

Microprobes were inserted into both sides of the spinal cord after conduction block with amethocaine gel, on the dorsal roots of the injured nerve, had been tested for effectiveness.

A. The mean image analyses (+/-s.e.m.) of microprobes inserted ipsilateral to the injured nerve, shows no change in optical density of those probes inserted after conduction block (n=45) compared to those inserted under basal conditions (n=33). The differences between the basal and post-conduction block groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord, at C1. There was no significant difference between these 2 groups, indicating no effect of proximal conduction block on spontaneous ir-NPY release.

B. The mean image analyses (+/-s.e.m.) of microprobes inserted contralateral to (n=41) the site of proximal conduction block and those microprobes inserted prior to this (n=27). There was no change in optical density of those probes inserted after conduction block compared to those inserted under basal conditions. The differences between the basal and post-conduction block groups of microprobes are shown as the *t* statistics relative to depth in the spinal cord at C2. As there was no significant difference between these 2 groups, proximal conduction block did not alter spontaneous ir-galanin release contralateral to the site of nerve injury.

It can be seen that proximal conduction block of neuronal input from the injured nerve had no effect on the pattern of spontaneous ir-NPY release on either side of the spinal cord.

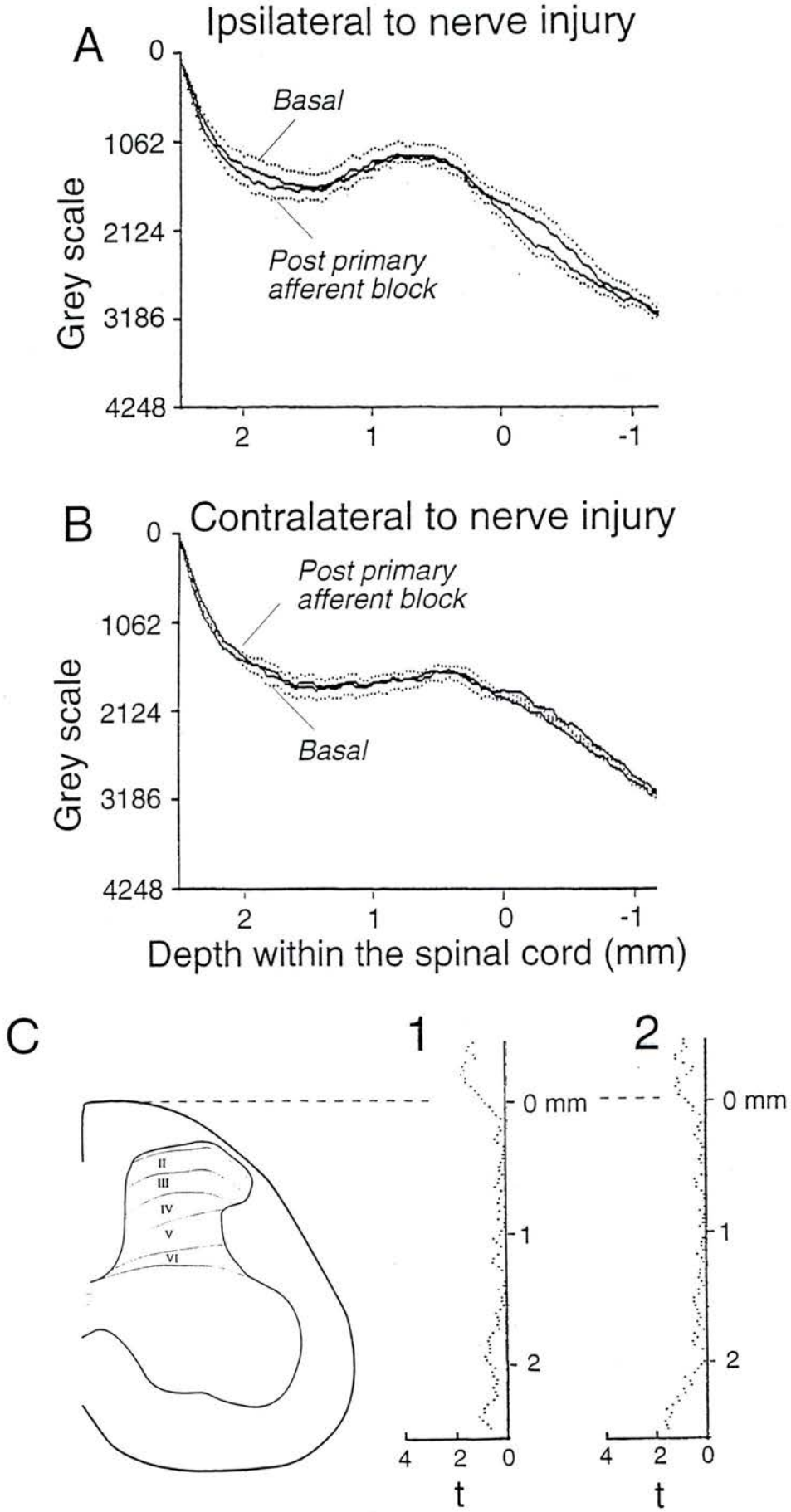
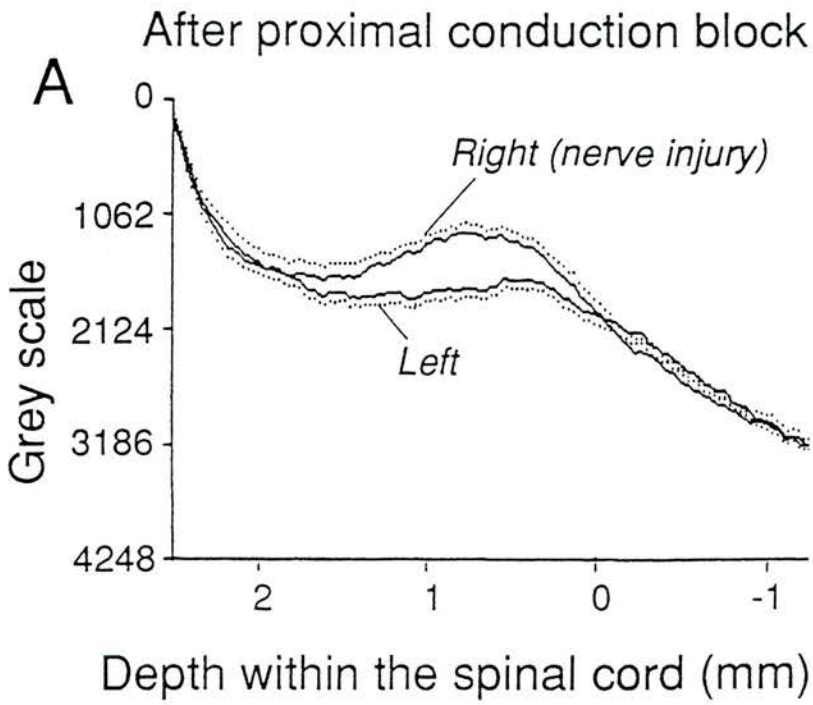

A
Ipsilateral to nerve injury
0
1062
2124
3186
4248
Grey scale
Basal
Post primary
afferent block
2
1
0
-1
B
Contralateral to nerve injury
0
1062
2124
3186
4248
Grey scale
Post primary
afferent block
Basal
2
1
0
-1
Depth within the spinal cord (mm)
C
1
2
II
III
IV
V
VI
0 mm
1
2
4
2
0
t
0 mm
1
2
4
2
0
t

Figure 31. Side to side comparison of ir-NPY release after proximal conduction block

A. The mean image of analyses (+/-s.e.m.) of microprobes inserted into the ipsilateral (n=45) and contralateral (n=41) side of the cord after blocking input from the injured nerve show that the deep zone of ir-NPY release that was found under basal conditions is still present.

B. The differences in ir-NPY release between the two sides of the cord, after proximal conduction block are shown as the *t* statistics relative to depth in the spinal cord. As there was no significant difference between these 2 groups, proximal conduction block did not alter the new area of spontaneous ir-NPY release found in the spinal cord of neuropathic rats, ipsilateral to the site of nerve injury. Thus, blocking neuronal input from the injured nerve has not altered the central release of ir-NPY in the spinal cord.


After proximal conduction block
A
0
1062
2124
3186
4248
Grey scale
Right (nerve injury)
Left
2
1
0
-1
Depth within the spinal cord (mm)

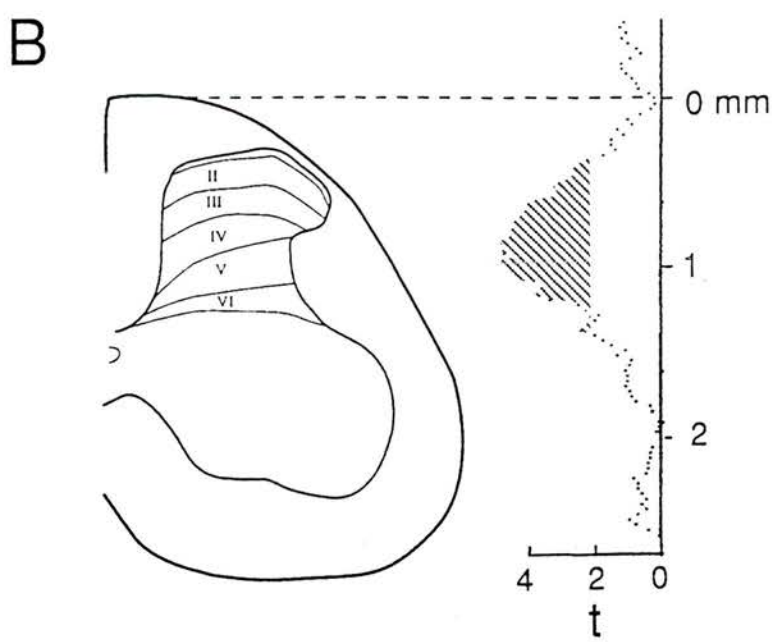
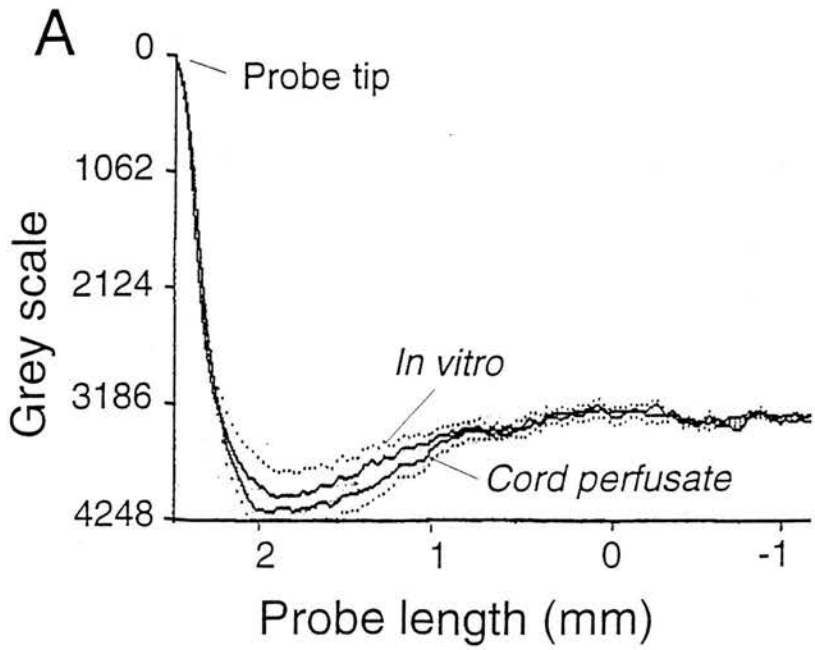

B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

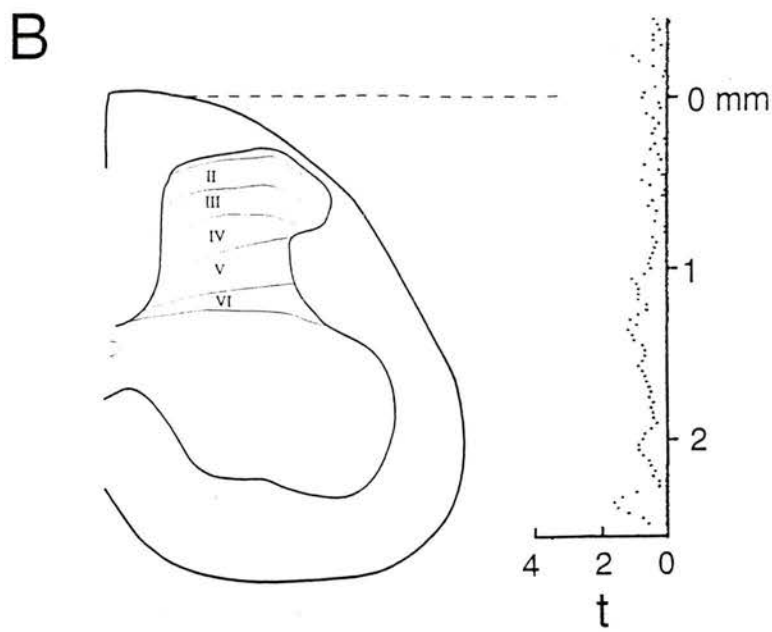
Figure 32. Testing for damage to NPY antibody coating on probe surface

A. The mean image of analyses (+/-s.e.m.) of microprobes incubated for 15 minutes in cord perfusate (n=23), followed by incubation in ^{125}I Fab, and *in vitro* probes incubated in ^{125}I Fab (n=11). There was no obvious deviation of the optical densities of these two groups, thus giving no evidence of damage to the NPY antibody coating on the probe surface.

B. The differences between the two groups of probes are shown as the t statistics relative to the length of the probe. At no point was there any significant difference between the two groups, confirming that there was no damage to the antibody coating.

Microprobes incubated in ^{125}I Fab





5.3.3. Effect of NT-3 infusion on behaviour

No attempt was made to formally quantify the characteristic signs seen with this model, but it was not possible to differentiate between the type of infusion that each rat had received, dependent on the general behaviour of the animal, such as hopping, foot position and grooming. There was no evidence of any difference in weight gain between the NT-3 and the saline groups over the time period studied, as shown in Table 9.

All rats that had undergone the CCI model did display evidence of mechanical allodynia and hyperalgesia on the side of nerve injury, regardless of the infusion type. This is shown in Figure 33 and the results are outlined in Tables 10 and 11. The paw withdrawal thresholds, as determine by von Frey hairs, and paw withdrawal duration to pin prick were compared at each time point using a Mann Whitney Rank Sum Test. The values obtained for those rats with a NT-3 infusion were compared to those with a saline infusion for right and left hindpaws. It was found that there was no significant difference at any of the time points tested except for on the right side (nerve injury), on the day of experiment at 10 to 14 days after the initial injury. At this point there was found to be a small but significant difference between those rats receiving NT-3 infusions sand those receiving saline infusions. The rats receiving NT-3 had a significantly higher paw withdrawal threshold to von Frey hairs ($p=0.005$) and a significantly shorter paw withdrawal duration to pin prick ($p=0.04$) than those receiving saline infusions. This would indicate that although NT-3 did not appear to have any role in induction of neuropathic pain, lack of NT-3 might be involved in the maintenance of the painful syndrome.

Table 9. Weight changes after nerve injury

The changes in weight of rats receiving either an infusion of saline (n=7) or NT-3 (n=13) over the study period is shown. At no point was there any difference between these two groups.

Group	Median weight of rat (g)	25%-75%
Pre-op NT-3	206	199-238
Pre-op Saline	206	192-252
Day3 NT-3	216	210-240
Day3 Saline	214	202-262
Day4 NT-3	222	214-247
Day4 Saline	232	206-273
Day5 NT-3	228	220-252
Day5 Saline	238	212-279
Day6 NT-3	234	228-267
Day6 Saline	270	219-292
Day7 NT-3	242	234-263
Day7 Saline	230	224-264
Exp day NT-3	288	265-295
Exp day Saline	266	247-319

Table 10. Response to von Frey hairs

The paw withdrawal threshold (g) is shown for rats receiving either a NT-3 infusion (A) or a saline infusion (B). Differences between right (nerve injured) and left hindpaws were detected from day 3 onwards in both groups ($p<0.001$), and a difference between the saline group and NT-3 group was detected on the right side on the day of experiment.

A. NT-3 infusion			
Group (n)	**Mean paw withdrawal threshold (g) (+/- s.e.m.)**	**Median paw withdrawal threshold (g)**	**Range**
PreopR (13)	65.01(+/-5.72)	75.86	47.02
PreopL (13)	65.01(+/-5.72)	75.86	47.02
Day3 R(13)	7.38(+/-0.98)	5.5	11.51
Day3 L (13)	61.39(+/-6.26)	75.86	47.02
Day4 R (13)	6.99(+/-0.91)	5.5	11.51
Day 4 L (13)	56.72(+/-7.06)	75.86	60.72
Day 5 R (13)	8.27(+/-1.84)	5.5	25.21
Day 5 L (13)	53.1(+/-7.16)	75.86	60.72
Day 6 R (11)	5.22(+/-0.6)	5.5	6.47
Day 6 L (11)	67.31(+/-5.73)	75.86	47.02
Day 7 R (11)	4.15(+/-0.59)	3.63	6.47
Day 7 L (11)	63.03(+/-6.62)	75.86	47.02
Day10/11 R (13)	4.03(+/-0.53)	3.63	7.03
Day 10/11 L (13)	72.72(+/-3.13)	75.86	47.02
Exp day R (13)	4.47(+/-0.73)	3.63	7.03
Exp day L (13)	65.01(+/-5.72)	75.86	47.02

B. Saline infusion			
Group (n)	**Mean paw withdrawal threshold (g) (+/-s.e.m.)**	**Median paw withdrawal threshold (g)**	**Range**
Preop R (7)	62.42(+/-8.67)	75.86	47.02
Preop L (7)	62.42(+/-8.67)	75.86	47.02
Day3 R(7)	8.53(+/-1.56)	8.51	11.51
Day3 L (7)	69.14(+/-6.72)	75.86	47.02
Day4 R (7)	6.02(+/-1.16)	5.5	8.12
Day 4 L (7)	62.42(+/-8.67)	75.86	47.02
Day 5 R (7)	5.09(+/-1.73)	3.63	13.1
Day 5 L (7)	42.27(+/-8.67)	28.84	47.02
Day 6 R (5)	3.74(+/-0.77)	3.63	3.45
Day 6 L (5)	47.65(+/-11.52)	28.84	47.02
Day 7 R (5)	15.28(+/-5.77)	11.75	27.36
Day 7 L (5)	57.05(+/-11.52)	75.86	47.02
Day10/11 R (2)	2.84(+/-0.79)	2.84	1.59
Day 10/11 L (2)	75.86(+/-0)	75.86	0
Exp day R (7)	1.6(+/-0.12)	1.48	0.84
Exp day L (7)	69.14(+/-6.72)	75.86	47.02

Table 11. Response to pin prick

The paw withdrawal duration (s) to pin prick is shown for each group. Form day three onwards there was a difference between right(nerve injured) and left hindpaws within each group ($p<0.001$). On the day of experiment the NT-3 group had a significantly shorted paw withdrawal duration than the saline group.

