Studies of Dynorphin and Cholecystokinin Release in the Rat Spinal Cord: Implications for Opioid Action

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ABSTRACT

Although much is now known of the cellular actions of exogenous opiate drugs and of endogenous opioid peptides, how these compounds modify interconnecting neuronal systems in the CNS is still poorly understood. The studies described in this thesis have addressed the latter by employing the antibody microprobe technique (Duggan A.W. and Hendry I.A., 1986, *Neurosci. Letts.* 68, 134-140) to investigate (a) the release of dynorphin A(1-8) in the spinal cord of the rat as inflammation develops in peripheral tissues, and (b) the release of cholecystokinin, a putative 'anti-opioid' neuropeptide, in the rat spinal cord following the administration of morphine.

(a) A dramatic increase in the spinal synthesis of prodynorphin derived peptides has been observed when inflammation develops in peripheral tissues. The functional significance of this increased synthesis is unclear and there have been no reports of the stimuli needed to produce dynorphin release *in vivo* nor of possible controls of such release. Microprobes bearing immobilised antibodies to the dynorphin A(1-8) derivative of prodynorphin, were inserted into the lumbar spinal cord of urethane anaesthetised normal rats and those with a peripheral inflammation, to determine whether dynorphins are being tonically released and how release is altered by manipulating the inflamed tissues. In the absence of any active peripheral stimulus the antibody microprobes detected minimal amounts of immunoreactive (ir)-dynorphin A(1-8) in two areas (lamina I and laminae IV-V) in the dorsal horn of the spinal cord of normal rats. With the development of unilateral ankle inflammation over 3 to 5 days, following subcutaneous injections of Freund's complete adjuvant, this was extended to the ventral horn of both sides of the spinal cord. Lateral compression of the ankles of the normal animals did not release ir-dynorphin A(1-8) during the period of stimulation, but this neuropeptide was detected in the ventral horn following the stimulus. By contrast, compression of inflamed ankles produced elevated levels of ir-dynorphin A(1-8) during the period of stimulus application at three major sites

in the spinal grey matter. The largest peak was in the deep dorsal horn/ upper ventral horn (laminae VI -VII), with further sites of significant release in the mid dorsal horn (laminae II-V) and the lower ventral horn. These levels persisted for at least one hour after the period of stimulation. At a cellular level dynorphins reduce transmitter release from nerve terminals, and hence the observation that ir-dynorphin A(1-8) is released in the ventral and deep dorsal horn in addition to the superficial dorsal horn of the rat by manipulation of inflamed tissues, implies that wide-spread spinal inhibitory controls of spinal neuronal firing are evoked by such stimuli. This may ultimately affect the perception of pain, but release in the ventral horn suggests an involvement in reducing motor responses to peripheral noxious stimuli.

(b) Of all possible candidates at the spinal cord level, the 'anti-opioid' activity of cholecystokinin (CCK) has been well characterised. This peptide has also been proposed to play a role in the development of tolerance to but not dependence on opiate drugs. Although the hypothesis that stimulation of opioid receptors may trigger a progressive compensatory increase in the activity of CCK containing neurones at the spinal cord level has received some indirect support, this has not been thoroughly investigated. Conflicting data have been obtained from experiments which have examined the spinal cord content of CCK and spinal release of CCK following the administration of opioids. Microprobes bearing immobilised antibodies to CCK-8, were inserted into the lumbar spinal cord of urethane anaesthetised normal rats under both basal conditions and following acute opiate administration. In the absence of any active peripheral stimulus an extensive presence of ir-CCK was detected in normal (drug naive) rats with three main zones. The largest peak was in the mid to deep dorsal horn/ upper ventral horn (laminae III -VII), with further major sites in the superficial dorsal horn (lamina I-II) and the mid/ lower ventral horn. Morphine administered intravenously over two hours (total dose 25mg/ kg) failed to alter this basal presence of ir-CCK. Enhanced release however, was observed in areas of the ventral horn of rats treated acutely with morphine following 1mg/ kg injections of naloxone. It is proposed that the

occupation of opioid receptors by morphine triggers the increased synthesis of CCK and predicted that following the development of tolerance to morphine, enhanced CCK release will be observed in the absence of naloxone administration.

DECLARATION OF AUTHORSHIP

I hereby declare that the studies outlined in this thesis are entirely my own work, under the supervision of Prof. A.W. Duggan, in the Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, The University of Edinburgh. This thesis has not been submitted for the purposes of obtaining any degree or qualification from any other academic institution.

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ABBREVIATIONS

ACTH:	adrenocorticotrophic hormone
AP5:	2-amino-5-phosphonovaleric acid
β-FNA :	β-funaltrexamine
BNTX:	7, benzylidenenaltrexone
BSA:	bovine serum albumin
CCD:	charged coupled device
CCK:	cholecystokinin
CGRP:	calcitonin gene-related peptide
CI977:	(5R)-(5α,7α,8β)-*N*-methyl-*N*-(7[1-pyrrolidinyl]-1-oxaspirol[4,5]dec-8-yl)-4-benzofuranacetamide monohydride
cDNA:	complementary DNA
Ci:	Curies
CLIP:	corticotrophin-like intermediate lobe peptide
CNS:	central nervous system
cpm:	counts per minute
CPP:	(±)-3-(2-carboxypiperazin-4yl)-propyl-1-phosphoric acid
CTOP:	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2
DADLE:	[D-Ala^2, D-Leu^5] enkephalin
[D-Ala^2]deltorphin I:	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2
[D-Ala^2]deltorphin II:	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2
dermorphin:	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2
DPDPE:	[D-Pen^2, D-Pen^5] enkephalin; where Pen = penicillamine
DPLPE:	[D-Pen^2, L-Pen^5] enkephalin
DAMGO:	[D-Ala^2, MePhe^4, Gly(ol)^5] enkephalin
DSBuLET:	[D-Ser(O-*ter*-butyl)^2, L-Leu^5] enkephalyl-Thr^6
DSLET:	[D-Ser^2, D-Leu^5] enkephalyl-Thr^6
DTLET:	[D-Thr^2, Leu^5] enkephalyl-Thr^6
DRG:	dorsal root ganglion
dyn:	dynorphin
EC:	extracellular loop
EKC:	ethylketocyclazine
end:	endorphin
FCA:	Freund's complete adjuvant

GABA:	γ-amino butyric acid
G-protein:	guanine nucleotide binding protein
(+)-HA966:	(+)-(1-hydroxy-3-amino pyrrolidine-2-one)
5-HT:	5-hydroxytryptamine
IC:	intracellular loop
ICI174864:	*N*-*N*-diallyl-Tyr-Aib-Aib-Phe-Leu
i.c.v.:	intracerebroventricularly
i.p.:	intraperitoneally
i.r.:	immunoreactive
i.t.:	intrathecally
i.v.:	intravenously
LE:	Leu-enkephalin
L365260:	3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N'*-3-methylphenyl urea
LPH:	lipotropin hormone
mRNA:	messenger RNA
ME:	Met-enkephalin
MERF:	Met-enkephalin-Arg-Phe
MERGL:	Met-enkephalin-Arg-Gly-Leu
MK-801:	dizocilpine maleate
morphiceptin:	Tyr-Pro-Phe-Pro-NH_2
MSH:	melanocyte stimulating hormone
NK:	neurokinin
NMDA:	*N*-methyl-D-aspartate
NalBzoH:	naloxone benzoylhydrazone
neoend:	neoendorphin
norBNI:	nor binaltorphimine
NTB:	naltriben (a benzofuran analogue of naltrindole)
5'-NTII:	naltindole-5'-isothiocyanate
PD117302:	(±) *trans*-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl] benzo[b]thiophene-4-acetamide
PBS:	phosphate buffered saline
PC:	prohormone converting
PL017:	Tyr-Pro-MePhe-D-Pro-NH_2
POMC:	proopiomelanocortin
proEnk:	proenkephalin
proDyn:	prodynorphin

RIA:	radioimmunoassay
TEA:	tetraethylammonium
TM:	transmembrane region
s.e.m.:	standard error of the mean
U50488H:	(±) *trans*-3,4-dichloro-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide
U69593:	(+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5) dec-8-yl]benzene acetamide
w/v:	weight/ volume

NOTE ON TERMINOLOGY

Throughout this thesis the term 'opioid' has been used for all ligands (peptide and non-peptide) producing effects at the μ, δ and κ 'opioid receptors', with a distinction made where considered necessary for the naturally occuring 'endogenous' opioid peptides. The term 'opiate', in contrast has only been applied to those ligands with a morphine-like structure (i.e. protonated amine juxtaposed to an aromatic ring).

Additionally unless otherwise stated 'CCK' refers to CCK-immunoreactivity or the sulphated octapeptide CCK-8, the most common derivative of procholecystokinin present in the vertebrate brain and spinal cord, including the rat

CHAPTER 1: General Introduction

1.1 OPIATES, OPIOID PEPTIDES AND OPIOID RECEPTORS: A HISTORICAL PERSPECTIVE

It is indeed a reflection of the achievements of modern pharmacology that over the past 20 years the mechanism of action of a group of compounds that have been in use for thousands of years, the 'opiates', have only now become to be significantly understood (recently reviewed by Snyder, 1986; Brownstein, 1993).

Opium is the Greek name given to the liquid ('opos') that appears on the unripened capsule of the poppy plant *Papaver somniferum* (Latin for the 'poppy that brings sleep') when it is notched. Writings of its use date back to at least the end of the third millennium B.C., the Sumerians of the Middle East being the first to cultivate the poppies, initially for use as a euphoriant in religious rituals, and then later for medicinal purposes, a potent analgesic and sedative. By the eighth century A.D., following the successful cultivation of large crops of the poppy by the Turks, Arab merchants were trading in opium with the peoples of India and China. Between the tenth and thirteenth centuries opium use spread to all parts of Europe. Opiate addition became an increasing problem, especially in China, where the practice of smoking raw opium had begun in the mid seventeenth century after tobacco smoking was banned. The availability of opium in Europe was limited until the eighteenth century, when the British trade with India and the Far East increased, the trade they forced with the Chinese culminating in the infamous 'Opium Wars'. Opium, however, only became a popular recreational agent in Europe during the early nineteenth century. The British Romantic writer Thomas de Quincey in his "Confessions of an English Opium Eater" (1821), provides an informative account of addiction to laudanum, which was a popular camphorated tincture of opium.

In 1806, a German scientist, Fredrich Serturner, isolated the active ingredient in opium. This proved to be a potent alkaloid, which he named 'morphine' (after Morpheus, the Greek god of dreams). This substance was to transform the practice of medicine, as well as the

experience of drug addition, especially following the development of the hypodermic syringe and the hollow needle in the 1850s, which meant that morphine could be injected directly into the bloodstream to produce a much more rapid and reliable effect than when taken orally. It began to be used for more surgical procedures, for post-operative and chronic pain, and as an adjuvant to general anaesthesia. The widespread use of injectable morphine as a painkiller in the American Civil War and Franco-Prussian war, resulted in so many veterans returning home as addicts, that addiction to morphine become known as the 'soldier's disease'. Efforts made to develop a more effective but less addictive opiate, were not successful, the Bayer group in 1898, introducing Heroin (diacetylmorphine) as a 'non-addicting' cough medicine. The addition of the two acetyl groups to morphine to form Heroin, increases the lipophilicity of the drug, enabling it to cross the blood-brain barrier more quickly than morphine, and so result in a more intense 'rush' of euphoria. In 1939, meperidine (Eisleb and Schaumann, 1939), the first opiate with a structure different from morphine was developed, atropine being the start compound. This was followed in 1941 (Scott and Chen, 1941), by the synthesis of methadone, which having a longer lasting effect compared to other opiates, was later used as a substitute for heroin to treat addicts. In 1942, nalorphine (Weijlard and Erikson, 1942) the first opiate with antagonist properties was produced, which although was later discovered to also have agonist properties lead in 1961, to the discovery of the pure opiate antagonist naloxone (Blumberg et al, 1961).

Endogenous opioid peptides and opioid receptors

By the late 1960's, it was becoming increasingly clear from the stereoselective requirements of opiate agonists and mixed agonist-antagonists, that receptors for these compounds must be present in the central nervous system (CNS). Initial attempts to demonstrate the existence of these receptors with radiolabelled drugs were unsuccessful (Goldstein et al, 1971). It was found that the relative affinities at which the opiate drugs bound to nervous tissue did not parallel their relative pharmacological potencies as opiates.

Due to the scarcity of opiate receptors in the brain, it was difficult to distinguish the binding of an opiate drug to a specific (receptor) site from the vast 'noise' of the more numerous non-specific binding interactions. By relative simple modifications of the radioassay procedure to increase the likelihood of detecting such specific binding, three groups (Pert and Synder, 1973; Terenius, 1973; Simon et al, 1973) succeeded in demonstrating high affinity, saturable binding sites for the opiate drugs in the CNS. These binding sites were also shown to have a non-uniform distribution in the brain (Kuhar et al, 1973). The search for the possible endogenous ligands of these receptors intensified.

In 1975, Hughes published that extracts of the pig brain contained activity which was opiate-like in the sense of inhibiting stimulation-induced contractions of the guinea pig ileum, and even more dramatically the mouse vas deferens, and that naloxone blocked these effects (Hughes, 1975). The opiate-like substances responsible proved to be two pentapeptides, Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin) and Tyr-Gly-Gly-Phe-Met (Met-enkephalin), differing only in one amino acid (Hughes et al, 1975). They were named 'enkephalin', (Greek for 'in the head'), subsequently often referred to by the more general term 'endorphin' (**end**ogenous m**orphin**e-like substance). The enkephalins proved to be the first of a series of endogenous opioid peptides to be isolated. In 1976, β-endorphin, a 31 amino acid fragment of β-lipotropin, that had been isolated from pituitary extracts several years earlier, was demonstrated to have the Met-enkephalin sequence in its NH_2-terminal, and to display high affinity for brain opiate receptors (Li and Chung, 1976; Bradbury et al, 1976; Chretien et al, 1976). Another group of potent peptides having opioid activity by bioassay was identified shortly afterwards and named the 'dynorphins' (from the Greek prefix 'dyn' signifying 'strength' or 'power'; Goldstein et al, 1979; 1981).

All the opioid peptides isolated were found to contain the NH_2-terminal pentapeptide amino acid sequence Tyr-Gly-Gly-Phe-X (where X = Met or Leu), and in mammals, belong to one of three families of peptides, derived from separate precursor proteins, referred to as proopiomelanocortin [POMC] (bovine: Mains et al, 1977; Roberts and Herbert, 1977;

Nakanishi et al, 1979. human: Whitfield et al, 1982. rat: Drouin and Goodman, 1980), proenkephalin [proEnk] (bovine: Noda et al, 1982; Gubler et al, 1982. human: Comb et al, 1982. rat: Rosen and Douglass, 1984.) and prodynorphin [proDyn] (porcine: Kakidani et al, 1982. human: Horikawa et al, 1983. rat: Civelli et al, 1985), encoded by individual genes, as confirmed by the subsequent cloning of the complementary DNA (cDNA) sequences of these opioid peptides (Weber et al, 1983).

That there were many potential ligands argued that there might be more than one opioid receptor. This had previously been proposed earlier by Martin and co-workers on the basis that naloxone showed different pA_2 values in antagonising the effects of different opiates in suppressing flexor reflexes in the chronic spinal dog, and that tolerance to one group did not result in cross tolerance to another class of opiates. The existence of three types of receptor was proposed and the types were named after the drugs that identified them; i.e. μ (**m**orphine sensitive), κ (ethyl**k**etocyclazocine sensitive) and σ (**S**KF10047 or *N*-allylnormetazocine sensitive) (Martin et al, 1976). From studies on the mouse vas deferens bioassay, Kosterlitz and co-workers (Lord et al, 1977) suggested the existence of yet another receptor type, δ (for **d**eferens), possessing a high affinity for the enkephalin peptides. They found that although the enkephalins were less effective than morphine in inhibiting the electrically induced contractions of the guinea pig ileum, they were more potent in inhibiting contractions of mouse vas deferens, this inhibition also being comparatively insensitive to naloxone. Similar work on the rat vas deferens led to the postulation of the ε receptor, which although has a high affinity for β-endorphin displayed a different pharmacological profile from the μ, δ and κ receptors (Wuster et al, 1978). Attempts to purify the opioid receptors to homogeneity were thwarted for many years due to their paucity in most tissues and their lability after detergent solubilization (Loh and Smith, 1990). As such the μ, δ and κ opioid receptors have only recently been cloned (μ: Chen et al, 1993; Wang et al, 1993; Thompson et al, 1993. δ: Evans et al, 1992; Kieffer et al, 1992, Knapp et al, 1994. κ: Yasuda et al, 1993; Meng et al, 1993; Minami et al, 1993; Mansson et al, 1994). These opioid receptors, as had been predicted

from biochemical and pharmacological studies, and by inference to other more fully characterised receptors, are guanine nucleotide binding (G)-protein coupled receptors, members of the rhodopsin receptor superfamily with seven transmembrane spanning domains, that appear to mediate their intracellular effects partially through the inhibition of adenylate cyclase.

Other groups of peptides have been described to exert effects at the μ, δ and κ opioid receptors, which have been collectively referred to as 'atypical' opioid peptides (Teschemacher, 1993). These peptides derived from precursor proteins other than POMC, proEnk and proDyn, have only a tyrosine residue in common, and in contrast to the 'typical' opioid peptides, often display extensions of sequence beyond this NH_2-terminal tyrosine residue, Tyr-X-X-X-X. Such 'atypical' opioid peptides include the β-caseomorphins generated by the proteolysis of the milk proteins β-casein (Bront et al, 1981), and the 'dermorphins' (**der**mal **morphin**e-like substance; Montecucchi et al, 1981) and 'deltorphins' (δ specific ligands; Kreil et al, 1989) secreted from the skin glands of *Phyllomedusa* amphibians. These *Phyllomedusa* peptides are quite unusual in that they contain D-amino acids, which have never before been found for natural peptides and which likely confers peptide stability. Along with these peptide ligands, there is also evidence that morphine may be synthesised endogenously. Initially isolated from toad skin (Oka et al, 1985), this alkaloid has also been found, though in very low concentrations, in mammalian tissue, including parts of the brain (Goldstein et al, 1985). However, whether this morphine is of dietry origin or represents true endogenous synthesis is unknown.

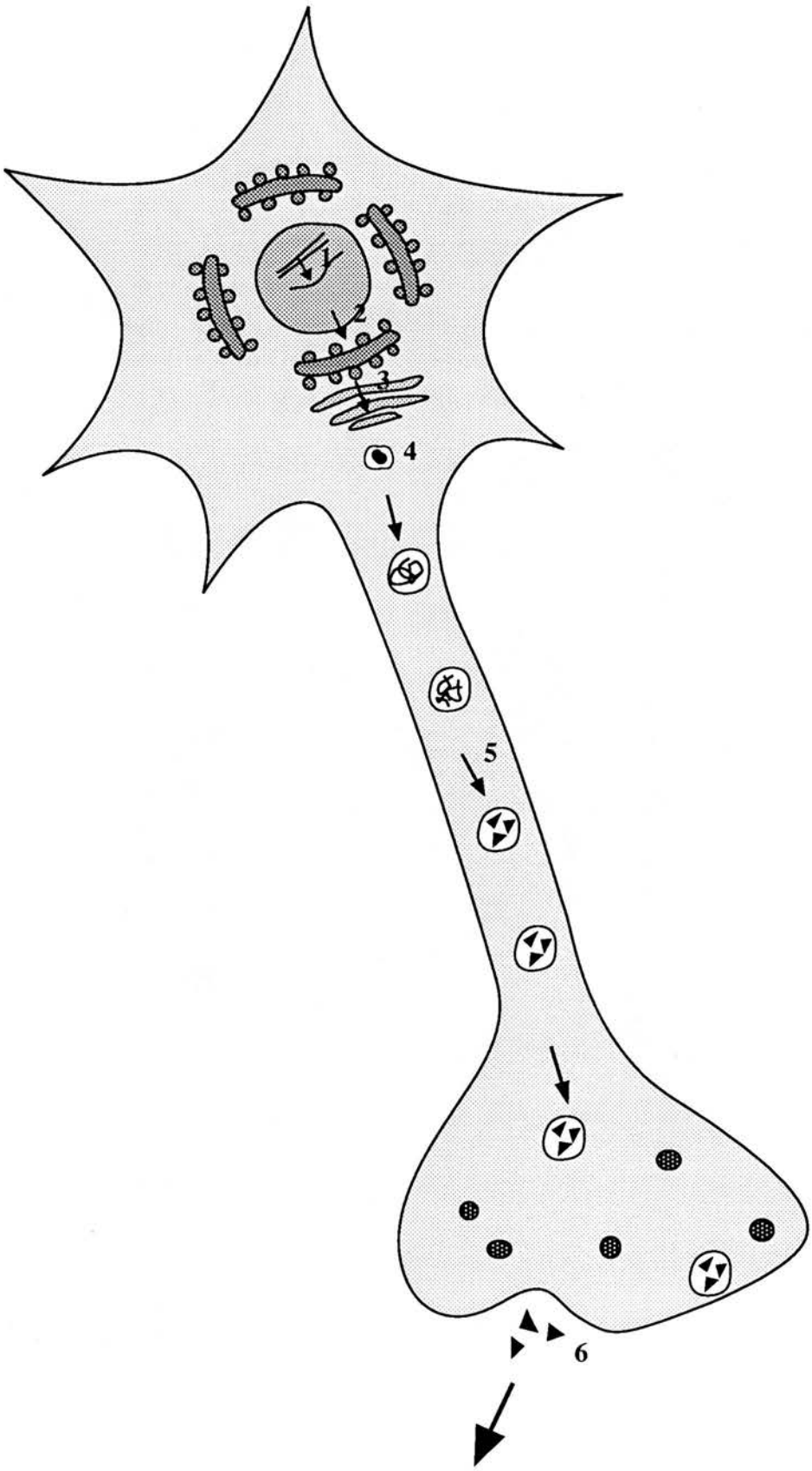
More recently the screening of genetic libraries has yielded numerous cDNA sequences which share considerable homology to members of recognised supergene families but with no known biological activity, the so-called 'orphan' proteins. Such screenings have notably identified a G-protein coupled receptor that is particularly abundant in the CNS, whose amino acid sequence closely resembles that of the μ, δ and κ opioid receptors, named amongst others ORL_1 (Mollereau et al, 1994) and LC123 (Bunzow et al, 1994). However, despite this

homology and being negatively coupled to adenylate cyclase, known opioid peptides or ligands do not appear to activate this receptor. A putative ligand for this receptor has been recently isolated from both rat (nociceptin; Meunier et al, 1995) and porcine (orphanin FQ; Reinscheid et al, 1995) brain. The peptide isolated displays a high degree of structural similarity to the opioid peptides, particularly the dynorphins, but appears to be pharmacologically and physiologically distinct, inducing hyperalgesia when administered intracerebroventricularly (i.c.v.) in mice.

1.2. OPIOID NEURONES: OVERVIEW OF PEPTIDE SYNTHESIS AND METABOLISM

Opioid peptides, like most peptide neurotransmitters and hormones known, are not synthesised directly but derived by the enzymatic cleavage of larger and generally inactive precursor polypeptides. The general stages involved in the neuronal synthesis of the opioid peptides are illustrated on figure 1. The gene encoding a particular family of opioid peptides is transcribed into messenger RNA (mRNA) in the nucleus of the opioid synthesising neurone, which in turn is translated into the characteristic pre-propeptide sequence by ribosomes on the surface of rough endoplasmic reticulum in the cell body. Each pre-propeptide sequence contains a hydrophobic NH_2-terminal signal sequence, that identifies the peptide as being destined for secretion, and directs its passage into the lumen of the endoplasmic reticulum, where it is then removed by a signal peptidase. The remaining propeptide is then directed to the Golgi apparatus, where it is moves from *cis-* to *trans-* Golgi compartments and to the *trans*-Golgi network by a series of vesicle fusion and budding steps. From within the *trans*-Golgi network the peptides are packaged into secretory granules (visualised as large dense core vesicles) and transported along the axon to the nerve terminal. Further biosynthetic processing occurs in the Golgi region as the secretory granules are forming and in the newly formed vesicles, involving cleavage of the peptide chain and

FIGURE 1. General stages involved in the neuronal synthesis of opioid peptides. The DNA encoding an opioid precursor is transcribed into mRNA in the nucleus of the opioid synthesising neurone (1). This mRNA is then translated by ribosomes on the surface of the rough endoplasmic reticulum to form a pre-propeptide (2). The NH_2-terminal signal peptide directs the passage into the lumen of the endoplasmic reticulum, where it is subsequently removed. The propeptide is then transported to the Golgi apparatus (3) where it is packaged into secretory granules (large dense core vesicles) (4). These are transported along the axon to the nerve terminal (5). During stages (4) and (5) the propeptide is subjected to various proteolytic steps and post-translational modifications to yield one or more biologically active peptides. These peptides are then released following an appropriate stimulus into the synaptic cleft by exocytosis (6).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modification of individual amino acid residues (as reviewed by Dockray, 1990; Bean et al, 1994). Sequential cleavage of the opioid family propeptide by a variety of endopeptidases (e.g. prohormone converting (PC) enzymes PC1 and PC2/PC3, adrenal trypsin-like enzyme, furin, dynorphin converting enzyme) yields one or more biologically active peptides. These endopeptidases selectively cleave the precursor at specific sites. Commonly, dibasic residues such as Lys-Arg, Arg-Arg, and Lys-Lys signal specific cleavage sites. However, not all such sites appear equally susceptible to processing indicating that the amino acids surrounding these residues must also confer some selectivity. Additionally a single arginine residue can often signal enzymatic cleavage, as in the case of prodynorphin processing. Following the action of the endopeptidases, a carboxypeptidase (e.g. carboxypeptidase E/H or enkephalin convertase) is required to remove the basic cleavage site residues from the COOH-terminus of the peptides (as reviewed by Dockray, 1990; Fricker et al, 1993). The timing of these cleavage events are crucial in determining whether the products will be packaged together or separately. If the cleavage takes place before packaging the resulting peptides may reside in distinct vesicle populations, whereas cleavage after packaging will result in colocalisation within the same vesicle, and hence corelease (Bean et al, 1994). In addition to these proteolytic steps some opioid peptides undergo other post-translational modifications in the endoplasmic reticulum or Golgi apparatus that may be required for the biological activity and/ or stability of the peptide. These include glycosylation, sulphation, phosphorylation, NH_2-terminal acetylation and COOH-terminal amidation (as reviewed by Mains et al, 1983; Dockray, 1990; Fricker et al, 1993).

Following release by exocytosis the activity of neuropeptides is terminated, in general, not by reuptake but by membrane bound peptidases that hydrolyse the active peptide to an inactive form. For the opioid peptides these include the ubiquitous relatively non-specific aminopeptidases, that inactivate the peptides by removing the NH_2-terminal tyrosine, as well as endo- or carboxy-peptidases (e.g. neutral endopeptidase[E.C.3.4] 24.11 or 'enkephalinase'). In addition to this degrading process, active peptides may also be converted

into smaller fragments with retained or changed biological activity. These so-called neuropeptide converting enzymes, capable of releasing bioactive fragments from their substrate peptides have been identified in various CNS tissues. For example, angiotensin converting enzyme converts Met-enkephalin-Arg-Phe to Met-enkephalin, metallo-endopeptidase 24.15 processes several precursors into Met- and Leu-enkephalin, and the so called 'dynorphin converting' enzymes convert prodynorphin derivatives especially dynorphin B(1-13) into Leu-enkephalin-Arg^6 (as reviewed by Dockray, 1990; Fricker et al, 1993; Persson et al, 1995; Csuhai et al, 1995).

Most, if not all, these intracellular and extracellular processing stages, appear to be open to modification, hence allow for a very 'plastic' system. The consequences of this for opioid neurotransmission will be highlighted in the following sections that discuss the multitude of opioid peptides synthesised and functionally active in the mammalian CNS. The extent of proteolytic processing and the peptide products ultimately released, appears to largely depend on the neurones in which they are localised, the physiological state of these neurones and their recent secretory demands. Since differential processing may lead to opioid peptides with distinct biological activity, the processing enzymes involved play a key role in opioid neuronal systems. However, it should be noted that most of these processes have been studied by *in vitro* methods using brain homogenates, and although immunocytochemical techniques have been used to identify the putative opioid neuronal products *in situ*, comparatively little is known of the extent to which these processes actually occur *in vivo*. Most of the enzymes reported to be involved in the biosynthesis and metabolism of the opioid peptides are also involved with processing other peptides.

1.3 OPIOIDS I: MULTIPLE OPIOID PEPTIDES AND OPIOID RECEPTORS

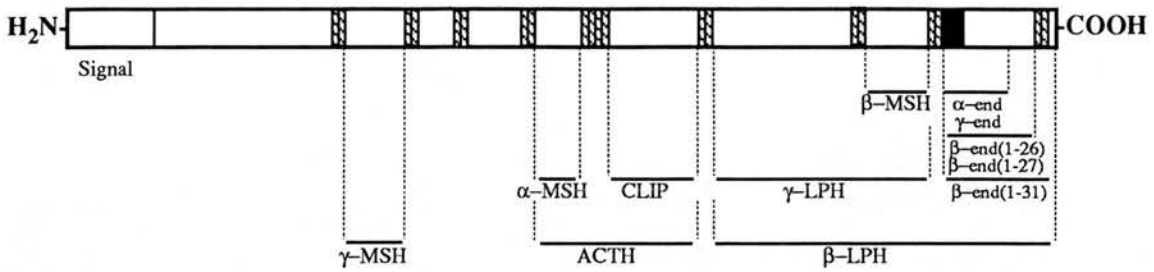
1. MULTIPLE OPIOID PEPTIDES

The majority of mammalian opioid peptides have been found by recombinant DNA techniques to be derived from three separate precursor polypeptides, POMC, proEnk and proDyn. The structure of these pre-propeptides along with their putative processing products are illustrated in figure 2. Though genetically distinct these precursors show a striking homology both at the genomic and pre-propeptide level, suggestive of a common ancestral gene, with translated regions being highly conserved throughout the hierarchy of species down to amphibia (Hollt et al, 1993). Each gene encoding a family of opioid peptides appears to be expressed uniquely, there being practically no examples of where opioid peptides from several genes are expressed in the same cell (Terenius, 1992). All three opioid peptide precursors can generate opioids of varying amino acid chain length. These proteolytic processing patterns appear to be differentially regulated in different tissues (Weber et al, 1983).

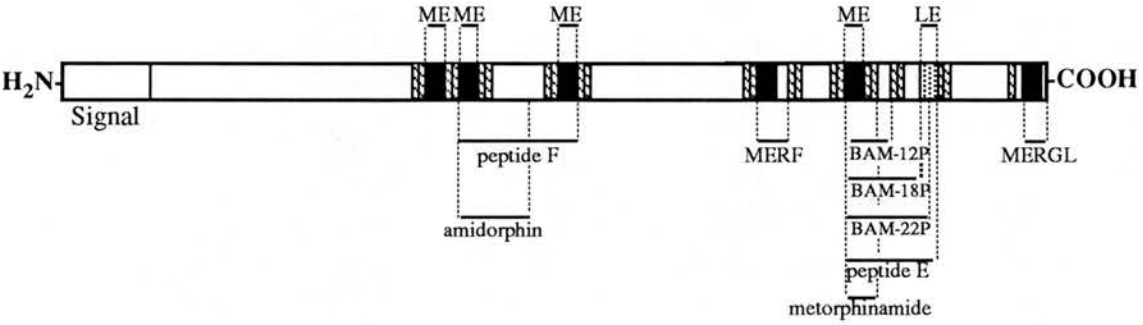
Proopiomelanocortin (POMC) is unique in that it contains only one copy of an opioid active peptide, β-endorphin (β-end). It is also unique in encoding other biologically active peptides, adrenocorticotrophic hormone (ACTH), corticotropin-like intermediate lobe peptide (CLIP), α-, β-, γ- melanocyte stimulating (MSH) and γ-, β- lipotropin (LPH) hormones. The proteolytic processing of POMC was initially analysed using pituitary tissue. It has been shown that POMC undergoes more cleavages and its products more post-translational modifications in the intermediate lobe than in the anterior lobe of the pituitary. In general the processing of POMC in the brain appears to be similar to that found in the intermediate pituitary, although less extensive in caudal brain regions. The cleavage of β-lipotropin to β-endorphin (1-31) is virtually complete in all rostral brain regions, with further cleavage to β-endorphin (1-27) and β-endorphin (1-26) also occurring to a significant extent in certain regions, notably the amygdala, hippocampus and nucleus accumbens. However, the

FIGURE 2. Schematic representation of the structure of the three opioid peptide precursors. Potential cleavage sites at dibasic amino acid residues on POMC (bovine), proEnk (bovine) and proDyn (porcine) are indicated along with known peptide products. Adapted from Millan et al (1986).

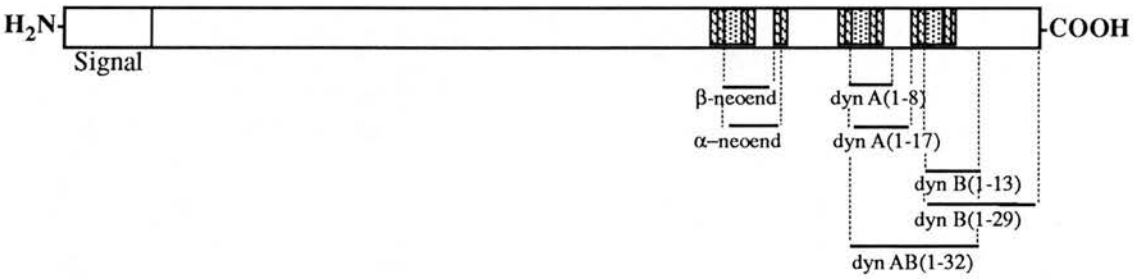
Pre-proopiomelanocortin [POMC] - 265 amino acids (Bovine)



Pre-proenkephalin [proEnk] - 263 amino acids (Bovine)



Pre-prodynorphin [proDyn] - 256 amino acids (Porcine)



Met-enkephalin (ME)

Leu-enkephalin (LE)

Lysine or arginine - pairs of these basic amino acids signal potential cleavage sites

NH_2-terminal acetylation of β-endorphin, which leads to a complete loss of its activity at opioid receptors, is much less pronounced in the brain than the pituitary. There is also debate whether other possible derivatives of β-endorphin, α-endorphin [β-endorphin (1-16)] and γ-endorphin [β-endorphin (1-17)] are physiological cleavage products of POMC (as reviewed by Weber at al, 1983; Khachaturian et al, 1985a; b; 1993; Hollt, 1986; 1993; Millan, 1986; Young et al, 1993).