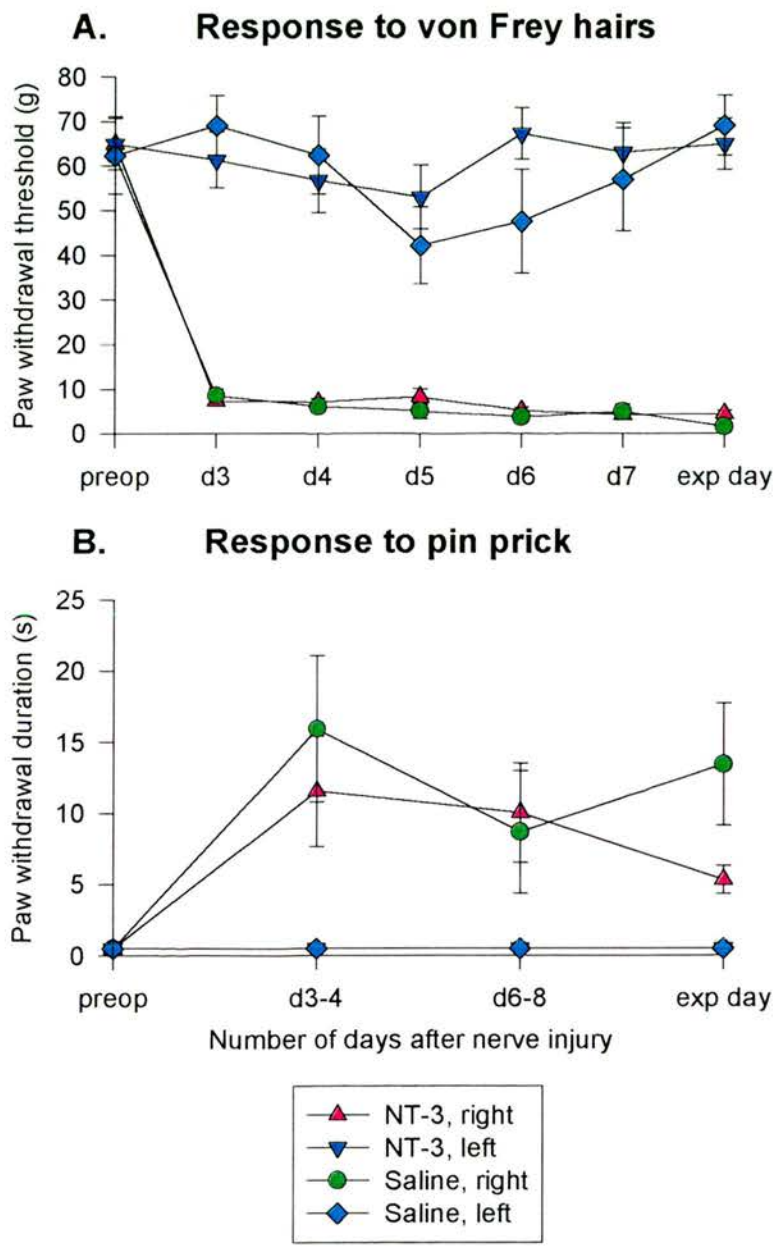
A. NT-3 infusion			
Group (n)	**Mean paw withdrawal duration (s) +/-s.e.m.)**	**Median paw withdrawal duration (s)**	**Range**
Preop R (13)	0.5 (+/-0)	0.5	0
Preop L (13)	0.5 (+/-0)	0.5	0
Day3-4 R (12)	11.5 (+/-4)	3	29
Day3-4 L (12)	0.5 (+/-0)	0.5	0
Day6-8 R (12)	10 (+/-3.5)	4.5	29.5
Day 6-8 L (12)	0.5 (+/-0)	0.5	0
Exp day R (13)	5.5 (+/-1)	5	13
Exp day L (13)	0.5 (+/-0)	0.5	0
B. Saline infusion			
Group (n)	**Mean paw withdrawal duration (s)(+/-s.e.m.)**	**Median paw withdrawal duration (s)**	**Range**
Preop R (7)	0.5 (+/-0)	0.5	0
Preop L (7)	0.5 (+/-0)	0.5	0
Day 3-4 R (7)	16 (+/-5)	12	29.5
Day 3-4 L (7)	0.5 (+/-0)	0.5	0
Day 6-8 R (7)	8.5(+/-4)	5.5	28
Day 6-8 L (7)	0.5 (+/-0)	0.5	0
Day 10-14 R (7)	13.5(+/-4)	7	25
Day 10-14 L (7)	0.5 (+/-0)	0.5	0

Figure 33. Response to behavioural testing

The results of behavioural testing for mechanical allodynia and hyperalgesia for rats with a CCI injury and either a saline or NT-3 infusion are shown. The paw withdrawal threshold to von Frey hairs is shown at A. and the paw withdrawal duration to pin prick is shown at B. The right side is the side of the nerve injury, with the left hindpaw being contralateral to the injury.

d=day

Figure 35. Response to behavioural testing



5.3.4 Effect of NT-3 infusion on ir-NPY release

A total of 256 probes were inserted into the spinal cord of rats with implanted osmotic pumps, containing either NT-3 or saline, all rats having undergone a CCI of the right sciatic nerve 10 to 14 days previously. A further 58 probes were used as *in vitro* controls to provide an estimate of probe shape in the absence of bound endogenously released ir-NPY.

NPY antibody-coated probes were inserted into the spinal cord ipsilateral (n=46) and contralateral (n=44) to the nerve injury, in rats that had a chronic infusion of 0.9% saline to the nerve injury site. There was found to be an area of increased spontaneous ir-NPY release on the side of the cord ipsilateral to nerve injury. This corresponded to previous studies of ir-NPY release [257](see section 5.3.2.), where there was a new area of ir-NPY release deeper in the dorsal horn than that found in normal rats. Thus, the surgery involved in placement of the osmotic pump, and the presence of the osmotic pump did not in itself alter the pattern of ir-NPY release in the spinal cord of neuropathic rats. This is shown in Figure 34.

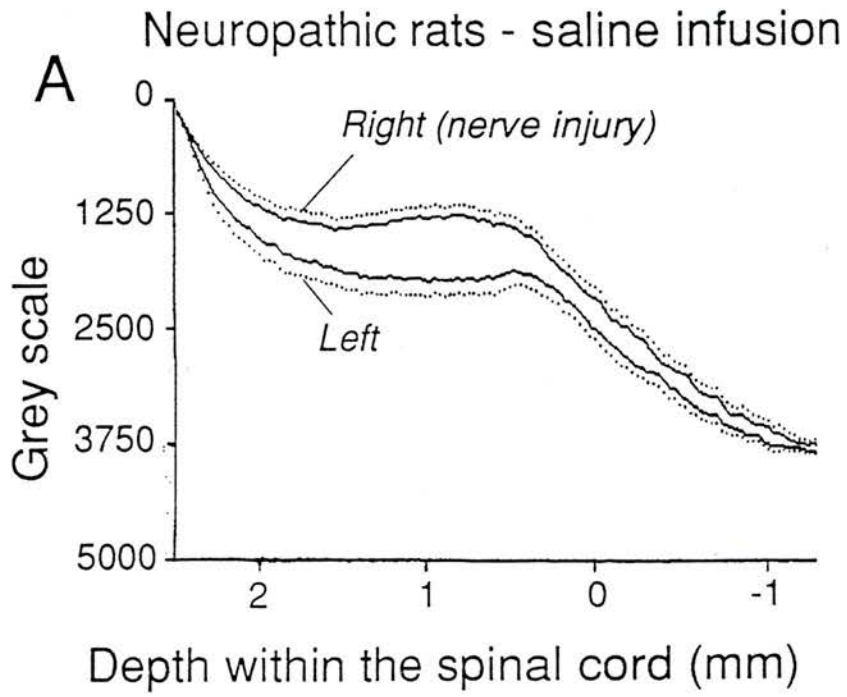
Microprobes were inserted into the spinal cord, ipsilateral (n=84) and contralateral (n=82) to the side of nerve injury, in rats that had a chronic infusion of NT-3 to the nerve injury site. There was an area of ir-NPY release in the deep dorsal horn ipsilateral to the side of nerve injury, corresponding to that previously found in neuropathic rats. The basal presence of ir-NPY release that was found in the contralateral side of the cord was also still present when compared to *in vitro* probes (n=58). This is shown in Figure 35. Thus, NT-3 infusion had no major effect on the deep zone of spontaneous ir-NPY release.

When microprobes that had been inserted into the ipsilateral side of the cord to the nerve injury in rats with either a saline infusion (n=46) or a NT-3 infusion (n=84) were compared, there was found to be no difference between these two groups. The same was true for probes inserted into the contralateral side of the cord for saline (n=44) or NT-3 (n=82) infused rats. This is shown in Figure 36. NT-3 did not have any effect on ir-NPY release from primary afferent fibres into the spinal cord of neuropathic rats.

Figure 34. Effect of saline infusion on ir-NPY release in the spinal cord of neuropathic rats

A. This shows the mean image analyses (+/-s.e.m.) of microprobes inserted ipsilateral (n=46) and contralateral (n=44) to a chronic constriction injury of the right sciatic nerve in rats with a mini-osmotic pump implanted at the time of nerve injury to infuse 0.9% saline until the day of ir-NPY release experiment. Similar areas of decreased optical density can be seen in those probes inserted ipsilaterally as that seen in Figure 29.

B. The t statistics obtained from comparison of probes inserted into both sides of the cord are plotted next to a diagram of the spinal cord. There is an area of significantly increased ir-NPY release in the ipsilateral side of the cord peaking in laminae III-V. This corresponds with results from previous studies of spontaneous ir-NPY release in neuropathic rats.


Neuropathic rats - saline infusion
A
0
1250
2500
3750
5000
Grey scale
Right (nerve injury)
Left
2
1
0
-1
Depth within the spinal cord (mm)

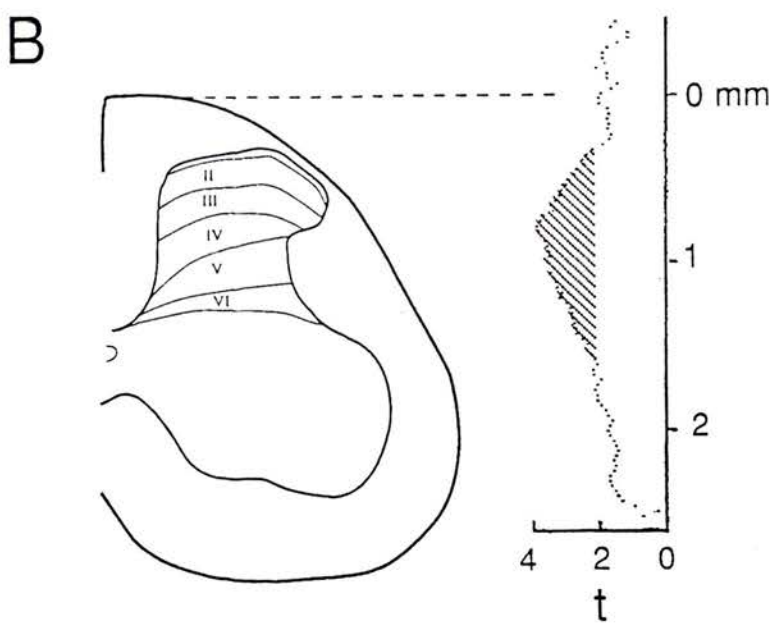
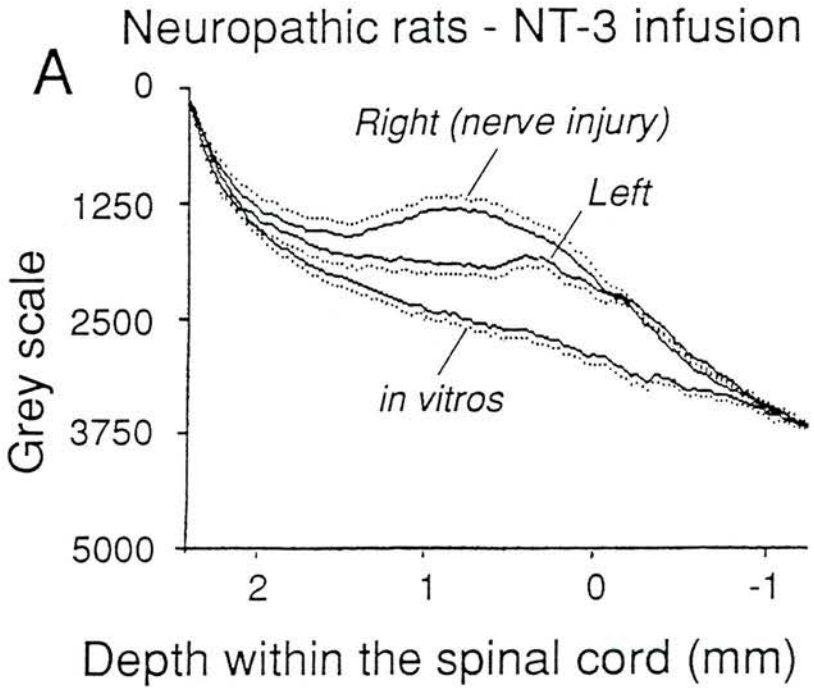

B
II
III
IV
V
VI
0 mm
-1
2
4
2
0
t

Figure 35. Effect of NT-3 infusion on ir-NPY release in the spinal cord of neuropathic rats

A. This shows the mean image analyses (+/-s.e.m.) of microprobes inserted ipsilateral (n=84) and contralateral (n=82) to a CCI of the right sciatic nerve in rats with a chronic infusion of NT-3. The mean image analysis of *in vitro* probes is also shown to demonstrate that there is a basal presence of ir-NPY found contralaterally, that is similar to that found in the normal situation [258].

B. The t statistics obtained from comparing probes inserted on each side of the spinal cord are shown next to a diagram of the spinal cord. A significantly increased area of ir-NPY release is apparent ipsilateral to injury, with a peak in laminae III-V, similar to that found in neuropathic rats without prior infusion of NT-3.


Neuropathic rats - NT-3 infusion
A
0
1250
2500
3750
5000
Grey scale
Right (nerve injury)
Left
in vitros
2
1
0
-1
Depth within the spinal cord (mm)

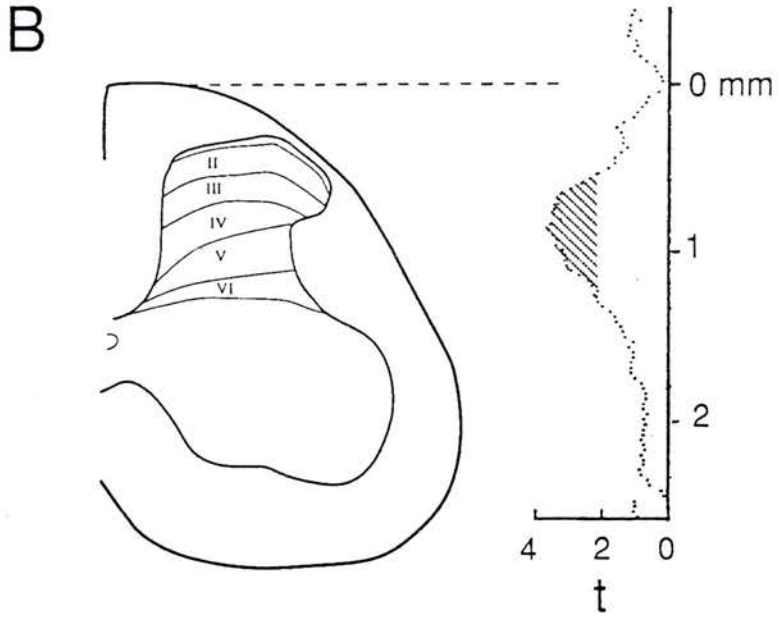
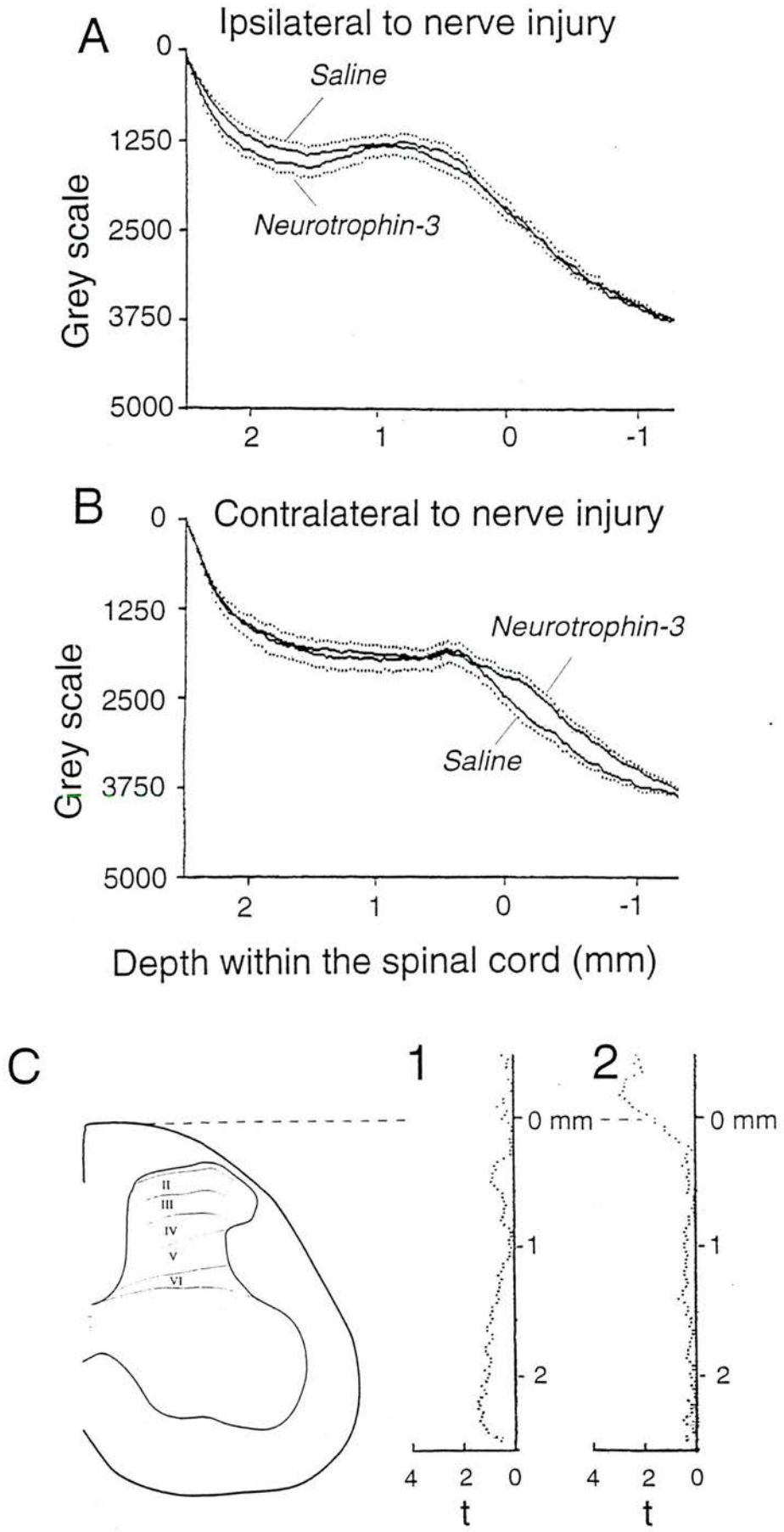

B
II
III
IV
V
VI
0 mm
1
2
4
2
0
t

Figure 36. Comparison of ir-NPY release in the CCI model in rats with a saline or a NT-3 infusion

A. This shows the mean image analyses (+/-s.e.m.) of probes inserted into the side of the spinal cord ipsilateral nerve injury in rats that had received a chronic infusion of either saline (n=46) or NT-3 (n=84). There is no major deviation of the two lines from each other, indicating no major difference in spontaneous sir-NPY release.

B. The mean image analyses (+/-s.e.m.) of probes inserted into the side of the spinal cord contralateral nerve injury in rats that had received a chronic infusion of either saline (n=44) or NT-3 (n=82). Again there is no major difference in optical density at the areas of the probe shown within the spinal cord.

C. The t statistics obtained from comparison of probes inserted into both ipsilateral (1) and contralateral (2) sides of the cord between saline or NT-3 infused rats, are plotted relative to depth within the spinal cord. At no point was there any significant difference between the saline and NT-3 groups, indicating that supplying exogenous NT-3 to the nerve injury site had no effect on central ir-NPY release.


A
Ipsilateral to nerve injury
Saline
Neurotrophin-3
Grey scale
0
1250
2500
3750
5000
2
1
0
-1
B
Contralateral to nerve injury
Neurotrophin-3
Saline
Grey scale
0
1250
2500
3750
5000
2
1
0
-1
Depth within the spinal cord (mm)
C
1
2
II
III
IV
V
VI
0 mm
1
2
4
2
0
t
0 mm
1
2
4
2
0
t

5.4 Discussion

The key new findings from the study of ir-NPY release in the spinal cord of the neuropathic rat are:

1. Conduction block proximal to the dorsal roots using amethocaine gel did not alter the new area of spontaneous ir-NPY release that had been found in the ipsilateral dorsal horn of neuropathic rats.
2. Despite a small decrease in mechanical allodynia and hyperalgesia 10-14 days after injury, chronic infusion of NT-3 did not alter the spontaneous release of ir-NPY in the spinal cord of neuropathic rats.

5.4.1. Stimulus for ir-NPY release

The stimulus for the deeper zone of ir-NPY release in the dorsal horn of neuropathic rats does not appear to be spontaneously occurring ectopic discharges from the injured nerve or the dorsal root ganglia, as complete conduction block did not alter this spontaneous ir-NPY release. As the adequacy of the block was checked by peripheral electrical stimulation to ensure no evoked activity within the cord, it is unlikely that the lack of effect on release was due to inadequate conduction block, particularly as ectopic discharges are inhibited at much lower doses of local anaesthetic than those required for conduction block [57,91]. There is increasing evidence that TTX-sensitive sodium channels are involved in the generation of ectopic discharges [327,328], and these are blocked by local anaesthetics [394,403]. There must therefore be an alternative stimulus resulting in the spontaneous release of ir-NPY ipsilateral to a peripheral nerve injury. Several possibilities must be considered:

1. Spontaneous neuronal activity not originating from primary afferent neurones may be involved. Activation of descending systems in the normal rat resulted in SP release from primary afferent terminals, via a NMDA receptor mediated mechanism, presumably by an action at a pre-synaptic site [242]. In both normal [146] and neuropathic [387] animals, there is evidence for descending systems influencing activity in the dorsal horn. In the CCI model, there was higher spontaneous activity within the dorsal horn itself, that was inhibited by stimulation of descending

systems [387], as well as an increase in inhibitory neurotransmitters from the descending systems [360]. Additionally, intrinsic spinal neurones may also act on the primary afferent terminals [461], possibly altering neuropeptide release, with further changes occurring after nerve injury [465]. Thus, general alterations in spinal excitability after nerve injury may alter neuropeptide release from primary afferent terminals.

2. The possibility that ir-NPY secretion and release is occurring without influence from neuronal activity must be considered. Peptides are normally packaged in large dense core vesicles (LDCVs), in the Golgi complex before transport to both synaptic and extra-synaptic sites [24,501]. Exocytosis of LDCVs is slower than that seen with classical neurotransmitters packaged in small synaptic vesicles (SSVs), but still requires an increase in intracellular calcium [259], and involves many of the same membrane proteins [34,81]. There is evidence for an increase in exocytosis of LDCVs after axotomy [501], but another factor may be alterations in the processing of peptides. Normally, large diameter primary afferent neurones do not contain many peptides, or LDCVs [490], and the rapid increase in peptide synthesis after nerve injury may be greater than the existing packaging systems can cope with. Thus some of the newly produced peptide may be secreted via the non-regulated, constitutive pathway, by which membrane components, enzymes, growth factors and extracellular matrix molecules are normally secreted [24,81]. There is some evidence that this may be the case for NPY, as it is found not only in LDCVs, but also diffusely in the cytoplasm of large diameter primary afferent neurones after nerve injury [490]. This is different from the situation with galanin, which is found mainly in small to medium sized primary afferent neurones, that even in the normal situation, synthesise and process peptides in LDCVs. There may also be changes in ion channel types after nerve injury, that could result in altered secretion patterns. There is evidence for changes in types of both sodium channels [322,445] and calcium channels [1,350] after nerve injury that may alter mechanisms of neuronal activation and neurotransmitter secretion.
3. Other substances that are secreted by the constitutive pathway, either from primary afferent neurones, local dorsal horn neurones or supraspinal systems may act

indirectly to stimulate ir-NPY release. Thus, brain derived neurotrophic factor (BDNF) has been shown to increase glutamate release and exocytosis from cortical neurones in culture [405], as well as modify the firing pattern of serotonergic neurones [55]. Both BDNF and NT-3 potentiated synaptic activity at the neuromuscular junction of developing synapses in culture [243], and there is evidence that neurotrophins can alter synaptic efficacy by effects on neurotransmitter release [213].

Role of NT-3 in the CCI model

NT-3 had a small but significant effect on the maintenance of pain-related behaviour, with a slight attenuation of both mechanical allodynia and hyperalgesia on the day of the ir-NPY release experiments, at 10-14 days after nerve injury. In a different model of a large fibre sensory neuropathy, NT-3 was found to alleviate the signs of neuropathy, although pain-related behaviour was not studied [158,238]. In addition, other neurotrophins such as NGF, have been shown to partially alleviate some of the neuropathic signs developing after nerve injury, both in the CCI model [349], and in diabetic sensory neuropathy [9]. Infusion of NT-3 into the rat midbrain had antinociceptive actions [380] and NT-3 inhibited SP release from an isolated spinal cord preparation, as well as inducing mechanical hypoalgesia after systemic administration [254].

It is clear from studies of knockout mice deficient in the genes for NT-3 or its high affinity receptor, the trkC receptor, that NT-3 and trkC are essential during development, as the homozygotes from both NT-3 and trkC studies die shortly after birth [3,123,124,209,378], with a marked decrease in large diameter sensory neurones. However, NT-3 is not necessary for survival of adult sensory neurones, but may have a role in the control of the adult neuronal phenotype [239]. Many large diameter neurones express trkC receptors [239] and there is evidence of rapid retrograde transport of trk receptors, from the periphery along the length of the axon to the cell body [37,38]. The effects of nerve injury on this retrograde transport and changes in the supply of NT-3 to the cell body may be important in alterations in the phenotype of NT-3 sensitive neurones [93]. Furthermore, although the effect of NT-3 on behaviour was limited, this is not surprising, as there is evidence that many of these factors act in a

synergistic fashion [238,266]. Although outwith the scope of the current study, the effects of NT-3 at time periods longer than 10-14 days after nerve injury, plus combination with other neurotrophins, deserve further investigation. It may be that, similar to the situation during development [5,237,384], the relative importance of different neurotrophins, either individually or in combination, changes as the pain syndrome develops.

The lack of effect of exogenously supplied NT-3 on ir-NPY release indicates that this neurotrophin is not an important factor in the spontaneous release of ir-NPY in the deep dorsal horn ipsilateral to nerve injury. In an immunohistochemical study of NPY-ir distribution, Ohara et al,1995 [326], found that after bilateral axotomy, exogenous NT-3 supplied to one of the transected sciatic nerves significantly attenuated the increase in NPY-ir in the dorsal horn and the gracile nuclei on that side of the cord. There is some discrepancy between the Ohara study of NPY levels in the spinal cord, and the current study on NPY release. There may be several reasons for this. Firstly, the Ohara study was of NPY distribution in post mortem specimens, as opposed to ir-NPY release *in vivo*. It does however seem unlikely that a decrease in NPY-ir in the dorsal horn was accompanied by no change in release. Secondly, a bilateral complete nerve transection was being studied, as opposed to a unilateral partial nerve injury. Rats with bilateral axotomy must find hindleg movement very difficult, with possible effects on feeding ability and increased stress due to an inability to move normally. Additionally, the contralateral side of the cord was used as the control group, and no evidence was presented that the effects of the contralateral saline infusion to the nerve had the same effect as simple axotomy on NPY changes. It may be that the silicone chamber around the nerve had effects on NPY distribution, either due to a chemical constituent of the chamber, or as a direct physical effect due to tissue irritation, and pressure. Other factors, such as differential changes in neurotrophin receptors, retrograde transport of NT-3 and alternative sources of NT-3 may also be involved when comparing a bilateral complete injury to a unilateral partial injury.

Although large fibres are thought to be mainly responsive to NT-3, because of the expression of its high affinity receptor, some of them also express the low affinity neurotrophin receptor, p75 [395], at which several neurotrophins can act, including NT-

3 and NGF. It may be therefore that the changes in ir-NPY release are regulated via other neurotrophins acting by this alternative pathway. The sprouting of large myelinated fibres into lamina II that occurred after nerve injury was not prevented by either intrathecal NT-3 [29] or NT-3 applied to the nerve injury site [120], whereas NGF did prevent this reorganisational change. Additionally, NGF partially prevented the increase in NPY synthesis in dorsal root ganglia, after axotomy [424]. Although the majority of neurones that increase NPY production after nerve injury do not have the high affinity NGF receptor, trkA, they do have the low affinity p75 receptor. It may be therefore that one of the stimuli resulting in spontaneous ir-NPY release is due to actions of NGF, or other neurotrophin. This is an area that requires furthers study. This current study has demonstrated that there was not a correlation between spontaneous ir-NPY release and behavioural changes induced by NT-3 infusion. The stimulus for spontaneous ir-NPY release was not due to lack of NT-3, but may be due to other secreted factors from primary afferent neurones, supraspinal sources or intrinsic dorsal horn neurones. Alternatively, ir-NPY may be released in the absence of any stimulus, via the constitutive pathway.

5.4.3. Functional effects of ir-NPY after nerve injury

It is important to consider the possible functional role of NPY released in the dorsal horn after nerve injury.

It may be acting to limit abnormal input from the damaged nerve and decreasing excitability in the dorsal horn, thus reducing abnormal pain transmission There is a considerable amount of evidence that NPY has an inhibitory action, both from studies of its electrophysiological actions [72,73,267,421] and its inhibition of SP release in the dorsal horn [110]. Additionally, studies of NPY knockout mice show an increased tendency to seizures [119], with intracerebroventricular NPY infusion preventing death from kainic acid induced seizures in these NPY deficient mice [19], indicating a possible generalised role in dampening neuronal excitability. There is evidence of antinociceptive actions [47,78,172], including depression of the nociceptive flexor reflex [478].

However, there is evidence that NPY may have excitatory actions, presumably due to actions at different receptor subtypes. Thus NPY has been shown to potentiate the

response to NMDA in the hippocampus [290,291]; to increase spontaneous activity of paraventricular neurones [10] and to potentiate the contractile effect of noradrenaline [36]. A few studies have also found that NPY administered either peripherally [417], or centrally [448] can result in mechanical hypersensitivity.

Overall, however, the evidence for inhibitory actions appears to predominate. NPY inhibited calcium currents [262], prevented increases in intracellular calcium [278] and inhibited cAMP production in the brainstem [318]. NPY also had inhibitory effect on other neurotransmitters, as it inhibited glutamate release in the hippocampus [147], transmitter release from capsaicin-sensitive terminals [141] and catecholamine release from sympathetic ganglia [323], as well as suppressing neurogenic inflammation [153] and having potential as a novel analgesic [78,301,433].

The fact that NPY appears to be released from primary afferent terminals without any neuronal activity, is rather strange if it is acting as an inhibitory neurotransmitter. An alternative explanation for its increased synthesis and release in response to peripheral nerve injury may be that it plays a part in the reorganisational changes that occur in the dorsal horn. It may be involved in the sprouting of the large diameter fibres from their deep termination into the superficial dorsal horn. Although the area where ir-NPY release was detected is deeper than where the newly sprouted fibres terminate, ir-NPY could be released from extra-synaptic sites [501], or there could be diffusion from its site of release to induce sprouting .It has already been demonstrated that ir-NPY persists for some significant length of time after its evoked release [257], and it could be that it induces growth along a diffusion gradient. There is evidence that NPY has growth inducing properties in neurones in culture [449], in vascular smooth muscle cells [122,373] and in fibroblasts [42]. Other peptides increased after nerve injury have also been shown to have growth stimulating properties, particularly VIP [126,212]. Thus, NPY may act, either alone or in combination with other substances, to induce the reorganisational changes occurring in the spinal cord after nerve injury, possibly with the additional function of inhibiting excess abnormal activity during the period of recovery.

Chapter 6. Concluding Remarks

6.1 Summary of significant results

The key findings from the present studies of the CCI model are:

1. There was an increase in spontaneous ir-galanin release in the superficial dorsal horn ipsilateral to nerve injury. Peripheral electrical stimulation of the injured nerve sufficient to activate both A and C fibres was required to further increase ir-galanin release in the dorsal horn, while stimulation of Aβ fibres alone did not alter spinal release of ir-galanin. Ir-galanin did not persist for any significant length of time after its evoked release. Blockade of all neuronal input from the injured nerve did not significantly alter basal ir-galanin release in the dorsal horn ipsilateral to nerve injury, but markedly increased ir-galanin release contralateral to nerve injury. This increase in basal ir-galanin release contralateral to the injured nerve, after conduction block, was such that the side to side difference in ir-galanin release seen in the neuropathic rats was eliminated.
2. Previous studies [257,258] of the CCI model, have shown the appearance of a new area of ir-NPY release in the deep dorsal horn ipsilateral to nerve injury. Ir-NPY release in this area was increased by electrical stimulation of the injured nerve sufficient to activate Aβ fibres. This ir-NPY could still be detected for up to one hour after its evoked release. Blockade of all neuronal input from the injured nerve, proximal to the dorsal root ganglia, had no effect on spontaneous ir-NPY release in this area of the dorsal horn. Chronic infusion of NT-3 to the site of nerve injury did not alter the spontaneous release of ir-NPY, although a minor alleviation of mechanical hypersensitivity was detected on the day of the ir-NPY release experiments.

6.2 Relation of findings to other studies on animal models of neuropathic pain

It is important to consider how the findings from these studies relate to other changes occurring in the spinal cord after nerve injury, that may result in the development of neuropathic pain. A major finding from studies of peripheral nerve injury was the change in adult sensory neuronal phenotype [308]. As discussed in section 1.2.8, this included dramatic alterations in neuropeptide synthesis [316]. Thus synthesis of neuropeptides normally thought to be involved in nociceptive transmission, such as SP and CGRP, was markedly decreased [164,183,280,425], whereas there was induction of neuropeptides not normally found in primary afferent neurones [308]. This change was seen in all sizes of primary afferent neurones, with neuropeptides such as galanin and VIP increasing in small to medium sized neurones [493,495] and NPY being found in large diameter neurones not normally associated with the production of neuropeptides [300,490]. A consequence of the altered neuropeptide production and distribution was a change in the pattern of coexistence of neuropeptides in primary afferent neurones [194,196,492,493]. Not only was there an increase in synthesis of galanin and NPY by different classes of primary afferent neurones, but the present release studies have demonstrated that there was also a change in their spinal release in the CCI model. There are some similarities between galanin and NPY changes after nerve injury:

1. Both galanin and NPY were up-regulated and released spontaneously in the ipsilateral dorsal horn of the spinal cord;
2. Whereas peripheral stimulation did not alter central release in normal rats, in the CCI model, release of both galanin and NPY could be evoked by stimulation of the injured nerve.

There were also, however, some quite marked differences between the patterns of ir-galanin and ir-NPY release, that may indicate different functional roles for these two neuropeptides.

It is apparent from the release studies, that different stimuli regulate the release of galanin and NPY. Low intensity stimulation of the injured nerve evoked only ir-NPY release in the deep dorsal horn, and it was only as the intensity of stimulation was increased in order to activate C as well as A fibres that ir-galanin release was evoked

in the superficial dorsal horn. This correlates well with the increased synthesis of NPY in large fibres and galanin in small to medium sized primary afferent fibres.

Blocking ectopic discharges did have an effect on basal ir-galanin release, mainly on the contralateral side of the spinal cord. Interpretation of this is complicated by the fact that there were probably at least two sites of origin of ir-galanin in the superficial dorsal horn of the spinal cord - small diameter primary afferent neurones, and intrinsic dorsal horn neurones. Nevertheless, alteration in neuronal input, either by blocking ectopic discharge or by high intensity stimulation, did alter central ir-galanin release, indicating that abnormal input from the injured nerve may play at least some role in regulating ir-galanin release. In contrast, the new area of spontaneous ir-NPY release, deep in the dorsal horn, was not altered at all by blocking neuronal input from the injured nerve. Additionally, unlike ir-galanin, ir-NPY released by low intensity stimulation, persisted for some time after its release. Thus, released ir-NPY may diffuse to sites distant from its release, with widespread actions on neuronal activity. There is evidence from studies of other neuropeptides that they may act in a paracrine fashion [109,167]. It has been proposed that some neuropeptides may not act by standard synaptic release-receptor interaction, but instead act by volume transmission, with many diffuse effects, by spreading from release sites [105,132,241]. This does not exclude the possibility of direct neuropeptide-receptor interaction at synapses, but instead may provide an additional mode of action, that may become more important as the amount of released ir-NPY is increased, for example after nerve injury.

Galanin, however, appears to act more like a classical neurotransmitter, with release in the area of its receptors, and little persistence after release. In addition, neuronal activity altered ir-galanin release, with bilateral effects on its release secondary to blocking neuronal conduction from the injured nerve. As discussed in section 1.3.6, inhibitory effects of galanin predominate after nerve injury [453,454,456,474]. It may be therefore that the ir-galanin released from small diameter primary afferent fibres in the superficial dorsal horn acts to reduce onward transmission of abnormal impulses from ectopically firing neurones. Abnormal impulses have been detected in all classes of neurones after nerve injury [187], with the possibility of interactions between ectopically

firing A fibres and the C fibres that may release ir-galanin. There is certainly evidence for interactions between ectopically firing cells at the level of the dorsal root ganglia [8]. As well as ir-galanin released from primary afferent fibres, ir-galanin released from intrinsic neurones must be considered. As demonstrated in normal rats, galanin is both produced by [497] and released from [168] intrinsic neurones in the superficial dorsal horn. There is no evidence that galanin synthesis is altered in these neurones after nerve injury, but changes in neuronal input may alter control of release from these cells. In neuropathic rats, there appears to be some form of bilateral tonic inhibition of ir-galanin release, due to impulses from the injured nerve, as revealed by the effects of conduction block. If galanin is indeed acting primarily as inhibitory neurotransmitter, and its release from intrinsic neurones is prevented from increasing after nerve injury, then this may result in a generalised increase in spinal cord excitability. Whether this results in perception of pain is unclear, but there has been shown to be a link between the rate of ectopic discharge and degree of spontaneous pain in both animal [407] and human studies [320]. Alternatively, the increase in spontaneous activity may be one of the factors involved in stimulating and guiding the regenerative changes in the spinal cord.