Proenkephalin (proEnk) in contrast, encodes for several peptides containing the opioid active core sequence, Tyr-Gly-Gly-Phe. These include four copies of Met-enkephalin [ME], one copy of Leu-enkephalin [LE] and one copy each of the COOH-terminally extended peptides the heptapeptide, Met-enkephalin-Arg-Phe [MERF], and the octapeptide, Met-enkephalin-Arg-Gly-Leu [MERGL]. Other possible opioid peptides include the large enkephalin containing peptides, peptide E and its COOH-terminal derivatives, metorphamide [adrenorphin; Met-enkephalin-Arg-Arg-Val-NH_2] and the BAM peptides [12P, 18P, 22P], as well as peptide F and amidorphin [peptide F(1-26)NH_2]. The proteolytic processing of proEnk was initially analysed in the adrenal medulla. In general, the processing of proEnk appears to be more complete in the brain, with free enkephalins forming the predominant products (as reviewed by Weber et al, 1983; Petruz et al, 1985; Millan, 1986; Khachaturian et al, 1985b; 1993; Hollt, 1986; 1993; Rossier, 1993)

Prodynorphin (proDyn) encodes several active opioid peptides, which are all COOH terminal extensions of Leu-enkephalin. The cleavage of proDyn at Lys-Arg sites results in the formation of dynorphin A(1-17), dynorphin B(1-29) [leumorphin], α- and β-neoendorphin, whilst additional cleavage at certain arginine sites results in the production of dynorphin A(1-8), dynorphin B(1-13) [rimorphin] and dynorphin AB(1-32) ['big dynorphin']. The proteolytic processing of proDyn has been extensively analysed using brain and pituitary tissue. Whereas dynorphin A(1-17) and dynorphin A(1-8) occur in similar amounts in posterior pituitary, dynorphin A(1-8) has been found to be the predominant peptide in the brain, particularly in the striatum and midbrain. Similarly dynorphin B(1-29) appears to be

completely processed to give dynorphin B(1-13). Although α-neoendorphin is a more common cleavage product since the Pro-Lys bond at the carboxy terminus of β-neoendorphin very resistant to proteolysis, significant quantities of β-neoendorphin are found in the hypothalamus and posterior pituitary. Other possible dibasic cleavage sites involving lysine and arginine do not appear to be responsible for the formation of proDyn peptides. Thus, although there are Arg-Arg sites that if cleaved would result in the formation of Leu-enkephalin, these sites appear to be rarely if ever used, with any Leu-enkephalin found in the brain and pituitary being commonly also localised with Met-enkephalin (as reviewed by Weber et al, 1983; Khachaturian et al, 1985, 1993; Hollt, 1986; 1993; Millan, 1986; Fallon and Ciofi, 1990; Day et al, 1993)

2. MULTIPLE OPIOID RECEPTORS

Although the existence of many types of opioid peptide binding sites have been postulated, only the μ, δ and κ have been shown by ligand binding autoradiography, and more recently studies employing antibodies to defined sequences of the cloned receptors, to exist as discrete opioid receptors in the mammalian CNS. The postulated σ receptor appears not to be an opioid receptor in the strict sense, since its mediated effects are not reversed by even very high concentrations of opioid antagonists, such as naloxone, and it also appears to be activated by the phencyclidines, another class of abused drugs (Zukin et al, 1984). As for the ε receptor, the question of whether this receptor type is present in the CNS has not yet been resolved, although it has recently been reported that such sites may have previously been classified as κ receptors (Nock et al, 1990).

Although opioid peptides were discovered due to their similarity to opiate-like compounds, there are substantial differences. Whereas morphine and its analogues are relatively selective for μ receptors, the endogenous opioid peptides tend to show a relatively poor preference for any one opioid receptor type (as will be discussed in detail in section 1.4). Likewise naloxone, whilst predominantly active at μ receptors has also some affinity for the other opioid receptor

types. A number of agonists and antagonists have been developed over recent years, that have a high affinity and/ or degree of selectivity for each opioid receptor type, as indicated on table 1. These have proved useful tools for investigating the functions of these receptors, especially those non-peptide ligands, which since they are relatively resistant to peptidase degradation, display prolonged biological activity. The use of some of these compounds in ligand binding, pharmacological and behavioural studies, have suggested that subtypes of the μ, δ and κ receptors exist (recently reviewed by Adler et al, 1990; Simon and Gioannini, 1993; Pasternak, 1993; Wollemann et al, 1993).

The **μ receptor** has high affinity for (a) the peptide agonists DAMGO (an analogue of enkephalin), morphine and its analogues such as ohmefentanyl, the naturally occurring morphiceptin and its analogue PLO17, and the dermorphins; (b) the competitive antagonists: naltrexone and its analogue naloxonazine, and CTOP; and (c) the irreversible antagonist β-FNA. From early pharmacological and behavioural studies a μ_1 subtype of receptor was proposed that binds morphine and enkephalins with a similar high affinity, in contrast to the μ_2 subtype at which enkephalins have a reduced affinity (Wolozin and Pasternak, 1981). These receptor subtypes have also been proposed to be distinguishable using the antagonist naloxonazine which displays some selectivity for the putative μ_1 subtype (Pasternak and Wood, 1986). However, the possibility that such a differentiation may simply reflect the binding of ligands to complexes of μ and δ receptors cannot be discounted from the findings of such studies. There is no evidence to date for such subtypes from cloning studies.

The **δ receptor** has high affinity for (a) the peptide agonists DPDPE, DPLPE, DTLET, DSLET and DSBuLET (all analogues of enkephalin), the naturally occurring deltorphins, [D-Ala^2]deltorphin I and II; and (b) the antagonists ICI-174864, BNTX (an analogue of naltrexone), naltrindole and its analogues NTB and 5'-NTII. Subtypes of the δ receptor have also been proposed from behavioural and pharmacological studies. Whereas the δ_1 subtype appears to be activated by DPDPE and blocked by BNTX but not NTB or 5'-NTII, the δ_2

TABLE 1. Selective ligands for opioid receptors. Agonists and antagonists developed to study the distribution and function of the different types of opioid receptor are listed, with the principal investigators responsible for the development of each ligand quoted in brackets (see abbreviations, pg xi, for details).

OPIOID BINDING SITE	AGONISTS	ANTAGONISTS
μ	**DAMGO** (Handa et al, 1981) **morphiceptin** (Chang et al, 1981) **Dermorphin** (Broccardo et al, 1981) **PL017** (Chang et al, 1983) **Ohmefentanyl** (Xu et al, 1985)	**naloxonazine** (μ_1; Pasternak and Hahn, 1980) **β-FNA** (Takemori et al, 1986) **CTOP** (Hawkins et al, 1989)
δ	**DPDPE** (δ_2; Mosberg et al, 1983) **DPLPE** (Mosberg et al, 1983) **DTLET** (Zajac et al, 1983) **DSLET** (Gacel et al, 1984) **DSBuLET** (Delay-Goyet et al, 1988) **[D-Ala^2]deltorphin I** (Erspamer et al, 1989) **[D-Ala^2]deltorphin II** (δ_1; Erspamer et al, 1989)	**ICI174 864** (Cotton et al, 1984) **naltrindole** (Portoghese et al, 1988) **5'-NTII** (δ_2; Portoghese et al, 1990) **NTB** (δ_2; Portoghese et al, 1991) **BNTX** (δ_1; Portoghese et al, 1992)
κ	**U50488H** (κ_1; Von Voigtlander et al, 1983) **U69593** (κ_1; Lahti et al 1985) **PD117302** (κ_1; Clark et al, 1988) **CI977** (κ_1; Hunter et al, 1990)	**norBNI** (Portoghese et al, 1987)

subtype is activated by [D-Ala^2]deltorphin II, and blocked by NTB and 5'-NTII (Jiang et al, 1991; Mattia et al, 1991; Sofuoglu et al, 1991; Portoghese et al, 1993).

The **κ receptor** has high affinity for (a) the benzeneacetamide agonists U50488H, U69593, PD117302 and CI977; and (b) the antagonist norBNI (an analogue of naltrexone). Subtypes of the κ receptor were suggested initially from the differing binding of DADLE and dynorphin A (Attalli et al, 1982; Morre et al, 1983), but more recently from that of the benzeneacetamide compounds (Su et al, 1985; Zukin et al, 1988; Hunter et al, 1989). The κ_1 subtype binds DADLE, dynorphin A and the benzeneacetamide agonists, in contrast to the κ_2 and κ_3 sites which display a relatively low affinity for these compounds, the κ_3 sites being distinguished using the naloxone derivative NalBzoH (Clark et al, 1989). However, there is debate as to whether the κ_3 subtype is not simply an isoform of a μ receptor, since it also displays a high affinity for μ selective agonists (Wollemann et al, 1993)

The recently cloned μ, δ and κ opioid receptors have been found to be highly homologous to one another both at a nucleic acid and amino acid level (recently reviewed by Uhl et al, 1994; Reisine, 1995; Pasternak and Standifer, 1995). The receptors appear highly conserved, the rodent (both mouse and rat) and human receptors that have been cloned displaying very similar amino acid sequences and ligand selectivities. They appear to form a subclass of the rhodopsin superfamily of seven transmembrane domained, G-protein coupled receptors, with a high sequence similarity to the somatostatin receptors. The rodent μ, δ and κ receptors are approximately 65% identical in amino acid sequence, with the highest similarity in the transmembrane spanning regions and intracellular loops (Reisine and Bell, 1993). The pharmacological profiles of these cloned receptors have been shown to be very similar to that of the endogenously expressed receptors, in particular the putative μ_2, δ_2 and κ_1 receptors (Raynor et al, 1994).

Further evidence to support the existence of the δ receptor subtypes *in vivo* has been provided by behavioural studies employing antisense oligodeoxynucleotides to the putative cloned δ_2 receptor. Whereas the intrathecal (i.t.) administration of the antisense probes to the

spinal cord was found to block the ability of both DPDPE and deltorphin II to induce analgesia (Standifer et al, 1994), the administration of these oligonucleotides i.c.v. was found to prevent only the deltorphin II induced analgesia, indicating an involvement of both the δ receptor subtypes at supraspinal sites (Lai et al, 1994). It is likely that similar investigations will proved fruitful to further discriminate subtypes of the μ and κ receptors.

1.4 OPIOIDS II: OPIOID PEPTIDE-RECEPTOR INTERACTIONS

Despite the opioid peptides being synthesised from independent precursors, no clear relationship between an opioid peptide family with a specific type of receptor appears to exist. The presence of the identical NH_2-terminal tetrapeptide sequence, places inherent limitations on the receptor selectivity of endogenous opioid peptides. Although early *in vitro* binding and pharmacological studies did reveal some selectivity of the μ receptors for the β-endorphins, the δ receptors for the Met- and Leu-enkephalins and the κ receptors for the dynorphins, these relationships are not exclusive. The β-endorphins derived from POMC display similar affinities for the μ and δ binding sites, and peptides are derived from proEnk and proDyn that can interact with all three opioid binding sites (Akil et al, 1981; Quirion and Pert, 1981; Wuster et al, 1981; Corbett et al, 1982; Magnan et al, 1982; Oka et al, 1983; Quirion and Weiss, 1983; Goldstein and James, 1984; Chavkin et al, 1985). Thus, any peptide selectivity appears more intimately related to the extent of peptide processing rather than the precursor from which it is derived.

Table 2 compares the binding relationships between some of these opioid peptides and the μ, δ and κ sites and figure 3 illustrates the peptide sequences. Since most early binding studies employed relatively non-selective radiolabelled ligands such as [^{3}H]-dihydromorphine, [^{3}H]-DADLE and [^{3}H]-ethylketocyclazine (EKC), and a variety of different tissue preparations, the data presented on table 2 is derived from a recent review by Corbett et al (1993) that considered only those inhibitory binding constant (K_i) values for peptide binding to homogenates of guinea pig brain determined using more selective ligands, such as

TABLE 2. Relative affinities of POMC, proEnk and proDyn derived peptides at μ, δ and κ opioid binding sites. The inhibitory effects (K_i, nM) of POMC , proENK and proDyn derived peptides on the binding at μ, δ and κ binding sites in homogenates of guinea-pig brain, obtained from a review by Corbet et al (1993), are compared. The μ binding sites were labelled with [^{3}H]-DAMGO or [^{3}H]-morphine, the δ sites with [^{3}H]-DADLE or [^{3}H]-DADLE in the presence of PL017, and the κ sites with [^{3}H]-bremazocine in the presence of DAMGO and DADLE or [^{3}H]-EKC in the presence of PL017 and DSLET. The relative affinity at each binding site was calculated using the equation K_i^{-1}(at μ, δ and κ) ÷ [K_i^{-1}(at μ) + K_i^{-1}(at δ) + K_i^{-1}(at κ)]. K_i^{-1} is the affinity constant, the reciprocal of the apparent dissociation constant inhibitory binding constant K_i (cited in brackets), determined from the IC_{50} values obtained from the binding competition studies between the radiolabelled ligands and the unlabelled opioid peptides.

		RELATIVE AFFINITY AT OPIOID BINDING SITES		
		μ	δ	κ
POMC DERIVED PEPTIDES	β-endorphin(1-31)	0.53 (2.1)	0.45 (2.4)	0.02 (96)
	β-endorphin(1-27)	0.44 (3.0)	0.55 (2.4)	0.01 (185)
PROENK DERIVED PEPTIDES	Leu-enkephalin	0.06 (19)	0.94 (1.2)	0 (8210)
	Met-enkephalin	0.09 (9.5)	0.91 (0.91)	0 (4442)
	Met-enkephalin-Arg-Phe	0.70 (3.7)	0.27 (9.4)	0.03 (93)
	Met-enkephalin-Arg-Gly-Phe	0.41 (6.6)	0.56 (4.8)	0.03 (79)
	peptide E	0.56 (0.53)	0.17 (1.7)	0.27 (1.1)
	metorphamide	0.66 (0.12)	0.03 (2.7)	0.31 (0.25)
	BAM 12P	0.66 (0.16)	0.08 (1.4)	0.26 (0.41)
	BAM 18P	0.68 (0.29)	0.08 (3.2)	0.26 (0.75)
	BAM 22P	0.57 (0.10)	0.09 (0.66)	0.34 (0.17)
	peptide F	0.46 (25)	0.39 (29)	0.15 (78)
PRODYN DERIVED PEPTIDES	dynorphin A(1-17)	0.14 (0.73)	0.04 (2.4)	0.82 (0.12)
	dynorphin A(1-8)	0.22 (3.8)	0.16 (5.0)	0.62 (1.3)
	dynorphin B(1-13)	0.15 (0.68)	0.03 (2.9)	0.82 (0.12)
	α-neoendorphin	0.10 (6.9)	0.33 (2.1)	0.57 (1.2)
	β-neoendorphin	0.11(1.2)	0.23 (0.57)	0.66 (0.20)

FIGURE 3. Comparison of opioid peptide sequences. Amino acid alignments of the opioid peptides derived from the POMC, proEnk and proDyn opioid peptide precursors, as illustrated in figure 2, and whose relative affinities at μ, δ and κ opioid binding sites are displayed on table 2. Adapted from Mansour et al (1995).

PEPTIDES	SEQUENCE
POMC derived peptides	
β-*endorphin (1-31)*	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His -Lys-Lys-Gly-Glu
β-*endorphin (1-27)*	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His
ProEnk derived peptides	
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu
Met-enkephalin	Tyr-Gly-Gly-Phe-Met
Met-enkephalin-Arg-Phe	Tyr-Gly-Gly-Phe-Met-**Arg**-Phe
Met-enkephalin-Arg-Gly-Phe	Tyr-Gly-Gly-Phe-Met-**Arg**-Gly-Phe
Peptide E	Tyr-Gly-Gly-Phe-Met-**Arg**-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu
metorphamide	Tyr-Gly-Gly-Phe-Met-**Arg**-Arg-Val-NH_2
BAM 12P	Tyr-Gly-Gly-Phe-Met-**Arg**-Val-Gly-Arg-Pro-Glu
BAM 18P	Tyr-Gly-Gly-Phe-Met-**Arg**-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln
BAM 22P	Tyr-Gly-Gly-Phe-Met-**Arg**-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly
Peptide F	Tyr-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Glu-Val-Leu-Gly-Lys-Arg-Tyr-Gly-Gly-Phe-Met
ProDyn derived peptides	
Dynorphin A(1-17)	Tyr-Gly-Gly-Phe-Leu-**Arg**-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
Dynorphin A(1-8)	Tyr-Gly-Gly-Phe-Leu-**Arg**-Arg-Ile
Dynorphin B(1-13)	Tyr-Gly-Gly-Phe-Leu-**Arg**-Arg-Gln-Phe-Lys-Val-Val-Thr
α-neoendorphin	Tyr-Gly-Gly-Phe-Leu-**Arg**-Lys-Tyr-Pro-Lys
β-neoendorphin	Tyr-Gly-Gly-Phe-Leu-**Arg**-Lys-Tyr-Pro

[^{3}H]-DAMGO and [^{3}H]-DPDPE. The affinity of each opioid peptide for the different opioid binding sites are compared in such a way that if a peptide interacts with only one binding site it will have a relative affinity of 1 at that site, whereas if it interacts equally well with all three binding sites it will have a relative affinity of 0.33 at each site. From table 2 both the long and short forms of β-endorphin can be seen to have similar affinities for the μ and δ binding sites with negligible affinity for κ sites, the removal of four amino acids from the COOH-terminus of β-endorphin(1-31) to give β-endorphin(1-27) causing only a small reduction in affinity for all three sites. By contrast, the opioid peptides derived from proEnk can be seen to display marked differences in their relative affinities for the μ, δ and κ binding sites. Whereas the pentapeptides Met-enkephalin and Leu-enkephalin have high affinities for the δ binding sites, lower affinities for the μ site and negligible affinity for the κ site, extensions of Met-enkephalin at the COOH-terminus can result in higher affinities at μ and/ or κ sites with either little change or a reduction in δ binding affinity. For example, extension of Met-enkephalin by an arginine residue with metorphamide, BAM 12, BAM 18, BAM 22, and peptide E, leads to a reduction in the affinity for the δ sites and an increase of that for the μ and κ sites. Peptide F with its Lys^6-Lys^7 extension by contrast displays comparatively little affinity for all three opioid receptors. Thus, complete processing of proEnk into Met- and Leu-enkephalins is associated with a change in binding preference towards the δ sites. Similarly whilst the proDyn peptides display a high affinity for κ binding sites they also bind to μ and δ sites, the smaller derivatives of proDyn, dynorphin A(1-8) and α-/ β-neoendorphin having a lower affinity for the κ site than the longer fragments, dynorphin A(1-17) and dynorphin B(1-13). These opioid peptide-receptor associations are in general agreement with those determined from pharmacological studies using a variety of smooth muscle assay systems (as reviewed by Hollt, 1986; Corbett et al, 1993), each with a different pharmacological profile. For example whereas the electrically evoked contractions of the mouse vas deferens are known to be inhibited by μ, δ and κ agonists, and those of the guinea-pig myenteric plexus-longitudinal muscle by μ and κ agonists, those of the rat, rabbit and hamster vas deferens appear to

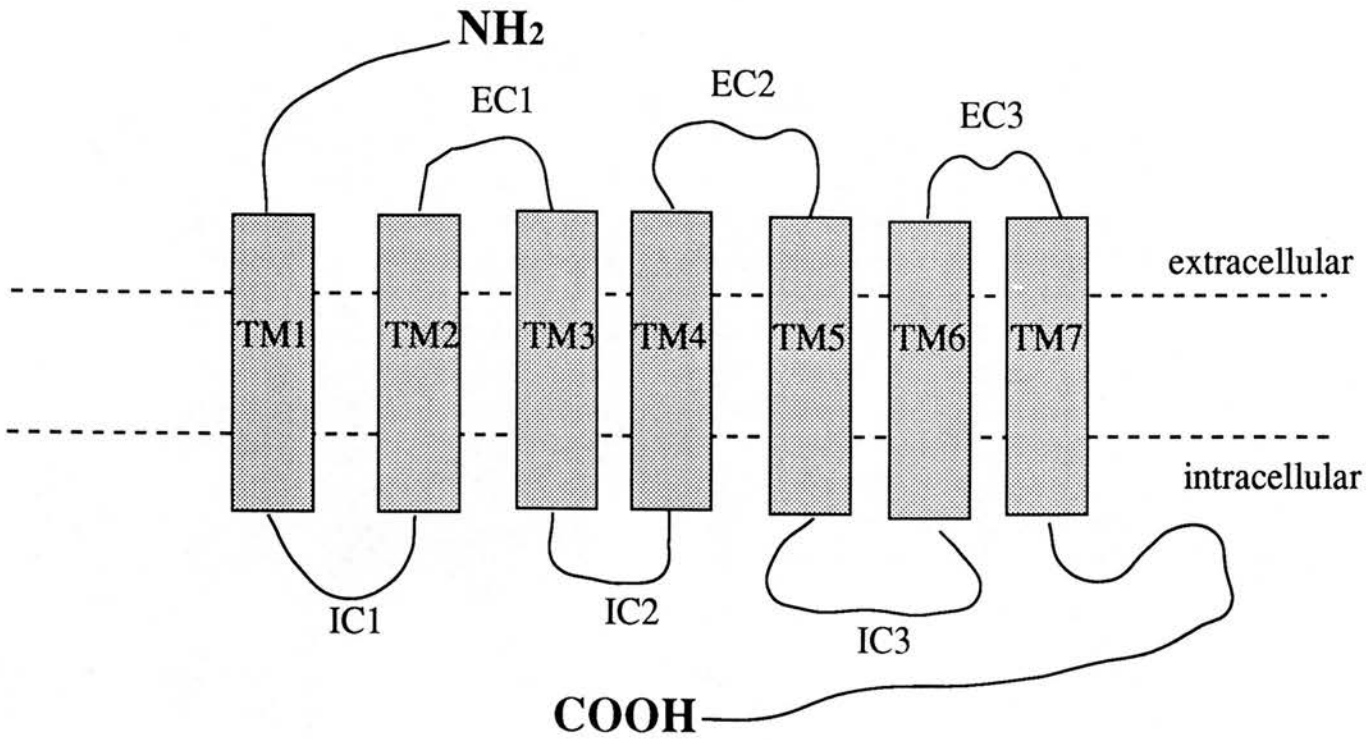
involve only one receptor type, being inhibited by μ, κ and δ agonists respectively (Smith and Leslie, 1993; Corbett et al, 1993).

Further support for such interactions has been provided more recently by competition studies examining the binding affinities of the newly cloned μ, δ, and κ opioid receptors expressed in transfected tumour Cos-1 cells (Mansour et al, 1995c). Of the three opioid receptors the κ receptor displayed the greatest degree of selectivity with respect to the endogenous ligands tested with approximately a 2400-fold affinity range, in contrast to the moderate (695-fold) and low range (20-fold) range of affinities of μ and δ receptors respectively for the same ligands. Importantly these studies also indicated how the endogenous opioid peptide selectivity profiles between the μ/ δ and κ receptors may result. This receptor discrimination appears to reside principally, with the κ receptor having additional sequence requirements, that the μ and δ receptors do not share. Based on the findings of the early pharmacological studies of the potencies of dynorphin fragments, receptor selectivity was proposed to be conferred by a 'message-address' configuration, the 'message' encoded by the tetrapeptide core sequence Try-Gly-Gly-Phe- and the 'address' by the C-terminal extensions (Chavkin and Goldstein, 1981). Mansour et al (1995) however, have proposed that whilst the Try-Gly-Gly-Phe-Met or -Leu peptide sequence is sufficient for μ and δ receptor binding, another core peptide sequence appears necessary to account for κ receptor affinity. In agreement with earlier studies (Chavkin and Goldstein, 1981; Corbett et al, 1982; Magnan et al, 1982; Oka et al, 1982; 1983; Yoshimura et al, 1982), they found that dynorphin A(1-8) appears to be the minimal sequence necessary to confer κ receptor affinity and selectivity, the longer forms appearing more potent due to their increased stability to peptidase degradation. Whereas Corbett et al (1982), had found that the extension of the COOH terminal sequence of Leu-enkephalin by the addition of an arginine in position 6, resulted in a marked (approximately 35-fold) increase in κ receptor affinity and a decrease in δ affinity (approximately 8-fold), further accentuated by the addition of another arginine in position 7, Mansour et al (1995) have proposed that a Try-Gly-Gly-Phe-Leu or -Met core

and an Arg^6-X^7 extension (where X = Arg or Lys) is required to achieve high affinity binding at the κ sites.

Clues as to how such receptor discrimination may result, may be gained from the findings of chimeric hybrid and site-directed mutagenesis studies of the cloned receptors. Whereas the cloned opioid receptors are highly homologous to one another on an amino acid level, the more limited selectivity range of the endogenous opioid peptides for the μ and δ receptors, especially β-endorphin, suggests that these receptors may be more structurally similar to one another than the κ receptors. A schematic representation of an opioid receptor is illustrated on figure 4. As indicated in section 1.3 the most divergent regions of the cloned receptors has been shown to be the extracellular NH_2- and intracellular COOH- termini, and the extracellular loops (EC). Studies examining the binding properties of μ-κ and δ-κ receptor chimeras, have shown that κ agonist selectivity is derived from the interaction with the fourth transmembrane domain (TM) and EC2 (Kong et al, 1994a; Wang et al, 1994; Xue et al, 1994; Meng et al, 1995). In contrast the agonist selectivity of the μ/ δ receptors appears to depend on interactions in the region of TM5 to TM7 domains, the δ receptor additionally requiring interactions with the COOH-terminal half of TM3 to EC2, while the μ receptor binding with the NH_2-terminus half of TM3 (Fukuda et al, 1995; Meng et al, 1995). Mutagenesis studies have also shown that the negative charges found in the EC2 loop of the κ receptor play a critical role. By interacting with the positively charged amino acids in positions 6 and 7 of COOH-terminally extended enkephalin peptides, such as dynorphin, these negative charges possibly serve to stabilise the peptide-receptor configuration, and hence allow the peptides to fit into the κ receptor binding pocket, in alignment to the NH_2-terminal tyrosine (Meng et al, 1993). Interestingly, such chimeric studies have shown that, in contrast to the μ, δ and most other known receptors, the κ receptor selective antagonists do not bind in the region of this agonist recognition site, but instead interact with entirely separate sites within the NH_2-terminus of the receptor (Kong et al, 1994b). Reisine (1995) has proposed that this NH_2-terminus must be able to fold upon the κ receptor, to move the separate

FIGURE 4. Schematic representation of an opioid receptor, illustrating the different extracellular (EC), transmembrane (TM) and intracellular (IC) domains.


NH2
EC1
EC2
EC3
extracellular
TM1
TM2
TM3
TM4
TM5
TM6
TM7
intracellular
IC1
IC2
IC3
COOH

recognition sites to be in close proximity, to allow for competition of agonist and antagonist binding.

A physiological consequence of the ability of an opioid peptide to potentially stimulate each class of opioid receptor, is that the functional expression of the activation of opioid synthesising neurones is dependent not only on the final peptide(s) forms that are synthesised and the concentrations following release, but also by the relative abundance of μ, δ and κ receptors present at the site of release.

1.5 OPIOIDS III: ACTIONS AT A CELLULAR LEVEL

Overall, opioids have been demonstrated to be inhibitory to neurotransmission, through a depression of neuronal firing rate and a reduction of neurotransmitter release. Where excitatory effects of opioids have been observed, for example in the olfactory bulb, hippocampus, ventral tegmental area and substantia gelatinosa of the spinal cord, they have been proposed to be the result of disinhibition (that is inhibition of inhibitory neurones) (Duggan and North, 1984). A number of *in vitro* preparations have been employed to investigate the actions of opioids at the cellular level. These include the guinea-pig myenteric (primarily μ and κ receptors) and submucous plexuses (predominantly δ receptors), the rat locus coeruleus (primarily μ receptors), various preparations of the spinal cord and dorsal root ganglion (DRG), and neuroblastoma derived cell lines. These studies have shown the activation of opioid receptors to result in at least one of three G-protein linked events, namely an increase in an outward potassium conductance, a reduction in a voltage dependent calcium conduction and an inhibition of the enzyme adenylate cyclase, each of which will now be considered in turn (recently reviewed by McFadzean, 1988; Loh and Smith, 1990; North, 1993; and Childers, 1993).

1. EFFECT OF OPIOIDS ON POTASSIUM CHANNELS

An increase in membrane potassium conductance following opioid administration has been demonstrated directly by measurement of membrane reversal potentials under voltage clamp conditions or indirectly via membrane resistance decreases and reductions in the opioid effect by potassium channel blockers, such as tetraethylammonium (TEA), barium or caesium. Increased potassium conductances have been observed following (a) μ receptor activation for example, in the guinea-pig myenteric plexus (North and Tonini, 1977), both the guinea-pig (Pepper and Henderson , 1980) and rat (Williams et al, 1982) locus coeruleus, the substantia gelatinosa of the rat spinal cord (Yoshimura and North, 1983), and mouse DRG cells (Werz and MacDonald, 1983); (b) following δ receptor activation in the guinea-pig submucous plexus (Mihara and North, 1986) and mouse DRG cells (Werz and MacDonald, 1983); and more recently (c) following κ receptor activation in the substantia gelatinosa of the guinea-pig spinal trigeminal nucleus (Grudt and Williams, 1993).

Potassium channels can either be affected by membrane potential, by the binding of intracellular messengers, or by both. Studies of the potassium conductance activated by opioid peptides in the rat locus coeruleus and guinea-pig submucous plexus, have suggested that an 'inwardly rectifying' type of potassium current is affected, the conductance becoming larger as the membrane is hyperpolarised (North et al, 1987). However, evidence that other potassium conductances can be affected has also been shown (North, 1993). Thus, there appears to be no species of potassium channel that is uniquely opened by opioid action, rather several distinct potassium channels can be activated. Pertussis toxin sensitive G-proteins, possibly including G_o, have been found to link the opioid peptides to these potassium channels. It has been proposed that the G-proteins might bind directly to the voltage dependent channels (Brown and Birnbaumer, 1990). The net result on the neurone of increasing the potassium conductance is likely to be a hyperpolarisation and reduction in the firing rate, whilst that on the terminal a reduction in calcium influx and an inhibition of transmitter release (North, 1993).

In some preparations, notably the locus coeruleus slice (Williams et al, 1982) and substantia gelatinosa of the spinal cord (Yoshimura and North, 1983), hyperpolarisations following opioid administration have been observed in a calcium-free medium, which implies a direct postsynaptic effect. Such postsynaptic actions of opioids have implications for the selective effects of opioids demonstrated in various parts of the CNS. For example, in the spinal cord opioids have been found to selectively inhibit C but not A fibre evoked neuronal activity (as will be discussed later in section 1.7).

2. EFFECTS OF OPIOIDS ON CALCIUM CHANNELS

μ, δ and κ receptors also appear to suppress calcium conductances. The most abundant type of calcium channels found in mammalian nerve cells are voltage dependent, classified as L, N, and T, according to whether their conductances are of a large, intermediate or transient nature. Reductions in a voltage dependent calcium conductance (independent of actions on potassium channels), were observed under voltage clamp conditions initially for κ receptors of cultured mouse DRG neurones (MacDonald and Werz, 1986), but have since been demonstrated (a) for μ receptors of the human neuroblastoma cell line SH-SY5Y (Seward et al, 1991) and adult rat dissociated DRG neurones (Schroeder et al, 1991); (b) for δ receptors of guinea-pig submucous plexus neurones (Surprenant et al, 1990); and (c) for κ receptors in rat cultured DRG (Bean et al, 1989) and spinal cord (Sah et al, 1990) neurones. Effects on calcium channels have also been inferred from reductions in action potential duration observed in the presence of voltage gated potassium channel blockers (Werz and MacDonald, 1985), although possible contributions from unblocked residual potassium receptors cannot be disregarded (North, 1993). From such studies on cultured DRG neurones has come the somewhat surprising observation that low concentrations (nM) of specific opioid receptor agonists (e.g. DAMGO, DPDPE or U50488H) may actually result in an prolongation of the action potential duration, by a mechanism distinct from that responsible for inhibiting the action at higher doses (μM) (Crain and Shen, 1990). However, support that this phenomenon

actually occurs *in vivo* is lacking. The calcium current inhibited appears to be of the N type (Seward et al, 1991; Surprenant et al, 1990). The opioid receptors appear to produce this effect again directly via pertussis sensitive G-proteins (Brown and Birnbaumer, 1990). It has been suggested that this G-protein binding may change the voltage dependent state of the calcium channel, thereby reduce the probability that it will open with membrane depolarisation (Bean et al, 1989). Whereas it has been proposed that such an action of opioids on calcium channels could underlie the presynaptic inhibition of neurotransmitter release, it is also as likely that an increased potassium conductance leading to earlier repolarisation and shorter action potential duration could also be involved in mediating such an effect, as previously described (North, 1993).

3. EFFECTS OF OPIOIDS ON ADENYLATE CYCLASE

Of all the second messenger systems which have been implicated with opioid receptor function, the inhibition of adenylate cyclase mediated by the G-protein G_i, is the best characterised. Although the first reports of opioid-inhibited adenylate cyclase were from brain membranes (Collier and Roy, 1974), much progress in understanding this mechanism has come from studies employing transformed cell lines such as the NG108-15 neuroblastoma x glioma hybrid cells (Sharma et al, 1975). This is now recognised as a sodium and pH dependent mechanism to which all three opioid receptors may be coupled (Schoffelmeer et al, 1987; Chrieiweiss et al, 1988; Dunman et al, 1988; Attali et al, 1989) although the evidence for the κ receptors is somewhat controversial (Childers, 1993). However, the relationship between opioid-inhibited adenylate cyclase and opioid-inhibited neurotransmitter release is unclear, most electrophysiological evidence to date suggesting that cyclic AMP plays no direct role in the effect of opioids on potassium and calcium conductances (Childers, 1993; North, 1993). For example, opioid responsive potassium (North et al, 1987) and calcium (Gross et al, 1990) currents recorded under whole cell voltage clamp conditions have been found to be unaffected by several procedures likely to enhance or mimic the actions of cyclic AMP, such

as the intracellular administration of forskolin or the catalytic subunits of cyclic AMP-dependent protein kinase (protein kinase A). An exception maybe the spinal cord, since evidence has been presented that the excitatory opioid effects on calcium conducance of mouse DRG cultures may be mediated by phosphorylation with protein kinase A via the G-protein G_s (Chen et al, 1988). Instead it has been proposed that opioid-inhibition of adenylate cyclase may play more of a long term regulatory role in modulating neuropeptide synthesis and receptor function, several neuropeptide genes, including proEnk, containing cyclic AMP-responsive promoter elements (CRE) and most G-protein coupled receptors, including opioid receptors, being regulated by phosphorylation (Childers, 1993).

The distinction between the opioid receptors at the cellular level is far from clear, no class of opioid receptor appears to be uniquely associated with a given ion channel or second messenger system, the μ, δ and κ opioid receptors having now been described to have effects on both potassium and calcium conductances. This along with evidence that these receptors can co-exist on the same neurone (e.g. DRG neurones, Werz et al, 1987) and the lack of ligand selectivity displayed by most opioids peptides reveals a somewhat bewildering complexity (or simplicity, if redundancy occurs) of the opioid systems. The action of opioids to increase membrane potassium conductance and inhibit neuronal calcium currents is shared by other members of a subgroup of the superfamily of G-protein coupled receptors to which the opioid receptors belong, indeed in many cases several receptors are expressed in the same cells and their activation involves the same conductances, as shown for noradrenaline α_2, somatostatin and δ receptors in the guinea-pig submucous plexus (Mihara et al, 1987; Suprenant et al, 1990). In contrast to these short term effectors, diffusible second messenger systems like cyclic AMP may be responsible for more long term changes of opioid action.

1.6 OPIOIDS IV: DISTRIBUTION IN THE MAMMALIAN CNS

As previously discussed (sections 1.3 and 1.4), three distinct types of opioid synthesising neurone exist in the mammalian CNS that may release a number of opioid active peptides, to exert their effects via at least three types of receptor. These neuronal systems have been found to be widely distributed throughout the CNS and to influence many functions including nociception and analgesia, cardiovascular regulation, gastrointestinal activity, respiration, appetite and thirst, thermoregulation, motor function, neuroendocrine and neuroimmune activity, learning and memory, reinforcement and reward behaviour (Khachaturian et al, 1993).

Some indication of the relative distribution of the opioid systems has been provided by comparing tissue levels of distinct opioid peptides and binding sites using radiolabelled specific antisera and ligands respectively. However, a more detailed anatomical description has been elucidated using immunocytochemical and *in situ* hybridisation techniques. Opioid peptides have been visualised to a cellular and subcellular level by light and electron microscopy, using a variety of labelled antisera. However, these studies may be limited by the sensitivity of the antibodies and their ability to penetrate to intracellular storage sites. Problems with the specificity of the immunological reaction can also occur. By detecting the mRNA for each precursor, the locations of the cell bodies of these opioid synthesising neurones can be identified with more certainty, the levels serving as a measure of the biosynthetic activity of the opioid system. Until recently autoradiography remained the only method for localising opioid ligand binding sites in the CNS. These studies have been limited by not only the availability of highly specific ligands but also on the ability to employ electron microscopic methods and other anatomical techniques sequentially. It is hoped now that either by using labelled antibodies raised to distinct amino acid sequences of the newly cloned receptors or *in situ* hybridisation oligonucleotide probes to visualise the receptors mRNA, the distribution of the opioid receptors will be determined to a higher degree of anatomical

resolution, including at an ultrastructural level, and perhaps even allow the putative subtypes to be differentiated.

The functional implications that can be derived from such anatomical studies is obviously limited, providing no evidence of whether a localised peptide will be released or whether a receptor site is functional. However, they do serve to highlight areas of potential interest. Thus, following a general overview of the distribution of the opioid peptide-receptor systems in the brain, that at the spinal cord level will be considered in greater depth.

1. DISTRIBUTION OF THE OPIOID SYNTHESISING NEURONES

POMC has been shown to be synthesised in the brain as well as the anterior and intermediate lobes of the pituitary gland in a number of species including the rat, monkey and man (Khachaturian et al, 1993). The pituitary is the major source of the POMC biosynthesis. In the brain immunocytochemical and *in situ* hybridisation studies have shown two distinct POMC cell groups to exist, namely in the arcuate nucleus of the hypothalamus (Bloom et al, 1978; Watson et al, 1978) and the nucleus tractus solitarus of the medulla (Joseph et al, 1983; Schwartzberg and Nakane , 1983; Gee et al, 1983). POMC mRNA has also been detected in the cerebral cortex, basal ganglia and amygdala (Civelli et al, 1982), and there is recent data to suggest that some β-endorphin containing cell bodies might be scattered caudally down the spinal cord (Gustein et al, 1992; Plantinga et al, 1992). Lesion and deafferentation studies have revealed the POMC cell bodies located in the arcuate have extensive projections throughout the brain to a diverse number of forebrain and midbrain structures. The main rostral targets include several hypothalamic nuclei, septum and preoptic area, where as the main lateral projections innervate several amygdaloid nuclei. Caudal targets include many areas considered to be associated with nociception, such as the periventricular nucleus of the thalamus, and the periaqueductal gray and raphe nuclei of the brain stem. Nerve fibres from the nucleus tractus solitarus in contrast appear to project mostly within the medulla itself and possibly into the spinal cord. Certain areas of the medulla oblongata including the nucleus

tractus solitarus, that are involved in respiration and cardiovascular regulation receive innervation from POMC cells in the arcuate as well as the nucleus tractus solitarus (as reviewed by Khatachurian et al, 1985a; b; 1993; Young et al, 1993) .

ProEnk synthesis has been shown widely throughout the neuroaxis from the cerebral cortex to the spinal cord in a number of species including the rat, monkey and man (Khachaturian et al, 1993). Immunocytochemical studies have found enkephalin containing neurones to form both short and long tract projections (Hokfelt et al, 1977a; b; 1979; Sar et al, 1978; Uhl et al, 1979; Finley et al, 1981; Conrath-Verrier et al, 1983; Standaert et al, 1986; Merchanthaler et al,, 1986; Menetrey and Basbaum, 1987; Nahin et al, 1987; Leah et al, 1988; Arvidsson et al, 1992). The use of the neurotoxin colchicine to inhibit microtubular axonal transport has enabled the cell bodies of smaller neurones and those synthesising relatively low amounts of proEnk to be visualised. Thus, proenkephalin containing cell bodies have been located in most regions of the telecephalon, including the cerebral cortex, amygdala, hippocampus, striatum, septum and preoptic area; most hypothalamic and thalamic nuclei in the diencephalon, such as the periventricular and lateral geniculate nuclei; in the midbrain, to the interpeduncular nucleus, the substantia nigra, the periaqueductal grey, and the superior and inferior colliculi; to a number brainstem nuclei including the raphe magnus, nucleus tractus solitarius, lateral reticular and spinal trigeminal nuclei; and at the level of the spinal cord, to the dorsal horn (as reviewed by Petrusz et al, 1985; Khachaturian et al, 1985b; 1993). *In situ* hybridisation studies performed more recently are in good agreement with these immunocytochemical results with even more proEnk containing cell bodies being observed in regions of the neocortex, nucleus accumbens, striatum and the cerebellum (Harlan et al, 1987). Since all these regions also contain enkephalin-positive fibres and terminals, this opioid system has the potential to influence a wide variety of CNS functions.

ProDyn, like proEnk, has been demonstrated to be synthesised in many neuronal systems throughout the brain and spinal cord of a number of species, including the rat, monkey and man (Khachaturian et al, 1993). In addition, proDyn is also synthesised in the anterior and

posterior lobes of the pituitary gland. Immunocytochemical studies have found dynorphin containing neurones to form both short and long tract projections (Vincent et al, 1982; Khachaturian et al, 1982; Menetrey and Basbaum, 1987; Nahin et al, 1987; Leah et al, 1988). Again the application of colchicine increased the ability to detect the presence of proDyn peptides in neuronal cell bodies. Such studies have shown proDyn peptides to be present in cell bodies in the cerebral cortex, amygdala, hippocampus, striatum, several hypothalamic and thalamic nuclei, the periaqueductal grey, numerous brain stem nuclei including the nucleus tractus solitarus, lateral reticular and spinal trigeminal nuclei, as well as in the dorsal horn of the spinal cord. Additionally fibres and terminals containing proDyn peptides are further found in many other regions of the CNS including the globus pallidus, substantia nigra and raphe nuclei (as reviewed by Khachaturian et al, 1985b, 1993; Fallon and Ciofi, 1990). This distribution has been confirmed to a limited extent by more recent *in situ* hybridisation studies (Morris et al, 1986; Ruda et al, 1988; Weihe et al 1989).

Thus, whilst the expression of the POMC is restricted to just a few brain areas, that of proEnk and proDyn appears to be widely distributed throughout the CNS. In general, the findings of both immunocytochemistry and *in situ* hybridisation studies suggest that although cells containing proEnk are more numerous than those of proDyn, their distribution throughout the brain and the spinal cord is often in parallel, although species differences have been found to exist. Notably considerable overlapping populations exist in regions implicated in the processing of nociceptive information, such as the periaqueductal grey and dorsal horn of the spinal cord. Although there is some evidence to support the coexistence of these two opioid peptide families in the CNS (Guthrie and Basbaum, 1984; Sasek and Elde, 1986; Weihe et al, 1988; Tuchscherer and Seybold, 1989; Fallon and Ciofi, 1990), this has still to be satisfactorily investigated, for example using double labelling techniques at the mRNA level. In contrast to proEnk, the distribution of the mRNA of proDyn has only been investigated in certain areas of physiological interest. Since the Leu-enkephalin sequence can be in both proEnk and proDyn derivatives there is the possibility of cross reactivity between

the two systems in immunocytochemical studies (Khatachurian et al, 1985b, 1993; Elde and Hokfelt, 1993)

2. DISTRIBUTION OF THE OPIOID RECEPTORS

The majority of studies to date have employed radiolabelled ligands to identify opioid binding sites which were equated with receptors. Whereas the anatomical distribution of the multiple opioid receptor systems is very similar across mammalian species from the rat to the monkey, the distribution and relative abundance of each opioid receptor type has been found to sometimes vary dramatically (as reviewed by Mansour et al, 1988; Adler et al, 1990; Mansour and Watson, 1993).

The **μ receptors** using relatively selective radioligands, such as [^{3}H]-DAMGO, have been shown to be widely distributed throughout the CNS of both the rat and monkey (Mansour and Watson, 1993). Particularly dense binding is observed in basal ganglia and limbic structures, thalamic and brain stem nuclei, including those associated with nociception, such as the periventricular region, periaqueductal grey and raphe nuclei, as well as the dorsal horn of the spinal cord. Marked species variations have been noted in the cerebral cortex and in the hypothalamus.

The **δ receptors** display the most restricted distribution of all the opioid receptors, with binding mainly concentrated in forebrain regions. The distribution of these receptors is also well conserved between mammalian species. For example, in both rat and monkey the overall similarity of δ receptor distribution using radioligands such as [^{3}H]-DPDPE is remarkable (Mansour and Watson, 1993). Here the δ binding appears densest in the cerebral cortex, striatum and amygdala, sparse to non-existent in the hypothalamus and thalamus, and poor in most brainstem regions where the δ binding is limited to a few structures such as the substantia nigra and the nucleus tractus solitarus, as well as the dorsal horn of the spinal cord.

The **κ receptors** demonstrate some of the most striking species differences in distribution observed amongst all the opioid receptor types. For example, in the rat these binding sites

comprise only 10% of the total number of opioid receptor sites, whilst in most other species, such as the guinea-pig, monkey and human, these sites represent at least a third of the opioid receptor population (Mansour and Watson, 1993). In all species examined so far, κ sites labelled with non-selective radioligands such as [^{3}H]-bremazocine or [^{3}H]-EKC in the presence of unlabeled DAMGO and DPDPE, and more recently using more selective ligands such as [^{3}H]-U69593, have been found to be widely distributed throughout the neuroaxis. Marked species differences are observed in forebrain areas such as the cerebral cortex, hippocampus and basal ganglia, but not those of the midbrain and hindbrain, such as the periaqueductal grey, superior and inferior colliculi, raphe and spinal trigeminal nuclei, and the dorsal horn of the spinal cord.

Overall, the distribution of the opioid receptor types is well conserved across species in brainstem and spinal cord areas, varying most notably in forebrain and midbrain structures, although that of the δ receptor sites appears to be well conserved throughout the entire neuroaxis. The distributions of the μ and κ receptors are parallel to some extent. Interestingly the distribution of the opioid receptors appears to be well conserved across species within certain functional systems. Notably aside from those regions considered important for the processing of nociceptive information, particularly dense binding is observed on structures of the limbic system such as the amygdala and hypothalamus, that regulate emotional behaviour and which may be responsible for the euphoric effects of opiates (Mansour et al, 1988, Mansour and Watson, 1993). Recent *in situ* hybridisation studies have visualised the distribution of the cloned receptors mRNA, and on the whole these distributions show a good correlation to those by autoradiography (as recently reviewed for the rat CNS; Mansour et al, 1995a). Such studies, however, allow the cell synthesising the receptors to be located but provide no information of the final distribution of the receptors within a cell. Some investigations using antibodies to defined sequences of the cloned opioid receptors have been performed (Arvidsson et al, 1995a; b; Mansour et al, 1995b; 1996) but much work remains to be done, particularly at the ultrastructural level and in combination with other anatomical

techniques such as tract-tracing and dual labelling procedures, to explore the relationship of the opioid receptors to other neurotransmitter systems. As discussed in section 1.4 no clear relationship exists between an opioid peptide family and a specific type of opioid receptor. This is further evident from the numerous reported 'mismatches' in opioid peptide-receptor distributions found in the mammalian CNS, with no clear parallelism between the distributions of a particular opioid peptide and receptor being found, including at the level of the spinal cord as will now be described.

3. DISTRIBUTION AT THE SPINAL CORD LEVEL

Members of the each of the three opioid peptide families have been identified in the mammalian spinal cord, but to a strikingly different extent. Whereas enkephalin and dynorphin immunoreactivity has been found in numerous cell bodies and axon varicosities, no neuronal cell bodies containing β-endorphin have been immunocytochemically labelled in the spinal cord of the adult rat and only a few scattered axon varicosities have been found (Tsou et al, 1986). However, more recent studies have indicated that some β-endorphin synthesis may occur at the spinal cord level, with reports of β-endorphin immunoreactivity measured by radioimmunoassay persisting in segments caudal to a thoracic spinal transection (Gustein et al, 1992), and of the detection of POMC mRNA in the rat spinal cord (Plantinga et al, 1992).

Enkephalin immunoreactivity has been described in cell bodies and fibres of laminae I to VII of the spinal cord (concentrated in lamina I-II) and in the region around the central canal, lamina X (rat: Hokfelt et al, 1977a; b; Sar et al, 1978; Uhl et al, 1979; Hunt et al, 1980; Finley et al, 1981; Stengaard-Pedersen and Larsson, 1981; Jansco et al, 1981; Gibson et al, 1981; Petrusz et al, 1985; Sasek and Elde, 1986; Miller and Seybold, 1987; 1989; Weihe et al, 1988. cat: Glazer and Basbaum, 1983; Bennet et al, 1982; Conrath-Verrier et al, 1983; Cruz and Basbaum et al 1985; Petrusz et al, 1985; Miller and Seybold, 1987; 1989. guinea-pig: Stengaard-Pedersen and Larsson, 1981). *In situ* hybridisation studies have confirmed this diffuse presence, labelled cell bodies being found in all laminae of the spinal cord except for

the motoneuronal cell groups (rat: Harlan et al, 1987; Noguchi et al, 1992), suggestive of a diverse role of this opioid system in spinal cord function. Although there is some evidence that enkephalin containing fibres may descend from the brainstem (rat: Hokfelt, 1979; Menetrey and Basbaum, 1987. cat/ monkey: Arvidsson et al, 1992) or originate from DRG (rat: Senba et al, 1982; 1989; Pohl et al, 1994. cat: Garry et al, 1989), most of the enkephalin immunoreactivity appears to reside in neurones intrinsic to the spinal cord. Some of these spinal neurones, notably those located around the central canal region in laminae X, appear to form ascending pathways to regions of the midbrain and diencephalon (rat: Standaert et al, 1986; Leah et al , 1988). There is evidence for co-localisation of enkephalin with substance P (rat: Senba et al, 1988; Ribeiro-da-Silva et al, 1991), γ-aminobutyric acid (GABA) (rat: Todd et al, 1992); 5-hydroxytryptamine (5HT) (cat/ monkey: Arvidsson et al, 1992) and somatostatin (rat: Todd and Spike, 1992), particularly in cells and terminals in the superficial dorsal horn .

ProDyn synthesising neurones have been found to display a considerably overlapping, although more restricted distribution to those containing proEnk, being located in cell bodies and fibres in laminae I-II, laminae IV-VI, and in the area around the central canal, lamina X (as will be considered in detail in section 4.4). Like enkephalin most of dynorphin immunoreactivity resides in neurones intrinsic to the spinal cord, with some evidence for coexistence with substance P (Tuscherer and Seybold, 1989) and as previously indicated co-localisation with enkephalin itself (Sasek and Elde, 1986; Weihe et al, 1988; Tuscherer and Seybold, 1989; Fallon and Ciofi, 1990). It is of note that although most studies have been performed at the lumbosacral level of the spinal cord, no significant rostrocaudal differences in the distribution of these peptides have been found. The localisation of proEnk and proDyn containing neurones in the superficical laminae and the neck of the spinal dorsal horn where small diameter primary afferents terminate has led to the function of these peptides at the spinal level to be discussed predominantly in context of the processing of nociceptive information (as will be further discussed in section 1.7).

All three types of opioid receptor have been localised to the dorsal horn of the mammalian spinal cord (rat: Gouardares et al, 1985; Morris and Hertz, 1987. guinea-pig: Gouardares et al, 1985). By autoradiography marked differences have been found to exist in the distribution of the μ, δ and κ binding sites in the rat lumbar spinal cord. Morris and Hertz (1987) found that whilst μ binding sites, labelled with [^{3}H]-DAMGO, were detected to a significant extent in the substantia gelatinosa (lamina II) and to a lesser extent laminae III-V and lamina VIII of the ventral horn, the levels of δ sites labelled with [^{3}H]-DADLE (in the presence of DAMGO) and κ sites labelled with [^{3}H]-bremazocine (in the presence of DAMGO and DADLE) were low and notable only in lamina I and lamina II respectively. This was later confirmed by studies employing quantitative densitometric analysis (Besse et al, 1991, Stevens et al, 1991). These studies found a remarkable degree of homology in the respective proportions of the three types of opioid receptor along the rostrocaudal axis of the spinal cord. For example, Besse et al (1991) showed for the rat that there is a high percentage of μ (70-74% of total opioid binding), an intermediate percentage of δ (18-20%), and a low percentage of κ (7-10%) binding sites at each of the spinal cord levels. Previous reports of rostrocaudal differences in the same or other species are believed most likely due to the use of poorly selective ligands and different tissue preparations (Lombard et al, 1995). As noted above, κ receptors have demonstrated some of the most striking species differences in the distribution of all the opioid receptor types. For example, in human studies, Gouarderes et al (1986) reported very low levels of the μ and δ opioid binding sites in the lumbosacral spinal cord, the only significant sites being those of κ. This is in contrast to the rat were only low levels of κ opioid binding sites are reported (Mansour et al, 1988). What this potentially means to the functioning of the opioid systems at the spinal level is yet unknown and the possibility exists that the rat may represent an atypical model for man with respect to the role of κ mediated opioid receptor systems. It should be noted however, for the purpose of studying the function of the endogenous opioid peptides themselves such a caveat is unlikely to exist due to their poor receptor selectivities and relatively minimal species variance in distribution.

There is evidence that a proportion of the opioid binding sites in the superficial layers of the dorsal horn are located near or on the spinal terminations of primary afferent fibres. Earlier binding and autoradiographic studies showed a clear decrease in opioid binding at this level following dorsal rhizotomy (monkey: La Motte et al, 1976; Ninkovic et al, 1982 rat: Ninkovic et al, 1981), or peripheral nerve section (rat: Fields et al, 1980). Such studies, however cannot exclude the possibility that trans-synaptic degeneration and damage to segmental arteries during rhizotomy might have occurred to produce cell death in the dorsal horn. However, the findings of electrophysiological studies on DRG neurones (as indicated in section 1.5) along with the reduction in binding observed in rats treated neonatally with capsaicin (a homovanillic acid derivative known to cause selective degeneration of small diameter primary afferent neurones; Jansco et al, 1977; Nagy et al, 1981) have also pointed to the preferential localisation of these receptors on a subpopulation of small diameter (mainly unmyelinated) primary afferent fibres (Gamse et al, 1979). Besse et al (1990) used the quantitative receptor autoradiographic technique to gauge the relative pre- and post- synaptic locations of the μ, δ and κ sites in the superficial layers of the rat cervical dorsal horn, 8 days after a large unilateral dorsal rhizotomy (C4 to T2), corresponding to the brachial plexus. Their results suggested a high percentage of the superficial laminae μ ([^{3}H]-DAMGO) and δ ([^{3}H]-DTLET) sites were located presynaptically, with decreases in binding of 76% and 61% respectively being observed at C7. However, the percentage decrease they observed for the κ sites (53%) needs to be considered with caution, since the non-selective ligand [^{3}H]-EKC was used to label these sites, and again the possibility of trans-synaptic degeneration cannot be excluded. Notably more recent studies employing antibodies to the cloned opioid receptors have reported that whereas μ (rat: Arvidsson et al, 1995b; Ding et al, 1995; Mansour et al, 1995b. monkey: Honda and Arvidsson, 1995) and κ (rat: Arvidsson et al, 1995c; Mansour et al, 1996) receptors appear to be present on both primary afferent axons and cell bodies and dendrites in the superficial dorsal horn, δ (rat: Dado et al, 1993; Arvidsson et al, 1995a;

Cheng et al, 1993. monkey: Honda and Arvidsson, 1995) receptors are mainly restricted to axons of presumed primary afferent nature.

Using *in situ* hybridisation techniques cells expressing μ, δ, and κ receptor mRNA have been detected in both the dorsal and ventral horns of the rat spinal cord, as well as DRG (as reviewed by Mansour et al, 1995a). Notably Maekawa et al (1994; 1995) found that in the rat lumbar spinal cord whereas the expression of μ and κ opioid receptor mRNAs was intense in cell bodies of laminae I-II, with additional moderate to intense signals for the μ receptor in laminae VII-VIII and for the κ receptor throughout lamina III-VIII, that of the δ receptor mRNA was very different. In contrast a marked expression of the δ receptor mRNA was found only in the ventral horn, with an intense signals observed on most of the large nuclei (likely motoneurones) in lamina IX.

1.7 OPIOIDS V: ROLE IN SPINAL CORD FUNCTION

An integral part of opiate drug action and the major reason for opiate use by man is their ability to alter sensations, from reducing the awareness of pain to producing states of euphoria. Since the opioid peptides were discovered due to their 'morphine-like' effects, considerable attention has been directed to the possible involvement of these peptides in the central transmission of sensory information. A major focus of interest has been the relationship of the opioids to the processing of peripheral stimuli normally perceived as painful, especially at the level of the spinal cord, where opiates appear to act to dramatically reduce the amount of nociceptive information reaching the brain. However, it is clear from the unwanted side effects of opiate drugs alone, that the endogenous opioid peptides likely have other actions. For example, the spinal administration of opiates in the intact and unaesthetised animal has revealed powerful receptor mediated effects on motor, cardiovascular, gastrointestinal and bladder function, suggestive of roles on somatomotor and autonomic systems (as reviewed by Yaksh and Nouiehed, 1985; Duggan and Fleetwood Walker, 1993;

Yaksh, 1993). Thus, in considering the role of opioids in spinal cord function although the major focus will be the processing of sensory information especially in relation to noxious stimuli, the association of these peptides with other spinal cord pathways must also be addressed. To do this the findings of electrophysiological and behavioural studies employing either systemic opiates or locally applied opioid receptor ligands will be reviewed for actions on nociceptive and non-nociceptive pathways. First a brief overview of the basic anatomy involved will be given.

Electrophysiological recordings of nerve fibres in animals, as well as psychophysical studies in man, have established that information concerning potentially damaging (or noxious stimuli), acting on cutaneous and deep tissue, is transmitted by small diameter myelinated (Aδ or group III) and unmyelinated (C or group IV) afferent fibres, whose cell bodies are located in ganglia outside the spinal cord. The central branches of these fibres run via the dorsal roots to terminate in the spinal grey matter of the dorsal horn. Rexed (1952) divided the spinal grey matter on a cytoarchitectonic basis into 10 laminae. A combination of electrophysiological and neuroanatomical techniques have shown that in general whereas cutaneous derived Aδ and C afferents terminate in the superficial dorsal horn in lamina I (marginal zone) and the outer lamina II (substantia gelatinosa) respectively (Light and Perl, 1977), the group III and IV afferents of deeper origin (i.e. muscle, visceral and joint) terminate to a greater extent in the deep dorsal horn laminae V-VI, as well as lamina I (Craig and Mense, 1983; Cervero and Connell, 1984; Craig et al, 1988). The non-nociceptive large diameter myelinated (cutaneous, Aα/ β or deep tissue, group I/ II) afferent fibres by contrast terminate in inner lamina II, lamina III-IV (nucleus proprius) and lamina V-VI of the dorsal horn (as reviewed by Besson and Chaoch, 1987). The dorsal horn neurones onto which the fine afferents terminate, provide the first point for the integration of these nociceptive signals and represent the first opportunity for modulation from intraspinal and supraspinal sources. These neurones either project to propriospinal or supraspinal destinations, or remain intrinsic to the same spinal cord segment, relaying the nociceptive message to higher order neurones that project to brain

centres involved in the perception of pain and/ or contact motoneurones that mediate spinal reflexes. Thus, although cells which fire to peripheral noxious stimuli (either mono- or polysynaptically) are widely distributed in the spinal cord, many are located in laminae I, II and V near the central terminals of peripheral nociceptors. The majority of lamina II cells are short, local circuit neurones (including the morphologically distinct 'islet' and 'stalk' cells) that make connections with other lamina II cells and dendrites that penetrate this lamina from neurones in laminae I, III-V, although the axons of some cells send their axons into lamina I (Bennet et al, 1979) and into the deeper laminae III-V (Light and Kavookjian, 1988). By contrast many lamina I and V cells are projection neurones, some lamina V cells having dendrites that penetrate into the superficial dorsal laminae making direct contacts with both small and large diameter primary afferents possible. Of the neurones that project supraspinally, transmitting nociceptive information to the brain, a considerable proportion ascend in the contralateral ventrolateral quadrant of the spinal cord. Although the origin and destination of these tracts are to some extent species dependent, they have been found in certain species (notably the rat, cat and monkey) to form a major part of the spinothalamic tract (laminae I, IV-VII), and some part of the spinomesencephalic (lamina I and V), spinoreticular (lamina VII and VIII), spinocervicothalamic (laminae III-V) tracts, as well as that of the postsynaptic dorsal column pathway (laminae III-V) (as reviewed by Besson and Chaoch, 1987). There is considerable evidence that nociceptive transmission is subject to control in the spinal cord, both segmentally (Melzack and Wall, 1965) and via descending fibres from areas of the brainstem (Reynolds, 1969), such as the periaqueductal grey matter, rostral ventromedial medulla (including the nucleus raphe magnus) and dorsolateral pontomesencephalic tegmentum (as reviewed by Fields and Basbaum, 1994).

1. OPIOIDS AND NOCICEPTIVE PATHWAYS

Evidence that opioids could exert direct effects on nociceptive pathways in the spinal cord came initially from the findings of electrophysiological studies that systemic opiates reduce

polysynaptic (flexor) but not monosynaptic (extensor) spinal reflexes in spinal animals (Wikler, 1950), and behavioural studies that intrathecally applied opiates produce regional analgesia without effecting voluntary motor activity (Yaksh and Rudy, 1976, 1977). The magnitude of this effect on reflexes was found to be most pronounced on motor neuronal activity evoked by high intensity electrical nerve stimulation or the application of noxious stimuli to spinal animals (cat: Koll et al, 1963; Bell and Martin, 1977. rabbit: Clarke and Ford, 1987. dog: Martin et al, 1976) and paraplegic man (Willer, 1985). Although there is relatively little data on the cell types affected by systemic opiates, an early study found that analgesic doses of morphine had a greater effect in reducing the spontaneous firing of neurones of lamina I and V, than any other laminae (Kitahata et al, 1974). Recordings in spinal cats revealed a preferential, naloxone reversible, suppression of Aδ and C fibre evoked excitations of dorsal horn neurones responsive to both innocuous and noxious inputs, following the systemic administration of a variety of opiates at analgesic doses (Le Bars et al, 1976; Duggan et al, 1980). Notably, such a selective inhibitory effect was demonstrated on the firing of ventrolateral tract neurones to C fibre input in the intact cat (Jurna and Grossman, 1976) and rat (Jurna and Heinz, 1979). In general, similar inhibitory effects have been observed when 'natural' noxious stimuli have been employed to excite spinal neurones, however the selectivity of this action was not always tested (as reviewed by Duggan and Fleetwood-Walker, 1993; Yaksh, 1993).

Electrophysiological studies have been performed to investigate sites of action of each opioid receptor type in the processing of nociceptive information at the spinal cord level. These studies involving the local administration of opioid receptor ligands (by microiontophoresis or pressure ejection) at defined spinal cord laminae, have revealed that μ, δ and κ opioid receptors are involved in the inhibition of nociceptive transmission at anatomically distinct sites (recently reviewed by Duggan and Fleetwood-Walker, 1993). Overall, **μ preferring ligands** (morphine, metorphamide, DAMGO) have been found to produce a selective, naloxone reversible antinociceptive influence in superficial lamina I/ II of

the cat (Duggan et al, 1976; 1977b, 1981; Zhao et al, 1986; Fleetwood-Walker et al, 1988) and rat (Hope et al, 1990a) spinal cord. Notably a highly selective action for morphine iontophoretically administered into lamina II was demonstrated by Duggan and co-workers, by simultaneously recording with a second microelectrode the nociceptive responses of neurones in laminae IV/ V. These effects were readily reversed by naloxone either administered systemically or iontophoresised into the substantia gelatinosa, and the specificity of the action lost when morphine was ejected closer to the lamina IV/ V cells being recorded (Duggan et al, 1976; 1977b; 1981). Similar double microelectrode studies were performed on identified spinocervical tract cells in laminae III-V using the highly selective μ agonist DAMGO (Fleetwood-Walker et al, 1988).

In contrast the conclusions that can be drawn form similar studies employing **δ preferring ligands** are more limited. Whereas early experiments showed that the stable enkephalin analogues [Met^5]enkephalinamide and DADLE produced similar naloxone reversible effects to morphine, on the nociceptive activation of laminae IV/ V neurones when ejected into the substantia gelatinosa (Duggan et al, 1977a; 1981; Davies and Dray et al, 1978; Sastry and Goh, 1983), repeating the experiments with the more δ selective agonists (DSLET and DPLPE), Fleetwood-Walker et al (1988) failed to reproduce this lamina II effect indicating that actions at μ receptors may be more responsible for the effect. However, non-selective actions of enkephalins were also described on the activity of lamina IV/ V neurones (Duggan et al, 1977a; Zieglgansberger and Tulloch, 1979) and identified primate spinothalamic tract neurones (Willcockson et al, 1986), that although were readily reversed by iontophoretically applied naloxone, were not reversed by even very high doses given systemically. This along with the lack of effect DADLE and the highly δ selective DPDPE had, when iontophoresised near these deep dorsal horn neurones in the cat (Fleetwood-Walker et al, 1988) and rat (Hope et al, 1990), indicates that these non-selective effects by the enkephalins are unlikely to be δ or even μ receptor mediated. In contrast in the rat, lamina I cells (some of which were identified as spinomesencephalic tract cells), were clearly inhibited by DPDPE applied in the superficial

dorsal horn (Hope et al, 1990). Since the inhibitory actions on these neurones (both non-selective and selective inhibitions being displayed), were antagonised by the local application of the δ selective antagonist ICI178864, these effects are likely be mediated by δ receptors. Although few electrophysiological studies have been performed using **κ preferring ligands**, the relatively selective agonists, dynorphin A and U50488H, were found to produce potent, naloxone reversible inhibition of the responses of identified cat spinocervical tract neurones and rat neurones in lamina IV/ V, when iontophoretically administered near to these deep dorsal horn neurones. However no effects were observed on the firing of either these lamina IV/ V neurones or the lamina I neurones of the rat when the agonists were applied in the substantia gelatinosa (Fleetwood-Walker et al, 1988; Hope et al, 1990).

The findings of studies employing the topical administration of opioids, although unable to localise sites of action to discrete spinal laminae and potentially limited by lipophilicity of drugs applied (which may result in a significant redistribution via systemic circulation), appear to be in general agreement with the those of the above iontophoretic studies. For example, whereas the μ receptor agonists DAMGO (Dickenson et al, 1987) and the naturally occurring dermorphin (Sullivan and Dickenson, 1988), and the δ receptor agonists DPDPE, DTLET (Dickenson et al, 1987) and DSBuLET (Sullivan et al, 1989) have been reported to partially inhibit C- but not A-fibre evoked responses in dorsal horn neurones when applied to the spinal cord surface, the topical administration of κ receptor agonists (U50488H, U69593, dynorphin A) has resulted in variable responses, with only the highest doses producing inhibitions that were insensitive to naloxone (Knox and Dickenson, 1987; Hylden et al, 1991) and norBNI (Sullivan and Dickenson, 1991). Whilst this may be a reflection simply of a deeper dorsal horn site of action, κ preferring ligands have also been found to display other 'non-opioid' effects at the spinal level. These will be detailed in a discussion of the functions of dynorphins in spinal processing in section 4.4. Overall electrophysiological and behavioural studies following the spinal administration of opioid receptor ligands have demonstrated clear analgesic responses (as reviewed by Millan, 1986; Millan et al, 1993).

Although the finding of early behavioural studies implied that κ preferring ligands act exclusively against non-thermal noxious stimuli (Schmauss and Yaksh, 1984), later studies employing 'match-intensity' stimuli revealed such modality selectivity to be artifactual (Millan et al, 1989). This was also supported at an electrophysiological level when the noxious thermal and mechanical stimuli were graded so as to produce equal firing in motoneurones (Parsons and Headley, 1989) and dorsal horn neurones (Dong et al, 1991) of the spinal rat, following the systemic administration of drugs.

The functional consequences of activating an opioid receptor in the spinal cord will differ depending on where the receptor is located; namely on the terminals of primary afferent fibres or descending pathways; or the dendrites/ cell bodies of local circuit neurones, projecting neurones (propriospinal or supraspinal) or motoneurones. The selective blockade of the nociceptive responses by the iontophoresis of μ receptor agonists in lamina I/ II suggests a presynaptic site of action via receptors located on nociceptor terminals or an action on specific local circuit neurones located in the superficial layers. In contrast, δ receptors appear to mediate more of a ubiquitous inhibitory mechanism on lamina I neurones, since both selective and non-selective actions are displayed. This likely involves a more complex role of these receptor sites in the modulation of nociceptive transmission, involving both pre- and post-synaptic actions. As indicated in section 1.5.1 the postsynaptic actions of opioids have implications for their selective effects, especially if they occur on the cell body. Whilst this could reflect a restricted distribution of the opioid receptors to certain local circuit neurones in the substantia gelatinosa, it could also reflect actions in selective areas of the dendritic tree of the neurone involved, or the selective 'antagonism' of a particular intracellular effect. Although the selective action of κ receptor agonists on lamina IV/ V neurones could equally involve presynaptic actions on the deeper terminating (group III and IV) nociceptors, it likely reflects an action at receptors on specific local circuit neurones in the deep dorsal horn. Notably the antinociceptive effect of dynorphin A(1-13) was effectively blocked by idazoxan (a selective α_2 adrenoceptor antagonist) (Fleetwood-Walker et al, 1988). Based on the

findings of previous electrophysiological studies that naloxone failed to reverse the inhibitions produced by 5-HT and noradrenaline on the firing of laminae IV/ V spinal neurones (Headley et al, 1978), and behavioural studies that pretreatment of the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine reduced the inhibition of the tail-flick response by intrathecal (i.t.) dynorphin (Von Voigtlander et al, 1984), it was proposed that the κ receptors in laminae IV/V region could control, by a 'disinhibition' mechanism, the inhibitory effects of these descending monoamine pathways on spinal cord processing of nociceptive information. The lack of antinociceptive effect displayed by the κ receptor ligands in the superficial laminae, where the majority of these κ receptors are located, is suggestive that unlike μ and δ receptors, a major role of these receptors in the spinal cord may be unrelated to the modulation of nociceptive inputs.