The changes in ir-galanin and ir-NPY release may also be influenced by changes in the pattern of co-existence of neuropeptides with other neurotransmitters after nerve injury. In normal rats, both galanin [379] and NPY [355] have been found to coexist with GABA in the dorsal horn. However, after nerve injury, there is bilateral decrease in GABA levels in the dorsal horn [175], as opposed to a unilateral increase in galanin and NPY. The pattern of neuronal firing may alter the type of neurotransmitter released, as shown in studies of noradrenaline and NPY in the sympathetic nervous system [82], with firing frequency influencing the relative release of neurotransmitter from the nerve terminal [246]. There is also evidence of feedback from released NPY altering subsequent release of noradrenaline [39]. Thus, there may be complex interactions between the pattern of peripheral neuronal input, neurotransmitter co-existence and regulation of release from both primary afferent neurones and dorsal horn neurones. The summation of these effects may contribute to the development of neuropathic pain.

Previous studies have found evidence of altered peripheral input, with increased activity evoked in the dorsal horn of neuropathic rats from Aβ fibre stimulation of the injured nerve [75]. This same stimulus resulted in increased release of ir-NPY in the deep dorsal horn. The question of whether or not stimulation of these fibres, which are normally concerned with light touch transmission, is related to the generation of mechanical allodynia, and the role of evoked ir-NPY remains to be answered. There is some evidence from inflammatory models that phenotypic switches can occur in large myelinated fibres. This may be related to the development of pain related behaviour, possibly arising from impulses in large fibres not normally involved in the transmission of nociceptive information [313].

There is considerable evidence for reorganisational changes occurring in the dorsal horn after nerve injury, with both degeneration and regeneration occurring. The stimuli driving this restructuring are not fully understood, but NPY may have a role as a growth inducing substance, possibly in conjunction with other substances produced in or transported to the spinal cord. Chronic administration of intrathecal NPY was shown to result in increased neurite outgrowth from cultured dorsal root ganglia neurones [449]. This study also found that NPY added to dorsal root ganglia cultures from normal rats had no effect alone ,but only increased neurite outgrowth when given in conjunction with dorsal spinal cord explant. Ir-NPY release in the deep dorsal horn may initiate sprouting of large fibres from this area to the superficial dorsal horn. The new site of termination of these sprouted fibres does correlate better with the area of ir-galanin release, and the location of C fibre-derived GAP-43 [71,97,467], but ir-NPY may still have stimulated the initial growth of these fibres. Thus NPY may act as inhibitory neurotransmitter, perhaps with a generalised damping of spinal cord activity, but may also have a role in the spinal cord reorganisation occurring in response to nerve injury.

The nature of the factors needed for central "remodelling" after peripheral injury is obscure, but neurotrophins may be important in the changes occurring. Not only are they essential for growth and survival during development, but changes in their supply may be important in the primary afferent changes seen after nerve injury [239]. Thus, NT-3 seems to modulate large myelinated fibres, although the present studies did not

demonstrate any relationship between ir-NPY release from large fibres and lack of NT-3 after nerve injury. The interaction between neuropeptide synthesis and release and neurotrophins is complex. Neurotrophins have been found to have rapid actions on neurotransmitter release at synapses [243,405], as well as effects on sensory neurone regrowth [38]. It may be therefore that after nerve injury both neuropeptides and neurotrophin are involved in the central consequences of peripheral injury, as neurotransmitters or neuromodulators, and as growth promoting substances.

As the present studies have demonstrated, the spontaneous release of ir-NPY in the deep dorsal horn is neither altered by primary afferent input, nor by supplying exogenous NT-3 to the nerve injury site. It may be therefore that this ir-NPY release is occurring by an alternative secretory pathway to that normally associated with neurotransmitters. If ir-NPY has a role as a growth-promoting substance, then it may be released from primary afferent neurones by a non-vesicular mechanism, utilising the constitutive secretory pathway by which membrane components and growth promoting substances are normally released [24,490]. In this hypothetical situation, compounds with neurotransmitter actions could be released without regulation from neuronal activity and contribute to the development of abnormal, and uncontrolled, activity in the spinal cord.

6.3 Future directions

The present studies have provided information about the factors influencing the spinal release of galanin and NPY, but certain areas require further investigation in order to understand the functional significance of these findings in context with other changes known to occur after nerve injury.

Further studies are needed to elucidate the factors regulating bilateral ir-galanin release, including investigation of the involvement of supraspinal systems. Although the origin of the increased ir-galanin in the superficial dorsal horn ipsilateral to nerve injury is likely to be from primary afferent neurones, this also needs further study.

Potentially, one of the most significant findings from the present studies is that the spontaneous release of ir-NPY does not appear to be regulated by primary afferent activity. Thus investigation of the nature of the stimulus resulting in release, and the

secretory mechanisms involved might provide valuable information about the effects of peripheral nerve injury on a novel central release pathway of neurotransmitters. For both galanin and NPY, it would be useful to relate release to receptor activation in the spinal cord before and after nerve injury. This would add to the knowledge of the functional roles of galanin and NPY, as well as the spatial relationship between sites of release and sites of action. This could be further investigated by using some of the newer galanin and NPY antagonists, with improved sensitivity and specificity, in electrophysiological studies in the CCI model.

Another area worthy of further study is the interaction between neurotrophins, neuropeptides and behaviour. The present studies found a very minor alleviation of mechanical allodynia and hyperalgesia on the day of ir-NPY release experiments, 10-14 days after nerve injury. It would be interesting to continue this study further, following the time course of the behavioural syndrome over a longer period, as well as extending the testing to include measurement of other responses. The effect of other neurotrophins, either alone or in combination could also be studied, in conjunction with studies of peptide release.

The ultimate aim of studies of animal models of neuropathy has to be improvements in the management of patients with neuropathic pain. This can only be achieved by understanding the responses to peripheral nerve injury that result in the development of pain, and subsequently targeting these changes in a logical fashion. This will include new analgesic agents, as those currently available are often ineffective. In view of the major changes in neurotransmitters and neuronal phenotype after nerve injury, this is not surprising. The exact functional roles of galanin and NPY still require further study, as does the complex interplay between neuropeptides, neurotrophins and other neurotransmitters in the spinal cord. Whether or not galanin and NPY are directly involved in modulation of nociceptive transmission, or simply involved in the central restructuring that occurs after peripheral nerve injury, it seems increasingly likely that analogues of galanin or NPY may be useful agents in the treatment of neuropathic pain.

Appendix

Publications

Colvin, L.A., Mark, M.A., Duggan, A.W., "Enhanced synaptic activity in the spinal cord in a rat model of peripheral mononeuropathy" Congress Abstracts, IASP Publications, 8th World Congress on Pain (1996) 29, A89.

Colvin, L.A., Mark, M.A., Duggan, A.W., "Antibody microprobe studies of immunoreactive galanin release in the spinal cord of the neuropathic rat." *British Journal of Anaesthesia,* 78 (1997) 462P.

Colvin, L.A., Mark, M.A., Duggan, A.W., "The effect of a peripheral mononeuropathy on immunoreactive (ir)-galanin release in the spinal cord of the rat." *Brain Research* 766 (1997) 259-261.

Colvin, L.A., "The effect of conduction block on basal immunoreactive-neuropeptide Y (ir-NPY) release in the spinal cord of the neuropathic rat." *British Journal of Anaesthesia* (in press).

Colvin, L.A., Mark, M.A., Duggan, A.W., "Immunoreactive (ir)-galanin release in the spinal cord of the neuropathic rat." *European Journal of Anaesthesiology* (in press)

Colvin, L. A., Duggan, A. W., "Primary afferent-evoked release of immunoreactive (ir)-galanin in the spinal cord of the neuropathic rat." Submitted to *British Journal of Anaesthesia.*

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Prizes

Scottish Society of Anaesthetists Registrar's Prize,1997 for paper entitled: "Release of immunoreactive-galanin in the spinal cord of the neuropathic rat."

First prize for free paper presentation entitled "Immunoreactive (ir)-galanin release in the spinal cord of the neuropathic rat" presented at the 19th Annual Meeting of the European Academy of Anaesthesiology. 1997.

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Congress Abstracts, IASP Publications, 8th World Congress on Pain (1996) 29, A89.

ENHANCED SYNAPTIC ACTIVITY IN THE SPINAL CORD IN A RAT MODEL OF PERIPHERAL MONONEUROPATHY

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Aim of Investigation: This study was designed to investigate the central mechanisms underlying mechanical allodynia. We used a rat model of peripheral mononeuropathy to investigate the functional changes which occur in the spinal cord in response to peripheral stimulation of large myelinated fibres.

Methods: A peripheral mononeuropathy was produced by placing four loose ligatures around the right sciatic nerve at mid thigh level, as described by Bennett and Xie (1988). All the experiments were carried out on urethane anaesthetized male Wistar rats that displayed mechanical allodynia, at ten to fifteen days after nerve ligation. Control groups consisted of normal animals and those in which a sham operation had been performed. Large myelinated fibres in each sciatic nerve were stimulated 3 times threshold and the resulting cord dorsum potentials and field potentials (from 100 to 1200 microns from the cord dorsum) were measured on both the ipsilateral and contralateral sides of the cord.

Results: Paired t-tests of the means revealed that there was a significant difference between stimulation of the normal nerve and of the ligated nerve for: 1. The afferent volley from the ligated nerve was smaller on the ipsilateral side of the cord. 2. When the cord dorsum potentials were measured, the smaller afferent volley from the ligated nerve evoked greater activity in the spinal cord than the normal nerve on both the ipsilateral and contralateral side of the cord. 3. The field potential measurements also showed greater evoked activity from the ligated nerve on the ipsilateral side of the cord. This difference was most marked at a depth of 400-600 microns from the surface of the cord.

Conclusions: This study provides new electrophysiological evidence that nerve injury can result in enhanced central responses to a peripheral stimulation of large myelinated fibres. The major site of this increase appears to occur at a specific area in the dorsal horn - a depth of 400-600 microns. This may reflect the anatomical changes which have been shown recently, of central reorganisation and sprouting of the large myelinated fibres in the dorsal horn. Mechanical allodynia may result from these altered spinal cord responses.

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ANTIBODY MICROPROBE STUDIES OF IMMUNOREACTIVE NEUROPEPTIDE Y IN THE SPINAL CORD OF THE RAT.

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Aim of Investigation: To study the spinal release of immunoreactive Neuropeptide Y (ir-NPY) in normal rats and determine the origin of this release. This was investigated by: 1/ determining the extracellular levels of ir-NPY in rats in the absence of any active peripheral stimulus. 2/ investigating whether peripheral stimulation releases ir-NPY. 3/ examining whether spinalisation alters ir-NPY basal levels to determine the involvement of the descending bulbospinal pathways.

Methods: Microprobes bearing immobilised antibodies to NPY were inserted 2.25mm into the lumbar spinal cord (at the site of the sciatic nerve distribution) of anaesthetised rats and remained insitu for 15 minutes. Following incubation in radioactivity, the autoradiographic image of each probe tip was analysed by an image analysis system.

Results: A surprisingly high basal presence of immunoreactive (ir)-NPY was found throughout the entire dorsal horn. Electrical stimulation of large diameter afferents of the ipsilateral sciatic nerve (1Hz, 20Hz both at 3 x threshold) and unmyelinated primary afferents (2 Hz, 20 volts) did not release NPY in the spinal cord. Spinalisation did not alter the extracellular levels of ir-NPY in the distal segment of the cord.

Conclusion: Failure of electrical stimulation of primary afferents and of spinalisation to alter the extracellular basal presence of ir-NPY suggests that this basal presence originates from intrinsic neurones.

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"IMMUNOREACTIVE (ir)-GALANIN RELEASE IN THE SPINAL CORD OF THE NEUROPATHIC RAT"

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Introduction

After peripheral nerve injury, galanin synthesis is significantly increased in dorsal root ganglia, with a corresponding increase in galanin levels in the superficial dorsal horn[1,2]. Galanin has an increased inhibitory action after nerve injury and may therefore act as an endogenous analgesic during nerve regeneration[3]. Previously extensive basal ir-galanin release has been found in normal rats, which is not affected by peripheral stimulation[4].

The aim of this study was to investigate the effects of peripheral stimulation on ir-galanin release after peripheral nerve injury. The persistence of stimulus-released ir-galanin was also studied.

Methods and Materials

A chronic constriction injury was produced in male Wistar rats (n=42), by placing four 4/0 chromic gut ligatures loosely around one sciatic nerve[5]. The guidelines for the care of experimental animals of the International Association for the Study of Pain were followed[6].

At 10-14 days after ligature placement, the lumbar spinal cords of urethane-anaesthetised rats were exposed. Microprobes bearing immobilised antibodies to galanin (Peninsula Labs) were inserted 2.25mm into the dorsal spinal cord. Following removal from the spinal cord and incubation in ^{125}I-labelled galanin (Peninsula Labs), autoradiographic images of each microprobe tip were analysed and compared using a computerised image analysis system. Binding of endogenous galanin to the microprobes resulted in deficits in binding of ^{125}I-labelled galanin. Differences between specified groups of microprobes were assigned statistical significance using Student's unpaired t-test.

Results

There was a new peak of ir-Gal release in the superficial dorsal horn, ipsilateral to nerve injury, when compared to the contralateral side. This area of spontaneous release was increased further by electrical stimulation of the injured nerve at a strength sufficient to activate A and C fibres, but not by stimulation of A fibres alone. Unlike other neuropeptides, such as neurokinin A, there was no evidence of persistence of the new areas of galanin release after the stimulation had finished.

Conclusions

These studies showed that ir-galanin was continuously released in the superficial dorsal horn ipsilateral to nerve injury. This release was increased further by stimulation of small primary afferent fibres. Although functional studies are required to elucidate the role of galanin after nerve injury, these studies of release suggest an important role in the modulation of nociceptive transmission. Its lack of persistence after stimulation would indicate that its main site of action is close to its site of release.

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"Antibody microprobe studies of immunoreactive (ir)- galanin release in the spinal cord of the neuropathic rat."

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Following peripheral nerve injury dramatic changes are seen in neuropeptide synthesis in the dorsal root ganglia [1], with significant increases in galanin and neuropeptide Y mRNA and a decrease in both CGRP and substance P mRNA [2]. The aim of this study was to investigate changes in central galanin release after peripheral nerve injury. Although the significance of galanin up-regulation is unknown, it has been postulated to play an inhibitory role in dorsal horn transmission.

Under sodium pentobarbitone anaesthesia, four 4/0 chromic gut ligatures were placed loosely around one sciatic nerve of male Wistar rats to produce a chronic constriction injury [3]. The International Association for the Study of Pain guidelines for the care of experimental animals were followed.

At 10-14 days after ligature placement, the lumbar spinal cords of urethane-anaesthetized rats (n=30) were exposed. Using a micromanipulator, microprobes bearing immobilised antibodies to galanin were inserted into the dorsal spinal cord to a depth of 2.25mm. Following removal from the spinal cord and incubation in ^{125}I-labelled galanin, autoradiographs of each microprobe tip were analysed using an image analysis system. *In vitro* microprobe images for each experiment were also analysed and normal rats were used as controls. Binding of endogenous galanin results in deficits in binding of ^{125}I-labelled galanin. Mean image analysis of defined groups of microprobes were compared, at 30μ intervals, and the differences between specified groups were assigned statistical significance using Student's t-test.

In normal animals, there was a fairly extensive presence of ir-galanin throughout the dorsal horn, with a peak in the superficial dorsal horn. In the neuropathic rats there was a significantly greater peak of ir-galanin release in the superficial dorsal horn ($p<0.05$). As there is significant up-regulation of galanin synthesis in small to medium sized dorsal root ganglia cells, the likely source of this new zone of central galanin release is from primary afferent fibres, affected by the peripheral nerve injury. Since this increased release occurred spontaneously, and there is evidence for spontaneous pain in this model, the two may be linked.

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THE EFFECT OF CONDUCTION BLOCK ON BASAL IMMUNOREACTIVE-NEUROPEPTIDE Y (IR-NPY) RELEASE IN THE SPINAL CORD OF THE NEUROPATHIC RAT

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In rats, ir-NPY is released spontaneously in the dorsal horn of the spinal cord, on the side ipsilateral to a chronic constriction injury of the sciatic nerve[1]. The likely site of origin is from primary afferent neurones[2], although the stimulus resulting in release in not known. After nerve injury, primary afferent neurones behave abnormally, with the generation of spontaneous ectopic activity both at the nerve injury site and the dorsal root ganglia[3,4]. If the spontaneous release of ir-NPY is due to ectopic discharges, then blocking these impulses should abolish the new zone of spontaneously released ir-NPY found in the spinal cord of neuropathic rats.

In male Wistar rats, a chronic constriction injury was induced by placing four 4/0 chromic gut ligatures loosely around the sciatic nerve, under sodium pentobarbitone anaesthesia[5]. The International Association for the Study of Pain guidelines for the care of experimental animals were followed. After 10-14 days, levels of ir-NPY release were determined in rats (n=12) showing behavioural evidence of neuropathic pain. Under urethane anaesthesia, the lumbar spinal cord was exposed and microprobes coated with immobilised antibodies to NPY were inserted bilaterally into the spinal cord to a depth of 2.25mm. Ir-NPY release was studied, firstly, under basal conditions, and then after conduction block with amethocaine gel proximal to the dorsal root ganglia. Following removal from the spinal cord and incubation in ^{125}I-labelled NPY, the microprobe tip was placed on X-ray monoemulsion film, to give an autoradiographic image for each microprobe, that was then analysed. Binding of endogenous NPY results in deficits in binding of ^{125}I-labelled NPY, which was detected as a decrease in the optical density by a computerised image analysis system. Mean image analysis of defined groups of microprobes were compared, at 30μ intervals, and the differences between specified groups were assigned statistical significance using Student's unpaired t-test.

Spontaneous release of ir-NPY was confirmed on the side of the cord ipsilateral to the nerve injury[6]. This spontaneous ir-NPY release was not altered by conduction block proximal to the dorsal root ganglia, when studied for up to three hours after conduction block. It is unlikely, therefore, that the spontaneous release of ir-NPY is due to ectopic activity developing after nerve injury. Indeed, NPY may not be acting as a neurotransmitter at all, but is instead involved in the regenerative changes that occur in the dorsal horn after peripheral nerve injury.

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Short communication

The effect of a peripheral mononeuropathy on immunoreactive (ir)-galanin release in the spinal cord of the rat

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Short communication

The effect of a peripheral mononeuropathy on immunoreactive (ir)-galanin release in the spinal cord of the rat

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Abstract

The pattern of ir-galanin release in the spinal cord of rats with a peripheral mononeuropathy was studied. On the side of the cord ipsilateral to the nerve injury enhanced ir-galanin release was found in the superficial dorsal horn. It is probable that, after nerve injury, some primary afferent neurons spontaneously release galanin from their central terminals. © 1997 Elsevier Science B.V.

Keywords: Chronic constriction injury; Dorsal horn; Galanin; Antibody microprobe; Spontaneous release

After a peripheral nerve injury, there are striking alterations in neuropeptide synthesis in primary afferent cell bodies in the dorsal root ganglia and in neuropeptide levels in the dorsal horn of the spinal cord [12,21]. Galanin, neuropeptide Y and vasoactive intestinal peptide (VIP) synthesis is markedly increased, while substance P, calcitonin gene-related peptide and somatostatin synthesis is decreased [13]. With galanin the largest increases were found in small to medium sized cells of the dorsal root ganglia [5,23] with an associated increase in ir-galanin distribution in the superficial dorsal horn [22].

The functional significance of these changes is unknown, with evidence of both inhibitory [14,18] and excitatory [8] effects. It has been postulated that galanin may be part of an endogenous analgesic system that becomes important after peripheral nerve injury [17,19].

In the spinal cord of the normal rat, Hope and coworkers [6] did find ir-galanin release in the dorsal horn, which was unaffected by primary afferent stimulation. They proposed that the main source of ir-galanin was from intrinsic neurons of the spinal cord.

The aim of this study was to examine the effects of a peripheral nerve injury on the central release patterns of ir-galanin.

A peripheral neuropathy was induced in 32 male Wistar rats (200–320 g, Charles River Ltd., UK) under sodium pentobarbitone anaesthesia (40–50 mg/kg, intraperitoneal), to produce a chronic constriction injury as described by Bennett and Xie [1]. Four 4/0 chromic gut sutures were placed loosely around the sciatic nerve at mid thigh level. The International Association for the Study of Pain guidelines for the care of experimental animals were followed [24].

Regular monitoring of the development of neuropathic pain behaviour was carried out in all animals post-operatively. The lowest threshold at which paw withdrawal to a series of von Frey hairs occurred provided an indication of the development of mechanical allodynia. The duration of paw withdrawal response to pin prick was used to detect mechanical hyperalgesia. An arbitrary value of 0.5 s was assigned to the rapid response normally seen [15]. The mean values, for each paw, obtained at each time point were compared using Student's paired *t*-test, for both paw withdrawal threshold and duration. Both paw withdrawal threshold to von Frey hairs and duration of paw withdrawal to pin prick showed a significant difference ($P < 0.001$) between ligatured and non-ligatured hindpaws from day 3 after ligature placement until the day on which ir-galanin release was studied.

Spinal release of ir-galanin was studied with antibody microprobes bearing immobilised galanin (rat) antiserum (Peninsula Labs., CA) The details of this technique are

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described in Duggan [3]. The microprobe technique detects bound endogenous galanin by the failure of binding of exogenous [^{125}I]galanin (rat, Peninsula Labs., CA) to the immobilised galanin antibody. Prior to commencing in vivo work, in vitro assays were carried out to ensure adequate specificity and sensitivity for the immobilised galanin antibody, using a range of related peptides and range of galanin concentrations. No evidence of cross reactivity was found between the galanin antiserum and neuropeptide Y, VIP and substance P. Sensitivity tests showed greater than 90% suppression of [^{125}I]galanin binding by 10^{-5} M galanin and approximately 40% suppression of binding with 10^{-9} M galanin.

Ten to 14 days after ligature placement, neuropathic rats were anaesthetized with urethane (up to 1.25 g/kg, intraperitoneal). Six normal rats were also used. A laminectomy was performed to expose the spinal segments, L2–L6, the dura removed and the exposed cord irrigated with sterile, warmed Ringer's solution. Using a micromanipulator, microprobes were inserted bilaterally, to a depth of 2.25 mm into the lumbar dorsal spinal cord. The probes were left in place for 15 min, then washed in cold 0.1% Tween/phosphate buffered saline, incubated for 24 h at 4°C in a solution of [^{125}I]galanin and then washed again. The distal portions were placed on a sheet of monoemulsion film (Kodak NMC) for 8 to 10 days to produce an individual autoradiographic image for each microprobe.

A computerised image analysis system (Imaging Technology PC Vision Plus frame grabber board) was used to analyse the autoradiographic images [4]. Each image was scanned to give a measure of optical density at 10-μm intervals along the length of the microprobe [2]. For each microprobe, a plot of grey scale (i.e. optical density) against depth of the probe in the spinal cord was obtained. Areas of endogenous galanin binding were shown by areas of low optical density and thus a low value on the grey scale. The means of defined groups of microprobes, were compared at 30 μm intervals along the length of the probes using Student's unpaired t-test.

Microprobes inserted into the spinal cord of normal rats in the absence of peripheral stimulation ($n = 57$) showed an extensive basal release of ir-galanin, when compared to in vitro microprobes ($n = 187$), confirming previous results from this laboratory [6]. In vitro microprobes had not been inserted into the central nervous system, but simply incubated in [^{125}I]galanin, thus representing zero endogenous galanin binding. There was no significant difference between the pattern of ir-galanin release between the right and left sides of the spinal cord in these normal rats.

The mean image analysis of microprobes ($n = 58$) inserted into the spinal cord of normal rats and the mean image analysis of microprobes ($n = 50$) inserted into the side of the spinal cord contralateral to the nerve injury, in neuropathic rats, were not significantly different. Thus, the initial extensive basal release of ir-galanin seen in the spinal cord of normal rats was unchanged in the spinal cord of neuropathic rats, contralateral to the peripheral nerve injury.

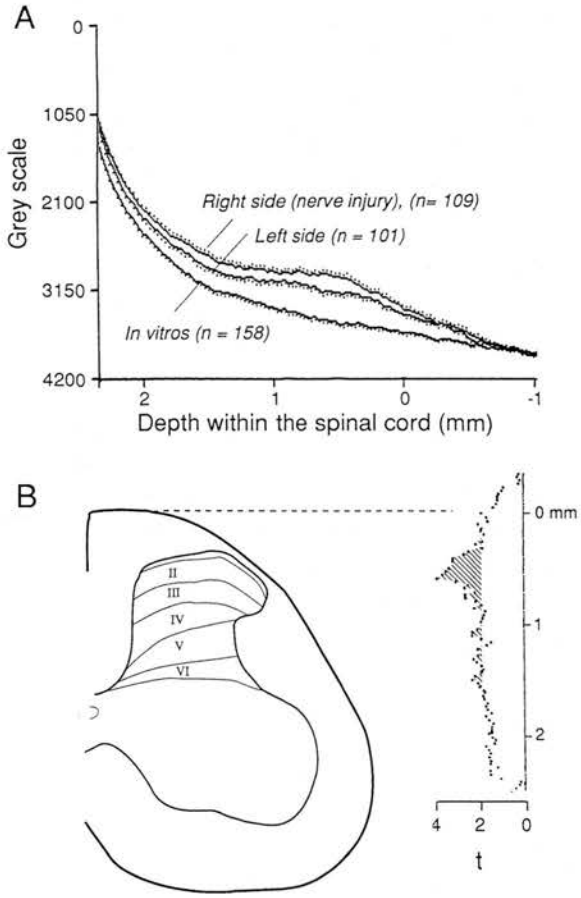

Fig. 1. Spontaneous ir-galanin release in the spinal cord of neuropathic rats. A: mean image analysis (±S.E.M.) of microprobes inserted into the ipsilateral (right, $n = 109$) and contralateral (left, $n = 101$) sides of the spinal cord relative to the nerve injury. An estimate of zero endogenous galanin binding is shown by the mean image analysis of in vitro microprobes ($n = 158$). B: the t statistics derived from the differences (at 30-μm intervals) between the groups of microprobes inserted into the right and left sides of the spinal cord of neuropathic rats (illustrated in A) are plotted with respect to a diagram of the spinal cord. The hatched areas representing where $t > 2$ and $P < 0.05$. The positions of the laminae are taken from Molander et al. [11].

By contrast, a new area of spontaneous ir-galanin release was detected with a peak in the superficial dorsal horn ipsilateral to the side of nerve injury, both when compared to the release pattern found in normal rats, and in the side of the cord contralateral to the nerve injury (see Fig. 1). This new zone of ir-galanin release is in the same area as the termination of the small to medium sized primary afferent fibres, that start synthesising galanin after nerve injury [5,12,13,23].

Normally, primary afferent fibres require a peripheral stimulus before central neuropeptide release occurs, in contrast to the spontaneous release of ir-galanin found in the neuropathic rat. However, recent work from this labo-

ratory has also demonstrated spontaneous release of neuropeptide Y in the dorsal horn of nerve injured rats [9]. There is an increasing amount of evidence that after nerve injury primary afferent neurones behave abnormally, with the generation of spontaneous ectopic activity both at the nerve injury site [10,16], and at cell bodies in the dorsal root ganglia [7,20]. This high level of spontaneous activity from primary afferents is likely to contribute to changes in somatosensory processing that occur centrally in response to peripheral nerve injury. Tal and co-workers [16] have indeed found that there was a correlation between spontaneous activity in primary afferent neurons and the development of thermal hyperalgesia.

As these afferents are firing spontaneously, it is possible that the central release of ir-galanin is due to these abnormal impulses. A corollary to this is that ectopic impulse-evoked release of galanin may be related to the generation of spontaneous pain.

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Research report

The release of immunoreactive neuropeptide Y in the spinal cord of the anaesthetized rat and cat

M.A. Mark, B. Jarrott [1], L.A. Colvin, S.J.A. MacMillan, A.W. Duggan *

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The release of immunoreactive neuropeptide Y in the spinal cord of the anaesthetized rat and cat

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Abstract

The release of immunoreactive (ir-) neuropeptide Y (NYP) was studied in the anaesthetized rat and cat by means of microprobes bearing immobilized antibodies to the C terminus of NPY. An extensive basal release of ir-NYP was detected throughout the dorsal and upper ventral horn of the rat. This spontaneous release was not significantly altered by sectioning the spinal cord at the thoraco-lumbar junction nor by electrical stimulation of peripheral nerves. Since NPY is virtually absent in primary afferents it is probable that spontaneous release within the spinal cord comes from active NPY-containing intrinsic spinal neurones. In the spinal cat spontaneous release of ir-NPY was detected in the mid-dorsal horn and this was unaltered by peripheral noxious thermal or noxious mechanical stimuli. As in the rat, release from intrinsic spinal neurones is most probable. The extensive spontaneous release of ir-NPY in both species suggests a widespread role in spinal cord function.

Keywords: Neuropeptide Y; Antibody microprobe; Spontaneous release; Rat spinal cord; Cat spinal cord; Spinal transection

1. Introduction

Structural and functional studies imply that neuropeptide Y (NPY) has a role in the spinal processing of information conveyed by primary afferent fibres. This was first suggested by histochemical studies of the distribution of NPY in the spinal cord. In the normal rat there is negligible NPY in primary afferents [47] but a dense plexus of NPY-containing fibres in laminae I and II of the dorsal horn [18,33] where many small diameter primary afferents terminate [26]. In the lamprey NPY-containing varicosities were observed closely opposed to ascending and descending branches of primary afferents [3] and in the cat an ultrastructural study observed NPY-containing synapses on both primary afferent terminals and axo-dendritic synapses in the superficial dorsal horn [9]. These observations in the cat suggest that NPY may control transmitter release from the central terminals of primary afferents. Supporting this are the reports that microinjection of NPY into the superficial dorsal horn of the cat reduced peripheral stimulus-evoked release of SP [13] and when added to the superfusate of cultured dorsal root ganglion neurones, NPY reduced high K^+-evoked release of SP [45]. Administered intrathecally, NPY produced behavioural analgesia in the rat [22] but had dose-dependent facilitatory or inhibitory effects on nociceptive rat spinal reflexes measured electrophysiologically [46].

There are, however, no reports of the release, and the processes controlling release, of NPY in the dorsal horn. The possible sources of release of NPY are intrinsic neurones of the spinal cord [6,40,44] and the terminals of brainstem neurones projecting to the spinal cord [15,16,20]. Although the near absence of NPY in dorsal root ganglion neurones implies that this compound cannot be released in significant amounts from the spinal terminations of primary afferents, peripheral inputs could still evoke intraspinal release of NPY by activating spinal or supraspinal processes. Hence in addition to studying basal release of immunoreactive (ir-) NPY in the spinal cord of the rat and the cat, the effects of peripheral nerve stimulation were studied in the rat and the effects of noxious peripheral thermal and mechanical stimuli were examined in the cat. Because of the known presence of NPY in the spinal

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terminations of fibres of supraspinal origin, the effect of spinal transection on spinal release of ir-NPY was also studied in the rat.

2. Materials and methods

2.1. Animal preparation

2.1.1. Rats

Experiments were performed on 25 male Wistar rats (weight range 300–400 g; Charles River, UK) anaesthetized with intraperitoneal urethane (1.25 g kg^{-1} initially, supplemented when necessary). Cannulae were inserted into the trachea, a carotid artery and an external jugular vein. The depth of anaesthesia was regularly assessed by continuous measurement of blood pressure and ensuring that the corneal blink and hind paw withdrawal reflexes remained absent. Blood oxygenation was assisted by directing a gentle jet of humidified oxygen towards the opening of the tracheal cannula. The animal's core temperature was maintained between 36 and 38°C using a controlled heating system. In experiments involving stimulation of high threshold afferents of the sciatic nerve, animals were artificially ventilated following neuromuscular paralysis with intravenous gallamine triethiodide (3 mg kg^{-1}) and the end-tidal CO_2 levels were continually monitored. The depth of anaesthesia was assessed prior to each injection of gallamine.

An extended laminectomy was made at vertebral levels T12 to L2 to expose the dura mater of the L2–L6 spinal cord segments. The animal was supported in a metal frame by six swan neck clamps positioned under the mammillary processes of the exposed vertebrae. The spinal cord was covered with Ringer-agar at the site of the laminectomy with a window over the lumbar spinal segments. Following opening of the dural sac with sterile forceps, the exposed spinal cord was irrigated with sterile Ringer's solution at 37°C continuously throughout the experiment. Unlike experiments on the cat it was not necessary to remove the pia arachnoid in the rat to allow microprobes to be inserted into the spinal cord. The sciatic nerve on one side was isolated and freed close to its trifurcation by separating the medial and lateral knee flexor muscles. This nerve was then gently mobilized for 1–2 cm to permit mounting on platinum electrodes in a liquid paraffin pool.

Before microprobes were inserted into the spinal cord, the suitability of potential penetration sites was examined functionally. A silver ball electrode was gently pressed onto the dorsum of the spinal cord just medial to the line of entry of the dorsal roots and the threshold stimulus current and voltage needed to evoke a field potential was measured. The ball electrode was positioned at several rostro-caudal locations as a measure of the distribution of afferent input from the sciatic nerve.

In four experiments, the spinal cord laminectomy was extended to include the lower thoracic cord. Prior to dividing the spinal cord a micromanipulator was used to inject, from a micropipette, 5 μl of lignocaine 1% solution in both sides of the spinal cord at segments T9–T10. The spinal cord was then teased apart using watchmaker's forceps, taking care to leave the dorsal vein intact.

2.1.2. Cats

Experiments were performed on six cats anaesthetized with pentobarbitone sodium (35 mg kg^{-1} initially and maintained by a constant intravenous infusion of 3 mg kg^{-1} h^{-1}). A carotid artery was cannulated to measure blood pressure and a tracheostomy performed for artificial respiration with humidified air. End-tidal CO_2 levels were monitored continuously. Body temperature was maintained at 37–38°C with a controlled heating pad.

The lumbar spinal cord was exposed over its whole length and the spinal cord was transected at the thoracolumbar junction after injection of 0.1 ml of 2% lignocaine. Each cat was suspended in a metal frame by means of pelvic and vertebral clamps. The lumbar dura mater was incised longitudinally and retracted laterally and a thin layer of Ringer-agar was placed on the dorsal surface of the spinal cord. This agar layer was removed at the area of proposed microprobe penetrations and the exposed spinal cord was continually irrigated wtih sterile Ringer's solution. The pia-arachnoid was removed at sites of microprobe insertion. Peripheral nerve stimulation was not performed in the cat experiments but noxious thermal or mechanical stimuli were applied to the hind paws. Noxious thermal stimulation consisted of immersing a hind paw in a water bath maintained at temperatures from 48°C to 52°C. The hind paw was immersed for 3 min and then removed for 2 min and this cycle was repeated for the total duration of the stimulus. Noxious mechanical stimulation was provided by alligator clips applied to the glabrous skin of a hind paw, on for 3 min and removed for 2 min.

2.2. Microprobe preparation

Antibody microprobes for the detection of ir-NPY were prepared as previously described [10]. Briefly, fine glass micropipettes, heat sealed at both ends, were incubated for up to 24 h in a 10% solution of γ-aminopropyltriethoxysilane in toluene. This procedure produced a fine, granular siloxane polymer layer on the outer surface of each micropipette. This polymeric coating possessed free amine groups to which Protein A (Sigma) was immobilized using glutaraldehyde coupling. Protein A is a staphylococcal derived protein which binds to the Fc region of some subclasses of IgG antibodies and this property was utilized to bind, to microprobes, immunoglobulins present in a polyclonal antiserum. Two antisera were used. In the rat experiments the antiserum was purchased from Peninsula Laboratories and had been raised against the C terminus of

human, rat NPY. Tests of specificity of the microprobes bearing this antibody are described later. In the cat experiments the antiserum was raised in rabbits against the C terminus of porcine NPY as described by Maccarrone and Jarrott [31]. Before insertion into the spinal cord, microprobes were broken back to give tip diameters of approx. 10 μm.

2.3. Rat protocol

All microprobes were inserted into the cord (two at a time) with a pair of stepping motor micromanipulators to a depth of 2.25 mm from the dorsal surface and left in situ for 15 min. In the absence of peripheral stimulation, microprobes were inserted into both sides of the cord (at a level determined by the electrophysiological recordings). Due to uncertainty on the persistence of any possibly released ir-NPY following peripheral stimulation, only one stimulation period of 15 min was used in each experiment. With electrical stimulation, microprobes were positioned only in the ipsilateral spinal cord. The stimulus parameters were: 0.5 ms pulses, either $\times 3$ threshold at 1 or 20 Hz or $> 100\times$ threshold at 2 Hz. Following electrical stimulation it was usual to insert microprobes for three periods of no stimulation.

In the rat experiments in which spinalization was performed midway through the experiment, four pairs of microprobes were inserted into both sides of the spinal cord before spinalization. After spinalization, this protocol was repeated with a further four pairs of microprobes thus ensuring that both sides of the cord were sampled equally. No peripheral stimulation was used in these experiments.

2.4. Cat protocol

In the cat experiments the sites of microprobe insertion were determined by recording extracellular neuronal responses (with a 4 M NaCl filled micropipette) to innocuous mechanical stimulation of the hind paws. Microprobes were inserted 3 mm into the spinal cord, two at a time, into the ipsilateral spinal cord and remained in place for 15 or 30 min. When peripheral stimulation was used it was usual to insert four control (no stimulus) microprobes both before and after each period of stimulation.

Following the insertion of microprobes, pontamine sky blue was ejected electrophoretically at several defined sites in the spinal cord. The cords were then removed, fixed, and sectioned to determine the location of resultant dye spots. These data were essential to allow locations on the probes to be related to the laminae of the spinal cord.

2.5. Microprobe treatment

The antibody microprobe technique detects bound endogenous ligand by the failure of binding of exogenous radiolabelled ligand. Thus, following removal from the spinal cord, microprobes were washed for 15 min in ice-cold phosphate-buffered saline (PBS) containing 0.1% Tween and then incubated for approx. 24 h at 6°C in a PBS-azide solution of ^{125}I-radiolabelled porcine NPY (approx. 2000 counts μl^{-1} min^{-1}) containing casein (0.1%, rat experiments) or bovine serum albumin (0.1%, cat experiments). In the rat experiments Bolton–Hunter-labelled peptide was purchased from Amersham (UK). In the cat experiments NPY was labelled by the iodogen method [7]. Probes were washed for 15 min in ice-cold PBS-Tween while continually drawing the solution through the tips to remove any radiolabelled NPY from within. The distal portions of the microprobes were broken off and mounted onto a sheet of paper, which was placed in an X-ray film cassette with a sheet of monoemulsion film (Kodak NMC) for 6–14 days. Two films were derived from each experiment.