As indicated in section 1.6.3, a proportion of μ and δ receptors in the dorsal horn appear to be located on the terminals of primary afferent fibres. However, there is limited electrophysiological evidence to support such a presynaptic inhibitory effect of opioids, and that reported may reflect an indirect action (Carstens et al, 1979; Sastry, 1980). Although as previously described in section 1.5, opioids active at μ, δ and κ receptors on a proportion of cultured or dissociated DRG neurones have been shown to exert actions on potassium and calcium channels to reduce the excitability of neurones and shorten the duration of their action potentials, little evidence to support such effects at the central terminals of small myelinated and unmyelinated primary afferents has been presented (Hori et al, 1992; Glaum et al, 1994). Instead opioids (at least μ receptor preferring ligands) have been reported to have direct hyperpolarising actions on dorsal horn neurones in slices of the rat spinal cord (Murase et al, 1982; Yoshimura and North, 1983; Jeftinija et al, 1988; Hori et al, 1992; Glaum et al, 1994) and trigeminal nucleus (Grudt and Williams, 1994), suggestive more of a postsynaptic mechanism. In addition, despite reports that the application of opioids (mainly μ and δ preferring) can reduce the evoked release of neuropeptides present within many small diameter primary afferents, such as substance P, when superfused onto *in vitro* slice preparations

(trigeminal nucleus: Jessel and Iversen, 1977. spinal cord: Mauborgne et al, 1987; Pohl et al, 1989) and the spinal dorsal horn *in vivo* (Yaksh et al, 1980; Go and Yaksh, 1987; Aimone and Yaksh, 1989), no such action has been found for analgesic doses of morphine administered systemically *in vivo* using the push-pull cannulae (substance P: Kuraishi et al, 1983) and antibody microprobes (substance P: Morton et al, 1990. CGRP: Morton and Hutchison, 1990. neurokinin A: Lang et al, 1991), the concentrations of the drugs applied topically being very high (10^{-3} to 10^{-5}M). Notably a recent study employing antibodies to the cloned μ receptor has reported that colocalisation with substance P is only occasionally seen in rat dorsal horn, suggestive that some nociceptive afferents do not possess μ opioid receptors (Ding et al, 1995). However, although a strong case has been presented against a presynaptic action of μ preferring ligands in mediating antinociceptive effects, this may not be equally true of δ preferring ligands, since immunological studies with antibodies directed against these receptors have found them to be predominantly located on axons in the spinal cord rather than on cell bodies or dendrites (Dado et al, 1993; Arvidsson et al, 1995a; Cheng et al, 1995). In addition recent whole cell voltage clamp recordings of visually identified lamina II neurones of the rat spinal cord slice appear to imply a presynaptic inhibitory role for the selective δ agonists DPDPE and [D-Ala^2]deltorphin II on excitatory glutamate mediated afferent transmission (Glaum et al, 1994). However, the source of such an opioid input is uncertain. Despite early proposals for axoaxonic synapses between enkephalin neurones and substance P containing primary afferents (Jessel and Iversen, 1977), such a synaptic arrangement has not been confirmed at the ultrastructural level (Hunt et al, 1980; Glazer and Basbaum, 1983). Instead enkephalin immunoreactive neurones have been described to contact spinothalamic tract neurones in lamina I and V (Ruda et al, 1984). Cho and Basbaum (1989) also failed to detect a significant number of dynorphin B immunostained structures presynaptic to degenerating primary afferents following dorsal rhizotomy. In a recent review Ribeiro da Silva and Cuello (1995) have proposed that since enkephalin containing neurones are sometimes presynaptic to the central element of synaptic glomeruli which do not display

peptide immunoreactivity in the central bouton, such neurones could act both pre- and post-synaptically in the modulation of non-peptidergic sensory neurones.

Studies have been performed in the cat and rat to determine whether the endogenous opioid systems exert a tonic influence on nociceptive pathways in the spinal cord. In general the effects of systemic naloxone on the basal and evoked firing of unidentified dorsal horn neurones have been rarely observed and variable. However, naloxone was found to consistently and selectively enhance the responses of ventrolateral tract neurones to electrical stimulation of peripheral C fibres in both the spinal rat (Bernatsky et al, 1983) and cat (Duggan et al, 1985). Additionally studies with naloxone revealed actions of the spinal segmental opioid systems in certain of the supraspinally mediated inhibitions in the rat (such as that evoked by electrical stimulation of the rostral ventromedial medulla; Rivot et al, 1979), but not the cat (as reviewed by Duggan and Fleetwood-Walker, 1993)

2. OPIOIDS AND NON-NOCICEPTIVE PATHWAYS

(i) Motor function

As previously discussed early electrophysiological evidence pointed to a relatively selective action of opioids in the suppression of nociceptive reflexes, exerting minimal influence on monosynaptic reflexes in extensor motoneurones induced by the application of a single stimulus to large diameter afferents. However, the reflex activity elicited by high frequency stimulation of these afferents has been found to be reduced by analgesic doses of opiate drugs (Jurna and Schafer, 1965; Krivoy et al, 1973), indicating that monosynaptic pathways can be influenced by activity induced in interneurones following such repetitive stimuli. Additionally, whereas the effects of systemic naloxone on dorsal horn neuronal activity have proved variable, ventral horn neurones have more reliably been shown to be under tonic control by endogenous opioids (as reviewed by Duggan and Fleetwood-Walker, 1993). This has demonstrated for both mono- and polysynaptic reflexes in the spinal cat

(Morton et al, 1982; Duggan et al, 1984) and rabbit (Clarke et al, 1989) following the electrical nerve stimulation. The mechanism underlying these spinal reflex inhibitions has yet to be established, but early intracellular studies involving the local iontophoretic administration of naloxone found no evidence for a direct effect on motoneurones (Zhao and Duggan, 1984). Of potential relevance to the function of such non-selective inhibitions is the finding that certain reflexes are only apparent when the opioid receptors are blocked. Thus, it is possible that the one of the functions of the endogenous opioid peptides at the spinal level is to help set the threshold for withdrawal reflexes, so that they are only activated by appropriate stimuli (Clarke et al, 1992).

(ii) Other functions

The spinal administration of opioids (mainly μ and δ preferring ligands) has also been observed to cause significant effects on certain genitourinary and gastrointestinal functions. For example, naloxone reversible inhibitions of the volume evoked micturation reflex and reductions in the velocity of intestinal transport have been observed in unanesthetised animals and man. Additionally, whilst the intrathecal injections of opioids fails to produce any detectable effect upon resting heart rate or blood pressure, there is a reduction of these sympathetic signs when evoked by high threshold stimuli (as reviewed by Yaksh and Nouiehed, 1985; Yaksh, 1993).

A full description of an 'opioid event' includes the stimuli producing the release of a particular opioid peptide, the spread and degradation of the peptide released, the receptors acted upon, and the functional consequences for neuronal processing following the activation of these receptors. Viewed in this way our present knowledge of opioid events in the spinal cord is limited. As highlighted in section 1.4 the opioid peptides do not display strong selectivities for the μ, δ and κ opioid binding sites. Thus, under physiological conditions opioid-receptor binding depends not only on the preferential affinity of the opioid peptide for a

given receptor but also on the amount of opioid peptide and receptor available in a given tissue. This is especially true of the spinal cord. Thus, whilst localising the action of opioid receptor agonists on the responses induced by defined stimuli to specific laminae of the spinal cord may be informative, such studies may also be misleading of the physiological situation. Instead it needs to be determined which sites are functionally active in response to a particular stimulus and which opioid peptides mediate the effects. However, the ability to perform such electrophysiological experiments was limited until recently by the lack of selective antagonists which could be administered locally to spinal tissue, and few such studies have been performed. Notably the selective κ receptor antagonist norBNI was recently employed to investigate the role of this endogenous opioid system in the spinal neuronal responses evoked under normal conditions and following the development of a peripheral inflammation (Stiller et al, 1993), the findings of which will be detailed in section 4.4. Whilst behavioural studies employing antisense oligodeoxynucleotides to the cloned opioid receptors have shown a relatively rapid turnover of receptors they have so far provided little additional insight into the roles of the opioid systems at the spinal cord level over and above studies administering selective opioid ligands (recently reviewed by Pasternak and Standifer, 1995). As for release studies, the majority of investigations performed *in vivo* have involved spinal perfusion, which is of obvious limited resolution in being able to detect the release of opioid peptides from the deep laminae of the spinal cord and few studies have been performed to investigate the effects of 'natural' stimuli (proEnk: Yaksh and Elde, 1980; 1981; Yaksh et al, 1983; Nyberg et al, 1983; Le Bars et al, 1987; Bourgoin et al, 1988. proDyn: Yaksh et al, 1983; Nyberg et al, 1983). Thus, there is a need to localise release to discrete laminae in response to physiological stimuli. This is central to the aim to the studies performed in this thesis. As will be subsequently described I have employed the antibody microprobe technique to study the release of dynorphins in the rat spinal cord under conditions of peripheral inflammation, in which this family of opioid peptides appear to play a special role.

1.8 RESPONSE OF THE OPIOID SYSTEMS TO PERIPHERAL INFLAMMATION

The role played by the opioid peptides in spinal cord function has been further investigated by studying the response of the endogenous opioid systems to different 'pain' states. A number of animal models have been employed to represent the persistent or chronic pain experienced in many clinically recognised pathological states involving inflammation and/ or trauma. In general, these models can be considered to represent two different though not exclusive 'pain' states, those involving tissue damage (i.e. 'nociceptive pain') and those involving nerve injury (i.e. 'neuropathic pain'). The response of opioid systems has been mainly investigated under inflammatory conditions. Through these studies a role of the proDyn family of peptides has been highlighted.

1. MODELS OF TISSUE INJURY AND INFLAMMATORY PAIN

These can be considered 'acute', in the sense of developing within minutes or 1-2 hours and resolving either within hours or 1-2 days, or 'chronic', developing over a longer time scale and maintained for days to weeks. Additionally acute inflammation may not resolve but may proceed to a chronic phase.

Acute inflammatory lesions have been produced by the intradermal injection of formalin (Dubuisson and Dennis, 1977) or carrageenan (Winter et al, 1962) into the plantar surface of rat hindpaw. Injections of formalin characteristically produce a biphasic behavioural response. This consists of an 'early transient' phase (related to the injection itself), that peaks at 5 min to subside by 10 to 15 min, and a 'late prolonged' phase (related to the ensuing inflammatory response), that occurs 20 to 60 min after the injection. Similar lesions have also been induced by the injection of sodium urate crystals (Okuda et al, 1984; Coderre and Wall, 1987), or kaolin and/ or carrageenan into the cavity of a joint, the latter more specifically to study joint 'pain' in non-recovery experiments (i.e. acute monoarthritis; Schaible and Schmidt, 1985). These lesions present histological and behavioural signs of inflammation, such as

oedema and hyperalgesia, within the first few hours and so allow changes of the peripheral and central processing of sensory information arising from the injected regions to be readily studied.

Chronic inflammatory lesions have more commonly employed the use of Freund's complete adjuvant (FCA), typically a suspension of heat killed bacteria, *Mycobacterium tuberculosum/ butyricum* in mineral oil. A diffuse persistent inflammatory state termed a 'polyarthritis' has been induced in rats by the intradermal innoculation of FCA at the tail base (Pearson et al, 1963; De Castro Costa et al, 1981). This produces a two stage inflammatory process characterised by an initial acute local reaction that develops within the first few hours and subsides after 3 to 5 days, and a diffuse inflammatory reaction that appears during the second week. This develops mainly in the hindlimbs and upper tail, peaking at 3 weeks and often takes up to 10 weeks to resolve. These polyarthritic animals have been shown to demonstrate characteristics typical of chronic pain in man; namely hyperalgesia, irritability, hyperventilation, decreased motility and loss of body weight. Further the rats are observed to self-administer opiate and inflammatory drugs (Millan et al, 1986; Colpaert, 1987; Millan, 1993). However, these animals suffer from a generalised disease state with lesions throughout the body rather than localised at a single joint or limb, that complicates the interpretation of data, making it difficult to relate observations to inflammatory pain alone. Thus, more limited peripheral inflammatory states are also employed with lesions appearing to be confined to a single joint or limb, that induce some of the neurophysiological, pharmacological and biochemical aspects of chronic pain states without the complication of multifocal lesions. These involve either the injection of small amounts of FCA or other inflammatory agents into a hindpaw, the tissue around a joint or into the joint cavity itself. For example, intradermal injections of FCA into the plantar surface of the rat hindpaw (i.e. unilateral adjuvent induced inflammation; Hargreaves et al, 1988; Iadarola et al, 1988a) have been described to produce a significant erythema, oedema and hyperalgesia of the injected paw (to both thermal and mechanical stimulation) within 4 hours, that peaks at 2 to 3 days post-inoculation, and persists

for 10 to 14 days. Other inflammatory agents, such as carrageenan, yeast or phorbol ester into the rat hindpaw have been found to induce similar states (Iadarola et al, 1988a). The injection of FCA into the periarticular tissue of the ankle joint of rats results a similar self-limiting and reproducible unilateral inflammation, that pertains more directly to joint pain (Grubb et al, 1988; Donaldson et al, 1993). More severe models involve the injection FCA (i.e. monoarthritis; Butler et al, 1992) into the ankle joint of the cat or rat. This produces along with the development of an arthritic joint, a much more prolonged inflammation that is well maintained for at least 6 weeks. The use of such unilateral models has allowed the establishment of more specific relationships between alterations in the peripheral and central processing of sensory information in response to the induction of prolonged inflammation states.

2. INFLAMMATION INDUCED CHANGES IN PRIMARY AFFERENT AND SPINAL CORD NEURONES

Experimental evidence supporting the sensitisation of thin myelinated and unmyelinated primary afferent nociceptive units following the development of a peripheral inflammation has been well documented, especially for the mechanosensitive group III and IV afferents found in the medial and posterior articular nerves (MAN and PAN) innervating the cat knee joint. The nociceptors become spontaneously active, and acquire lowered thresholds and increased responsiveness to suprathreshold stimuli as inflammation develops (Coggeshall et al, 1983; Guilbaud et al, 1985; Schaible and Schmidt, 1985,1988; Grigg et al 1986; Grubb et al, 1991). The recruitment of previously mechanoinsensitive afferents or 'silent nociceptors' has also been observed (Schaible and Schmidt, 1985; 1988; Grigg et al 1986; Meyer et al, 1991). A variety of mediators released from neighbouring inflammatory cells, as well as afferent and efferent axons, are thought to participate in the sensitisation processes including prostaglandins, leukotrienes, bradykinin, and 5HT amongst others (recently reviewed by Schaible and Grubb, 1993). An increased afferent input might reasonably result in

hyperactivity of spinal neurones receiving such input, but it has been proposed that additional changes occur centrally resulting in increased responses to a given input, a state called by some investigators 'central sensitisation' (recently reviewed by Coderre et al, 1993). Since these may contribute to the allodynia, hyperalgesia, and spontaneous or persistent pain associated with tissue injury, by altering the processing of somatosensory information at the spinal level and changing the message transmitted from the spinal cord to the brain, I shall now briefly describe some of the evidence supporting this proposal.

It had long been postulated from clinical observations, that injury and noxious stimulation of the periphery could induce changes centrally to alter pain sensitivity. Hardy et al (1950) recognised that cutaneous injury resulted in two distinct types of hyperalgesia; primary and secondary. The former primary zone, represented by the increased sensitivity to both thermal and mechanical noxious stimulation at the site of an injury, they proposed was mediated by peripheral mechanisms (i.e. neurogenic inflammation). The latter secondary zone, represented by the increased sensitivity to mechanical but not thermal stimuli observed outside of this region, they proposed must also involve central processes, since it was found to occur beyond the recognised area of neurogenic induced 'flare' response (recently reviewed by Treede et al, 1992). More recently human microneurography studies involving the intradermal injection of capsaicin have supported this classification, demonstrating how cutaneous hyperalgesia once established requires minimal input from the injured peripheral tissue (La Motte et al, 1992). Direct experimental evidence for enhanced central responses was initially provided by Mendell (1966) who showed how repeated activation of C fibre afferents sequentially increased dorsal horn activity to result in prolonged discharges in spinal neurones that lasted seconds to minutes poststimulus, termed 'windup'. The potential importance of such 'central hyperexcitability' to post-injury pain hypersensitivity states was popularised by Woolf and coworkers. They demonstrated how brief 'conditioning' stimulation of afferent C fibres either by direct electrical stimulation of cutaneous and muscle afferent nerves (Wall and Woolf, 1984) or by cutaneous and deep tissue injury (Woolf, 1983; Woolf and McMahon, 1985),

could increase the excitability of flexor efferent motoneurones to biceps femoris/ semitendinosus muscle in response to noxious mechanical stimulation of the hindpaw, for a period of 5-10 minutes (e.g. cutaneous/ sural nerve stimulation) to up to an hour (e.g. muscle/ gastrocnemius-soleus nerve stimulation). Notably reductions in flexion reflex thresholds to noxious mechanical and thermal stimulation were also observed in the limb contralateral, as well as ipsilateral to the site of tissue injury (Woolf, 1984; Cook et al, 1987), and were shown to occur independently of changes in the excitability of the afferent terminals in the dorsal horn and of the flexor motorneurones involved (Cook et al, 1986).

Increases in the spontaneous activity, reduced thresholds and increases in the responses to afferent inputs of nociresponsive spinal neurones have been subsequently observed following an intense C fibre mediated barrage of activity, elicited by the electrical stimulation of peripheral nerves (Schouenborg and Dickenson, 1985), 'natural' stimulation (Laird and Cervero, 1989; Woolf and King, 1990; Simone et al, 1991; Dougherty and Willis, 1992) or tissue injury (Menetrey and Besson, 1982; Schaible et al, 1987; Neugebauer and Schaible, 1988, 1990; Doughterty et al, 1992, Grubb et al, 1993). Expansions of the cutaneous receptive fields of these spinal neurones have also been observed beyond the focus of the inflammatory lesion and hence the range of innervation primary afferents to adjacent ipsilateral and even into contralateral regions (Menetrey and Besson, 1982; Calvino et al, 1987; Neugebauer and Schaible, 1988, 1990; Hyden et al, 1989; Hoheisel et al, 1993; Grubb et al, 1993).

A variety of compounds released from the terminals of both primary afferents and intrinsic spinal neurones have been implicated in the hyperexcitability of spinal neurones following inflammation, including the excitatory amino acids, L-glutamate and L-aspartate. Notably under these conditions a special role of the N-methyl-D-aspartate (NMDA) class of glutamate receptor has been proposed from a variety of electrophysiological (kaolin and carrageenan: Schaible et al, 1991a; Neugebauer et al, 1993. FCA: Doughterty et al 1992; Neugebauer et al, 1994; formalin: Haley et al, 1990) and behavioural (carrageenan: Ren et al,

1992. formalin: Coderre and Melzack, 1992) studies. These receptors along with the non-NMDA classes of glutamate receptors (that mediate the transmission of both innocuous and noxious stimuli under normal physiological conditions), appear to play a primary role in the generation and maintenance of central sensitisation states in response to tissue injury, with evidence for the enhanced spinal release of the excitatory amino acids as inflammation develops peripherally (Skilling et al, 1988; Sorkin et al, 1992; Sluka and Westlund, 1993) . For example, the 'wind-up' of dorsal horn neuronal activity by repetitive C fibre stimulation has been shown to be significantly reduced following the application of either competitive (AP5: Dickenson and Sullivan, 1987; 1990; Thompson et al, 1990. CPP: Woolf and Thompson, 1991) and non-competitive (ketamine: Davies and Lodge, 1987. MK-801: Woolf and Thompson, 1991) excitatory amino acid receptor antagonists. The findings of biophysical studies (Mayer et al,, 1984) that the NMDA receptor linked ion channel, unlike that of the other ionotrophic non-NMDA receptors, is blocked by Mg^{2+} at hyperpolarised resting membrane potentials (hence unique in being both ligand and voltage gated) appears to partly explain why these receptors require such high threshold prolonged neuronal activation to become involved in spinal processing of sensory information.

Alterations have also been shown to occur in the synthesis (Noguchi et al, 1988; Minami et al, 1989; Kurashi et al, 1989; Smith et al, 1992; Hanesch et al, 1993a) and release of a number of neuropeptides contained primarily within primary afferent fibres such as the tachykinins substance P (Oku et al., 1987; Schaible et al., 1990; Garry and Hargreaves, 1992) and neurokinin(NK) A (Hope et al., 1990b), and CGRP (Garry and Hargreaves, 1992; Collin et al, 1993; Schaible et al., 1994). The tachykinins via actions at NK1 and NK2 receptors respectively have been shown to elicit slow depolarisations of dorsal horn neurones *in vitro* (Murase and Randic, 1984; Urban and Randic, 1984) and to enhance excitatory nociceptive responses of spinal neurones *in vivo* (Hendry et al, 1976; Fleetwood-walker et al, 1990; De Konnick and Hendry, 1991; Dougherty et al, 1994; Neugebauer et al, 1995), resulting in

prolonged facilitation of nociceptive flexor reflexes when applied intrathecally (Woolf and Wiesenfeld-Hallin, 1986; Xu et al, 1991a).

Significant differences in the timing of the enhanced release of these neuropeptides as inflammation develops, suggest that there are evolving processes influencing the spinal processing of nociceptive information. For example, antibody microprobe studies have been performed to study the release of some of these peptides following the induction of an acute inflammation in the knee joint of the cat by intra-articular injections of kaolin and carrageenan. Whereas a spinal release of immunoreactive(ir)-substance P was not observed until 3 up to 8 hours post-inoculation, and then only after joint manipulation (Schaible et al, 1990), neurokinin A was detected immediately after joint injection (Hope et al, 1990). A more recent antibody microprobe study also found evidence that CGRP release is enhanced within the first few hours of an acute inflammation (Schaible et al, 1994). Interestingly nerve growth factor has recently been implicated in the inflammation evoked increased expression of tachykinins and CGRP (Donnerer et al, 1993), which suggests that increased neuronal activity is not the only critical factor in the induction of hyperexcitability states. The action of excitatory amino acids in the spinal dorsal horn has been found to be enhanced pre- and post-synaptically by these primary afferent neuropeptides *in vitro* (Randic et al, 1990; Kangra and Randic, 1990; Rusin et al, 1992; 1993) and *in vivo* (Dougherty and Willis, 1991; Dougherty et al, 1993; Song and Zhao, 1993), with evidence for modulation of release by substance P (Kangra and Randic, 1990; Sluka and Westlund, 1993). Notably there is evidence for coexistence of substance P/ CGRP with L-glutamate in a subpopulation of primary afferent neurones (DeBaisi and Rustioni, 1988). Although the increased involvement of NMDA under inflammatory conditions receptors may be simply explained by a removal of the Mg^{2+} block by a sustained small tachykinin-induced depolarisation, other mechanisms possibly involving protein kinase mediated phosphorylations are proposed (as reviewed by Randic et al, 1995).

3. THE RESPONSE OF OPIOID SYSTEMS AT THE SPINAL CORD LEVEL

Whilst most emphasis has been focused on the facilitation of excitatory influences on spinal cord neurones following the development of a peripheral inflammation, changes have also been described in inhibitory systems active at the spinal cord level, both intrinsic to the spinal cord and supraspinally located. For example, it has been shown that the effectiveness of tonic descending inhibition on spinal cord neurones (notably in laminae IV-VIII) is increased during the first hours of a developing inflammation in a knee joint (Schaible et al, 1991b). The inhibitory systems segmentally active in the spinal cord involve either GABA/ glycine or opioid peptide releasing neurones. Although increases have been observed in GABA neuronal systems at the spinal cord level (Nahin and Hylden, 1991; Castro-Lopes et al, 1994), the most dramatic changes involve the endogenous opioid peptides. Notably a significant enhancement in the synthesis of the proDyn family of opioid peptides has been observed, the consequences of which will be considered later in section 4.4. However, since there is evidence that some of the dynorphin neurones project to supraspinal areas (Standaert et al, 1986; Nahin et al, 1987; 1989; 1992; Leah et al, 1988) the consequences of activating these opioid containing neurones appears complex, likely exerting an analgesic influence at the spinal cord level as well as modulating the transmission of nociceptive information in certain regions of the diencephalon and brainstem. A tonic activity of the spinal cord opioid systems under inflammatory conditions was suggested by the increases in the spontaneous firing and the responses of dorsal horn neurones to electrical stimulation of C fibres observed for spinalised polyarthritic rats following the systemic administration of naloxone (Lombard and Besson, 1989).

As previously indicated POMC synthesising neurones do not appear to exert a significant influence in the spinal cord. In polyarthritic rats increases in the plasma levels of β-endorphin have been observed, that parallel an upregulation of POMC mRNA and ir-β-endorphin in the anterior but not intermediate lobe of the pituitary (Millan et al, 1986). However, since these changes are not observed following the induction of a unilateral inflammation (Millan et al,

1988) but are observed following chronic foot shock (Akil et al, 1986), they are thought to more likely reflect a state of chronic stress than chronic pain (Millan, 1993).

The extent of the involvement of proEnk synthesising neurones in the responses of spinal neurones to peripheral inflammation remains controversial. Early studies in polyarthritic rats employing radioimmunoassay techniques reported enhanced levels of ir-Met-enkephalin in the spinal cord but not brain of these animals (Cesselin et al, 1980, Millan et al, 1986). However, this response has proved small and inconsistent, and appears unaccompanied by any obvious elevation in the levels of proEnk mRNA (Millan, 1993). The induction of a unilateral inflammation has similarly been shown to exert a mild influence upon proEnk neurones. Although reports by an early radioimmunoassay study of increased enkephalin levels after the injection of FCA into the rat hindpaw (Faccini et al, 1984) were not confirmed by immunocytochemical studies using the same inflammatory agent (Iadarola et al, 1988b; Millan et al, 1988), significant though variable increases in the expression of proEnk mRNA have been found by RNA blot analysis (Iadarola and Draisci, 1988; Iadarola et al, 1988b; Draisci and Iadarola, 1989; Draisci et al, 1991). These increases occur rapidly within 4 hours of inducing the inflammation and persist for about 5 days post-inoculation (Iadarola et al, 1988b). More consistent changes have since been observed by *in situ* hybridisation (Noguchi et al, 1992; Przewlocka et al, 1992). The response appears to involve only a subpopulation of proEnk neurones, since the increases in proEnk mRNA observed were limited to laminae I-II, V-VI and VII. These increases in proEnk expression parallel those previously observed following the injection of formalin into the rat hindpaw (Noguchi et al, 1989) or high intensity electrical stimulation of primary afferent neurones (Nishimori et al, 1989). Studies of the spinal release of ir-Met-enkephalin into a surface perfusate following the development of such peripheral inflammation states have likewise observed variable responses. Whereas a reduction in the spontaneous but not evoked release of ir-Met-enkephalin was observed in the spinal perfusate of polyarthritic rats *in vitro* (Cesselin et al, 1984) and *in vivo* (Bourgoin et al, 1988), an initial elevation (12 to 24 hours post-inoculation) in the spontaneous but not

evoked release of ir-Met-enkephalin-Arg-Gly-Leu has been reported using spinal cord slices of rats with a unilateral inflammation (Przewlocka et al, 1992). In these studies release was evoked either by raising the potassium concentration of the perfusing fluid, or (where applicable) by manipulating the inflamed limbs. However, the release of ir-proEnk derivatives into spinal perfusate has been consistently observed *in vivo,* following the high intensity electrical stimulation of primary afferent fibres of the normal rat and cat (Yaksh and Elde, 1980; 1981; Yaksh et al, 1983; Nyberg et al, 1983).

In contrast to proEnk, a dramatic increase in the synthesis and spinal content of proDyn derived peptides has consistently been observed following the development of a peripheral inflammation. Pronounced elevations in the spinal cord levels of proDyn peptides have been detected by radioimmunoassay and immunocytochemistry in polyarthritic rats (Millan et al, 1985; 1986; Weihe et al, 1988; 1989), as well as those with a developed unilateral inflammation (Iadarola et al, 1988a; b; Ruda et al, 1988; Millan et al, 1988; Weihe et al, 1989; Takahashi et al, 1989; 1990; Nahin et al, 1989; 1992; Noguchi et al, 1991; Przewlocka et al, 1992). These were accompanied by reports of striking increases in the levels of the mRNA encoding proDyn as measured by RNA blot (Hollt et al, 1987; Iadarola et al, 1988a, b; Iadarola and Draisci, 1988; Draisci and Iadarola, 1989; Draisci et al, 1991) and *in situ* hybridisation (Ruda et al, 1988; Weihe et al, 1989; Noguchi et al, 1991; Przewlocka et al, 1992; Parker et al, 1993; Persson et al, 1994; Tolle et al, 1994). These changes have been localised to neurones in the superficial (laminae I-II) and the deep (laminae IV-VI) dorsal horn, as well as dorsolateral to the central canal in lamina VI, VII and X (as will be described in detail in section 4.4). A time course analysis has revealed a significant enhancement of the proDyn mRNA in the spinal cord within 24 hours of the injection of FCA into the hindpaw, detectable by RNA blot as early as 4 hours, with a peak six- to eight-fold increase between days 2 and 5, and approaching control levels within 10 to 14 days. This paralleled the development and time course of the oedema and behavioural hyperalgesia in the hindpaw of these animals. Increases in the dynorphin peptide were apparent by radioimmunassay, 2 to 3

days post-inoculation, peaking with a three-fold increase around day 5 (Iadarola et al, 1988a; b).

Immediate-early genes such as c-fos and c-jun, are thought to regulate initial genetic events that lead to prolonged functional changes in the CNS (as reviewed by Hanley, 1988; Morgan and Curran, 1989; Sheng and Greenberg, 1990; Munglani and Hunt, 1995; Herdegen and Zimmermann, 1995; Hughes and Dragunow, 1995). These proto-oncogenes (so called due to their homology to retroviral oncogenes) encode nuclear phosphoproteins (e.g. Fos, Jun), that act as transcription factors by binding to specific DNA consensus sequences in the promoter region of target genes (such as AP1 sites), as homo- or heterodimeric complexes. In this way these phosphoproteins are proposed to act as 'third' messengers in the stimulus-transcriptional coupling cascade, to alter gene expression in response to protein kinases activated by second messengers such as diacylglycerol, cyclic AMP and Ca^{2+}. It has been proposed that these immediate early genes play a role in the upregulation of proDyn (and also proEnk) following the development of a peripheral inflammation. Increases in the levels of ir-Fos have been observed in the dorsal horn of the rat, predominantly in laminae I-II and V-VI, following the induction of an inflammatory lesion in the periphery (FCA: Menetry et al, 1989; Hylden et al, 1992; Abbadie and Besson, 1992; Lanteri-Minet et al, 1993; Tolle et al, 1994. carrageenan: Draisci and Iadarola, 1989; Noguchi et al 1991; Chapman et al, 1995a; b. formalin: Presley et al, 1990; Williams et al, 1990; Gogas et al, 1991; Leah et al, 1992; Abbadie et al, 1992; Hunter et al, 1995). RNA blot analysis has revealed a rapid and pronounced elevation in c-fos mRNA within 30 minutes after the injection of carrageenan into the rat hindpaw. A three-fold increase was seen to remain for up to 2 hours post-injection, decreasing to approximately half these levels by 4 hours to give nearly complete recovery to control levels by 8 hours (Draisci and Iadarola, 1989). As well as this close temporal link, there is also evidence to support co-localisation of c-fos and proDyn genes within single neurones in the spinal cord (Naranjo et al, 1991; Noguchi et al, 1991; Lucas et al, 1993). Additionally a AP1-like binding site has been found in the promoter region of the proDyn gene

(Naranjo et al, 1991). Similar evidence exists that c-fos and related proteins are involved in the increase in proenkephalin synthesis (Sonnenberg et al, 1989; Noguchi et al, 1992). Thus, although not specifically involved in regulation of any one neuropeptide gene, these nuclear phosphoproteins serve as good markers for activation of spinal neurones under such conditions of peripheral inflammation. Notably the recent demonstration that pre- and post-administration of the NMDA receptor antagonist (+)-HA966 (a partial agonist at the strychnine-insensitive glycine site of the NMDA receptor complex) reduced spinal c-fos expression evoked by intraplantar carrageenan into the rat hindpaw (Chapman et al, 1995a), illustrates a contribution of NMDA receptor activation to inflammation evoked c-fos expression (and perhaps proDyn/ proEnk expression).

Despite the upregulation in the synthesis of opioid peptides there appears to be no significant modification in the number of opioid receptor sites, nor their binding affinity, in the spinal cord of polyarthritic rats (Cesselin et al, 1980; Millan et al, 1986; Delay and Goyet, 1989; Besse et al, 1992) or that of rats with an inflamed hindpaw (Iadarola et al, 1988a; Millan et al, 1988). However, complex changes, have been observed in the superficial laminae of the rat spinal cord when the more persistent states of unilateral inflammation have been employed, such as monoarthritis (Besse et al, 1992). Using quantitative receptor autoradiography and the selective opioid ligands [^{3}H]DAMGO and [^{3}H]DPDPE bilateral increases in the number of μ and δ binding sites were observed in laminae I-II of the lumbar spinal level at 2 weeks, followed by a bilateral decrease in the κ binding sites labelled with [^{3}H]U69593 at 4 weeks. Similar bilateral increases in the number of μ binding sites were observed using a model of chronic pain induced by unilateral foot rot in sheep but no change in that of δ binding sites (Brandt and Livingston, 1990). No alteration in the affinity of the opioid receptors was evident in either study. These changes along with some evidence for a reduction in the density of κ receptor binding in polyarthritic rats (Millan et al, 1986), are suggestive that opioid receptor alterations may occur only in more long-term persistent states of noxious stimulation. In the polyarthritic rat any changes in opioid binding specifically

related to peripheral inflammation may be masked by alterations induced by the generalised disease state produced. The interpretation of such studies however requires that possible binding by endogenous ligands be completely dissociated prior to incubating a tissue with the exogenous labelled peptide. Notably a recent *in situ* hybridisation study found significant increases in the expression of μ and κ opioid receptor mRNA in cell bodies located in laminae I-II of the rat lumbar dorsal horn ipsilateral to a unilateral hindpaw inflammation at day 11 (one day after an additional injection of FCA), but no change in expression of δ opioid receptor mRNA in any laminae (Maekawa et al, 1995). However, no examination was made of the expression of opioid receptor mRNAs in the DRG of the adjuvant injected rats.

Taken as a whole these experimental findings are indicative of a significant involvement of the proDyn releasing neurones in spinal cord function following a peripheral inflammation. The contribution from proEnk releasing neurones however cannot be readily dismissed. There is evidence that a large 'constitutive' level of proEnk mRNA exists in the normal state. This has been measured by RNA blot analysis to be approximately 4 times that of the proDyn and so may be sufficient to meet requirements for the spinal proEnk neurones under inflamed conditions (Ruda et al, 1995). For example, Iadarola and coworkers calculated that the pool of proEnk derived peptides measured by RIA in the normal state is 10-20 times that of the proDyn peptides (Iadarola et al, 1988b). In the face of such a normal high level of activity, it is perhaps not surprising that small increases have been difficult to detect, and it is has been suggested that those neurones that display an increase may be without such a high constitutive expression of proEnk mRNA (Noguchi et al, 1991). Potentially relevant to the functional consequences of such an enhancement of opioid peptide synthesis at the spinal cord level is the reduction in the activity of a dynorphin converting enzyme in the cerebrospinal fluid (CSF) observed in the acute phase of polyarthritis (Persson et al, 1992a) and at 2 weeks post-injection in monoarthritc rats (Persson et al, 1992b), as well as in the spinal cord tissues of rats with an inflamed hind paw (Silberring et al, 1992).

As discussed previously, the enhanced excitability of spinal neurones at the spinal cord level following inflammation may partially underlie the pain experienced in many disease states involving such tissue damage. Whilst this may be triggered by the enhanced release of excitatory amino acids and excitatory neuropeptides such as substance P, little is known of the involvement of the opioid systems in the development of such states. An enhanced release is implied from the dramatic upregulation of the synthesis of the proDyn peptides but little information exists about the mechanisms of their spinal release. The κ receptor preferring dynorphins as already indicated have proved enigmatic peptides to study, with both facilatory and inhibitory roles in the processing of sensory information at the spinal level having been proposed (as will be discussed further in section 4.4). It was hoped by studying the effect of the development of peripheral inflammation on the release of these peptides in the spinal cord, as will be described in this thesis, that clues to the functional significance of the dynorphins in such pain states could be obtained.

1.9 ANTI-OPIOID MECHANISMS: ROLE OF CHOLECYSTOKININ

Chronic exposure to opiate drugs, such as morphine, results in the development of tolerance, characterised as a decline in the response to a given dose of opiate so that progressively higher doses (or concentrations) become necessary to elicit the same intensity of opioid effect, evident as a shift to the right on a concentration-response curve. This process is equally seen for analgesia, respiratory depression, bradycardia and the hypotensive actions of opiates. Following the cessation of the opiate or administration of a suitable opiate antagonist, such as naloxone, physical dependence may also be demonstrated. This is manifestested as a characteristic withdrawal (abstinence) syndrome, many aspects of which appear to be a 'rebound overshoot' from the opiate depressed state (recently reviewed by Johnson and Fleming, 1989; Cox, 1993; Way, 1993; Schulz, 1993). It is now generally accepted that tolerance to and dependence upon opioids are reflections of cellular adaptations,

investigations into the nature of which have ranged from cultured cell clones to whole animals. However, despite considerable research, the mechanisms underlying these phenomena and the relationship between them is still poorly understood.

The close coincidence observed between the development of tolerance and of physical dependence in animals and man led many early investigators to propose mechanisms linking the two phenomena. Notably influential amongst these was the 'adenylate cyclase' hypothesis, that emerged from the finding by Collier and Roy (1974) that opiates in rat brain homogenates inhibited the synthesis of cyclic AMP induced by the prostaglandins PGE_1 and PGE_2 in a concentration dependent, naloxone reversible, manner. They suggested that inhibition of cyclic AMP formation was the fundamental basis of the actions of opioids, and that tolerance and dependence could be mediated by a compensating hypertrophy of the cyclic AMP synthesis mechanisms within the opiate sensitive neurone (Collier, 1980). This theory received considerable support by the demonstration that NG108-15 neuroblastoma x glioma hybrid cells cultured in the presence of an opiate (and hence chronically exposed) expressed an increased capacity to synthesise cyclic AMP (Sharma et al, 1975). However, investigations on isolated tissue preparations placed doubt on such 'unitary' theories, showing that tolerance and dependence may be separable under certain circumstances (Wuster et al, 1985). For example, the mouse vas deferens has been demonstrated to display high degrees of opioid tolerance without any signs of dependence (Schulz et al, 1980a). Similar studies also demonstrated a remarkable lack of cross tolerance among μ, δ and κ receptor agonists (Schulz et al, 1980b). Further, electrophysiological investigations using rat locus coeruleus slices obtained from morphine pre-treated animals display a significant degree of tolerance to opioids but no sign of dependence (Andrade et al, 1983). Evidence has also been presented for the occurrence of naloxone precipitated withdrawal hyperexcitability of spinal cord neurones of cats treated acutely with morphine in the absence of any tolerance (Johnson and Duggan, 1984).

The occurrence of opioid tolerance but not dependence may be explained by a reduction in the number of functional receptors, either as a result of 'desensitisation' due to functional uncoupling of opioid receptor-effector systems, or 'down regulation' due to internalisation of the opioid receptors themselves. Although the evidence for such changes are not consistent especially from studies *in vivo* (as reviewed by Johnson and Fleming, 1989; Cox, 1993), they cannot be readily dismissed. In contrast the development and expression of dependence necessitates that some opioid receptors remain functional, and the involvement of additional receptor system mechanisms mediating excitatory neurotransmission to allow a hypersensitive state to develop. As an integrated circuitry appears to be essential for full expression of a dependent state, this may due to compensatory hypertrophy of intra- or extra-cellularly localised mechanisms which are involved in the functioning of an opioid neuronal pathway (Wuster et al, 1985).

Since endogenous opioid peptides were discovered many physiological 'antagonists' of their actions have been proposed, the so-called 'anti-opioid' peptides (recently reviewed by Rothman, 1992; Bhargava, 1994; Cesselin, 1995). These include cholecystokinin, neuropeptide FF (a Phe-Met-Arg-Phe[FMRF] -NH_2-like peptide), thyrotropin-releasing hormone and MSH release-inhibiting factor. It has been proposed that these peptides are released in response to activation of opioid circuitry either by exogenously administered opiates or by the endogenous opioid peptides. Of all possible candidates at the spinal cord level, the anti-opioid activity of cholecystokinin (CCK) has been well characterised, specifically with respect to the analgesic actions of opioids (as recently reviewed by Baber et al, 1989). This peptide has also been proposed to play a role in the development of tolerance to but not dependence on opiate drugs. Following a general description of the neurotransmitter functions of CCK in the CNS, the evidence for CCK for acting as an 'anti-opioid' at the spinal level will be considered.

1. CHOLECYSTOKININ - A HORMONE AND A NEUROTRANSMITTER

The term 'cholecystokinin' was first used by Ivy and Olderg (1928) to describe the hormonal factor in extracts of intestinal mucosa which mediated gall bladder contractions. When later isolated by Mutt and Jorpes (1966) from the purified intestine of the pig, this factor was found to also correspond to 'pancreozymin', the hormonal factor in intestinal extracts that had been described by Harper and Roper (1943) to stimulate pancreatic secretion. First characterised as a 33 amino acid peptide, CCK was found to share an identical COOH-terminal pentapeptide sequence with gastrin, Gly-Trp-Met-Asp-Phe-NH_2 (Mutt and Jorpes, 1968). Several longer and shorter molecular forms of cholecystokinin have since been identified including CCK-58, CCK-39, CCK-8 and CCK-4, all of which are amidated and except for CCK-4, sulphated (Goltermann, 1985; Rehfeld, 1985). These peptides have been shown to arise by post-translational modification from a common precursor molecule, pre-procholecystokinin (rat: Deschenes et al, 1984; pig: Gubler et al, 1984; human: Takahasi et al, 1985). It has been proposed that the CCK and gastrin families of peptides share a common ancestral gene (Larsson and Rehfeld, 1977), along with the caerulein-peptides found in the skin of amphibians, which also share the same COOH terminal pentapeptide sequence (as illustrated on figure 5), more recent comparative studies indicating that CCK is phylogenetically older than gastrin (Johnson and Rehfeld, 1992). Evidence that CCK might also exist in the brain was first indicated by Vanderhaeghen et al (1975), who described the presence of a 'brain gastrin immunoreactive peptide, BGP' within the cortex of a number of vertebrate species from amphibians to mammals. The majority of this immunoreactive material was subsequently demonstrated to represent CCK-8 in its biologically active sulphated form (rat: Dockray, 1976; 1980; Beinfeld, 1981. rabbit: Straus et al, 1977. sheep: Dockray et al, 1978. pig: Dockray, 1976; Muller et al, 1977; Rehfeld, 1978a. dog: Dockray, 1976. man: Rehfeld, 1978a; Robberecht et al, 1978).

Cholecystokinin has since been described by radioimmunoassay and immunocytochemical studies to be widely distributed throughout the mammalian nervous system. Although species

FIGURE 5. Amino acid sequences of the COOH-terminal regions of CCK and related peptides

Cholecystokinin

$$\overset{SO_3H}{|}$$
-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2

Caerulein

$$\overset{SO_3H}{|}$$
-Asp-Tyr-Thr-Gly-Trp-Met-Asp-Phe-NH_2

Gastrin

$$\overset{SO_3H}{|}$$
-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2

differences exist, a significant presence has been observed in general in the cerebral cortex, amydala, hippocampus, olfactory lobes, basal ganglia, hypothalamus, thalamus, various midbrain structures such as the substantia nigra, ventral tegmental area, periaqueductal grey matter, raphe nuclei, the medulla oblongata and nucleus of tractus solitarius, and the spinal cord (rat: Innis et al, 1979; Loren et al, 1979; Vanderhaeghen et al, 1980; Stengaard-Pedersen and Larsson, 1981b; Beinfeld et al, 1981; Tuchscherer and Seybold, 1983; Fuji et al , 1985; Vanderhaeghen, 1985; Falon and Seroogy, 1985; Gall et al, 1987. guinea-pig: Larsson and Rehfeld, 1979; Stengaard-Pedersen and Larsson, 1981b. pig: Rehfeld, 1978a. man: Emson et al, 1982; Rehfeld et al, 1978a). Moreover, in some of these brain regions (notably in the cerebral cortex, amygdala, hippocampus, and septum of the rat), CCK has been reported to be the most abundant peptide with levels 10 to 100 times greater than that of other peptides studied (Crawley et al, 1985). This is in contrast to the true gastrins that were found only in significant quantities in the neurohypophysis (pig: Rehfeld, 1978a; b. rat: Loren et al, 1979; Vanderhaeghen et al, 1980. guinea-pig: Larsson and Rehfeld, 1979. man: Rehfeld, 1978a). Other molecular forms of CCK if present are always found in much lower amounts than CCK-8 (Dockray et al, 1976; Robberecht et al, 1978; Muller et al, 1977; Rehfeld, 1978a; Larsson and Rehfeld, 1979; Beinfeld, 1981; Frey, 1985). Despite some speculation about the specificity of COOH-terminally directed antisera to CCK-gastrin commonly employed in immunocytochemical studies (Ju et al, 1986; 1987a, Wiliams et al, 1987; Hokfelt et al, 1988; as will be considered in detail in section 5.4), the distribution of CCK determined in this way has been confirmed in most areas of the CNS by biochemical methods (Rehfeld, 1978a; Larsson and Rehfeld, 1979; Beinfeld et al, 1981), by the use of sequence specific monoclonal or polyclonal antisera directed at NH_2-terminal sites (Fuji et al, 1985; Hokfelt et al, 1985; 1988), and more recently by RNA blot (Voigt and Uhl, 1988; Iadarola et al, 1989; Lindefors et al, 1991) and *in situ* hybridisation studies (Siegel and Young, 1985; Savasta et al, 1988; Lanaud et al, 1989; Ingram et al, 1989; Schiffman and Vanderhaeghen, 1991; Lindefors et al, 1991; Rattray et al, 1992).

Early binding studies in rat using [^{125}I]CCK-33, along with other CCK related peptide fragments, demonstrated differences between brain and peripheral tissues. In the brain CCK-8, desulphated CCK-8, CCK-4 and pentagastrin were bound with nearly equal affinity, in comparison with binding sites in the pancreas and gut, that display a high selectivity for CCK-8 (Innis and Synder, 1980a, b; Saito et al, 1980). Although electrophysiological studies involving the iontophoretic administration of CCK peptides and analogues into specific brain regions suggested that all CCK receptors within the CNS were not identical (Chiodo and Bunney, 1983; Hommer et al, 1985), early homogenate binding and autoradiographic studies failed to provide any evidence of receptor heterogeneity (Sankaran et al, 1980; Hays et al, 1980; Saito et al, 1991; Gaudreau et al, 1983a; b; Zarbin et al, 1983; Van Dijk et al, 1984; Williams et al, 1985). Later however, Moran et al, (1986) in more detailed studies based on the differential binding by sulphated and desulphated CCK-8, demonstrated by autoradiography that binding sites existed in a few nuclei in the rat brain (namely the area postrema, nucleus of tractus solitarius, dorsal hypothalamic nuclei and interpendunclear nuclei), that displayed the same ligand selectivity as those in the periphery. They classified these receptors as type A (for Alimentary i.e. pancreas and gastric) to distinguish them from the majority found, type B (for Brain), that are more diffusely distributed and are relatively non-selective to the multiple forms of CCK. However, the differentiation of receptor subtypes based on agonist affinity is problematical and it was only on the development of the non-peptide CCK receptor antagonists, such as MK329 (Devazepide or L365718) with a high selectivity for peripheral CCK-A receptors (Chang and Lotti, 1986; Evans et al, 1986), that a clearer differentiation between these classes of CCK receptor could be achieved in biochemical and pharmacological studies. The development of similar antagonists for the CCK-B receptor, such as L365260 (Bock et al, 1989; Lotti and Chang, 1989), has allowed the locations of these CCK-A and CCK-B receptors in the CNS to be even more precisely defined. For example, these antagonists (displayed on table 3) have confirmed the limited presence of CCK-A receptors in the rodent (Hill et al, 1987a, b; Hill and Woodruff, 1990)

TABLE 3. CCK receptor antagonists. Selective antagonists developed to study the distribution and function of the different types of CCK receptor are listed, with the principal investigators responsible for the development of each antagonist quoted in brackets (see abbreviations, pg xi, for details).

NON-SELECTIVE	**proglumide** (Hahne et al, 1981) **benzotript** (Hahne et al, 1981) **lorglumide (CR1409)** (Makovec et al, 1985)
CCK-A SELECTIVE	**MK329 (L365718 or devazepide)** (Evans et al, 1986; Chang and Lotti, 1986) **L365031** (Evans et al, 1986)
CCK-B SELECTIVE	**L365260** (Bock et al, 1989; Lotti and Chang, 1989) **CI988 (PD134308)** (Hughes et al, 1990)

and primate (Hill et al, 1990; Hill and Woodruff, 1990) brain, and allowed differences between species to be revealed, such as in the spinal cord where the CCK-B receptors are the prominent type in the rodent but not the primate, including man (Hill et al, 1988; Hill and Woodruff, 1990; Ghilardi et al, 1992). The CCK-B receptor binding sites appear the densest in forebrain structures such as cerebral cortex, hippocampus, amygdala, septum, olfactory bulbs, and basal ganglia and generally low in the midbrain and rest of brainstem (Hill and Woodruff, 1990, Moran and McHugh, 1990). The CCK-A receptors, suggested to be phylogenetically older (Vigna et al, 1984), since they are localised in the area postrema (a chemosensitive trigger zone where the special epithelial cells of the blood-brain barrier are absent) and the nucleus of tractus solitarius (the site of entry of vagal afferents), are likely to play an important role in satiety following food ingestion, being possibly receptive to peripherally circulating CCK (Moran et al, 1986; Moran and McHugh , 1990). Notably the recent cloning of the rat CCK-A and CCK-B receptors has shown them to be members of the G-protein coupled seven transmembrane spanning superfamily of receptors, and to be identical in both nervous and peripheral tissue (Wank et al, 1992a, b).

From such anatomical studies, along with the finding of neuropharmacological, electrophysiological and behavioural studies, CCK has been proposed to play a role in a wide variety of CNS functions, including nociception and analgesia, appetite and thirst, thermoregulation, neuroleptic activity, anxiety, arousal, motor behaviour, and neuroendocrine activity (as reviewed by Vanderhaeghen, 1985; Baber et al, 1989; Cooper and Dourish, 1990; Harro et al, 1993; Crawley and Corwin, 1994). Aside from their potential usefulness as adjuvants in opiate mediated analgesia (as will now be considered in detail), the main focus of interest in developing effective CCK receptor agonists and antagonists has come from the prominent role of CCK in satiety following food ingestion and anxiety (particularly of the 'panic-like' nature), the latter notably appearing to involve CCK-4.

2. ROLE IN NOCICEPTION AND ANALGESIA

Cholecystokinin is localised in regions of the mammalian CNS associated with nociception and pain modulation, notably the superficial layers of the spinal cord dorsal horn (see references in section 5.4), the periaqueductal grey (Maciewicz et al, 1984; Skirboll et al, 1982; 1983; Lanaud et al, 1989; Rattray et al, 1992; Liu et al, 1994) and raphe nuclei (Mantyh and Hunt, 1984) of the brain stem, and intralaminar nuclei of the ventromedial thalamus (Hunt et al, 1986; Lanaud et al, 1989). In these regions CCK has been described to display a similar distribution to the opioid systems (Stengaard-Pedersen and Larsson, 1981c; Gall et al, 1987; Pohl et al 1990), with even evidence for colocalisation with enkephalin (Gall et al, 1987) and dynorphin (Gibbins et al, 1987). CCK has been notably also been described as coexistent with substance P (Dalsgaard et al, 1982; Skirboll et al, 1982; 1983; Gibbins et al, 1987; Ju et al, 1987a; Tuschsherer et al, 1987).

Evidence that CCK plays a role in the processing of nociceptive information at the spinal level, was originally proposed on the basis of early findings that CCK-8 (ng doses) produced a powerful, naloxone reversible, analgesia when injected intrathecally (Jurna and Zetler, 1981), and that ir-CCK was released into the spinal superfusate by bilateral electrical stimulation of the sciatic nerve only at Aδ/ C fibre intensities (Yaksh et al, 1982). However, the analgesic effect of i.t. CCK-8 appears to be dependent on the animal species, the behavioural test performed and doses of CCK-8 administered (recently reviewed by Baber et al, 1989). These analgesic effects are now considered to be mediated at high levels and possibly represent more of a pharmacological than physiological effect. More potent analgesic effects were obtained when CCK was administered supraspinally, namely in the periaqueductal grey and ventromedial thalamus, and observed more consistently using analgesia tests such as the hot plate, that are not simple spinal reflexes such as the tail-flick assay (Zetler, 1980; Jurna and Zetler, 1981; Barbaz et al, 1986; Hill et al, 1987; Pittaway et al, 1987). Also even high doses of i.t. CCK-8 (approximately 10 times the ED_{50} obtained in the tail flick test), failed to reduce the activity in ascending axons in the spinal cord of rats evoked by the electrical stimulation of

primary nociceptive afferents (Doi and Jurna, 1982). By contrast in the spinal cord predominantly excitatory effects have been recorded electrophysiologically following the local administration of CCK-8 (Jeftinija et al, 1981; Salt and Hill, 1982; Willetts et al, 1985), as in many other regions of the CNS (Dodd and Kelly , 1981; Skirboll et al, 1981; Chiodo and Bunney, 1983; Brooks and Kelly, 1985; Hommer et al, 1985; Jaffe et al, 1987; Liu et al, 1994), most of these spinal neurones being also excited by substance P (Willetts et al, 1985). Notably it has been proposed that CCK acts as a physiological 'antagonist' of the antinociceptive effects induced by opioids at the spinal level, the evidence for which will now be considered in detail.

Following observations that CCK under certain conditions displays actions opposite to those of opioids, such as food intake, Faris et al (1983) hypothesised that CCK would block opioid mediated (naloxone reversible) analgesia. Using the tail flick test, they observed that pre-treatment with CCK-8 significantly reduced the analgesia mediated by both exogenous morphine (administered either intrathecally or systemically), and that induced by brief electrical shocks to the rat forepaw ('foot shock induced analgesia', FSIA), for which there is evidence for mediation by endogenous opioids (Watkins et al, 1982). Significantly lower doses of CCK-8 were employed (approximately 10 times lower than those reported to elicit an analgesic response) which alone produced no effect on the tail flick latencies to radiant heat (Faris et al; 1983; Faris, 1985). Faris and co-workers also showed that rats immunised against CCK-8 conjugated to bovine serum albumin (BSA), displayed heightened analgesic responses compared to controls that had been treated with BSA alone (Faris et al, 1984; Faris, 1985). A multitude of behavioural evidence has since been presented in favour of CCK acting as a endogenous inhibitor of opioid actions at the spinal level, determining the magnitude of the opioid analgesic response, some of the more convincing being studies where the effects of CCK receptor antagonists were examined. These are considered below. It is interesting that many behavioural studies have found central effects following the peripheral administration of CCK-8, despite reports this derivative of procholecystokinin does not cross

the blood-brain barrier (Banks et al, 1980), although it may to a limited extent along with even lower molecular weight forms such as CCK-4. Additionally there is also a possibility that some of the central effects of systemic CCK could be indirect via actions in the periphery.

Early studies by Watkins and co-workers in rats using the weak non-selective CCK receptor antagonist proglumide (co-administered either intrathecally or systemically together with morphine), indicated that endogenous CCK can act to attenuate the analgesia induced by morphine and forepaw shock, as well as that mediated by the injection of a stable enkephalin analogue, as assessed by the tail flick assay (Watkins et al, 1984; 1985a; b). A potentiation of the analgesic response was observed with low doses of the antagonist (up to 0.1μg given intrathecally), followed by a reduction of the response with high doses (1 and 5μg), a 'bell-shaped' dose-response curve being produced. This phenomenon has been observed with other CCK receptor antagonists (Dourish et al, 1988; 1990) and as yet remains unexplained, though it may be the result of actions at multiple CCK neuronal sites or systems. The specificity of the response to the opioid systems was supported by the lack of enhancement by proglumide of non-opioid mediated analgesia (i.t. noradrenaline, hindpaw foot shock). Again there was no evidence that CCK was producing an independent hyperalgesic effect, since proglumide had no effect in the absence of opioids. Thus, the CCK pathways appear not to be tonically involved in nociception but rather activated by opioids (endogenously released or exogenously administered), and may function to return pain perception back to normal. A similar reciprocal role of CCK and opioid peptides has also been revealed by studies of the analgesia induced by i.c.v. β-endorphin in the hot plate test (Itoh et al, 1982; Katsuura and Itoh, 1985) found to be mediated by the spinal release of Met-enkephalin (Tseng et al, 1986; Suh et al, 1992), and by the demonstration that proglumide administered into the periaqueductal grey of rats potentiated analgesia from morphine given either by the same route (Watkins et al, 1984, 1985a) or systemically (Li and Han, 1989).

Other studies with proglumide and benzotript (another weak CCK receptor antagonist) have pointed to a role of CCK in opiate tolerance. Thus, the chronic coadministration of the

CCK antagonists with morphine was found to inhibit the development of tolerance to morphine analgesia in rats (Watkins et al, 1984, 1985a; b; Tang et al, 1984; Rovati et al, 1985; Panerai et al, 1987). It has been proposed that tolerance to the analgesic effect of opiates may in part result from a progressive compensatory increase in the activity of CCK systems in response to prolonged opiate administration. No such involvement of CCK in the occurrence of opioid dependence was evident, as assessed by observing the withdrawal syndrome precipitated by graded doses of acutely injected naloxone (Rovati et al, 1985; Panerai et al, 1987).

Using the tail flick (Dourish et al, 1988; 1990) and paw pressure (O'Neill et al, 1989; Rattray et al, 1988) tests in rats, the more potent and selective CCK-A receptor antagonists, MK329 or L365031, given systemically have been similarly found to enhance morphine analgesia, and prevent the development of tolerance to morphine. Even more potent effects have been observed in rats using the CCK-B receptor selective antagonist L365260 (Dourish et al, 1990; Zhou et al, 1993b), indicating that the CCK-opioid interaction most likely occurs through CCK-B receptors in the rodent. Although CCK receptor antagonists alone had not been found to produce an effect on behavioural nociceptive thresholds, this CCK-B receptor antagonist when administered systemically into monkeys was shown to induce analgesia, as revealed by increased tail withdrawal latencies to immersion in warm (55°C) water (O'Neill et al, 1990). Due to the lack of CCK-B receptors in the primate spinal cord, however, this effect is likely to be mediated at a higher CNS level, such as the periaqueductal grey. More recently another CCK-B receptor antagonist CI988 (PD134308) has been shown to potentiate the analgesic effect of morphine (Wiesenfeld-Hallin et al, 1990), to block the development of tolerance to morphine (Xu et al, 1992), as well as reverse the tolerance induced (Hoffman and Wiesenfeld-Hallin, 1994) in rats to repeated morphine administration, as assessed by the hot plate test. There is also considerable evidence for an involvement of CCK in the electroacupuncture analgesia reported to be mediated by the release of endogenous opioid peptides (Han et al, 1981; 1985; 1986; Zhou et al, 1993a). It is of note that CCK has also

been reported to exert effects on other neuronal systems implicated in antinociception at the spinal cord level, namely GABA (Rodriguez et al, 1987), galanin (Wiesenfeld-Hallin et al, 1990) and noradrenaline (Sullivan et al, 1994), although such actions could be indirect via actions on opioid systems.

Although behavioural research points to a possible physiological role of CCK as an endogenous modulator of opioid analgesia, the mechanism of interaction remains obscure, and much remains to be done at a single cell and membrane level. Electrophysiological studies have tended to support the findings of these behavioural investigations. CCK does not appear to play a direct role in nociceptive transmission. The spinal application of CCK-8 (Jeftinija et al, 1981; Magnuson et al, 1990; Kellstein et al, 1991; Stanfa and Dickenson, 1993; Sullivan et al, 1994) and of CCK receptor antagonists (Suberg et al, 1985; Kellstein et al, 1991; Stanfa and Dickenson, 1993; Sullivan et al, 1994) have been shown to produce minimal effects on C fibre evoked discharges of dorsal horn neurones, and when effects have occurred with selective antagonists (Wiesenfeld-Hallin and Duranti, 1987; Wiesenfeld-Hallin et al, 1990) they have been attributed to indirect opioid mechanisms, namely a blockade of the tonic activity of the endogenous CCK system. In contrast however, CCK peptides and antagonists have been found to reduce and enhance respectively the depressive action of morphine on C fibre evoked responses at the spinal cord level (Suberg et al, 1985; Kellstein et al, 1991; Stanfa and Dickenson, 1993; Sullivan et al, 1994) and on the nociceptive flexion reflex (Wiesenfeld-Hallin and Duranti, 1987; Wiesenfeld-Hallin et al, 1990). A recent study by Chapman et al (1995b) also revealed a reciprocal relationship between the CCK and opioid peptides on the expression of c-fos in the spinal cord, following the development of a peripheral inflammation. A normally ineffective dose of morphine was found to reduce the expression c-fos in superficial and deep laminae of the spinal cord when administered systemically with the CCK-B antagonist L365260, which alone induced no effect.

Thus, along with evidence that i.t. morphine causes the release of ir-CCK from the rat spinal cord *in vivo* (Tang et al, 1984; Zhou et al, 1993b), it has been suggested that CCK

may be released in response to opioid receptor activation in the spinal cord and act to modulate the analgesic effects of opioids. Prolonged repetitive activation of opioid antinociceptive pathways would then induce a compensatory increase in the activity of anti-opioid CCK circuitry and hence cause progressively greater antagonism of opioid activity, culminating in the development of tolerance to the analgesic effect of opioids. Standard pharmacological texts describe such a process as a 'physiological antagonism' (Rang et al, 1991). Such an hypothesis appears to be supported by the demonstration that larger amounts (20 fold) amounts of the CCK antagonists (proglumide and its more potent analogue lorglumide) are required to reduce the tolerance that develops to a higher dose (10 fold) of morphine co-administered intrathecally, than that which is induced by a lower dose (Kellstein and Mayer, 1991).

However, the possible modulatory effects of opioid receptor activation on CCK neuronal systems has not been thoroughly investigated. Despite an early study reporting that spinal levels of ir-CCK are significantly increased in rats following morphine treatment (Watkins et al, 1985b), more recent investigations have observed either no effect (Pohl et al, 1992) or significant increases (Ding and Bayer, 1993) in the spinal cord content of CCK following acute and chronic morphine treatment. Moreover, conflicting data have been obtained from experiments which have examined spinal release of CCK following the administration of opioids *in vivo* (Tang et el, 1984; Rogriguez and Sancristan, 1989; Zhou et al, 1993) and *in vitro* (Benoliel et al, 1991; 1994a; b). It was hoped by examining the effect of acute and chronic opiate administration on the release of CCK in the spinal cord, as will be discussed in this thesis, that information on the mechanism of this interaction could be obtained.

1.10 PRINCIPLES OF THE ANTIBODY MICROPROBE TECHNIQUE

The antibody microprobe technique was developed by Duggan and Hendry (1986) as a means of improving the spatial precision with which sites of neuropeptide release can be

determined in the CNS under *in vivo* conditions. The principles of the antibody microprobe technique are illustrated in figures 6 and 7.

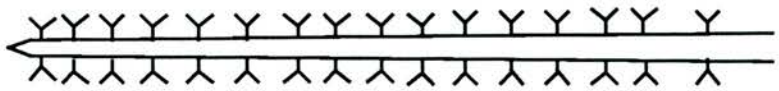
Basically, antibody microprobes are fine glass micropipettes, with a siloxane polymer coating on their outer surfaces, to which antibodies to a neuropeptide of interest are immobilised. These are inserted into the brain or the spinal cord and left *in situ* for a defined period of time, so that a proportion of the neuropeptide if released, may bind to the adjacent microprobe surface. Following withdrawal from the CNS, any binding of the endogenous peptide can be detected as the failure of regions of the microprobe to bind a radiolabelled form of the peptide, in which it is subsequently incubated and autoradiographic images obtained. These microprobe autoradiographs can be analysed quantitatively with a computer assisted image analysis system, as illustrated on figure 7. The distribution of the amounts of bound radiolabelled peptide is determined by changes in the image density (or grey scale value) along the length of the autoradiographs, with any deficits in the tracer binding being represented graphically as comparatively low grey scale values. A sorting program can select groups of microprobes according to stimulus/ experimental parameters and allow pairs of such grouped or ' mean' image analyses to be compared and the significance of their regional differences evaluated statistically. The binding pattern of the endogenous peptide detected on the antibody microprobes, under defined physiological conditions, can then be related back to discrete structures in the CNS, such as the individual laminae of the spinal cord. Release is equated with a significant increase in the extracellular levels of a compound following a defined stimulus.

The antibody microprobe technique has so far been employed in the cat and rat spinal cord to study the release of substance P (cat: Duggan and Hendry, 1986; Duggan et al, 1987; 1988a; b; 1991a; 1992; 1995; Hutchison and Morton, 1989; Morton et al, 1990; Schaible et al, 1990; 1992; Zhao et al, 1992; Lang et al, 1994. rat: Lang and Hope, 1994), neurokinin A (cat: Duggan et al, 1990; 1991b; Hope et al, 1990 b; c; Lang et al, 1991), CGRP (cat: Morton and Hutchison, 1989; 1990; Schaible et al, 1994. rat: Schaible et al, 1994),

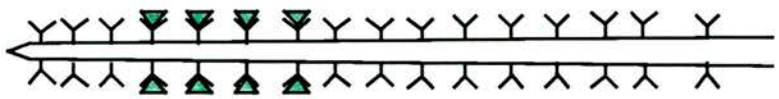
FIGURE 6 Principles of the antibody microprobe technique for the *in vivo* detection of neuropeptide release I. From antibody immobilisation to the production of autoradiographic images.

PRINCIPLES OF THE ANTIBODY MICROPROBE TECHNIQUE I

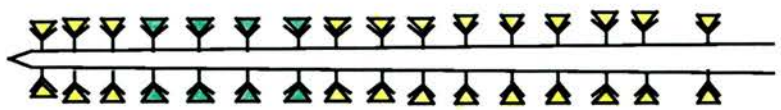
1. Antibodies (Y) to a neuropeptide of interest are immobilised onto the outer surfaces of glass micropipettes.



2. Locally released neuropeptide (▽) binds to adjacent antibodies *in vivo*.



3. Radiolabelled form of the peptide (▽) in which microprobes are subsequently incubated *in vitro* binds to free antibody sites.



4. After washing microprobes are placed on X-ray film to obtain an autoradiographic image.

5. Autoradiographs indicate sites of extracellular neuropeptide presence.

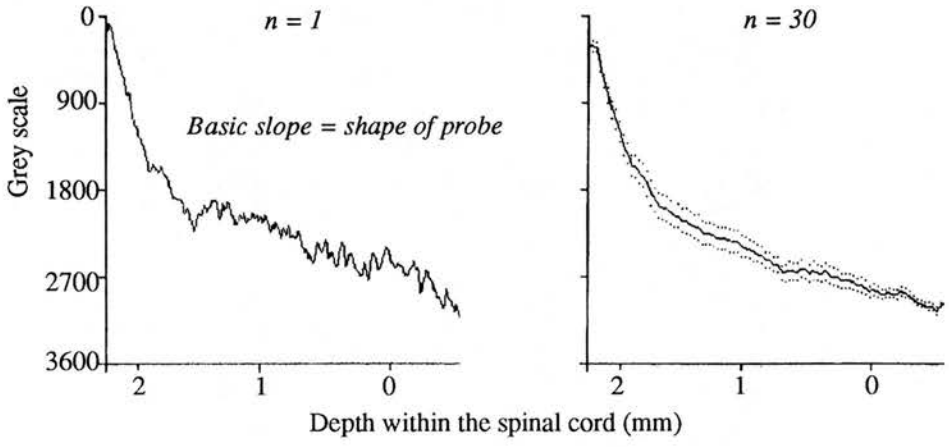
Area of reduced image = area of reduced radioligand binding
= area of endogenous ligand binding

6. Groups of microprobes present under defined physiological conditions are compared. Release is equated with increased extracellular levels produced by a defined stimulus

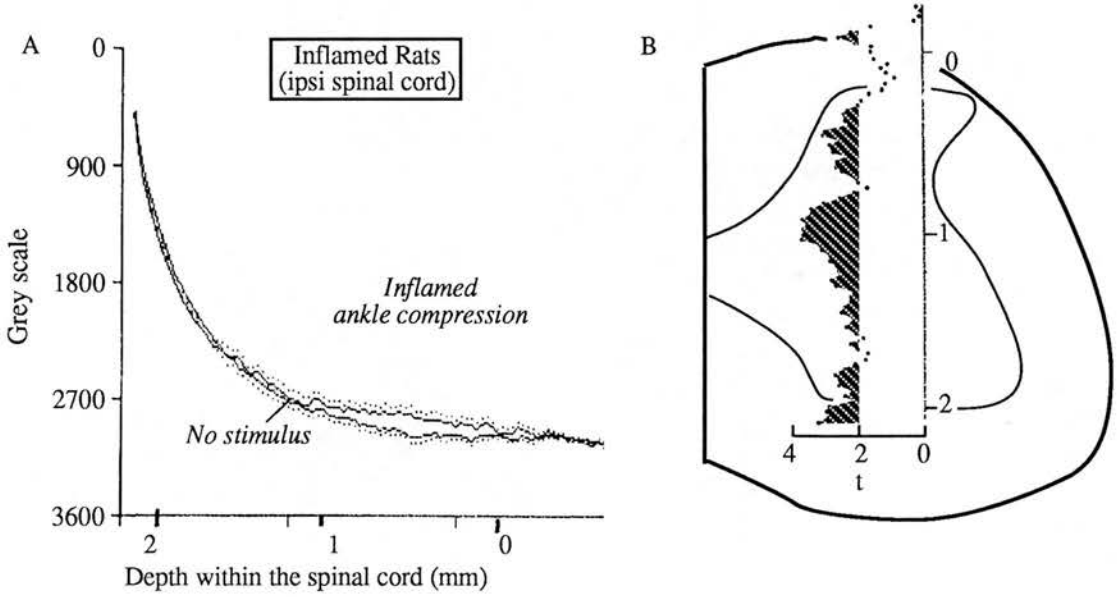
FIGURE 7 Principles of the antibody microprobe technique for the *in vivo* detection of neuropeptide release II. Image analysis of the autoradiographic images to reveal sites of endogenous peptide binding.