The resultant microprobe autoradiographs were analysed with an image analysis system employing an Imaging Technology PC Vision Plus frame grabber board operating in a Data Control System 286e (AT based) computer, as previously described [19]. A charged coupled device camera scanned each image starting at the tip and following background subtractions, a transverse integration of optical density on a scale of 0–255 was performed for each microprobe at defined intervals. With the magnification of the system used, and the resolution of the image analysis system (512×512 locations per frame) this corresponds to a 10 μm interval for transverse integrations. However, this is beyond the biological resolution of the microprobe method [10] and therefore the average of three successive integrals was taken to give a final resolution of 30 μm. For each microprobe, the resultant plot of integrated optical density (grey scale) with respect to length together with coded information which described the experimental conditions for that particular microprobe were stored on a computer file. Regions of bound endogenous ir-NPY along the length of each microprobe have been equated with relative deficits in the tracer binding, that are represented graphically as comparatively low grey scale values.

A sorting programme was used to retrieve groups of microprobes which met stated criteria and produced for each group a plot of the mean grey scale values ($\pm$S.E.M. in 30 μm steps) with respect to depth within the spinal cord. Each site is treated independently and hence, for selected groups of microprobes, statistical significance can be assigned to differences between mean optical densities at each site. The significance of the differences at each 30 μm analysis point was estimated using Students' t-test. It should be noted that the pooling of microprobe images introduces errors in determining sites of release since between-animal variations in anatomy are not accurately accounted for and even within one experiment not all probes are introduced at the same distance from the midline. With large numbers of microprobes it is likely that the peak sites of release are accurately determined but that

the spread of sites of release around such peaks is magnified by the pooling of images.

2.6. In vitro tests

Because the antisera used were C terminus directed they recognized porcine, human and rat NPY equally as these forms of NPY differ by only one amino acid at position 17. The sequence for cat NPY is unknown but a C terminus directed antiserum was considered most appropriate because of the known sequences of NPY variants [14]. Data from the manufacturer stated that the antiserum used in rat experiments recognized peptide YY but not prepro NPY(68–97). In parallel with present experiments, the sensitivity of the prepared antibody microprobes was regularly assessed by incubating ten to 15 either directly in ^{125}I-NPY or in a range of concentrations (10^{-5}–10^{-9} M) of unlabelled NPY at 37°C for 30 min prior to the incubation in a solution of ^{125}I-NPY for approx. 24 h at 6°C. Following washing in PBS-Tween, the distal portions of these microprobes were broken off, mounted on small pieces of cardboard and placed in tubes for processing by a γ-counter. In vitro tests indicated that a 10^{-7} M solution of NPY suppressed such binding by greater than 50% with a 10^{-5} M solution resulting in greater than 80% suppression.

3. Results

3.1. Microprobes present in the spinal cord in the absence of peripheral stimulation

3.1.1. Rat

A basal extracellular presence of a neuropeptide can be inferred by observing differences in the mean image analysis of microprobes not inserted into the central nervous system (but simply incubated in the radiolabelled peptide, termed in vitros in Fig. 1) and that of microprobes present in the nervous system in the absence of any peripheral stimulus and then incubated with the labelled peptide (termed no stimulus microprobes in Fig. 1). With the latter it was relatively common for autoradiographs to show sharply defined zones of failure of binding of ^{125}I-NPY and a density scan of one is shown in Fig. 1A. This zone corresponds to the upper dorsal horn. Fig. 1B compares the mean image analysis of 56 in vitro microprobes and that of 73 no stimulus microprobes. The zone of maximal difference between these groups corresponds to the zone of reduced binding in the microprobe of Fig. 1A but the total area of significant differences encompasses a much broader area. This is shown in Fig. 1C where the t-statistics derived from the differences between these groups are plotted in relation to a diagram of the spinal cord. The hatched area indicates where the differences are significant at the $P < 0.05$ level and this area includes the whole of

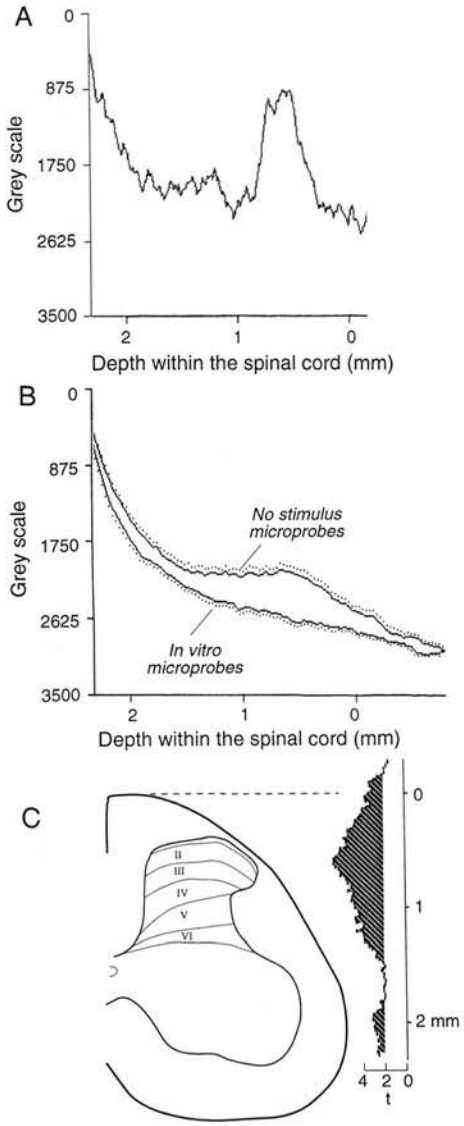

Fig. 1. Spontaneous release of immunoreactive (ir-) NPY in the spinal cord of the rat. A: density scan of the autoradiograph of a single microprobe inserted 2.25 mm into the spinal cord of a rat and which remained in place for 15 min in the absence of any applied stimulus. B: the mean image analysis of 56 microprobes simply incubated in ^{125}I-NPY (in vitro microprobes) and that of 73 microprobes inserted 2.25 mm into the spinal cord and which remained there for 15 min in the absence of any applied stimulus (no stimulus microprobes). A line joins the mean grey scale values and +S.E.M. is plotted at each analysis point for the no stimulus group of microprobes and −S.E.M. is plotted for the in vitro microprobes. C: the t-statistics derived from the differences (in 30 μm intervals) between the groups of microprobes illustrated in B are plotted with respect to a diagram of the rat spinal cord. The hatched areas indicate where $t > 2$ and hence where the difference is significant at $P < 0.05$. The positions of the spinal laminae are taken from Molander et al. [34].

the dorsal horn and dorsal columns merging into the upper ventral horn and a further zone in the deep ventral horn extending into the ventral white matter. The site of maximal difference corresponds to the upper dorsal horn.

The high basal levels of ir-NPY found in intact animals could represent release from the spinal terminations of

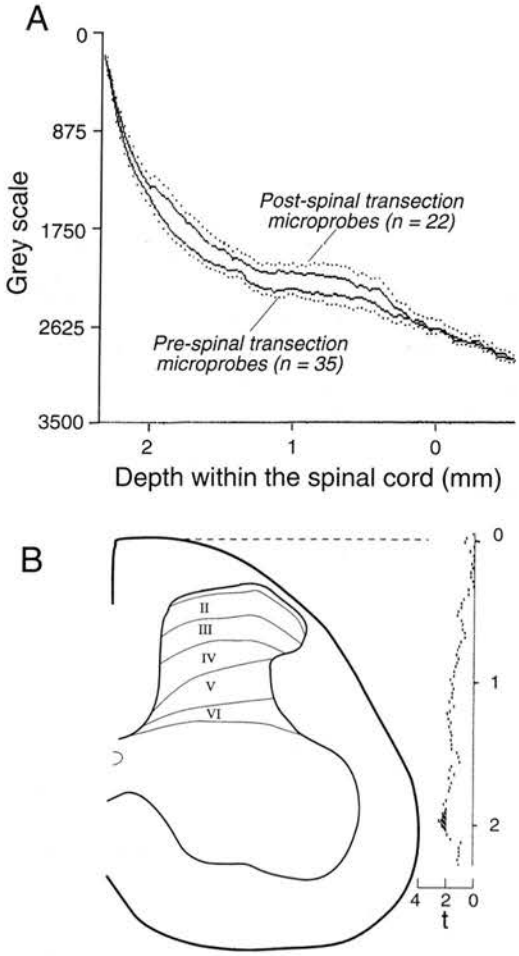

Fig. 2. Spinal transection and the spontaneous release of ir-NPY in the rat spinal cord. A: the two plotted image analyses were derived from 35 microprobes present in lumbar spinal cord in the absence of peripheral stimulation and prior to transection of the lower thoracic spinal cord (pre-spinal transection microprobes) and 22 comparable microprobes inserted after spinal cord transection (post-spinal transection microprobes). B: the *t*-statistics derived from the differences of the image analyses shown in A are plotted in relation to a diagram of the rat spinal cord.

fibres of supraspinal origin. To examine this four rats were spinalized midway through an experiment for comparison of basal levels of ir-NPY found by microprobes inserted into the spinal cord before and after spinalization at a mid-thoracic level. Fig. 2A illustrates the mean image analyses of these two groups. Although the post-spinalization microprobes ($n = 22$) are displaced above the pre-spinalization group ($n = 35$), suggesting higher extracellular levels of ir-NPY following sectioning of the spinal cord, the differences are not significant (Fig. 2B), except for a restricted area of the ventral horn.

3.1.2. Spinal cats

Microprobes inserted into the spinal cord of previously spinalized cats also detected a basal presence of ir-NPY. Fig. 3A compares the mean image analysis of 31 such microprobes and that of 22 microprobes not inserted into the spinal cord but simply incubated in radiolabelled NPY. The plot of the former is uniformly displaced above that of the in vitro microprobes, but Fig. 3B shows that this attains significance at the $P < 0.05$ level only in the upper dorsal horn.

3.2. Microprobes present in the spinal cord during electrical stimulation of large and small diameter fibres of the ipsilateral sciatic nerve of the rat

3.2.1. Stimulation at ×3 threshold (large myelinated fibres)

In eight of these experiments large myelinated fibres in the sciatic nerve were stimulated electrically at 1 Hz for one 15 min period. Probes were inserted before, during and after stimulation. The mean image analyses of 26 microprobes inserted 2.25 mm into the spinal cord and left in situ for 15 min in the absence of any active peripheral stimulus and 16 microprobes inserted for the same time and same depth, but during electrical stimulation of large

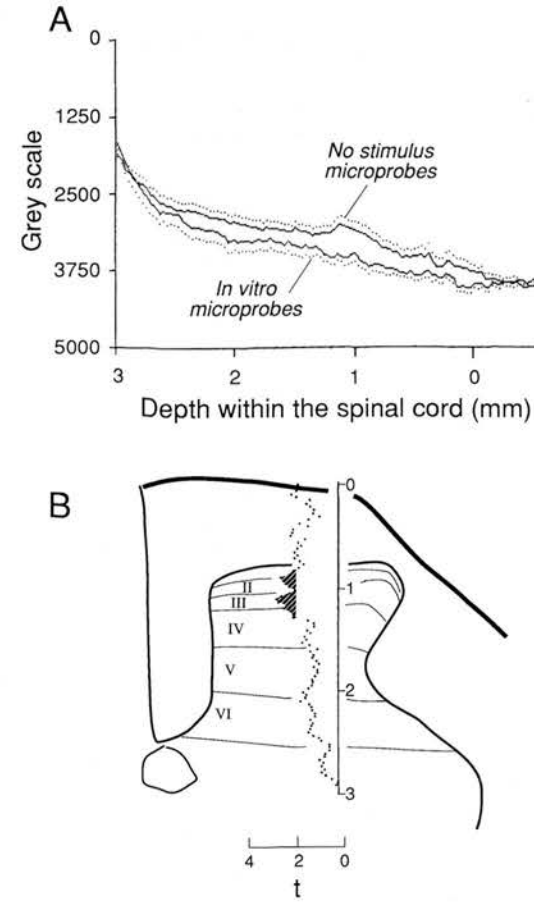

Fig. 3. Spontaneous release of ir-NPY in the spinal cat. A: the mean image analysis of 22 in vitro microprobes is compared with that of 31 no stimulus microprobes inserted 3 mm into the spinal cord and in the absence of any peripheral stimulus (no stimulus microprobes). B: the *t*-statistics derived from the differences between the two groups shown in A are plotted with respect to a diagram of the cat lumbar spinal cord.

myelinated fibres at 1 Hz, showed no significant differences. Microprobes inserted after a period of electrical stimulation at 1 Hz also failed to show significant alterations from pre-stimulation microprobes (not illustrated).

In six experiments large myelinated fibres in the sciatic nerve were stimulated at 20 Hz for one 15 min period. The mean image analyses of 22 microprobes inserted 2.25 mm into the spinal cord and left in situ for 15 min in the absence of any active peripheral stimulus and 11 microprobes inserted for the same time and same depth in the same experiments but during electrical stimulation of large myelinated fibres at 20 Hz are illustrated in Fig. 4. The plots for both groups are very similar and there are no statistical differences between them at any 30 μm interval. Both groups are displaced above the plot of the mean image analysis of the in vitro microprobes from these experiments.

3.2.2. Stimulation at $> \times 100$ threshold

In six experiments the stimulus intensity was 20 V ($> 100 \times$ threshold) and hence both myelinated and unmyelinated fibres of the sciatic nerve were stimulated. The stimulus frequency used was 2 Hz since unmyelinated fibres do not follow high stimulus frequencies.

The mean image analyses of 24 microprobes inserted 2.25 mm into the spinal cord and left in situ for 15 min in the absence of any active peripheral stimulus and 12 microprobes inserted for the same time and to the same depth during electrical stimulation at 2 Hz ($> 100 \times$ threshold) showed no significant differences (not illustrated).

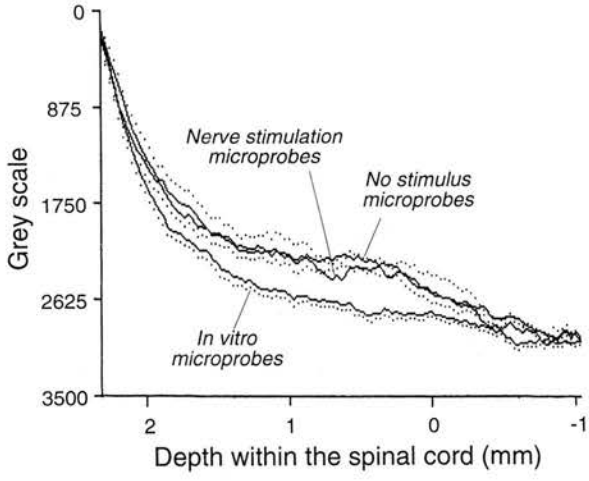

Fig. 4. The failure of electrical stimulation of large diameter primary afferent fibres to release ir-NPY in the rat. The three mean image analyses plotted were derived from 21 in vitro microprobes, 22 microprobes present in the spinal cord prior to peripheral nerve stimulation (no stimulus microprobes), 11 microprobes present in the spinal cord while the ipsilateral sciatic nerve was stimulated at 20 Hz, 0.5 ms pulses, stimulus strength $\times 3$ threshold for producing a dorsal column volley (nerve stimulation microprobes). All microprobes are derived from the same experiments.

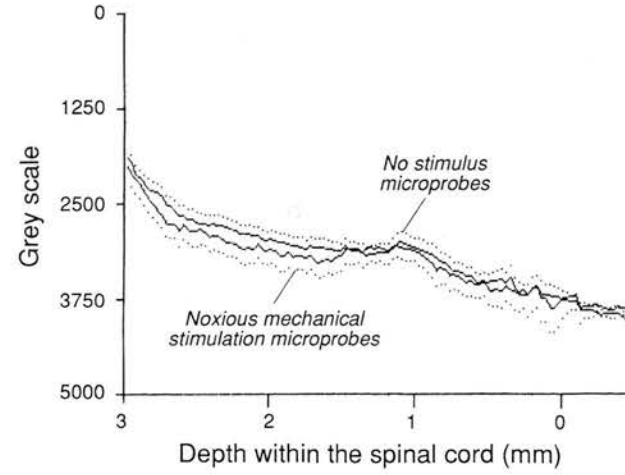

Fig. 5. The failure of noxious mechanical stimulation to release ir-NPY in the cat spinal cord. The mean image analyses of 31 microprobes present in the spinal cord without peripheral stimulation (no stimulus microprobes) is compared with that of 22 microprobes present in the spinal cord while noxious mechanical stimuli were applied intermittently to the ipsilateral hind paw (noxious mechanical stimulation microprobes).

3.2.3. Microprobes present in the spinal cord of the spinal cat during noxious peripheral stimulation

A total of 35 microprobes remained in the spinal cord while noxious peripheral stimuli were applied to the ipsilateral hind limb (24 noxious mechanical stimulation, 11 noxious thermal stimulation). The images of these microprobes showed no significant differences from those microprobes inserted in the absence of any peripheral stimulation. Fig. 5 illustrates the near coincident mean image analyses of 31 no stimulus microprobes with that of 22 microprobes present in the spinal cord during noxious mechanical stimulation.

4. Discussion

The most significant finding of the present study was the relative ease with which ir-NPY was detected in the spinal cord of both the rat and the cat in the absence of any applied peripheral stimulus. This is in marked contrast to other peptides studied in the spinal cord of the rat and cat, such as substance P [11,25], dynorphin [39] and galanin [21,36]. This probably indicates a significant role for NPY in spinal cord function in both species but requires some consideration of what is being detected by microprobes and of the possible sources of spontaneously released ir-NPY.

The antibodies immobilized to the microprobes of the present experiments were C terminus directed and hence, as well as detecting NPY, it is possible that shorter fragments resulting from enzymic degradation might also bind to microprobes. Little is known of the degradation of NPY following its release within the central nervous system. Indeed, there has been difficulty in demonstrating any

degradation of NPY by peptidases found in the central nervous system [29]. This is believed to result from the unique structure of NPY: a polyproline-like helix lying antiparallel to an amphiphilic α helix and the possession of an amidated C terminus [43]. Degradation by the non-brain-occurring peptidases endopeptidase 24.18, aminopeptidase P and dipeptidylpeptidase IV has been shown [32,38]. With cultured cortical neurones slow degradation by presumed plasmin and plasminogen activator activity occurred [28]. It is probable, therefore, that with microprobes remaining in the spinal cord for 15–30 min what was bound, and subsequently prevented the binding of radiolabelled NPY, was predominantly NPY. Although the immobilized antibodies used in the present experiments did recognize peptide YY, analysis of chromatographic and radioimmunoassay data led De Quidt et al. [8] to conclude that NPY is the main, if not the only, member of the pancreatic polypeptide family in the central nervous system of the rat, pig and human.

Although microprobes are fine diameter micropipettes, they do detect very small quantities of neuropeptides in their immediate environment [10]. Thus it is possible that NPY-containing cell bodies or fibres, ruptured by the passage of microprobes, contributed to the basal levels detected. We do not consider this to be a major contribution since very differing patterns of basal extracellular presence have been detected for differing neuropeptides both in the spinal cord of the rat [21,25,39] and cat [11,12,37,41] despite the passage of microprobes through areas enriched in the relevant neuropeptide.

Microprobe studies of release of neuropeptides in the spinal cord are performed on animals which have been anaesthetized and subjected to considerable surgery. Despite general anaesthesia such animals could have a continued spinal input of impulses in nociceptors of both superficial and deep origin. This has been proposed as the source of the small basal presence of ir-SP in the superficial dorsal horn of cats [11] and rats [25] since SP is readily released in this area by peripheral noxious stimuli particularly as inflammation develops peripherally [42]. In the present experiments, however, no evidence was obtained for an alteration in basal levels of extracellular ir-NPY by impulses in small diameter primary afferents (intact rat) or nociceptors (spinal cat). Hence it is improbable that the large basal presence of ir-NPY can be attributed to continued activity in peripheral nociceptors.

The spinalization experiments were performed in the present series to test whether continued activity in descending fibres was contributing to the basal release of ir-NPY in the rat. This cannot have been a major source since the shift in the mean image analysis of post-spinalization microprobes was in the direction of increased release (implying inhibition of release) but the measured differences did not attain statistical significance at any of the dorsal horn sites. By a process of exclusion, it appears that spontaneous firing of intrinsic spinal neurones was predominantly responsible for the large basal presence of ir-NPY detected in the present experiments.

We were unable to perform experiments on the persistence of NPY following release since the stimuli used did not produce a spinal release of ir-NPY. The ability of a neuropeptide to resist rapid degradation following release is an important determinant of its ability to access receptors and influence neurones remote from sites of release [1,17,27]. As discussed previously, there is evidence that NPY is not degraded rapidly within the central nervous system and hence the wide distribution of basal extracellular ir-NPY may represent wide diffusion rather than release from a multiplicity of sites. Our finding of a basal presence of ir-NPY in the dorsal and ventral white matter may also relate to diffusion after release.

Electrophysiological studies [2,4,5,24] and transmitter release studies [13,45] indicate that NPY inhibits transmitter release at a number of sites, probably by an action on calcium channels [30]. Additionally in the central nervous system, potentiation of excitation of hippocampal neurones by NMDA [35] and of inhibition by noradrenaline [23] has been reported. How these actions apply to events in the spinal cord is currently unknown but the present results suggest that NPY is a widely used neuropeptide in spinal cord function.

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SPONTANEOUS RELEASE OF IMMUNOREACTIVE NEUROPEPTIDE Y FROM THE CENTRAL TERMINALS OF LARGE DIAMETER PRIMARY AFFERENTS OF RATS WITH PERIPHERAL NERVE INJURY

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Abstract—Microprobes bearing immobilized antibodies to the C-terminus of neuropeptide Y were used to measure the release of this neuropeptide in the spinal cords of rats with a unilateral peripheral neuropathy and in sham-operated animals. All neuropathic animals showed the characteristic behavioural syndrome and were studied at 14 days postsciatic nerve loose-ligation. An extensive spontaneous release of immunoreactive neuropeptide Y was detected in the spinal cords of the neuropathic rats and, compared to sham-operated rats, a new zone of release was found in the deep dorsal horn. Electrical stimulation of large diameter primary afferents proximal to the nerve ligature produced widespread release of neuropeptide Y in the dorsal horn which persisted for up to 1 h poststimulation.

It is possible that ectopic impulses arising in the injured nerve were responsible for the spontaneous central release of neuropeptide Y and this neuropeptide may play a role in the central response to peripheral nerve injury. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: neuropeptide Y release, spinal cord, neuropathic rat, antibody microprobes.

When the peripheral axons of primary afferent neurons of the rat are damaged or divided changes occur in the neuroactive compounds which these cells synthesize. Notable among these changes are decreased numbers of cells containing substance P and calcitonin gene-related peptide and increased numbers immunoreactive for galanin, vasoactive intestinal polypeptide and neuropeptide Y (NPY).[19,23,26,35,47–50,54] Studies of mRNA expression indicate that these changes result from altered synthesis.[37,38] The synthesis of NPY is remarkable since this neuropeptide is virtually absent from the dorsal root ganglion neurons of normal rats.[57] Quantitative studies of cell body size suggest that it is predominantly neurons with large myelinated peripheral fibres which synthesize NPY following peripheral axotomy and that this occurs in approximately 25% of the total neurons of a dorsal root ganglion.[38] The functional significance of these neuropeptide changes is unknown. If the newly-synthesized peptides are released in the spinal cord during nerve regeneration they could produce widespread changes in neuronal excitability relevant to recovery of function in the deafferented spinal neurons or could even promote structural changes also relevant to functional recovery.[32,45,53,55] Rats with a peripheral nerve constriction injury which damages a proportion of fibres within the nerve have a behavioural syndrome likened to a complex regional pain syndrome of humans.[4,24,44] Thus there is evidence for the occurrence of spontaneous pain, allodynia and an alleviation of the syndrome by sympathectomy.[56] These rats have neuropeptide changes in the spinal cord and dorsal root ganglia similar to those produced by nerve transection.[22,37,49]

Nerve constriction differs from complete transection in that there is a partial loss of fibres distal to the site of injury[3,34] and hence an afferent input (albeit reduced) from the periphery can occur. It is possible that it is an interaction between these impulses and the altered central state relevant to reorganization following complete transection that contributes to the dysesthetic state of partial nerve injury. Such considerations indicate the need to study spinal release of neuropeptides from primary afferent neurons in rats with peripheral nerve injury.

We have previously studied release of immunoreactive NPY (IR-NPY) in the normal rat and found evidence for considerable basal release resulting from activity in intrinsic spinal neurons.[29] The present experiments have examined release of IR-NPY in rats with a unilateral constriction injury of the sciatic nerve.

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Abbreviations: IR, immunoreactive, NGF, nerve growth factor, NPY, neuropeptide Y, NT-3, neurotrophin-3, PBS, phosphate-buffered saline.

There are several aspects of release in this model requiring investigation. The first is whether the newly-synthesized NPY contained within dorsal root ganglion neurons can be released from such structures since release of NPY from primary afferents does not occur in normal rats. If release occurs, does this occur spontaneously or is it necessary to evoke impulses in NPY-containing neurons by peripheral stimuli to produce release? Since microprobes can localize sites of release with some accuracy it needs to be determined whether release of NPY from primary afferents is at different sites in the spinal cord from those in normal rats, since with the latter there is evidence that intrinsic cord neurons are the main sources of release. Finally, persistence and diffusion after release occurs with some neuropeptides and this needs to be investigated for NPY.

EXPERIMENTAL PROCEDURES

The nerve injury model

For the production of nerve injuries adult male Wistar rats (weight range 300–450 g, Charles River Ltd, U.K.) were anaesthetized with sodium pentobarbitone (50 mg/kg i.p.). Using full aseptic technique, four 4/0 chromic gut loose ligatures were placed around the right sciatic nerve, proximal to its trifurcation.[4] In the sham group, the right sciatic nerve was exposed and manipulated but not ligated. Postoperatively, the International Association for the Study of Pain guidelines for the care of experimental animals were followed.[58] The animals used displayed the changes associated with this model such as hopping, licking, everted paw and ventroflexed toe. An analgesymeter (Ugo Basile) was used to test for the development of mechanical allodynia. Rats were tested prior to surgery and then at one to three day intervals over the next two weeks. A difference score was calculated for paw withdrawal thresholds obtained from right and left hind paws, as this gives a more consistent value.[4]

Microprobe preparation

Antibody microprobes for the detection if IR-NPY were prepared following the technique previously described.[11] Briefly, fine glass micropipettes, heat sealed at both ends, were incubated for up to 24 h in a 10% solution of γ-aminopropyltriethoxysilane in toluene. This procedure produced a fine, granular siloxane polymer layer on the outer surface of each micropipette possessing free amine groups to which Protein A (Sigma) was immobilized using glutaraldehyde coupling. Protein A then bound immunoglobulins present in a polyclonal antiserum raised in rabbits against the carboxy terminus of NPY (Peninsula Laboratories). Tests of specificity of the microprobes bearing this antibody were carried out prior to *in vivo* experiments. Before insertion into the spinal cord, microprobes were broken back to give tip diameters of approximately 10 μm.

Experimental protocol

For microprobe experiments rats were anaesthetized with intraperitoneal (i.p.) urethane (1.5 g/kg initially, supplemented when necessary). Cannulae were inserted into the trachea, a carotid artery and an external jugular vein, to aid unobstructed breathing, and to permit direct measurements of arterial blood pressure and the intravenous injection of substances respectively. The depth of the anaesthesia was continually assessed by the corneal blink reflex, hindpaw withdrawal and blood pressure response to pinching the paw. Blood oxygenation was assisted by directing a gentle jet of humidified oxygen towards the opening of the tracheal cannula. The animal's body temperature was monitored with a rectal probe connected to a controlled heating system and maintained between 36–38°C. In experiments involving stimulation of the sciatic nerve, animals were artificially ventilated following paralysis with vecuronium injected intravenously (1 mg/kg) and the end tidal CO_2 levels were continually monitored and maintained approximately at 4%. Vecuronium was only given as neuromuscular paralysis declined which enabled regular assessment of depth of anaesthesia. An extended laminectomy was performed at vertebral levels T_{12}–L_2 to expose the dura mater of the lumbar spinal cord (spinal segments L_2–L_6). The animal was supported in a metal frame and six swan neck clamps were positioned under the mamillary processes of the exposed vertebrae. Agar was allowed to set over the exposed spinal segments and a window was made at sites of proposed microprobe entry. Following opening of the dural sac with sterile forceps, the exposed spinal cord was irrigated with sterile Ringer solution at 37°C continuously throughout the experiment. This continuous irrigation minimized the collection of exudates on the surface of the cord and excess fluid was removed by suction at an edge of the agar window. The sciatic nerve on the operated side of each rat (both sham-operated and nerve-ligatured animals) was isolated and gently mobilized proximal to the ligated or sham-manipulated area to permit mounting on platinum electrodes. To support the leg a nerve pool was constructed and filled with paraffin oil at 37°C.

Before microprobes were inserted into the spinal cord, the suitability of potential penetration sites was examined. A silver ball electrode was gently pressed on to the dorsum of the spinal cord just medial to the line of entry of the dorsal roots and the threshold stimulus current and voltage needed to evoke a field potential was measured. The ball electrode was positioned at several rostrocaudal locations to determine the distribution of afferent input from the sciatic nerve and hence to select sites of insertion for microprobes.

All probes were inserted into the cord (two at a time) with a pair of stepping motor micromanipulators to a depth of 2.25 mm from the dorsal surface and left *in situ* for 15 min with or without an active peripheral stimulus. When a peripheral nerve was stimulated, only one period of 15 min stimulation was used in each experiment due to uncertainty on the persistence of released IR-NPY. In these experiments the first two pairs of microprobes were inserted into both sides of the spinal cord in the absence of any nerve stimulation. Electrical stimulation at either 20 Hz ($3 \times$ threshold) or 2 Hz ($>100 \times$ threshold) began when the next pair of microprobes were inserted into the spinal cord. The effects of unilateral nerve stimulation were studied bilaterally in the spinal cord. Following peripheral nerve stimulation it was usual to insert microprobes into both sides of the spinal cord for three periods of no stimulation.

At the end of each experiment Pontamine Sky Blue was ejected ionophoretically at a defined site in the spinal cord. The cords were then removed, fixed and sectioned to determine the location of resultant dye spots. This data was essential to allow locations on the probes to be related to sites within the spinal cord. The antibody microprobe technique detects bound endogenous ligand by the failure of binding of exogenous radiolabelled ligand. Thus, following removal from the spinal cord, microprobes were washed for 15 min in ice-cold phosphate-buffered saline (PBS) containing 0.1% Tween 20 and then incubated for approximately 24 h at 6°C in a PBS–azide solution of ^{125}I-radiolabelled NPY (Amersham) containing casein (0.1%). The final dilution of the radiolabelled peptide resulted in approximately 2000 counts/min/μl. Probes were then washed for 15 min in ice-cold PBS–Tween 20 while continually drawing the solution through the tips to remove any radiolabelled NPY which may have entered. The distal portions of the

microprobes were broken off and mounted on to a sheet of paper, which was placed in an X-ray film cassette with a sheet of monoemulsion film (Kodak, NMC) for six to 10 days. Two films were derived from each experiment.

The resultant microprobe autoradiographs were analysed with an image analysis system employing an Imaging Technology PC Vision frame grabber board operating in a Data Control System 286e (AT based) computer, as previously described.[18] A charged coupled device camera scanned each autoradiograph and, following background subtraction, transverse integrations of optical density on a scale of 0–255 were performed along the length of each microprobe at defined intervals. With the magnification of the system used, and the resolution of the image analysis system (512 × 512 locations per frame) this corresponds to a 10 μm interval for transverse integrations. However, this is beyond the biological resolution of the microprobe method (see Ref. 10) and therefore the average of three successive integrals was taken to give a final optical resolution of 30 μm. For each microprobe, the resultant plot of integrated optical density (grey scale) with respect to length along the microprobe, together with coded information which described the experimental conditions for that particular microprobe, were stored in a computer file.

A sorting program was used to retrieve groups of microprobes which met stated criteria and produce for each group a plot of the mean integrated grey scale values (±S.E.M. in 30 μm steps) with respect to depth within the spinal cord. Each site was treated independently and statistical significance was assigned to differences between mean optical densities at each site when comparing selected groups of microprobes. The significance of the differences at each 30 μm analysis point was estimated using the Student's *t*-test.

In vitro *tests*

The antiserum used was polyclonal and directed against the C terminus of NPY (porcine). The manufacturer stated that this antibody detected human and rat NPY and porcine and rat peptide YY equally well. In parallel with present experiments, the sensitivity of the prepared antibody microprobes was regularly assessed by incubating a small number either directly in [^{125}I]NPY or a range of concentrations (10^{-5}–10^{-9}M) of unlabelled NPY *in vitro* at 37°C for 30 min prior to the incubation in a solution of [^{125}I]NPY for approximately 24 h at 6°C. Following washing in PBS–Tween 20, the distal portion of these microprobes were broken off, mounted on small pieces of cardboard and placed in tubes for processing by gamma counter. X-Ray images of these *in vitro* probes were also obtained.

The counts of total radioactivity of microprobe tips indicated that over 10% of the total radioactivity in which they had been incubated bound to the microprobes. *In vitro* tests indicated that a 10^{-7} M solution of NPY suppressed such binding by greater than 50%, with 10^{-5} M resulting in greater than 80% suppression. Hence it can be assumed that the non-specific binding for these microprobes accounts for less than 20% of the total binding.

RESULTS

Spontaneous release of immunoreactive neuropeptide Y in sham-operated rats

A basal presence of IR-NPY was found in the spinal cord of sham-operated rats. Figure 1A illustrates the mean image analysis of 42 microprobes inserted into either side of the spinal cords of these animals and that of 23 *in vitro* microprobes derived from the same experiments. Figure 1B plots the *t*-statistics derived from the differences between these

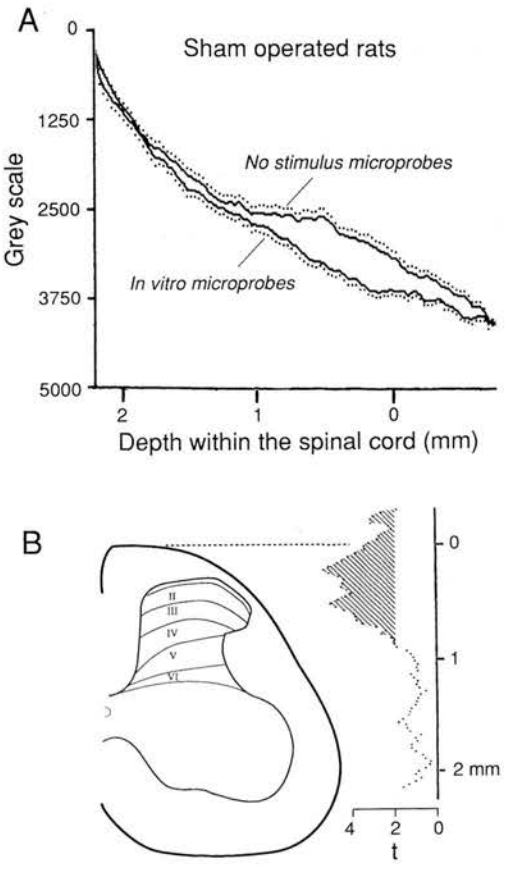

Fig. 1. The basal presence of IR-NPY in the spinal cord of sham-operated rats. (A) The mean image analysis of two groups of microprobes is plotted with respect to depth within the spinal cord: those present in both sides of the spinal cord of sham-operated rats for 15 min in the absence of any active peripheral stimulation (no stim, n=42) and those which were not inserted into the spinal cord but simply incubated in [^{125}I]NPY (*in vitros*, n=23). For each group the mean grey scale was determined in 30 μm intervals and a line joins these points. At each analysis point the standard error of the mean (S.E.M.) is also plotted (+) for no stimulus and (−) for *in vitros*. (B) A plot of the "*t*"-statistics derived from the standard errors of the differences of the means at each analysis point for the two groups shown in (A), is related to an outline of a transverse section of the lumbar spinal cord. The hatched area indicates where these sites are significant at the $P<0.05$ level.

two groups and the hatched areas indicate where these differences are significant at the $P<0.05$ level. The latter includes all of the dorsal horn, dorsal columns and extends into the perfusate of the spinal cord for 0.5 mm. We have previously observed the latter in studies of IR-NPY release in normal rats. The zone of most significant release of IR-NPY extends from 0.3 mm to 0.6 mm from the cord dorsum and this approximates to laminae I, II and III of the dorsal horn.

A separate analysis (not illustrated) showed no differences in the mean image analyses of microprobes inserted into the left side and into the right side of the spinal cord of animals subjected to the sham operation (which was always on the right side).

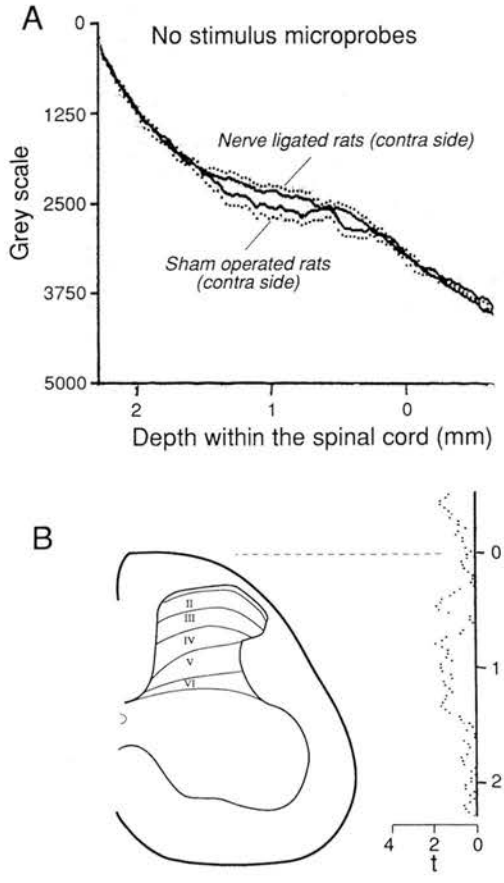

Fig. 2. The basal presence of IR-NPY in the contralateral spinal cord of sham-operated and nerve-ligated rats. (A) The mean image analysis of 23 microprobes present for 15 min in the side of the spinal cord contralateral to the leg incision and in the absence of any active peripheral stimuli of sham-operated animals (sham-operated rats, contra side) is compared with that of 55 microprobes present for the same time period in the contralateral side of the spinal cord of the nerve-ligated rats and in the absence of any active peripheral stimuli (nerve-ligated rats, contra side). (B) The "*t*"-statistics derived from the differences between the two groups of microprobes are plotted with respect to an outline of the spinal cord. These differences are not significant at the $P<0.05$ level.