PRINCIPLES OF THE ANTIBODY MICROPROBE TECHNIQUE II

1. Using a computer-assisted image analysis system, autoradiographs are converted into plots of image density (or grey scale values) v.s. length at 30μm intervals (A). The analysis system allows data from a group of microprobes to be pooled and a plot of mean image density (+/- s.e.m.) at each 30μm interval to be produced (B). The means are joined to give a continuous line but the s.e.m. at each analysis point is plotted separately.



2. Mean image analysis for two groups of microprobes can be statistically compared at each 30μm interval, using multiple Students' t-tests (A). Areas of statistically significant differences (i.e. where $t>2$, $P<0.05$) in level of neuropeptide detection can be referred to depth within spinal cords (B).



somatostatin (cat: Morton et al, 1989), galanin (cat: Morton and Hutchison, 1989. rat: Hope et al, 1994), dynorphin (cat: Hutchison et al, 1990) and neuropeptide Y (rat: Mark et al, current study) in response to a range of peripheral stimuli and systemically administered drugs. It has also been used in the brain to measure the release of substance P (cat: Williams et al, 1994a. rat: Furmidge et al, 1993); neurokinin A (rat: Furmidge et al, 1995), β-endorphin (rat: Duggan et al, 1993. cat: Williams et al, 1993), neuropeptide Y (cat: Williams et al, 1993), thyrotrophin releasing hormone (rat: Waterfall et al, 1994), and enkephalins (cat: Williams et al, 1994b).

The amounts of neuropeptide which can be detected on autoradiographs of microprobes, as displacement of the radiolabelled ligand, can be extremely small. For example, complete inhibition of the binding of [^{125}I]Bolton-Hunter substance P with a specific activity of approximately 2000 Ci/ mmol, has been estimated to correspond to the binding of 3 x 10^{-18} mole of substance P, over a 100μm of the length of microprobe (Duggan, 1991). The amount of time the microprobes need to remain in the CNS to detect the endogenous peptide varies according to local neuropeptide concentrations and the sensitivity of the particular microprobes, ranging in these previous studies from 5 to 30 minutes. A recent study showed that 150 stimuli delivered at 0.5Hz for a 5 minute duration, resulted in just detectable release of immunoreactive (ir)-substance P in the spinal cord by microprobes (Duggan et al, 1995). Although more of a qualitative than quantitative technique, some estimate of the concentrations in the vicinity which produce a given inhibition of binding is possible. With nearly all studies performed, it has been shown *in vitro* that the neuropeptide to which the immobilised antibodies have been raised, at a concentration of 10^{-7}M for 30 minutes at 37^{0}C, has suppressed the binding of the radiolabelled form of the peptide by greater than 50% (Duggan and Furmidge, 1994). With β-endorphin such suppression occurred with 10^{-9}M (Duggan et al, 1993). Morton et al (1989) also made some estimate of the concentrations of somatostatin to which the microprobes were exposed to *in vivo*, by relating images of

microprobes exposed to differing concentrations of the neuropeptide *in vitro* to those of microprobes that had been inserted into the spinal cord.

Since the antibody microprobe technique determines peptide release with a biological resolution of the order of 100μm, it can provide far greater insight to the role of a neuropeptide in a particular system than is possible with non-invasive, surface perfusion methods. Also since each microprobe has a diameter of only 5 to 10μm, the microprobes cause minimal trauma to the tissue into which they are inserted, and hence minimal disturbance to the structures releasing the peptide. This contrasts to the trauma associated with the other invasive methods that attempt to localise areas of release *in vivo*, such as those involving the introduction of push pull cannulae and microdialysis fibres with diameters of 300-500μm into the CNS. These other invasive techniques give poor biological resolution, for example in the spinal cord they localise sites of release to little better than the dorsal or the ventral horn, and have a tendency towards low recovery rates. Thus, the antibody microprobe technique remains the least damaging invasive method for the *in vivo* detection of neuropeptide release.

1.11 AIM OF THESIS

To employ the antibody microprobe technique to study release of neuropeptides, as a means of investigating the involvement of the endogenous opioid peptide dynorphin in spinal cord function using an inflammatory model of 'chronic' pain, and the possible role of cholecystokinin as an endogenous opioid 'antagonist'.

CHAPTER 2: General Methodology I: Animal Models and Surgical Procedures

2.1 ANIMALS

Male Wistar rats (weight range 250-275g) obtained initially from Bantin and Kingman, UK, and then later from Charles River,UK, were used in the studies described in thesis. Charles River rats due to their higher pathogen-free status were preferred, since this likely affords them a greater resistance to the respiratory infections which rats are particularly prone when being housed. No other differences were observed between the animal sources.

The rats were acclimatised on arrival for at least 48 hours before being used in any procedure/ experiment. They were kept under controlled conditions, at an ambient temperature of 19-23^{0}C, in 12 hour light/ dark cycle, and had free access to food and water.

2.2 ANAESTHESIA

1. RECOVERY PROCEDURES

(i) Diethyl ether

Chronic ankle inflammations were initially induced under diethyl ether anaesthesia. This involved placing a rat on a metal grid suspended in a sealed glass bell jar in a fume cupboard, beneath which cotton wool balls soaked in diethyl ether had been positioned, until a suitable level of anaesthesia was obtained. When the procedure was complete the animal was observed for complete recovery before being rehoused.

Diethyl ether was later deemed as too irritant for both the rat and the handler to be used in this procedure.

(ii) Halothane

The majority of the recovery procedures performed were carried out under halothane anaesthesia (initially started at 4% to induce anaesthesia then reduced to between 0.5 and 1%).

This involved placing the animal in a specifically designed chamber through which halothane gas could be passed at a controlled concentration and any excess scavenged away into a special storage vial. When anaesthetised the rat was removed from the chamber to allow the induction of a chronic ankle inflammation. Anaesthesia was maintained throughout by attaching the rat directly to the halothane supply via a mask into which both the nose and mouth of the rat could be sealed. When the procedure was complete the halothane supply was removed, but the oxygen supply continued until the animal had regained consciousness.

2 NON-RECOVERY PROCEDURES

Urethane (ethyl carbamate)

For all the non-recovery experiments described in this thesis, rats were anaesthetised with urethane (25% weight/volume[w/v] solution; Sigma Chemical, UK). A single intraperitoneal (i.p.) injection of 1g/ kg urethane in Ringers' solution was sufficient to induce complete anaesthesia. Following induction, the depth of the anaesthesia was assessed by direct measurement of the mean arterial blood pressure (systolic pressure maintained at between 100 and 120mmHg), via a catheter inserted into a carotid artery and by regularly testing for corneal blink and hindpaw withdrawal reflexes which had to be absent. Further injections of urethane were given i.p to maintain anaesthesia sufficient to abolish both of the reflexes measured. In addition, blood pressure levels and changes induced by peripheral stimuli were closely monitored to ensure that noxious stimuli would induce no more than approximately a 5mmHg rise in blood pressure.

At the end of each experiment the anaesthetised animal, was killed by an intravenous (i.v.) injection of a mixture of concentrated KCl and urethane, which resulted in cardiac arrest within seconds.

2.3 INDUCTION OF PERIPHERAL INFLAMMATION

1. DEVELOPED ANKLE INFLAMMATION

For the purpose of the experiments performed in this thesis a unilateral adjuvant induced inflammation of the periarticular tissues (Grubb et al, 1988; Donaldson et al, 1993) was employed as a model of chronic inflammation. This was used primarily since it resulted in reliable and reproducible unilateral inflammation that remained stable over several weeks, but also since it probably pertains more directly to the joint 'pain' that most electrophysiological investigations of inflammation have studied under more acute conditions (as described in section 1.8).

To induce such an inflammation animals received 4 subcutaneous injections of a Freund's complete adjuvant (FCA) suspension (1.0 mg/ ml heat killed and dried *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide manooleate; total volume of 0.15ml; Sigma Chemical, UK) around one ankle joint, using a sterile 26½ gauge needle and a 2ml syringe, on Hibitane (0.5% in 70% ethanol; Zeneca Ltd., UK) cleaned skin. This was performed initially under diethyl ether and then for later experiments under halothane anaethesia (as described in section 2.2.1). These animals were then left for between 3 to 5 days before being used in an experiment. Throughout this time the animals were monitored for the development of swellings at other peripheral locations. On the day of the experiment the circumferences of both ankles were measured. These injections resulted in a self-limiting inflammation that was well developed by 3 to 5 days, the ankle region swelling from an average diameter in normal animals of 2.7cm to a mean diameter of 4.0cm within this time period. Injected animals showed little or no apparent discomfort, gait impairment or change in weight gain.

Previous histological studies by Hanesch et al (1993b) have shown that the inflammation resulting from this procedure involves both articular (capsule and cartilage) and periarticular tissues (connective tissue, tendons and skin). Using this dose of FCA no signs indicative of a

generalised disease state, such as the development of swellings at other peripheral sites, were ever observed. This was in agreement with the studies of Donaldson et al (1993).

2 ACUTE ANKLE INFLAMMATION

In addition some rats received the same adjuvant injections on the morning of the experiment, to allow study of the release of peptides at an early phase of the inflammatory response. The injected ankle usually showed some sign of swelling over the period of the study.

2. 4 ACUTE ADMINISTRATION OF MORPHINE

Morphine was administered intravenously to rats, over a two hour period, in as high doses as possible without causing significant respiratory depression, bradycardia or hypotension. It was determined that for a rat of approximately 400g this could be achieved by administering 2.5 or 5 mg/ kg of morphine every 15 minutes over the two hour period, each dose being followed by sterile Ringers' solution to flush the cannula of the drug solution. Morphine HCl (Martindale Pharmaceuticals, UK) was dissolved from powder form in sterile Ringers' solution into a 25mg/ml stock solution, divided into 1ml volumes in Eppendorf tubes and stored at -20°C until use.

To block the effect of the morphine, naloxone was administered as an adequate single injection of 1mg/kg, again followed by sterile Ringers' solution to flush the cannula of the drug solution. Naloxone HCl (Sigma Chemical, UK) was dissolved from powder form in sterile Ringers' solution into a 10mg/ml stock solution, divided into 1ml volumes in Eppendorf tubes and stored at -20°C until use.

2.5 SURGICAL PROCEDURES

Initial surgery involved the cannulation of a carotid artery, an external jugular vein and the trachea, to permit direct measurements of arterial blood pressure, to allow the intravenous injection of drugs and to aid unobstructed breathing respectively. The anaesthetised rat was laid on its back on a homeothermic heating pad. The neck area was closely shaved with electric clippers and a longitudinal incision made along the midline of the exposed skin. A jugular and then a carotid artery were exposed using blunt dissection through the musculature, connective tissue and fat pads. A section of each vessel was completely cleared using small curved forceps allowing three fine silk ligatures to be positioned, one of which was tightened rostrally to allow the vessel to be stretched slightly by attached artery forceps. An opening was then made in each vessel, using iris scissors under a binocular microscope. The carotid artery was first securely clamped at its caudal end. Portex cannula (3FG) attached to plastic three way taps were filled with either sterile Ringers' solution alone for the vein, or with Heparin (12.5units/ ml; CP Pharmaceuticals Ltd, UK) for the artery. These cannulae were then introduced caudally into each vessel in turn, for a distance of approximately 1.5cm, and secured in position by tightening the remaining ligatures. The trachea was then exposed and an opening was made to allow the narrow end of a fluted cannula to be inserted. These tracheal cannulae were manufactured in the heat of a bunsen flame from polythene tubing and were fluted to maximise air flow. Notches were made at their widest point to hold a ligature formed from a linen thread that had been previously placed beneath the trachea. From this stage efforts were made to ensure the trachea remained unobstructed by intermittent suction. Blood oxygenation was also assisted by directing a gentle jet of humidified oxygen towards the opening of the tracheal cannula.

An extended laminectomy was then made at vertebral levels T_{12} to L_2 to expose the dura mater of the lumbar spinal segments L_1-L_6, that receive an input from the ankle joint area. This involved removal, by blunt dissection, of the muscle groups attached to the dorsal surface

of the vertebral column, approximately 2cm either side of the rats most caudal ("floating") rib which is attached to T13. The exposed bone was cleared of all tissue with a blunt scapel blade. The rat was then positioned in a stereotaxic frame (Preclinical Veterinary Sciences, The University of Edinburgh), by means of ear bars and a jaw vice, and six swan neck clamps were placed under alternate mammillary processes of the exposed vertebrae. The incised skin was reflected towards the frame and secured to each corner by means of linen thread. The animals temperature from this point was continually monitored with a rectal probe and maintained between 36-38^{0}C using a controlled heating system, which consisted essentially of a metal plate, with inserted controlled heating elements, that partially surrounded the animal (Preclinical Veterinary Sciences, University of Edinburgh). This was found to be more satisfactory than commercially available heating blankets which tended to obstruct chest movement in the spinal frame which was used. The heated plate was controlled by a Devices control box of the type normally used with heating blankets. Any exposed parts of the rat were covered with a foil blanket, in particular it was found that heat loss was greatly assisted by covering the tail. The dorsal vertebrae were removed with micro-rongeurs at the required segments under a binocular microscope. The edges of the remains of the laminae were lined with sterile haemostatic gauze (Sterispon) to prevent any subsequent bleeding. A sterile solution of purified agar in Ringer's solution (4% w/v; Unipath Ltd., UK) was then used to cover the muscle and nervous tissue beneath and to form a solid pool, into which a window over the lumbar spinal segments was then made. Following opening of the dural sac with sterile forceps, the exposed spinal cord was irrigated via a pump and heating jacket with sterile Ringers' solution at 37^{0}C, continuously throughout the experiment, to prevent drying of the cord surface and ensure reasonably sterile conditions around the spinal cord. Any excess fluid was removed by suction at an edge of the opening. Although continuous irrigation minimised the collection of inflammatory exudates on the surface of the cord, it was often necessary to remove any small accumulations of fibrinous material from archnoid/ pia openings with sterile fine forceps and hand controlled suction.

CHAPTER 3: General Methodology II: Preparation and Use of Antibody Microprobes

3.1 STORAGE OF ANTIBODIES AND PEPTIDES

Certain precautions were taken in storage and handling of the antibodies and peptides employed in the antibody microprobe studies, to ensure that they performed optimally. This is in contrast to the inorganic compounds commonly employed, which were more robust and whose storage was more straightforward.

1. ANTIBODIES

The antibodies to dynorphin and CCK, were purchased commercially from Peninsula Laboratories Europe Ltd. They were supplied in a stable form, lyophilised from a buffer solution of 0.1M sodium phosphate buffer (pH7.4), 0.05M NaCl, 0.1% bovine serum albumin (BSA), 0.01% NaN_3 and 0.1% Triton X-100. In practice it has been found that microprobes were better made from such preparations if the lysophilisate was reconstituted at double the concentrations for radioimmunoassay recommended by the manufacturer, with the recommended diluent. Thus, in these studies, the lyophilised antiserum was reconstituted with 25ml of distilled water and then divided into 250µl and 500µl volumes in Eppendorf tubes. The resultant aliquots were then stored at -20°C until use. No reconstituted antibodies were ever refrozen after being thawed, as repeated thawing/ refreezing damages protein structure.

2. PEPTIDES

All peptides used in the antibody microprobe studies described in this thesis, were stored in Eppendorf tubes treated with Sigmacote (Sigma Chemical, UK). This coating that minimises the binding of peptides to surfaces, was also (more importantly) employed to coat the inner surface of the glass microcapillaries, that were used in various stages in the *in vitro* and *in vivo* experiments as a convenient way of incubating the microprobes in small quantities of the unlabelled and radiolabelled peptides. As with the antibodies, no peptides were ever refrozen after being thawed from the reconstituted state.

(i) Unlabelled peptides

These were purchased in freeze-dried form from Peninsula Laboratories or Sigma Chemical, and reconstituted according to the suppliers recommendations with the appropriate dilutent, commonly a solution of phosphate buffered saline (PBS) containing 0.1% sodium azide. The bulk of the peptide was reconstituted as a 10^{-4}M solution. These were then used to make up 100µl dilutions of 10^{-5}, 10^{-7} and 10^{-9}M either at the same time as stock solutions or as required in the Sigmacoted Eppendorf tubes. All aliquots were stored at -20^{0}C or -70^{0}C.

(ii) Radiolabelled peptides

Commercially [^{125}I]-labelled peptides were purchased in a lyophilised form in 10µCi batches, from Peninsula Laboratories or Amersham International plc, UK. These were reconstituted immediately on delivery with the recommended dilutent, distilled water for dynorphin A(1-8) and CCK, then divided into suitable volumes. These aliquots were stored in a designated, lead screened freezer at -20^{0}C until required. On the day of an experiment a single aliquot was diluted in a solution of PBS-azide containing 0.5% BSA (Sigma Chemical, UK) to give a solution with a gamma emission of 2000 counts per minute (cpm) per µl and stored on ice (but not frozen) until required. The BSA serves to block non-specific binding of the radiolabelled peptide to the microprobe surface. Since [^{125}I] has a half life of 60 days, batches of the radiolabelled peptides were usually only used up to approximately 6 weeks after their specified activity date.

3.2 PREPARATION OF ANTIBODY MICROPROBES

The preparation of the antibody microprobes has changed in various subtle ways since the most recent detailed published account by Duggan (1992) and indeed since the work in this thesis was initiated. It is of note that other forms of antibody microprobes

have been prepared involving evaporative organic aminosilane, polycarbonate or epoxylite resin-activated charcoal coatings (Waterfall et al, 1994), however these will not be discussed further in the context of this thesis. The following is the current method of antibody microprobe production used in our laboratory.

1. SILOXANE POLYMER COATING OF GLASS MICROPIPETTES

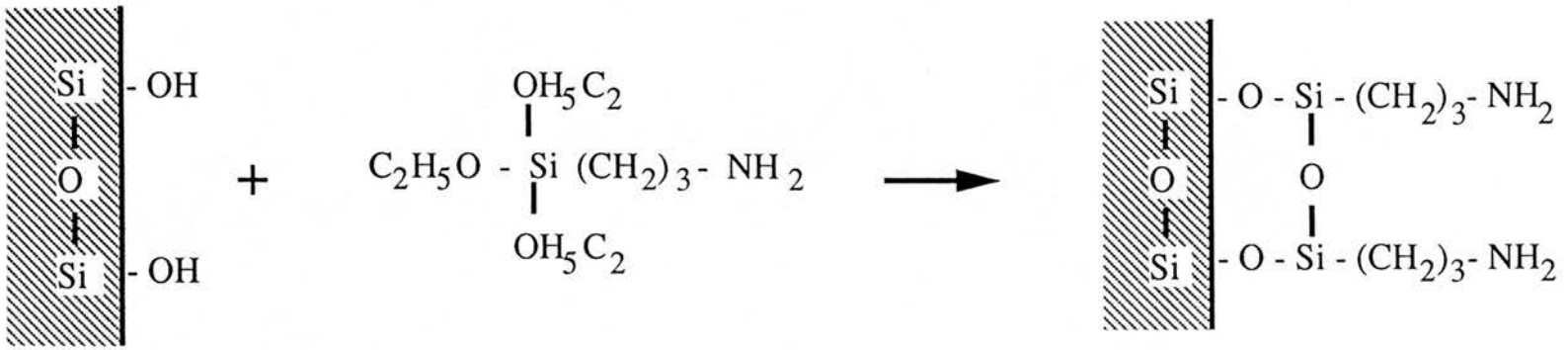
Proteins can be immobilised to glass by placing organic groups onto the glass surface (Weetall, 1970). With the antibody microprobes this is done by reacting a substituted silane (γ-aminopropyltriethoxysilane) with silanol groups and hydroxyl groups of adsorbed water, on the outer surface of the glass micropipettes. The initial reaction is shown schematically in figure 8A. The hydroxyl groups interacting with the substituted silane are shown as part of the glass, but there is evidence that much of the reaction depends on chemisorbed water molecules. Provided there is sufficient adsorbed water on the surface of the glass, there is a tendency for a cross-linked polymer to build up. Such a polymeric coating can be readily visualised under the microscope and assessed for the quality needed for the future uniform coating with antibodies, which is necessary if any degree of spatial precision is to be obtained.

(i) Preparation of the glass micropipette

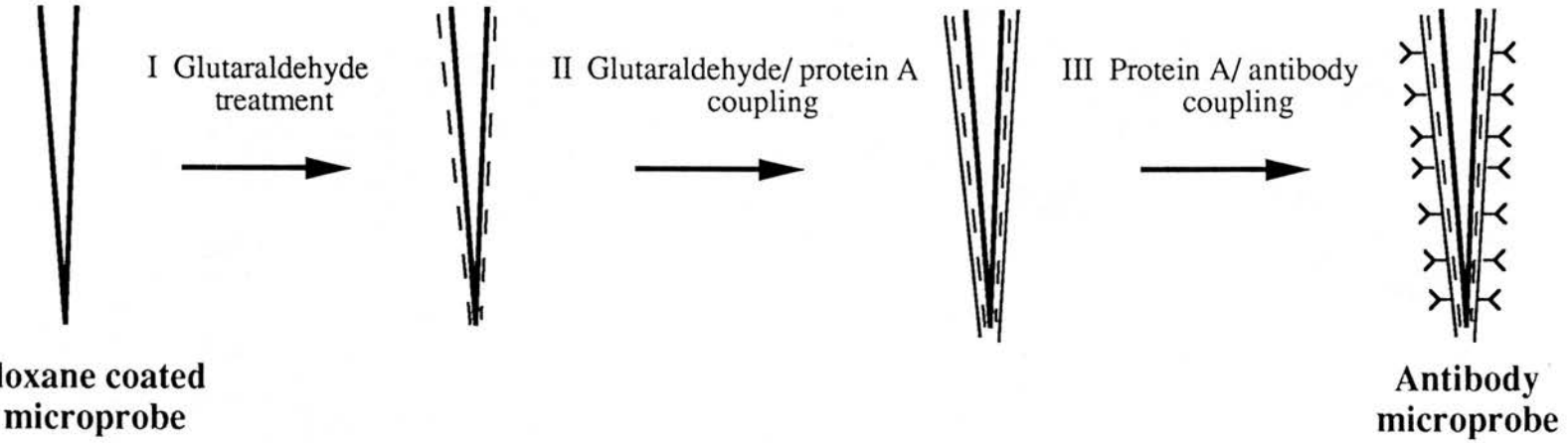
Hollow, non-filament borosilicate capillary glass (GC300-10; Clark Electrochemical Instruments, UK; external diameter 3mm, internal diameter 1.62mm), was used to manufacture the glass antibody microprobes. The capillary glass was cleaned thoroughly before use by incubating it overnight in a container of xylene and then serially in three different containers of absolute alcohol, for periods of 30 minutes. After drying in a clean oven, the glass was then stored in covered beakers until required.

FIGURE 8. Preparation of antibody microprobes. (A) The interaction between γ-aminopropyltriethoxysilane and the glass surface of a micropipette to produce alkylamine (siloxane coated) glass. (B) Stages in the preparation of the antibody microprobes. The sequence of reagents used to couple antibodies to siloxane coated microprobes.

A


Glass
γ–Aminopropyltriethoxysilane
Alkylamine glass
Si
- OH
O
Si
- OH
+
OH5C2
C2H5O - Si (CH2)3- NH2
OH5C2
Si
- O - Si - (CH2)3- NH2
O
Si
- O - Si - (CH2)3- NH2

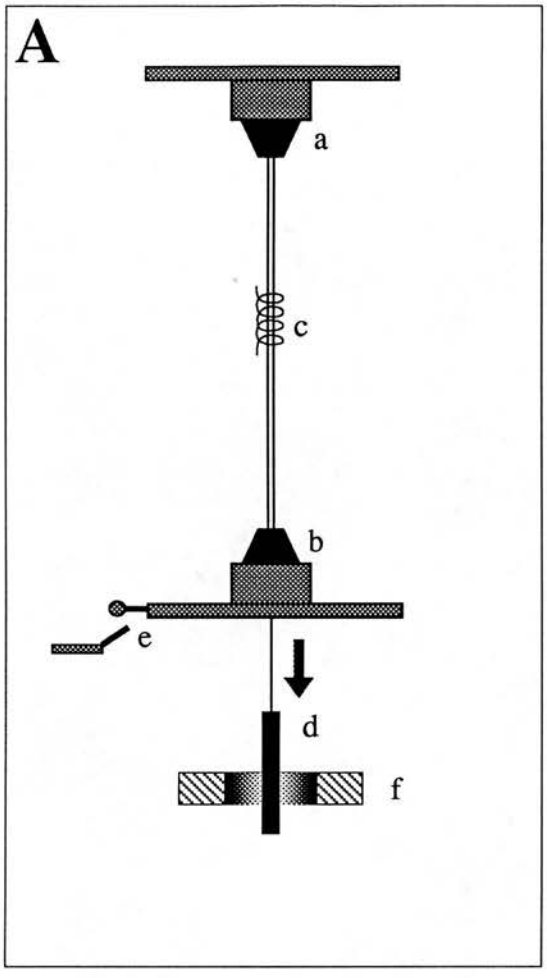
B

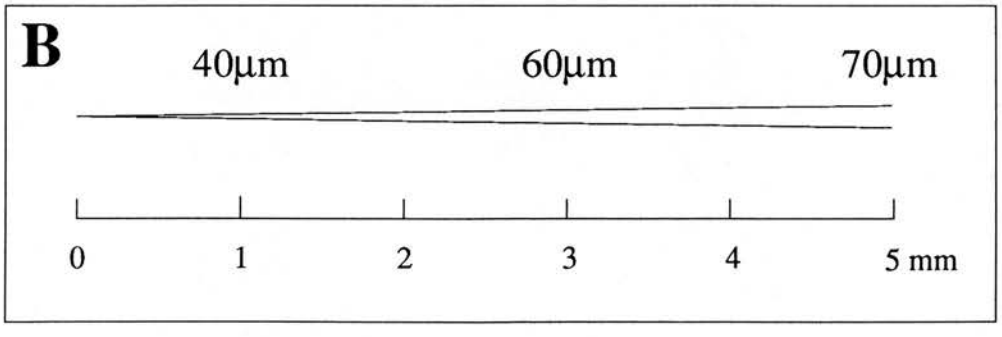

I Glutaraldehyde treatment
II Glutaraldehyde/ protein A coupling
III Protein A/ antibody coupling
Siloxane coated microprobe
Antibody microprobe

The capillary glass was then drawn out to a very gradual taper by heating in the coil of a vertical microelectrode puller (Preclinical Veterinary Sciences, The University of Edinburgh). The coil extends over 10mm of the capillaries, thus ensuring the melting of a large area of glass. The principles of operation of this type of puller and the factors that influence the shape of the micropipettes, are illustrated on figure 9A. Figure 9B shows the typical dimensions of a pulled micropipette used in the studies described in this thesis. Since in these studies microprobes were inserted 2.25mm into the rat spinal cord, the shape of the micropipettes had to remain approximately constant up to 5mm from the tip. The pulled micropipettes were then heat sealed at both ends; the unpulled end in a gas flame and the tip, by touching it briefly against an electrically-heated coil mounted in a micromanipulator under a binocular microscope to allow accurate sealing. The resultant micropipettes although very flexible and fairly resistant to accidental breakage were stored horizontally in specially prepared grooved metal racks held within covered plastic boxes throughout most of the production.

The micropipettes (usually in groups of approximately twenty) were then tightly packed into glass carrying buckets with small perforations in the base, to allow easy handling and permit draining throughout microprobe preparation during the many washing procedures. Micropipettes, in buckets, were then immersed in 50% nitric acid for 30 minutes and then given 3 x 10 minute washes in distilled water. This is believed to increase the number of free silanol groups on the glass surface (Weetall, 1970). The buckets of micropipettes were suspended by their hooked upper ends in a clean oven and dried at 200^0C for at least 2 hours, or until further use. This entire process resulted in clean, dry micropipettes with some degree of surface etching, on which subsequent chemical reactions could proceed.

FIGURE 9. Manufacture of glass micropipettes for antibody coating. (A) Principles of operation of micropipette puller. The capillary glass is suspended between the two holders (a, b) running through a heated metal coil (c) at mid-length. The lower holder is attached to a solid metal weight (d) by means of a robust metal cable. As the glass is heated by the coil, it stretches and the lower holder drops. At a particular distance a switch is activated, which triggers a strong electromagnetic field (f) , this rapidly pulls the weight down and draws the molten glass out to a very gradual taper before separating to give two micropipettes. The heat of the coil, drop distance until magnetic pull triggered and pull force are all factors which determine microprobe shape. (B) Typical dimensions of antibody microprobes used for detection of neuropeptides in the rat spinal cord in the studies described in this thesis.


A
a
c
b
e
d
f


B
40µm
60µm
70µm
0
1
2
3
4
5 mm

(ii) Preparation of the reagents

The following method is based on having a measured amount of water in the solution of substituted silane, in an attempt to control the amount of siloxane polymerisation at the surfaces of the micropipettes, and hence maximise the coating success rate. Excessive water in the solution results in excess polymerisation within the bulk of the silane/ toluene solution.

Clean boiling tubes (24/29; Quickfit) were placed in the same oven as the micropippettes and dried for 2 hours. Whilst still warm the boiling tubes were placed in a fume cupboard and filled with 30 ml of reagent grade toluene (Aldrich, UK), 10ml aliquots of γ-aminopropyltriethoxysilane (APTES; Aldrich) and 5µl of distilled water The boiling tubes were then stoppered with polythene caps and vortexed to ensure complete mixing of the solutions. The toluene had been previously been treated with molecular sieves (4A, 4-8 mesh; Aldrich) to absorb as much water as possible (150g of molecular sieves are added to a 2.5 litres of the reagent grade toluene and left for at least 24 hours), to control the amount of water in the substituted silane solution.

(iii) Exposure of the glass micropipettes to the reagents

The buckets of glass micropipettes were carefully removed from the oven with metal forceps and allowed to cool slightly before each was added to a tube of APTES/ toluene mixture. The boiling tubes were then restoppered and placed in centrifuge holders, that are balanced in pairs using lead pieces. The boiling tubes were then placed in a centrifuge and spun at 4^0C, 2000rpm for 1 hour. This centrifugation results in a more even build up of siloxane polymer on the surface of the micropipettes. This is thought to be due to either centrifugation of free water away from the tips of the micropipettes or to the removal of small particles of debris from the solution around the tip, which may act as foci for the polymerisation reaction. The stoppered boiling tubes were then removed from the centrifuge, placed in a rack and left to stand overnight in a refrigerator at 4^0C. The buckets of microprobes were then removed from the boiling tubes in a fume cupboard and washed by a

brief immersion in the toluene solution, after which they were allowed to dry at room temperature. The APTES/ toluene mixture was then discarded. Sample microprobes from each bucket were then inspected under a binocular microscope for adequacy of coating. A satisfactory coat is one that is readily visible and even. If too light, it was necessary to repeat the above procedure to build up the intensity of the siloxane coat. Once the micropipettes were deemed to be satisfactorily coated, they were cured, still within the carrier, in an oven at 200^0C for 24 hours. This increased the cross-linking within the polymer and rendered it insoluble.

(iv) Assessment of siloxane coating

Following curing, each microprobe was checked for evenness of coat by placing in a micromanipulator mounted beneath a binocular microscope and examined under incident light at x12.5 magnification. A good polymer coat on the surface of the microprobes will appear as a milky deposit, white prior to heat curing and yellowish afterwards. Following curing the coat should be resistant to a vigorous wiping with a clean soft tissue. It was important to identify microprobes with uneven or patchy distribution of siloxane coating, which were disregarded, and also to identify microprobes with small focal aggregations of polymer. In some cases these 'lumpy' microprobes can be made usable by the wiping procedure. Microprobes were generally sorted into three groups; those with light, medium and heavy polymer coating. Although the analysis of microprobes (as will be described in section 3.5) is performed in such a way as to minimise the effects of coat density on the final result, it was deemed appropriate to utilise microprobes with similar densities of siloxane.

2. COUPLING OF ANTIBODIES TO MICROPROBES

It takes a minimum of 48 hours for microprobes to be prepared for an *in vivo* experiment. Siloxane coated microprobes can be stored indefinitely, but once proteins have been linked

they probably have relatively short lifespan, and have usually been used within approximately 72 hours of being completed.

There are three stages in the coupling of antibodies to the siloxane coated microprobe. The basic reagents used at each stage are illustrated figure 8B. Proteins are covalently bound to the amine groups of the siloxane coated microprobe surfaces via the bifunctional reagent glutaraldehyde. The antibodies to the neuropeptides studied in this thesis were only available in polyclonal antiserum, raised by the active immunisation of rabbits to either porcine dynorphin A(1-8) or CCK-8, and hence full of unwanted proteins. Thus, protein A, a staphylococcal derived protein which has a high selectivity for the Fc region of some immunoglobulins of the IgG class (namely those raised in rabbits and guinea-pigs) whilst leaving the peptide binding site free (Goding, 1978), was used as an intermediate to bind the required immunoglobulins from a polyclonal antiserum to the surfaces of the microprobes.

(i) Glutaraldehyde treatment

The desired number of siloxane coated microprobes to be used in an experiment, were selected and placed in clean, siloxane-free glass carrying buckets, their coatings having been given a final check under the microscope. They were then immersed in a solution of 2.5% glutaraldehyde (BDH Laboratory Suppliers, UK) in distilled water for 30 minutes. The buckets of microprobes were then washed for 3 x 10 minute washes in distilled water.

(ii) Glutaraldehyde/ protein A coupling

Under a dissecting microscope, the tips of the glutaraldehyde treated microprobes were then immediately inserted into 5µl glass microcapillaries (Blaubrand; UK) filled with a solution of protein A (*Staphylococcus aureus*; Sigma Chemical, UK). The protein A had been diluted in PBS-azide solution, to give a final concentration of 0.1mg ml^{-1}. Each microprobe, capped with a capillary, was placed horizontally in a slot on a perspex rack. When all the microprobes were treated, the racks were placed in a covered plastic tray and placed in a cold

room at 6^0C for at least 24 hours. Paper towels soaked in PBS-azide were placed in the base of the tray to minimise evaporation during this period.

(iii) Protein A/ antibody coupling

Following incubation the microprobes were removed from the protein A containing capillaries and placed in a glass dish containing sodium borohydride 2.5% w/v (Sigma Chemical, UK) in a borate buffer for 10 minutes. This mixture reacts to produce free hydrogen, that reduces the Schiff bases, formed by aldehyde coupling to amino groups on the protein A. The microprobes were then placed in further clean, siloxane-free glass carrying buckets and given 3 x 10 minute washes in PBS-azide solution. The microprobes were then inserted into 20µl glass microcapillaries (Camlab, UK) containing a solution of the antibody to the neuropeptide under study. The microprobes were then again placed on perspex racks in covered trays and placed in a cold room at 6^0C for a minimum of 24 hours, depending on the dilution of the antibody.

At least 15 minutes before their use in an experiment, the microprobes were stored in glass carrying buckets in solutions of PBS-azide, to wash away any excess antibody and allow the microprobes to acclimatise to room temperature (20 to 23^0C).

3.3 *IN VITRO* TESTING OF ANTIBODY MICROPROBES

In vitro testing of the antibody microprobes occurs both before and periodically throughout studying the release of a particular neuropeptide in the CNS.

1. PRIOR TO *IN VIVO* EXPERIMENTATION

Prior to use *in vivo* the sensitivity and specificity of the antibody microprobes for the neuropeptide being studied, needs to be determined. When purchasing a new antibody, great attention is paid to the manufacturers claims regarding the cross-reactivity of the antisera with

other peptides. From such radioimmunoassay data, those antisera displaying the greatest specificity for the neuropeptide of interest, were initially chosen for testing (see individual studies for details of specific antiserum). As previously stated these were polyclonal antisera and hence contained immunoglobulins of a ranging affinity and specificity.

The typical format of an *in vitro* assay testing both the sensitivity and specificity of antibody microprobes is illustrated on table 4, using the gamma counter results for microprobes bearing antibodies to dynorphin A(1-8).

(i) Measurements of antibody microprobe sensitivity and non-specific binding of the radiolabelled ligand

This involved incubating microprobes in known concentrations of the neuropeptide being studied. Antibody microprobes (at least five per peptide concentration to be tested, plus an additional five microprobes which were simply incubated in the radiolabelled peptide) were prepared as previously described. Approximately 15 minutes prior to use in an assay, the microprobes were stored in glass carrying buckets in solutions of PBS-azide at room temperature. Then the majority were inserted into 5µl Sigmacoted capillaries filled with various concentrations of peptide solution, identified accordingly by colour coding with an indelible marker pen, and placed for 30 minutes at 37^0C in a humidified incubator, to mimic to some extent the conditions to which comparable microprobes will be exposed to *in vivo*. On removal from the incubator the microprobes were placed in a holder and their tips lowered vertically into a magnetically stirred, ice-cold solution of PBS-azide containing 0.1% Tween 80 (Pierce and Warriner Ltd), for 15 minutes (including those not exposed to any concentration of peptide solution), to remove any unbound peptide and debris from their surfaces. After washing the tips, all the microprobes were placed in 5µl Sigmacoted capillaries filled with the radiolabelled form of the neuropeptide under study, for 24 hours at 6^0C.

TABLE 4. The results of an *in vitro* assay testing both the sensitivity and specificity of microprobes bearing antibodies to dynorphin A(1-8). Microprobes mounted on card were placed in tubes (corresponding to POS no.1 to no.36) and assessed for amount of radioactive emission (in cpm) using a Beckmann RIA scintillation well gamma counter. The mean ± s.e.m. were obtained for microprobes that had been incubated under the same conditions, using cpm values (or 'counts') that had been corrected for background emissions (cor.cpm).

Microprobes no. 1 to no.5: were not exposed to any concentration of peptide solution but simply incubated in [^{125}I]-dynorphin A(1-8). The mean ± s.e.m. of the cor.cpm obtained for these '0M' microprobes represent 100% binding of the radiolabelled ligand.

Microprobes no. 6 to no.9: were incubated in a 10^{-9}M solution of dynorphin A(1-8) prior to [^{125}I]-dynorphin A(1-8). This resulted in an overall suppression of the mean counts obtained for microprobes no. 1 to 5 (i.e. the total counts) of 36%.

Microprobes no. 10 to no.15: were incubated in a 10^{-7}M solution of dynorphin A(1-8). This resulted in an overall suppression of the total counts of 56%.

Microprobes no. 16 to no.21: were incubated in a 10^{-5}M solution of dynorphin A(1-8). This resulted in an overall suppression of the total counts of 84%.

Microprobes no. 22 to no.26: were incubated in a 10^{-5}M solution of dynorphin A(1-17). This resulted in an overall suppression of the total counts of 5%.

Microprobes no. 27 to no.31: were incubated in a 10^{-5}M solution of dynorphin A(1-13). This resulted in an overall suppression of the total counts of 18%.

Microprobes no. 32 to no.36: were incubated in a 10^{-7}M solution of Leu-enkephalin. This resulted in no apparent overall suppression of the counts.

DYNORPHIN A(1-8) *IN VITRO* [21/ 4/ 94]

PAT	DET	POS	TIME	COUNTS	COR.CPM	COR.CPM MEAN± S.E.M.	PROBE TYPE [PEPTIDE]
0	1	1	60	914	880.0		
0	1	2	60	1422	1396.7		
0	1	3	60	1148	1118.0	1105.2	0M
0	1	4	60	1057	1025.4	± 75.4	
0	1	5	60	1136	1105.8		
0	2	6	60	621	583.0		
0	2	7	60	779	741.0	711.5	Dyn A(1-8)
0	2	8	60	791	753.0	± 37.4	10^{-9}M
0	2	9	60	807	769.0	(↓36%)	
0	3	10	60	582	570.3		
0	3	11	60	421	403.0		
0	3	12	60	489	473.7	485.5	Dyn A(1-8)
0	3	13	60	634	596.0	± 31.0	10^{-7}M
0	3	14	60	433	395.0	(↓56%)	
0	3	15	60	513	475.0		
0	4	16	60	208	161.7		
0	4	17	60	316	271.6		
0	4	18	60	284	239.1	175.2	Dyn A(1-8)
0	4	19	60	168	121.1	± 24.7	10^{-5}M
0	4	20	60	179	152.4	(↓84%)	
0	4	21	60	133	105.0		
0	5	22	60	1035	1033.8		
0	5	23	60	1216	1220.1	1046.9	Dyn A(1-17)
0	5	24	60	819	811.4	± 63.3	10^{-5}M
0	5	25	60	1010	1008.0	(↓5%)	
0	5	26	60	1151	1161.3		
0	6	27	60	875	874.6		
0	6	28	60	983	986.8	908.0	Dyn A(1-13)
0	6	29	60	945	947.3	± 32.9	10^{-5}M
0	6	30	60	784	780.1	(↓18%)	
0	6	31	60	989	951.0		
0	7	32	60	1266	1191.0		
0	7	33	60	1230	1155.0	1180.44	Leu-Enk
0	7	34	60	1036	998.5	± 46.4	10^{-7}M
0	7	35	60	1333	1297.1	(↓0%)	
0	7	36	60	1273	1260.6		

Following incubation in the radiolabelled peptide, the microprobes were again washed for 15 minutes in a magnetically stirred, ice-cold solution of PBS Tween 80. The tips of the microprobes were then carefully broken off, between a thumb and forefinger, to give a final length of approximately 15mm. It is important during this procedure that none of the tip is lost. The distal portions of these microprobes were then mounted on narrow strips of cardboard by applying a small amount of typist correction fluid (Liquid paper/ Tippex) to the broken end. These strips of card were then placed in tubes, and the amount of radioactive emission (in cpm^{-1}) was determined for each tube using a Beckmann RIA scintillation well gamma-counter.

Autoradiographic images of these *in vitro* probes were also obtained. This was done by fixing the strips of card bearing the microprobe tips onto a sheet of paper, again using correction fluid, and placing it into a standard (non-screen) X-ray film cassette next to a sheet of monoemulsion X-ray film (CEA, MRB) for up to 7 days.

(ii) Measurements of the specificity of the antibody

Although the antisera used have been examined by conventional radioimmunoassay before purchase, it is also important to verify some of the manufacturers claims for specificity prior to commencing *in vivo* work. Such measurements were carried out at the same time as the sensitivity and non-specific binding of the radiolabelled ligand was tested and involved incubating microprobes in equivalent concentrations of those peptides most likely to produce some cross-reactivity, in an identical manner to that described in (i).

2. CONCURRENT WITH *IN VIVO* EXPERIMENTATION

Periodically throughout the use of a batch of the radiolabelled peptide, a less intensive check of the functioning of the antibody microprobes was made. Typically this involved suppression with only two concentrations of the unlabelled neuropeptide being studied *in vivo*, and intermittently that of another peptide had been previously been shown to result in some

cross-reactivity. This was to confirm that the sensitivity and specificity of the microprobes being employed were still as determined from the initial *in vitro* investigations.

3.4 *IN VIVO* USE OF ANTIBODY MICROPROBES

Before microprobes were inserted into the spinal cord, the suitability of potential penetration sites in relation to the proposed peripheral stimuli are examined. In the present experiments this was achieved by recording with a 4M NaCl filled microelectrode, the excitatory responses of neurones in the deep dorsal horn (approximately 0.6 to 1.0mm from the dorsal surface of the spinal cord) to light mechanical cutaneous stimuli. All the studies described in this thesis, involved placement of microprobes into regions of the lumbar spinal cord that responded to such stimulation of the ankle region. A sketch of the vascular system on the dorsum surface of the spinal cord exposed was made for future reference of the microprobe penetration sites chosen. In order to permit microprobe entry into the spinal cord it was often necessary to remove the pia mater at these sites using sterile fine forceps.

Microprobes were removed from the PBS-azide solution in which they had been stored immediately prior to use and both of the sealed ends were removed. The unpulled end was removed by a glass cutter and the tip, by gently bumping it against the surface of a metal coil under a binocular microscope, to give tip diameters of approximately 10μm. At this stage some of the microprobes were filled with potamine sky blue 2% (w/v) in 1.2M sodium acetate solution to allow easy visualisation of the tips for accurate placement in the cord, and also to eject dye for future determination of tip placement. Pairs of microprobes were introduced into the same side of the spinal cord to a depth of 2.25mm from the dorsal surface using two stepping motor driven micromanipulators. One microprobe was orientated vertically to the spinal cord surface and the other at 10^0 from the vertical, both approximately 0.6mm mediolaterally from the centre of the dorsal vein. When the tips of the microprobes were visualised as just touching the cord surface, under a binocular microscope, the manipulators

were re-zeroed, and the microprobes were lowered into the spinal cord. Microprobes were initially kept in the cord for 5, 15 or 30 minutes, until preliminary analysis could be performed, to determine the optimum period of time required for that particular release study. During these periods either no stimulus was applied, the animal was subjected to the application of a peripheral stimulus, or a drug was given intravenously (see individual studies for details). On average 24 microprobes were placed in the spinal cord of any one animal.

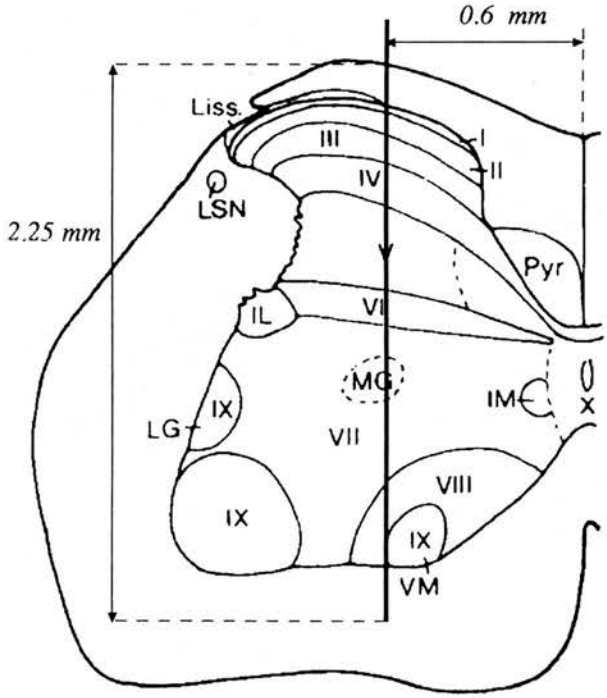
At the end of each experiment pontamine sky blue was ejected iontophoretically at several defined sites and depths in the lumbar spinal cord, some of which corresponded to sites of prior microprobe insertion sites. The spinal cord was then removed and fixed in paraformaldehyde (10%). This was later sectioned and processed histologically to locate the resultant dye spots. This data was essential to verify the placement of microprobes during an experiment, and allow locations on the probes to be related to positions within the spinal cord. An example of such a spinal cord section is shown in figure10. On the opposing figure legend is a schematic drawing depicting the lamination of the spinal cord grey matter of the third lumbar spinal segment of the adult rat, taken from Molander et al (1984). This can be seen to bear close resemblance to the spinal cord section illustrated.

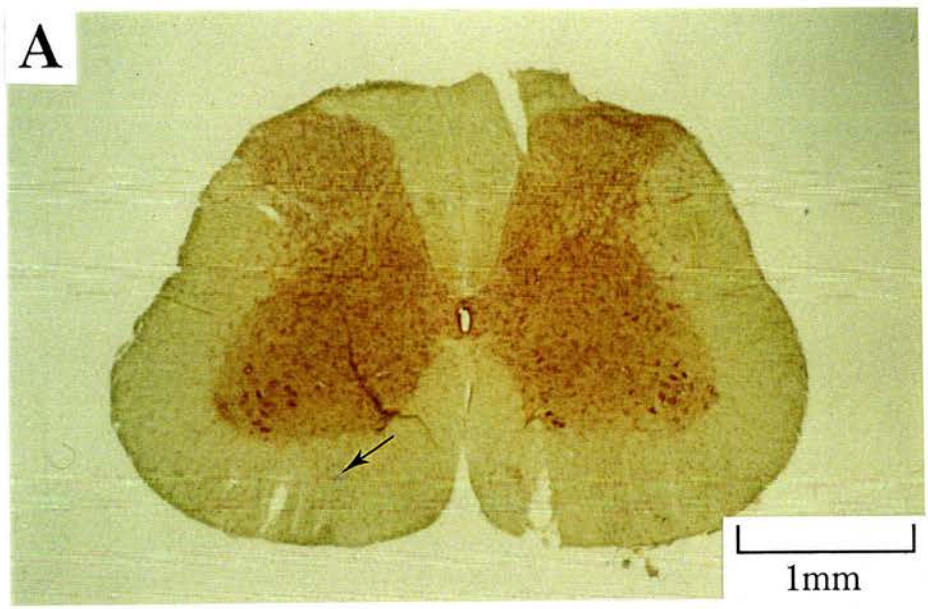
Upon removal from the spinal cord, microprobes were washed for 15 minutes in ice-cold PBS Tween 80, and then incubated for approximately 24 hours at 6^0C in Sigmacoted capillaries containing the radiolabelled form of the peptide under study. Following this period the microprobes were again washed for 15 minutes in an ice cold solution of PBS Tween 80, but this time with suction applied to the inside of the microprobes, as a precaution in case the sealed electrode tips had been broken and any radiolabelled peptide that might have been transported by capillary attraction into the tip. Suction was obtained by mounting the thick ends of the microprobes in a sealed perspex block, which was in turn connected to a vacuum pump. The distal portions (10 to 15mm) of the microprobes were then carefully broken off, mounted on a sheet of paper and placed in a X-ray film cassette next to monoemulsion X-ray film for 3 to 5 days for dynorphin study and 5 to 7 days for CCK study, in a manner

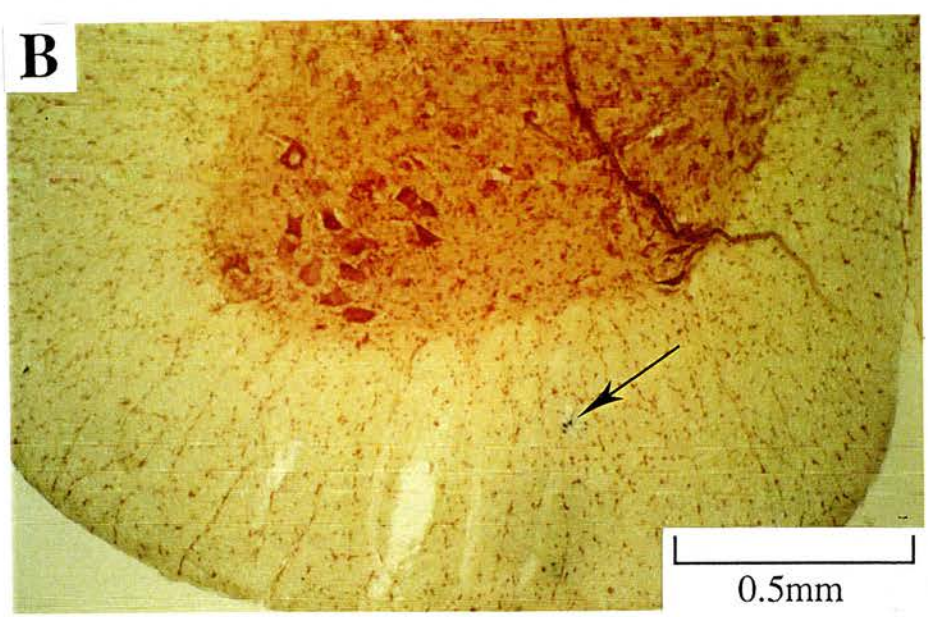
FIGURE 10. Histological section of the rat spinal cord. (A) Transverse 52μm section of the lumbar region of the rat spinal cord (stained with neutral red) from an antibody microprobe experiment studying the release of ir-dynorphin A(1-8). To verify the placement of microprobes, pontamine sky blue was ejected iontophoretically at defined depths near some of the sites of prior microprobe insertion, which in this particular section was 2.25mm below the surface of the cord dorsum (marked by an arrow-head). This can be seen in greater detail in (B).

Below is a schematic drawing depicting the lamination of the spinal grey matter of the third lumbar spinal segment of the adult rat (derived from Molander et al, 1984). This can be seen to bear a close resemblance to the spinal cord section illustrated opposite. **Liss**, Lissauer's tract; **LSN**, lateral spinal nucleus; **IL**, intermedio-lateral nucleus; **Pyr**, pyraminal tract; **LG**, lateral group of large cells in the dorsolateral part of the ventral horn; **IM**, intermedio-medial nucleus; **MG**, medial group of large neurones in the intermediate zone; **VM**, ventro-medial nucleus.

As depicted by the solid vertical line microprobes were inserted approximately 0.6mm mediolaterally from the centre of the dorsal vein on the exposed surface of the spinal cord to a depth of 2.25mm.




A
1mm


B
0.5mm

analogous to that previously described for the *in vitro* tests. At least two films were derived from each experiment.

A satisfactory X-ray film was considered to be one with unblurred, dark images for all microprobes, but in which it was clear that exposure was submaximal. In some experiments it was necessary to expose a second film to the microprobes for a longer time than that used for the first exposure. Occasionally, single microprobe images would be blurred due to poor apposition of the microprobes to the film and in this case a minor repositioning of the affected microprobe was made prior to repeated exposure. The autoradiographs were labelled on each film with a number corresponding to the order of placement in the spinal cord during the course of the experiment. An example of such an autoradiographic image is included on figure 6.4.

3.5 IMAGE ANALYSIS OF ANTIBODY MICROPROBE AUTORADIOGRAPHS

The principles employed in the analysis of the antibody microprobe autoradiographs obtained, are based on those described by Hendry et al. (1988). Quantitative microdensitometric analysis was achieved using a computer based image analysis system that quantifies the microprobe images as changes in image density (or degrees of blackness) along the microprobe length.

The developed film chosen to represent a particular experiment was cut into small strips each containing a single microprobe image. Following the removal of any surface dust and grease by wiping with a clean soft tissue, each was placed in turn onto a microscope stage in a light proof box and illuminated. Uniform illumination was achieved by a controlled light source (two 25W microscope halogen bulbs, connected to a Farnell 0.25V power supply), located beneath the stage, through diffusion plates and a narrow slit, set at a width just larger than the microprobe image itself. The film was then scanned by a charged coupled device camera (CCD; Panasonic), using an enlarger lens held in extendable bellows. Although the

automatic gain control of the CCD camera was deactivated, it was also necessary to restrict the field of the camera to just that of the microprobe image, to make it a satisfactory microdensitometer. A constant magnification was maintained throughout the studies described in this thesis, by setting the distance from the camera lens to the film so that only the last 5mm of the microprobe tip occupied the monitor screen.

Image analysis of the camera image employed an Imaging Technology PC Vision Plus frame grabber board operating in an AT based computer (DCS 286e). Using a memory map of 512 x 512 locations this image board converted the optical density of the video image to an arbitrary 'greyscale' value over the range of 0 to 255 degrees of brightness recognised by the CCD camera, where 0 is black and 255 is maximum light. If the light intensity is increased above 255 no further change in the grey scale reading can be obtained and so linearity is lost. Thus, to ensure that the entire range of values was being accessed, the light intensity was maintained at 0 in the absence of any X-ray film and once set, the lighting level for any particular experimental film was left unchanged, while all the microprobe images on that film were entered. Since the length of microprobes scanned was 5mm, each memory location in the image board map corresponded to 5000μm ÷ 512, which has been regarded as 10μm.

The values obtained for the microprobe image were corrected for background. Prior to scanning the microprobe images from an experiment, a background value for the entire field had been calculated and stored as a disk file. Due to the particulate nature of X-ray film, this had been done by scanning 10 times, two different unexposed parts of the X-ray film, and taking the average sum of the digitalized values obtained for the 512 x 512 pixels. However, since the object of the image analysis was to quantify the microprobe autoradiographs as changes in degrees of blackness, a new value was assigned to each pixel from a white to black 'look-up table', so that 0 now represents maximum light and 255, black. A coloured image of each microprobe was displayed on the monitor, the corrected intensities having been converted to 16 false colours by red, green and blue look-up tables.

Transverse integrations of these background corrected greyscale values were then performed by the computer, for each vertical column of pixels across the restricted part of the field which contained the microprobe image. Thus an array of numbers were obtained which represented image blackness in 10μm intervals. These were then stored in a file on the hard disc of the computer, together with 37 coded values that represent various details of the experimental conditions relevant to that microprobe. The coded values which were entered to define the experimental conditions in the studies of this thesis, are outlined in table 5. Thus, each file entry for a microprobe image has 549 numbers, 37 for experimental conditions and 512 representing the 5mm of microprobe image length analysed. The integral values obtained can be displayed graphically as a function of the image density (or grey scale value) changes along the microprobe length, to a resolution of 10μm (corresponding approximately to the width of a single pixel). Although in theory the microprobes detect continuously along their length, the resolution of the method is determined mainly by the scattering of radiation emitted by bound radioactive molecules, and hence the distance of the source from the detecting film. This is in turn determined by probe diameter and hence increases with the distance from the tip. So although the image analysis is performed in 10μm steps, this is beyond the biological resolution of the microprobe method. This has instead been calculated to be nearer to 100μm, since 100μm sources of release are readily detected at a distance of 3mm from the tip (Duggan, 1992). For display purposes the average of three successive integrals was taken to give a final plotted resolution of 30μm. Such an image density scan is represented on figure 7.1 (n=1). The slope in such a graph results from the progressive increase in probe diameter starting from the tip. Any inhibition of binding of the radiolabelled peptide (representing binding of the endogenous peptide) is displayed as areas where the grey scale values are lower than those at the corresponding points on control microprobes that have only been exposed to the radiolabelled peptide.

For every microprobe study described in this thesis, the coded values for each microprobe image were entered into one large file. This enabled a sorting program to retrieve groups of

TABLE 5. Examples of the coded values entered to define the experimental conditions in the antibody microprobe studies described in this thesis. These values are used to select groups of microprobes conforming to a particular experimental criteria from a file of images so that mean image analyses can be prepared. Some codes (i.e. no.1 to no.17) apply to all microprobes in an experiment, whereas others (i.e. no.18 to no.37) vary for each individual microprobe, in all instances 99 represents non-applicable criteria. Record i.d. numbers 151, 203, 666, 670 are microprobes from the dynorphin study (code 14 = 91, code 26 = 9) and 166, 173 microprobes from the CCK study (code 14 = 80, code 26 = 81).

Microprobe no.151: was present in the spinal cord of a normal rat (code 18 = 1), for 30 minutes (code 21 = 30), prior to any mechanical stimulation (code 18 = 1, code 19 = 99).

Microprobe no.203: was not inserted into an animal (i.e. *in vitro*) but exposed to [^{125}I]-dynorphin A(1-8) alone and processed concurrently with those used *in vivo* (code 18 = 90).

Microprobe no.666: was present in the ipsilateral side of the spinal cord of a rat with unilateral ankle inflammation (code 18 = **6**4), during the 30 minute period of noxious mechanical stimulation (code 18 = 6**4**, code 19 = 6).

Microprobe no.670: was present in the contralateral side of the spinal cord of the same rat that microprobe no.666 had been inserted (code 18 = **7**1), for the 35-65 minute period post compression of the uninflamed ankle (code 18 = 7**1**, code 19 = 99, code 30 = 3).

Microprobe no. 166: was present in the spinal cord of a rat during the third 30 minute period of the acute intravenous administration (code 18 = 10) of morphine (code 19 = 10), the total dose given in this time period being 5mg/ kg (code 35 = 5).

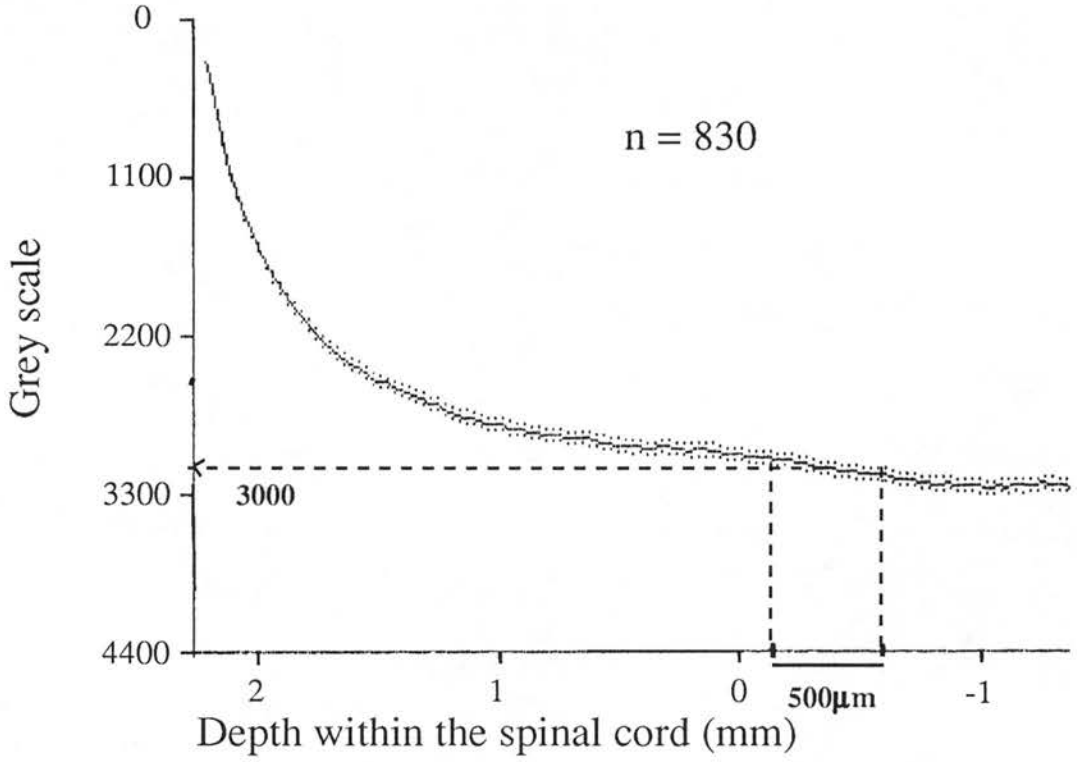
Microprobe no. 173: was present in the spinal cord of the same rat that microprobe no.166 had been inserted during the first 30 minute period post injection (code 18 = 80) of naloxone (code 19 = 11), the total dose given at the start of this time period being 1mg/ kg (code 35 = 1). However, since this microprobe displayed problems with binding [^{125}I]-CCK it was disregarded in any subsequent analysis (code 14 = 99).

1	Record i.d. number	151	203	666	670	166	173
2 3 4	Experimental date: (day/ month/ year)	7 7 93	7 7 93	17 5 94	17 5 94	21 2 95	21 2 95
5	Experimental number	18	18	46	46	10	10
6	X-ray film cassette type (screen/ no screen)	2	2	2	2	2	2
7	X-ray film type (monolayer/ bilayer)	1	1	1	1	1	1
8	Hours on film (hours)	96	96	72	72	120	120
9	Microprobe glass (hollow/ solid)	2	2	2	2	2	2
10	Species (rat/ cat)	2	99	2	2	2	2
11	Anaesthetic type (urethane etc.)	3	99	3	3	3	3
12	Intact spinal cord/ spinalised	1	99	1	1	1	1
13	Not paralysed/ paralysed with gallamine	2	99	2	2	2	2
14	Antibody used (type and manufacturer)	91	91	91	91	80	99
15	Antibody dilution	7	7	7	7	7	7
16	Concentration of peptide for *in vitro* test	7	0	7	7	7	7
17	Method of spinal cord irrigation	3	99	3	3	3	3
18	Stimulus type (none, mechanical etc.)	1	90	64	71	10	80
19	Stimulus parameters (none, pinch, flexion etc.)	99	99	6	99	10	11
20	Probe number within experiment	1	99	3	7	9	17
21	Time in spinal cord (in minutes)	30	99	30	30	30	30
22	Depth of insertion into spinal cord	22	99	22	22	22	22
23	Estimated distance from tip to cord surface	22	99	22	22	22	22
24	Estimated distance of substantia gelatinosa from tip	16	99	16	16	16	16
25	Estimated distance of lamina V form tip	10	99	10	10	10	10
26	Radiolabelled ligand used (type and manufacturer)	9	9	9	9	81	81
27	Wash treatment on removal from cord	2	2	2	2	2	2
28	Incubation time in radiolabelled ligand (hours)	1	1	1	1	1	1
29	Special features of probes/ autoradiographic images	0	0	0	0	0	0
30	Total previous stimuli given	0	99	0	3	3	1
31	Previous noxious stimuli for this patch/ section of cord	0	99	0	0	0	0
32	Age of patch (if applicable)	0	99	0	0	0	0
33	Probe order in patch/ section of cord (1st, 2nd, etc.)	99	99	99	99	99	99
34	Type of antibody on companion probe	99	99	99	99	99	99
35	Drug type and dose	99	99	99	99	5	99
36	Time (minutes) since first dose of drug (any route)	99	99	99	99	99	99
37	Extra comments	99	99	99	99	99	99

microprobes conforming to a particular experimental criteria, and produce a plot of the mean grey scale values (± s.e.m.) in 30μm steps. An example of such a mean image density scan is represented on figure 7.1 (n=30). The mean greyscale values for a particular grouping of microprobes are plotted out as a solid line graph, with the values of the s.e.m. as dotted lines on both sides of the mean line. To minimise any differences due to variability in physical factors such as X-ray film exposure times, the analysis programme has a facility to 'normalise' the values obtained for each microprobe image scan. This involved determining from the mean image density scan of the total group of microprobes from a particular study, the mean grey scale value of a relatively constant 500μm section of the grouped microprobes (approximating to the width of 50 pixels), located in a region not inserted into the spinal cord. This was then taken as a standard to calculate a variability constant for each image density scan, to adjust the coded integral values for each microprobe image that had been previously entered into a file on the hard disc, and thereafter referred as 'normalised data'. For example the standard grey scale value of the image density scans for microprobes used to study the release of ir-dynorphin A(1-8), was taken to be 3000 along the 500μm region 2.4 to 2.9mm from the tip (see figure 11). The readjusted integral values for each microprobe image were then entered into a new file on the hard disc.

Using this normalised data, the data sorting program was used to compare the differences between groups and estimate the significance of the differences at each of the 30μm analysis points using the Students' t-test. This is represented on figure 7.2. Here the 't' statistics, derived from the differences between the mean image analyses of the two groups, are plotted in 30μm intervals against a schematic representation of the spinal cord. The cross hatched areas representing areas where the presence of the neuropeptide under study, in this case dynorphin A(1-8) was found to be statistically significant at the $P < 0.05$ level (i.e. $t>2$). It should be noted that taking differences between two groups of microprobes (each with the shape produced by increasing thickness when progressing away from the tip) eliminates the slope inherent in the plot of each group. There is still the problem however, that a zone of

FIGURE 11. Determination of a standard grey scale value to 'normalise' the values obtained for each microprobe image scan in the dynorphin study. This is an example of the type of mean image density scan of dynorphin A(1-8) microprobes used to decide the standard grey scale value of a relatively constant 500μm section of the grouped microprobes located in a region distal to that inserted into the spinal cord. The 500μm region 2.4 to 2.9mm from the tip was chosen, which approximates to a grey scale value of 3000. This was used as a standard to 'normalise' the values obtained for each microprobe image scan in the dynorphin study. Such a process serves to minimise any differences between microprobes due to variability in physical factors such as X-ray film exposure times.


0
1100
2200
3300
4400
Grey scale
n = 830
3000
2
1
0
500µm
-1
Depth within the spinal cord (mm)

complete inhibition of binding near the tip will look smaller than a zone on the thicker part of the microprobe (the absolute difference is greater with the latter). As the variance in the difference is smaller near the tip (smaller absolute values), a plot of the t-statistics (in 30μm steps) is a better illustration of where significant differences in levels were detected by the two groups.

CHAPTER 4: Studies of the Spinal Release of Dynorphin - Effect of the Development of Peripheral Inflammation

4.1 INTRODUCTION

In 1979 Goldstein isolated a peptide from porcine pituitary that performed so favourably to other opioid peptides in the guinea pig ileum assay, that he gave it the Greek prefix 'dyn-', signifying strength or power, describing it as 'an extraordinary potent opioid peptide' (Goldstein et al, 1979). The peptide isolated, dynorphin A(1-17), is now known to be prodynorphin 209-225 (Goldstein et al, 1981), derived by the sequential proteolytic cleavage of the much longer genetically distinct precursor, pre-proDyn, with additional cleavage of this region resulting in dynorphin A(1-8) (prodynorphin 209-216; Minamino et al, 1980). Other common dynorphins found in the mammalian nervous system are α-neoendorphin (prodynorphin 175-184; Kangawa et al, 1981) and dynorphin B(1-13) (prodynorphin 228-240; Kilpatrick et al, 1982).