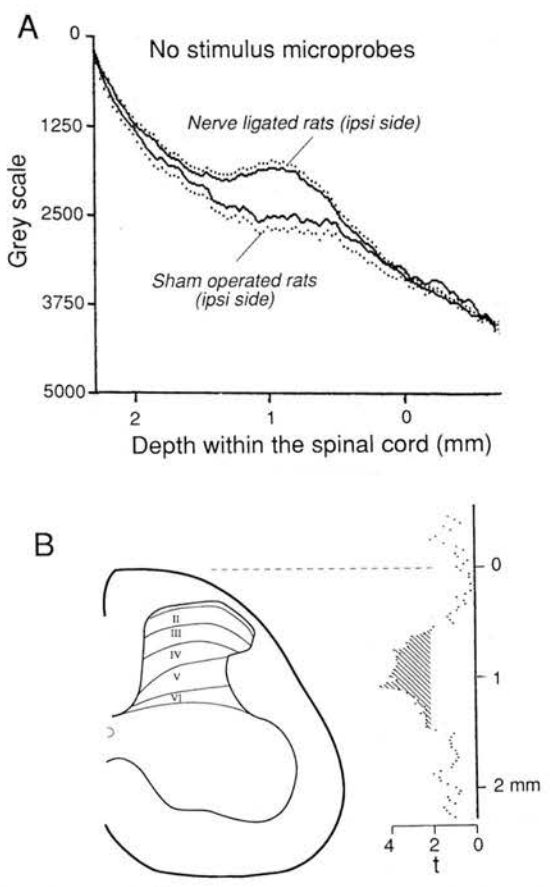

Fig. 3. An additional zone of release of IR-NPY in the ipsilateral side of the spinal cord of rats with unilateral sciatic nerve injury. (A) The mean image analysis of two groups of microprobes present in the spinal cord for 15 min in the absence of any active peripheral stimuli are plotted with respect to depth within the spinal cord: those present in the side of the spinal cord ipsilateral to the nerve ligation (nerve-ligated rats, ipsi side, $n=53$) and those present in the side of the spinal cord ipsilateral to the sham operation (sham-operated rats, ipsi side, $n=19$). The "*t*"-statistics derived from the differences between the two groups of microprobes are plotted with respect to an outline of the spinal cord. The hatched areas indicates where these differences are significant at the $P<0.05$ level.

Spontaneous release of immunoreactive neuropeptide Y in rats with a unilateral constriction nerve injury

Figure 2 compares the mean image analysis of microprobes inserted into the spinal cord of nerve constricted rats, but on the side (L) contralateral to the nerve injury with that of comparable microprobes used with sham-operated animals. Although the mean analysis of the neuropathic rats is displaced above that of the sham-operated animals at several sites (suggesting increased basal levels), none of these differences were significant at the $P<0.05$ level.

On the side of the spinal cord ipsilateral to the injured nerve however, there were significant differences from the sham-operated rats. This is illustrated in Fig. 3. The plot derived from the microprobes used with the neuropathic group is clearly displaced above that of the sham group over a restricted area. Figure 3 shows that this was significant from 0.5 mm to 1.5 mm from the dorsal surface with a broad peak of greatest difference from 0.7 to 1.1 mm from the dorsal surface. The latter zone approximates to laminae IV, V and VI of the dorsal horn and the total area of significantly increased release includes laminae III, IV, V, VI and upper lamina VII. It should be noted that these areas were not the only sites of significant levels of extracellular IR-NPY in this side of the spinal cord of nerve ligatured animals, but that they were superimposed on that equally present in sham operated rats. Figure 4 has been compiled to illustrate this point. This compares the mean image analyses of microprobes inserted into the side of the spinal cord ipsilateral to the

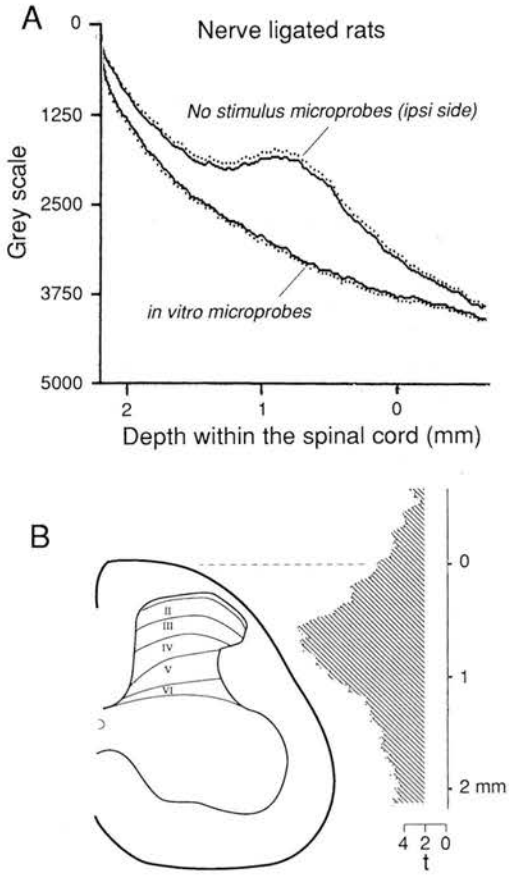

Fig. 4. The basal presence of IR-NPY in the ipsilateral side of the spinal cord of rats with a peripheral sciatic nerve injury. (A) The mean image analysis of 53 microprobes present for 15 min in the side of the spinal cord ipsilateral to a peripheral nerve injury and in the absence of any active peripheral stimulus (no stimulus microprobes, ipsi side) is compared with that of microprobes simply incubated in [^{125}I]NPY (*in vitro* microprobes). (B) The "*t*"-statistics derived from the differences between the means of the two groups shown in (A) are plotted with respect to a transverse section of the rat spinal cord. The hatched areas indicate where these differences are significant at the $P<0.05$ level.

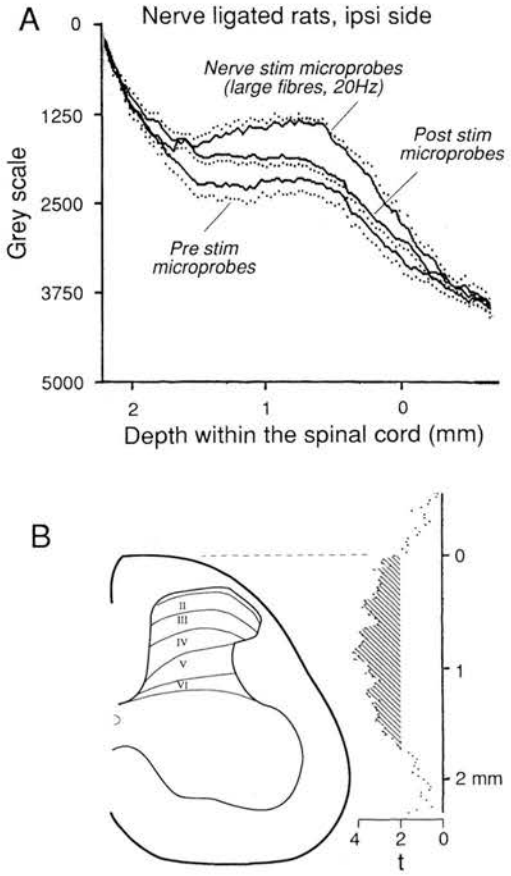

Fig. 5. Release and persistence of IR-NPY in the ipsilateral spinal cord of nerve ligated rats by large myelinated fibre stimulation proximal to the ligature at 20 Hz. (A) The mean image analysis of three groups of microprobes present in the spinal cord of nerve-ligated rats for 15 min were plotted with respect to depth within the spinal cord: those present in the side of the spinal cord ipsilateral to the nerve ligation in the absence of peripheral stimuli (*ipsi*, $n=21$), those present in the same side of the cord during stimulation of large myelinated fibres at 20 Hz, $3\times$T (nerve stim microprobes, large fibres, 20 Hz, $n=14$) and that of microprobes present in the same side of the spinal cord but 5–60 min following a period of large fibre stimulation (post-stim microprobes). A plot of the "*t*"-statistics is related to an outline of the lumbar spinal cord. Significant differences at the $P<0.05$ level are indicated by the hatched areas.

ligatured nerve with that of the *in vitro* microprobes derived from the same experiments (Fig. 4A). The differences between these groups are shown in Fig. 4B. This shows that significant levels of IR-NPY occurred throughout the whole of the spinal cord, extending into the perfusate, and that this was greatest at an area approximating to laminae III and IV.

Release of immunoreactive neuropeptide Y following peripheral nerve stimulation in nerve ligatured rats

Our previous experiments found no spinal release of IR-NPY following electrical stimulation of the sciatic nerve in the normal rat.[29] By contrast, in the present experiments IR-NPY was readily released by electrical stimulation of the sciatic nerve proximal to the site of the nerve ligatures.

Figure 5A illustrates the mean image analyses of microprobes present in the side of the spinal cord ipsilateral to the injured nerve, before, during and after electrical stimulation of large diameter afferents of the ligatured nerve with a frequency of 20 Hz. The plot for microprobes present during nerve stimulation is displaced above that of the prestimulus microprobes throughout the whole of the spinal cord areas sampled. Figure 5B shows that the differences between these groups are significant 0.3–1.5 mm from the dorsal surface. Not illustrated is that stimulation of large fibres at 2 Hz gave comparable results to stimulation at 20 Hz.

Figure 5A also contains the mean image analysis of microprobes present in the spinal cord for up to 1 h

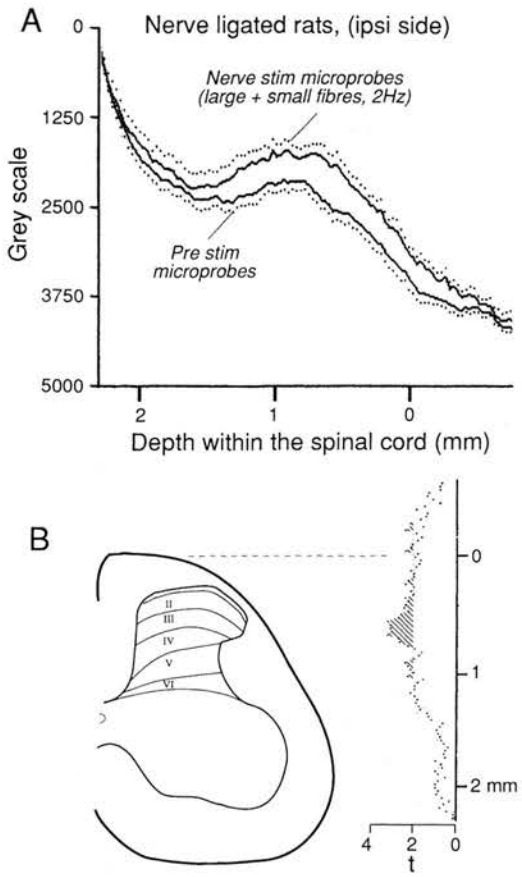

Fig. 6. Release of IR-NPY in the ipsilateral spinal cord of the nerve ligated rat during stimulation of large myelinated fibres and unmyelinated fibres proximal to the ligature. (A) The mean image analysis of two groups of microprobes present in the spinal cord of nerve-ligated rats for 15 min was plotted with respect to depth within the spinal cord: those present in either side of the spinal cord in the absence of any active peripheral stimuli (pre-stim, ipsi, n=18) and nine microprobes present in the side of the spinal cord ipsilateral to the nerve ligation during stimulation at 2 Hz, 100 × T (nerve stim microprobes, large+small fibres, 2 Hz). (B) A plot of the "t"-statistics derived from the differences of the means of the two groups shown in A at each 30 μm interval is related to an outline of the lumbar spinal cord. The hatched area indicates where these sites are significant at the P<0.05 level.

after a period of prior electrical stimulation and this is still clearly displaced above the prestimulus group at many sites in the spinal cord.

The effects of electrical stimulation with a strength adequate to excite unmyelinated as well as myelinated fibres is shown in Fig. 6. Because unmyelinated fibres do not follow high stimulus frequencies for prolonged periods, the stimulus frequency used was 2 Hz. The mean image analysis of microprobes present in the spinal cord during stimulation is almost uniformly displaced above that of the prestimulus group but Fig. 6B shows that the differences between these groups only attained significance at P<0.05 over laminae III, IV and V, with the peak approximating to the III/IV junction. This is a relatively restricted area when compared with large fibres-alone stimulation. Microprobes inserted after stimulation of both large and small fibres found elevated levels of IR-NPY relative to prestimulus microprobes which was significant at all three time periods examined (5–20 min, 25–40 min and 45–60 min).

DISCUSSION

Our previous experiments found an extensive basal release of IR-NPY in normal anaesthetized rats, but concluded that this was predominantly derived from activity in intrinsic spinal neurons.[29] Comparable basal release in sham-operated animals and on the side of the spinal cord contralateral to the ligatured nerve was found in the present experiments but release in the ipsilateral spinal cord in nerve ligatured animals with a behavioural neuropathy showed significant departures from the normal pattern. These can be summarized as:

(i) superimposed on the normal pattern of spontaneous IR-NPY release there was an additional zone of spontaneous release resulting in raised extracellular levels in the mid and deep dorsal horn (approximating to laminae IV, V and VI);

(ii) whereas peripheral nerve stimulation did not result in spinal release of IR-NPY in normal rats, electrical stimulation of large myelinated afferents proximal to the site of nerve ligature in neuropathic rats did elicit release.

We believe that release from the terminals of large diameter primary afferent fibres is the most likely source of the additional zone of extracellular IR-NPY detected by microprobes. Thus electrical stimulation of these fibres did not alter the spatial distribution of extracellular IR-NPY but simply elevated these levels in a pattern similar to that present prior to electrical stimulation. The peak of IR-NPY following large fibre peripheral nerve stimulation remained in the deep dorsal horn, upper ventral horn.

Within dorsal root ganglia, the increased presence of NPY which follows either axotomy[38,48,50] or nerve constriction[35,49] occurs predominantly in a population of large- to medium-sized dorsal root ganglion neurons. This correlates with our finding that electrical stimulation restricted to low threshold (large diameter) fibres proximal to the constriction site was adequate to elicit release of IR-NPY. Large diameter dorsal root ganglion neurons are functionally a very heterogeneous group, but there is recent evidence on the relevant phenotypes. Administration of neurotrophin-3 (NT-3)[41] or nerve growth factor (NGF)[46] at a site of nerve section reduces by at least 50% the ensuing synthesis of NPY by dorsal root ganglion neurons. This implies that *de novo* synthesis of NPY is a response to deprivation of retrogradely transported NT-3 and NGF. Mice deficient in the gene for NT-3 have impaired development of

cutaneous slowly adapting mechanoreceptor[1] fibres which project to laminae III and IV of the spinal cord.[6] Supporting this is a study of mice lacking the trkC receptor believed to be the receptor acted upon by NT-3.[25] The animals were deficient in afferents projecting to the ventral horn although deep dorsal horn projections appeared normal. Another group[15] have found that mice deficient in the gene for NT-3 have an absence of proprioceptive (muscle spindle and tendon organ derived) afferents. The latter project predominantly to the ventral horn.[6] Amongst large diameter dorsal root ganglion cells there is no evidence linking the development of a particular functional phenotype to a dependence on NGF. Indeed only a small proportion of these cells express the high affinity trkA recptor for NGF.[2,31] Although it is an assumption that dependence during development can be linked to the response of neurons to deprivation of growth factors following peripheral injury, if the phenotypes of large neurons synthesizing NPY following peripheral axon damage include both proprioceptive and large fibre mechanoreceptors, then impulses in these fibres would result in release of NPY in the mid and lower dorsal horn and the upper ventral horn.

Immunocytochemical studies of IR-NPY distribution in the dorsal horn following either transection[40] or partial injury[35] have found increases predominantly in laminae III and IV. In the transection study, this increase was prevented by dorsal rhizotomy implying an origin in primary afferent fibres.[40] An increase in laminae III/IV is consistent with NPY contained within slowly adapting mechanoreceptors[6] but not with a presence within muscle spindle afferents as these terminate more deeply. The reasons for this discrepancy are unknown but it is possible that the roles of the neurotrophins differ during development and regeneration after injury.

It has been proposed that NPY acts to inhibit the transmission of impulses from primary afferents during the regenerative phase[36] since an inhibition of transmitter release by NPY has been shown by both electrophysiological studies[5] and direct measures of transmitter release.[13] An alternate hypothesis is that NPY is responsible for some of the reported organizational changes in the dorsal horn following periphery axonal damage[55] since the dendritic outgrowth property of cultured dorsal root ganglion neurons has been shown to be increased by added NPY.[53] It has been shown that following peripheral nerve injury, a proportion of large diameter afferents migrate dorsally within the dorsal horn of the spinal cord and established connections within lamina III the major site of termination of small diamter cutaneous afferents. This migration has been established on the basis of transported markers applied to whole nerves[27,55] or injected into single dorsal root ganglion neurons.[55] An important consideration is whether these fibres contain and release NPY. We consider this improbable. As cited previously, increases in NPY following peripheral nerve injury occur in laminae III, IV and not in lamina II[35,40] and the present study found that the major change in where extracellular NPY was detected was in laminae IV to VI.

Where a neuropeptide is found following release, however, is not necessarily coincident with sites of release, since, if the compound is slowly degraded, considerable diffusion can occur following release.[12,16,43] In the present studies the increases in extracellular IR-NPY evoked by nerve stimulation did persist for up to 1 h after release suggesting a slow degradation of released NPY. Thus, it is possible that the new zone of presence of IR-NPY revealed in the present studies is more extensive than the area of releasing neurons. Supporting this is the extensive presence of IR-NPY in the dorsal columns and even the surface perfusate which almost certainly resulted from diffusion from the adjacent gray matter.

CONCLUSIONS

An important conclusion from the present experiments is that IR-NPY is spontaneously released from the central terminals of large diameter primary afferents in the absence of added peripheral stimulation. It is improbable that impulses originating from the periphery in undamaged, myelinated fibres contributed to such release since there is extensive loss of myelinated fibres distal to the ligatures in the procedure of Bennett and Xie.[3] A possible explanation for this extensive presence of IR-NPY in the spinal cord of nerve ligatured rats is release produced by ectopic impulses arising in regenerating or damaged fibres. Ectopic impulses have been described in dorsal roots of rats with sciatic nerve ligatures[20,21] with evidence that such impulses arise at or near the site of the ligatures or dorsal root ganglion neurons. Ectopic impulse generation has been most extensively studied following complete nerve transection and spontaneous impulses in large diameter primary afferents have been recorded by several laboratories.[7–9,33,42,51,52] There is evidence for such impulses arising both at the site of nerve injury[9,20,51] and from cell bodies in dorsal root ganglia.[9,21,30] Several authors have proposed that spontaneous activity in primary afferent fibres may be responsible for spontaneous pain following nerve injury in man[8,39] and for the development of allodynia and hyperalgesia in animals with an experimental nerve injury.[17,20] An obvious sequela to the present experiments is to examine the effects of local anaesthetic applied either proximally or distally to dorsal root ganglia, on spinal release of NPY. We cannot exclude the possibility of spontaneous release occurring in the absence of impulse invasion since there is structural evidence for neuropeptide-containing dense core vesicles fusing with non-junctional areas of axon terminal membranes.[14] In the present experiments,

however, peripheral nerve stimulation did result in release of IR-NPY. Spontaneous release could also occur through a continued depolarization of the spinal terminals of the relevant fibres. Such a mechanism has been proposed as important for release from the terminals of small diameter primary afferents.[28] Irrespective of the mechanisms, the finding of a relatively large spontaneous release of IR-NPY of probable primary afferent origin, suggests a role for this neuropeptide in the phase of regeneration which follows injury to these fibres.

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