As indicated in section 1.8, this family of opioid peptides shows dramatic changes in synthesis and spinal content with inflammation, with increases in dynorphin A(1-8) (Iadarola et al., 1988a, b; Ruda et al., 1988; Nahin et al., 1989, 1992), dynorphin A(1-17) (Millan et al., 1985, 1986, 1988; Weihe et al., 1988, 1989) and α-neoendorphin (Przewlocka et al., 1992) having all been described. Evidence that these rises in neuropeptide levels are preceded by increases in the mRNA levels for proDyn has also been presented (Iadarola and Draisci, 1988; Ruda et al., 1988; Iadarola et al., 1988a, b; Weihe et al., 1989; Draisci and Iadarola, 1989; Przewlocka et al.,1992).

Early behavioural studies implied a close association of the regulation of these opioid peptides with sensory alterations, those polyarthritic rats showing the greatest mechanical hyperalgesia in the paw pressure test displaying the greatest rise in dynorphin immunoreactivity (Millan et al, 1985). The changes in proDyn synthesis were found to be localised to the spinal segments receiving the afferent input from the inflamed limbs, occurring bilaterally in the polyarthritic rats and ipsilaterally in animals with a unilateral inflammation (Millan et al, 1988). Indirect evidence of the nature of this afferent input was provided by a

study employing capsaicin neonatally to destroy almost all the unmyelinated (the majority likely nociceptive), primary afferent fibres (Hylden et al, 1992). These investigators found dramatic reductions in the number of neurones exhibiting prodynorphin mRNA when a hindpaw inflammation was induced in the neonatally capsaicin-treated rats in adulthood compared to those treated neonatally with vehicle alone, implying that dynorphin gene expression is partially dependent on input from capsaicin sensitive primary afferents. This could be mono- or poly-synaptic but there is anatomical evidence to suggest that dynorphin containing spinal neurones are contacted monosynaptically by primary afferents (Cho and Basbaum, 1989), including putative nociceptive afferents that contain CGRP and substance P (Takahashi et al.,1988, 1990; Carlton and Hayes, 1989). These neuropeptides were found to be reduced following neonatal capsaicin treatment (Hammond and Ruda, 1991). It is notable that although such neonatal capsaicin treatment resulted in some reduction in thermal sensitivity in normal rats, its lack of effect on FCA-induced oedema and thermal hyperalgesia implies that the primary afferents affected are not directly involved in the inflammatory response (Hammond and Ruda, 1989). A close association of the regulation of these spinal dynorphin containing neurones with sensory processes is also suggested by the finding that the marked increase in proDyn mRNA expression following injections of carrageenan into the rat hindpaw was inhibited by the presence of tachykinin receptor antagonists (Parker et al, 1994).

Although an increased RNA message for proDyn could simply be a response to prior release, it has been proposed that the immediate stimulus to elevated levels of dynorphins is increased transcription of the prodynorphin gene through immediate early genes, such as c-fos. As outlined in section 1.8.3, a close temporal link between the increases in c-fos protein (ir-Fos) and mRNA levels for proDyn has been observed in the spinal cord of the rat on the induction of a peripheral inflammation (Draisci and Iadarola, 1989), along with evidence to support the co-localisation of c-fos and the proDyn message within single neurones of the spinal cord (Naranjo et al., 1991; Noguchi et al., 1991; Lucas et al., 1993). Since the c-fos protein is expressed by many neurones of the spinal cord in response to the arrival of impulses

in nociceptive primary afferents, following noxious mechanical (Bullit et al, 1990; Leah et al., 1992; Cruz et al, 1994), thermal (Hunt et al., 1987; Bullit et al, 1990; Williams et al, 1990; Naranjo et al, 1991; Evans et al., 1994; Gillardon et al., 1994; Abbadie et al., 1994a; b), and chemical (Hunt et al., 1987; Menetrey et al, 1989; Cruz et al, 1994) peripheral stimulation, as well as electrical nerve stimulation at C fibre intensity (Wei and Zhao, 1995; Willcockson et al, 1995; Herdegen et al, 1991), it is possible that the increased synthesis of dynorphins is a primary response to this input. Notably inflammation induced increases in c-fos expression were greatly attenuated by neonatal capsaicin treatment (Hylden et al, 1992). Additional support for this proposal has been provided by the demonstration that intrathecal pretreatment with an antisense oligodeoxynucleotides to c-fos mRNA inhibits the increase in the synthesis of proDyn by spinal neurones following peripheral inflammation (Hunter et al., 1995).

Although an enhanced spinal release of the prodynorphin derived peptides as inflammation develops peripherally is implied from these biochemical and immunohistochemical findings, few studies have been performed to investigate this phenomenon directly. Using spinal cord isolated from the lumbar region of rats with unilateral induced hindpaw inflammation, Przewlocki et al (1992) found evidence for an enhancement of both the basal and potassium evoked release of α-neoendorphin within 12 hours of inoculation, the ability to evoke release declining thereafter. Such studies, however provide no localisation of the possible sites of release and little information about the physiological controls of such release. Little is even known about the mechanisms of the spinal release of dynorphins in normal animals. Reports concerning the release of the proDyn peptides into artificial CSF fluid superfusing the subarachnoid space of the spinal cord of the normal rat and cat, have provided evidence that the levels of dynorphin A in a spinal perfusate are increased by high intensity electrical stimulation of peripheral nerves (Yaksh et al, 1983; Nyberg et al, 1983), which has been suggested by some groups as evidence of its central role in the analgesia of high frequency electroacupuncture (rabbit: Han and Xie, 1984). Studies of the release of proDyn peptides from spinal cord *in vivo* (Xie et al, 1986) and *in vitro* (Przewlocka et al, 1990) of normal rats

suggest that spinal proDyn neurones are under excitatory (noradrenaline/ α_2 adrenoceptor) and inhibitory (GABA) influences. Additionally, Hutchinson et al (1990) used the antibody microprobe technique to study the release of dynorphin A(1-17) in the lumbar spinal cord of the normal cat. They found a basal presence of ir-dynorphin A(1-17) in lamina I of the superficial dorsal horn, which was increased by high frequency (50 to 100Hz) electrical stimulation of the ipsilateral tibial nerve at C but not A fibre strength. There was also evidence for the release of ir-dynorphin A(1-17) deeper in the dorsal horn (laminae V-VI). However, the effect of more natural stimulation was not investigated. Evoked release in lamina I was abolished by spinal transection which was interpreted that such release came from intrinsic neurones activated by supraspinally derived fibres. Possibly relevant to this proposal are the findings that adding corticotrophin releasing factor to a perfusate of the isolated mouse cord produced a release of ir-dynorphin A(1-17) (Song and Takemori, 1992) and that depletion of 5HT fibres descending from raphe nuclei to the spinal cord by the intrathecal administration of the neurotoxin 5,7-dihydroxytryptamine dramatically reduced the expression of prodynorphin mRNA in the superficial dorsal horn (Lucas et al, 1993).

Hence, when I commenced my studies there was a need to determine the adequate stimuli producing a spinal release of dynorphins under *in vivo* conditions both in normal animals and in those with peripheral inflammation and specifically to determine (a) whether dynorphins are tonically released in both normal and inflamed animals and (b) if such release is increased or decreased by manipulation of the inflamed tissues. In the experiments described in this thesis I have employed microprobes bearing immobilised antibodies to dynorphin A(1-8), to study the release of ir-dynorphin in the spinal cord of normal rats and those with a unilateral ankle inflammation. Dynorphin A(1-8) was chosen since there is evidence that this peptide is the major end product of the processing of proDyn in several areas of the central nervous system, including the spinal cord (Weber et al.,1982; Sweetnam et al.,1986) and it is this form of the dynorphin molecule which has been most extensively studied in relation to peripheral inflammation (Iadarola et al, 1988a; b; Ruda et al, 1988; Nahin et al, 1989; 1992)

4.2 MATERIALS AND METHODS

1. MICROPROBE PREPARATION

Antibody microprobes were prepared as previously described (section 3.2), using a polyclonal antiserum [Peninsula Laboratories] that had been raised in rabbits against the COOH-terminus of porcine dynorphin A(1-8). Data supplied from Peninsula Laboratories indicated that this antiserum had negligible cross reactivity (<0.01%) with the porcine prodynorphin derived peptides dynorphin AB(1-32), dynorphin A(1-17), dynorphin A(1-13), dynorphin B(1-13), α-neoendorphin and zero cross reactivity for other tested opioid peptides, porcine β-endorphin(1-31), Met-enkephalin and Leu-enkephalin-Arg [dynorphin A(1-6)].

2. EXPERIMENTAL PREPARATION

A total of 40 male Wistar rats (weight range 250 to 422g; mean ± s.e.m. = 356±6g) were used in this study. Twenty-two animals received injections of FCA around one ankle joint (as outlined in section 2.3) and were used in experiments 3 to 5 days later. An additional six rats received FCA injections on the morning of the experiment, to allow study of the release ir-dynorphin A(1-8) at an early phase of the inflammatory response.

Anaesthesia was induced, cannulae were inserted and the lumbar spinal cord exposed as previously described (sections 2.2.2 and 2.5). All microprobes were inserted 2.25mm into the dorsal spinal cord. Initially, microprobes were kept in the cord for 5, 15 or 30 minutes but as preliminary analysis indicated that the probability of binding any dynorphin A(1-8) increased with length of time in the spinal cord, all later experiments employed incubation times of 30 minutes. During these periods either no stimulus was applied or the ankle joint was mechanically manipulated.

Since the most likely stimulus producing pain from inflamed skin, joints or tendons is that resulting either from direct physical contact with an environmental object or movement of the damaged tissues, the ankle joint region was only subjected to mechanical stimulation. A

typical protocol was 3 minutes of mechanical stimulation followed by 2 minutes of no stimulation. In the initial experiments the inflamed tissues were compressed laterally, with a modified strong spring clip. In later experiments this stimulus was quantified by the application of a pneumatic compression device incorporating a strain gauge for measuring applied force and employing electronic control of the times of application. The jaws of the pneumatic compression device were coated in a smooth heat shrink plastic cover to minimise skin damage. Typically, a force of 14.7N was applied, the surface area of the compression device contacting the rat ankle being $20mm^2$. In one series of experiments involving five animals (three of which had a developed ankle inflammation), the ankle joint area was flexed by pulling the limb out to near its full extent and then pushing the hindpaw gently until it was in close apposition to the knee. This flexion of the ankle joint was repeated every 5 seconds throughout each 3 minute period of mechanical stimulation. In another series of experiments involving four animals (all of which had a developed ankle inflammation), the laminectomy was extended to vertebral level T9, the region where the spinal cord which naturally bends when animal was positioned in the stereotaxic frame used. Following the injection of lignocaine (2% solution, total volume of 0.05ml; Xylocaine, Astra, UK) at the proposed site of incision, the exposed spinal cord was completely transected at this level.

Although inflammation was induced unilaterally, pairs of microprobes were inserted into both sides of the spinal cord, comparable stimuli being applied to the inflamed and uninflamed hind limbs. With each side of the spinal cord, periods of no stimulation usually preceded those of mechanical stimulation to the ipsilateral ankle region but also often followed them. Due to uncertainty on the persistence of any possibly released ir-dynorphin A(1-8) following peripheral stimulation, it was usual to perform periods of peripheral stimulation (and associated periods of no stimulation) only twice, once on each side of the spinal cord. When in eleven experiments, however, the chosen protocol was carried out more than once on one side of the cord, a time of at least 2 hours elapsed between periods of stimulation. Blood pressure, breathing rate and end tidal CO_2 were monitored throughout.

After removal from the spinal cord microprobes were washed and then incubated for approximately 24 hours in porcine [^{125}I]-dynorphin A(1-8) [Peninsula Laboratories] and prepared for analysis as previously described (sections 3.4 and 3.5).

3. *IN VITRO* TESTS

Prior to use and throughout use *in vivo*, the sensitivity of the prepared antibody microprobes were tested (as outlined in section 3.3).

4.3 RESULTS

A total of 559 microprobes coated with antibodies to dynorphin A(1-8) inserted into the rat spinal cord form the basis of this analysis. An additional 582 microprobes were used for *in vitro* sensitivity tests.

1. *IN VITRO* TESTS

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 porcine dynorphin A(1-8) suppressed such binding on average by 55%, with a 10^{-5}M solution resulting on average in 80% suppression. Hence, it can be assumed that the non-specific binding for these microprobes accounted for less than 20% of the total binding. Information supplied by Peninsula Laboratories on the specificity of the antiserum was confirmed on microprobes by demonstrating minimum suppression of binding of [^{125}I]-dynorphin A(1-8) by a range of concentrations of the porcine derived peptides dynorphin A(1-17), dynorphin A(1-13) and Leu-enkephalin [Peninsula Laboratories].

2. BASAL LEVELS OF ir-DYNORPHIN A(1-8)

Since the primary aim of this study was to study the release of ir-dynorphin A(1-8) in animals with a developed peripheral inflammation, it was necessary to make comparisons between normal rats and those with unilateral ankle inflammation. In the case of the latter group the release of ir-dynorphin A(1-8) was examined in both sides of the spinal cord.

A basal presence of a neuropeptide at a particular site in the nervous system is inferred by differences between the mean image analysis of microprobes not inserted into the nervous system but simply incubated in the radiolabelled ligand and that of microprobes placed in the relevant area for a comparable time but in the absence of any active stimulus. Such comparisons indicated that ir-dynorphin A(1-8) was present in the spinal cord of normal rats

and both sides of the spinal cord of those with developed unilateral ankle inflammation under these 'basal' conditions. As microprobes present in the spinal cord for 5 or 15 minutes failed to detect ir-dynorphin(1-8) consistently, the data presented below was derived from microprobes present for 30 minutes in the spinal cord only.

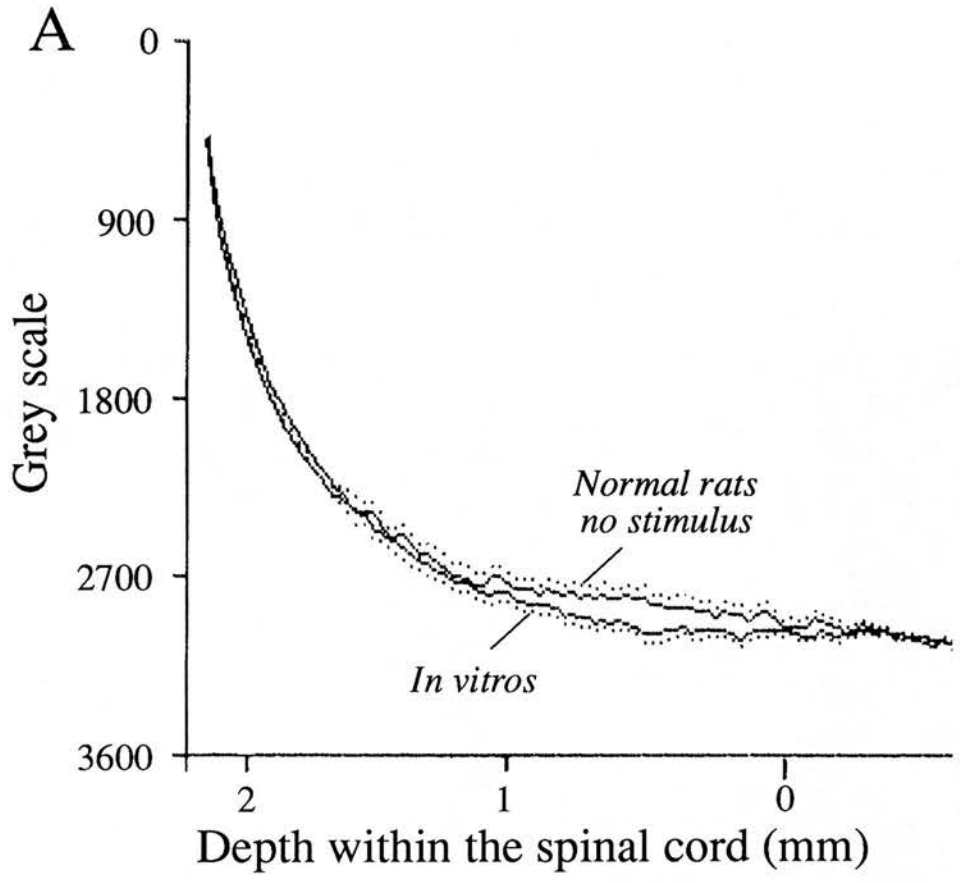
(i) Normal animals

Figure 12A compares the mean image density scan of microprobes (n=77) present in both sides of the spinal cord of normal rats for periods of 30 minutes and prior to any mechanical stimulation of the corresponding ankle regions, with that of *in vitro* microprobes (n=98) that had only been exposed to [^{125}I]-dynorphin A(1-8) but processed concurrently with those used *in vivo*. Figure 12B plots in 30μm intervals the 't' statistics derived from the differences between the mean image analyses of the two groups. The hatched area indicates where these differences are significant at the $P<0.05$ level ($t>2$) and this is approximately from 0.5 to 1.0mm from the dorsal surface of the spinal cord. Thus, in normal animals extracellular ir-dynorphin A(1-8) was present in zones of both the superficial and deep dorsal horn approximating to lamina I and laminae IV-V.

(ii) Animals with developed unilateral ankle inflammation: ipsilateral side of the spinal cord

A more extensive basal presence of ir-dynorphin A(1-8) was found in the ipsilateral side of the spinal cord (58 microprobes) in animals with unilateral ankle inflammation than in normal animals. When compared with *in vitro* microprobes significant levels of ir-dynorphin A (1-8) were found from the dorsal surface of the spinal cord down to 2.0mm from the dorsal surface (not illustrated). This approximates to the whole of the dorsal horn and ventral horn. Figure 13 illustrates differences between the mean image analysis of microprobes in this group and that of microprobes present in the spinal cord of normal animals but without added stimulation.

FIGURE 12. Basal presence of ir-dynorphin A(1-8) in the spinal cord of normal rats. (A) The mean image analyses of two groups of microprobes are plotted with respect to length: those present in the spinal cord of drug naive animals for 30 minutes in the absence of any peripheral stimulation (*normal rats, no stimulus*, n=77) and those which are not inserted into the spinal cord but simply incubated in [^{125}I]-dynorphin A(1-8)) (*in vitros*, n=98). With each mean image analysis the mean grey scale was determined in 30μm 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 means at each analysis point in the mean image analyses shown in (A), is related to an outline of the spinal cord at the area sampled. The hatched areas indicate the sites where these differences are significant at the $P<0.05$ level.


A
0
900
1800
2700
3600
Grey scale
Normal rats
no stimulus
In vitros
2
1
0
Depth within the spinal cord (mm)

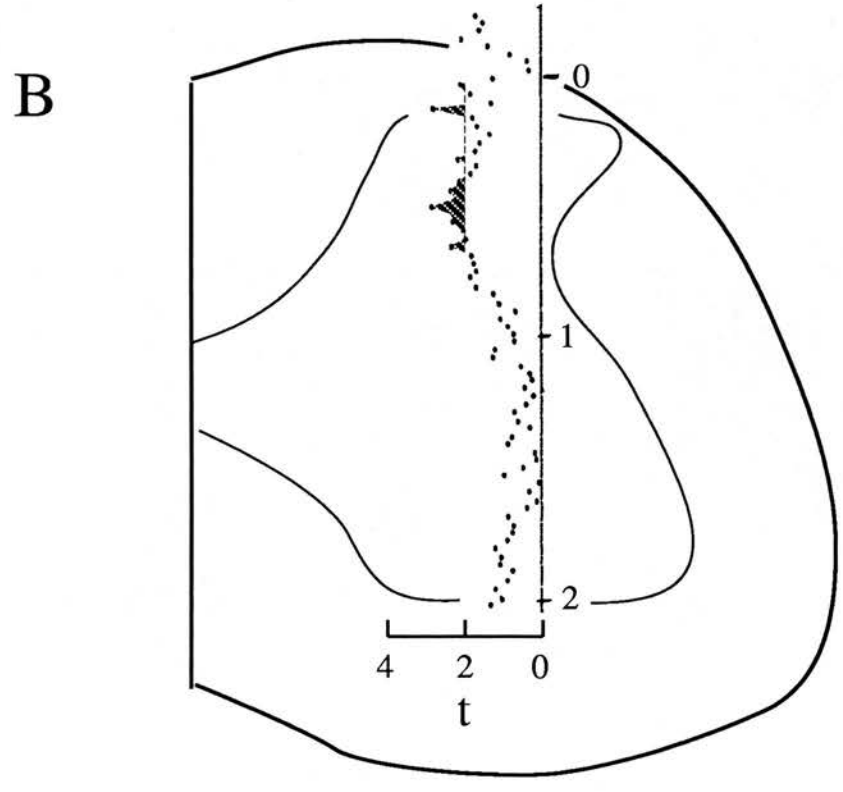
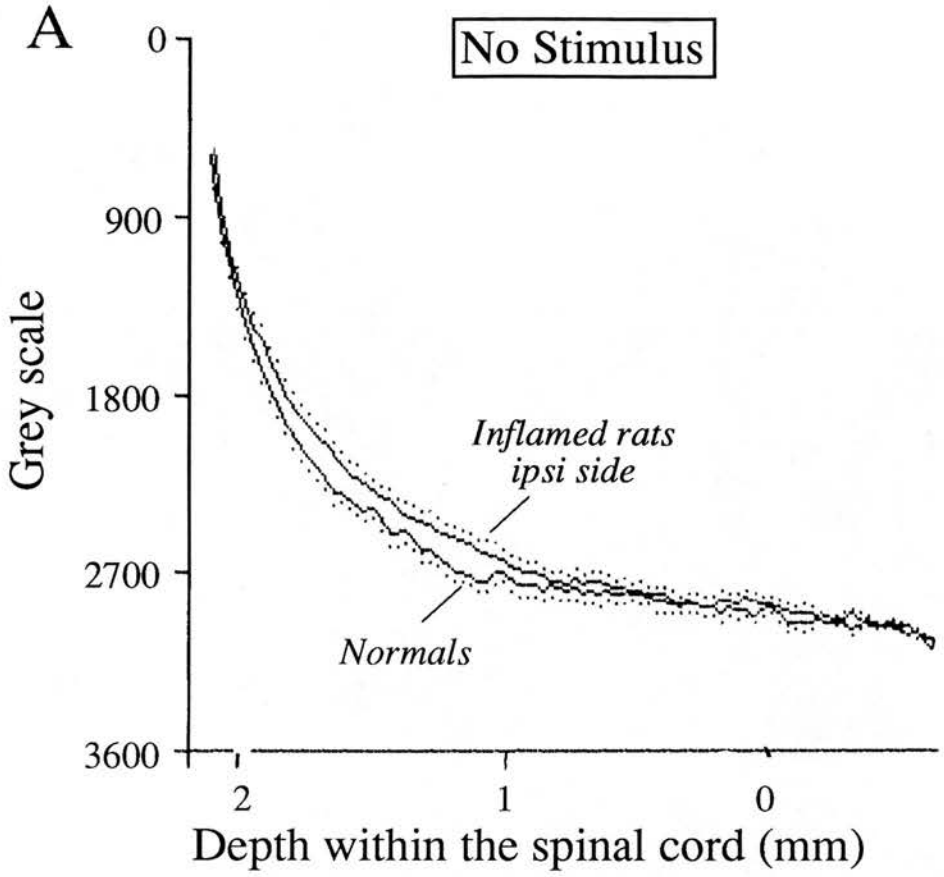
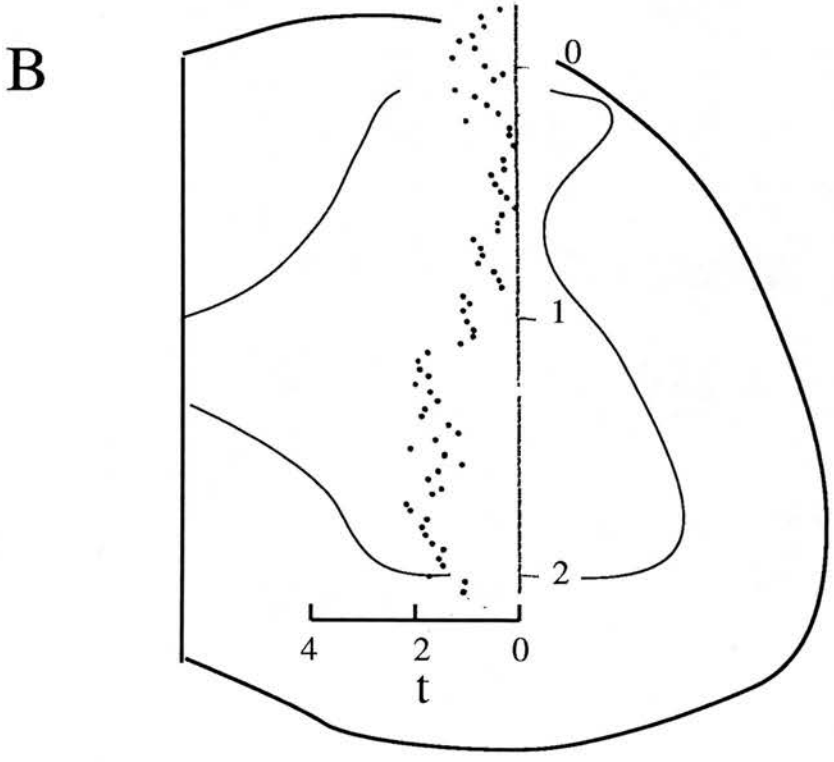

B
0
1
2
4
2
0
t

FIGURE 13. Effect of peripheral inflammation on the basal levels of ir-dynorphin A(1-8) in the ipsilateral side of the spinal cord. (A) The mean image analysis of 58 microprobes present for 30 minutes in the ipsilateral side of the spinal cord of rats with a unilateral ankle inflammation in the absence of any added stimulation (*inflamed rats, ipsi side*) is compared to that of microprobes present under similar basal conditions in normal animals (*normals*, n=77). (B) The differences between the two groups of microprobes are plotted with respect to depth in the spinal cord. Since only isolated points attain significance at the $P< 0.05$ level, there is no cross hatching as in other figures.


A
No Stimulus
0
900
1800
2700
3600
Grey scale
Inflamed rats
ipsi side
Normals
2
1
0
Depth within the spinal cord (mm)


B
0
1
2
4
2
0
t

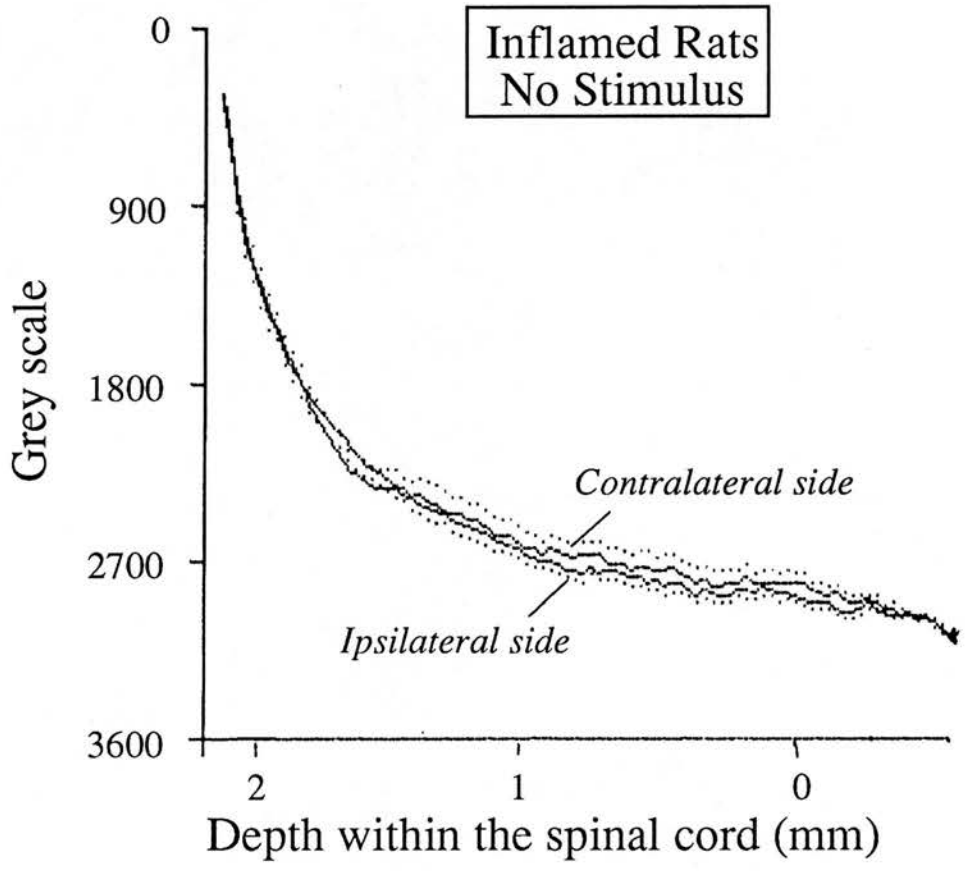
This shows comparable basal levels of ir-dynorphin A(1-8) in the dorsal horn of normal and inflamed animals but apparently greater levels of this neuropeptide at many sites in the ventral horn of rats with peripheral inflammation.

(iii) Animals with developed unilateral ankle inflammation: contralateral side of the spinal cord

The basal extracellular levels of ir-dynorphin A(1-8) in the contralateral side (34 microprobes) of the spinal cords of animals with developed ankle inflammation were found to be comparable to those present in the ipsilateral side under these basal conditions. Figure 14 illustrates the nearly completely coincident mean image analyses of these two groups of microprobes.

Attempts to study the influence of supraspinal pathways on the basal levels of ir-dynorphin A(1-8) found in animals with a developed unilateral ankle inflammation by spinalising a group of animals, were thwarted due to problems with the condition of the animals being used at this stage of work. This consisted in the development of 'stiff legs' during the course of an experiment followed by death. This problem could not be attributed to the actual spinalisation itself since it also occurred with other non-spinalised animals, both normals and those with developed ankle inflammation. No satisfactory explanation for this problem was afforded although a possible contributing factor could have been the use of a new stereotaxic frame.

FIGURE 14. Comparable basal presence of ir-dynorphin A(1-8) in both sides of the spinal cord of rats with unilateral inflammation. This figure compares the mean image analysis of microprobes present for 30 minutes in the ipsilateral side of the spinal cord of rats with unilateral ankle inflammation (*ipsilateral side*, n=58) to that of microprobes present in the contralateral side of the same animals in the absence of any added stimulation (*contralateral side*, n=34).


Inflamed Rats
No Stimulus
0
900
1800
2700
3600
Grey scale
Contralateral side
Ipsilateral side
2
1
0
Depth within the spinal cord (mm)

3. THE EFFECT OF LATERAL COMPRESSION OF THE ANKLE REGION

3.1. Microprobes present in the spinal cord during stimulus application

Although significant basal levels of ir-dynorphin A(1-8) were present both in normal rats and those with unilateral ankle inflammation, these two groups differed markedly in their responses to lateral compression of the ankle.

(i) Normal animals

The application of lateral compression to the ankle joint and surrounding tissue of normal rats failed to increase extracellular levels of ir-dynorphin A(1-8) during the period of stimulus application. No significant differences were found between the mean image density scans of microprobes (n=33) present in the ipsilateral spinal cord during the application of the mechanical stimuli and those microprobes (n=67) present in the same animals in the absence of any prior stimulation, as illustrated in figure 15.

(ii) Animals with developed unilateral ankle inflammation: ipsilateral side of the spinal cord

Lateral compression of the ankle with a developed inflammation, significantly increased the extracellular levels of ir-dynorphin A(1-8) above those found under basal conditions in the ipsilateral side of the spinal cord. This is illustrated in figure 16A in which the mean image analysis of microprobes (n=30) present in the ipsilateral side of the spinal cord during the 30 minute period of noxious mechanical stimulation can be seen to be displaced above that of microprobes (n=47) present for an identical time period prior to this stimulus in the same animals, but in the absence of any added peripheral stimulation. These enhanced extracellular levels of ir-dynorphin A(1-8) were found to be statistically significant at the $P<0.05$ level at nearly all sites sampled from 0.25mm to 2.2mm from the dorsal surface of the spinal cord. As figure 16B shows, there were three main sites of release. The largest peak was in the deep dorsal/ upper ventral horn (laminae VI-VII), with further major sites in

FIGURE 15. Failure to release ir-dynorphin A(1-8) during a period of ankle compression in normal animals. The mean image analyses of the 33 microprobes present in the spinal cord for 30 minutes during the application of the mechanical stimuli (*lateral ankle compression*) is compared to that of microprobes present in the same animals in the absence of any prior stimulation (*no stimulus*, n=67).

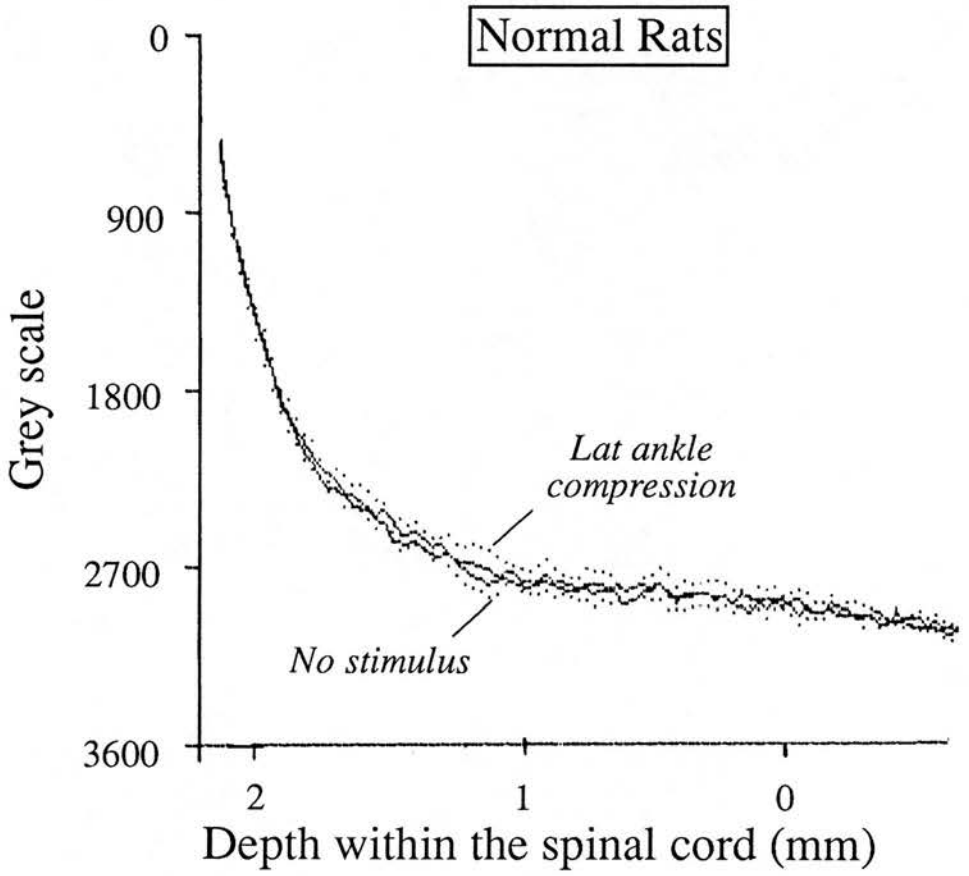
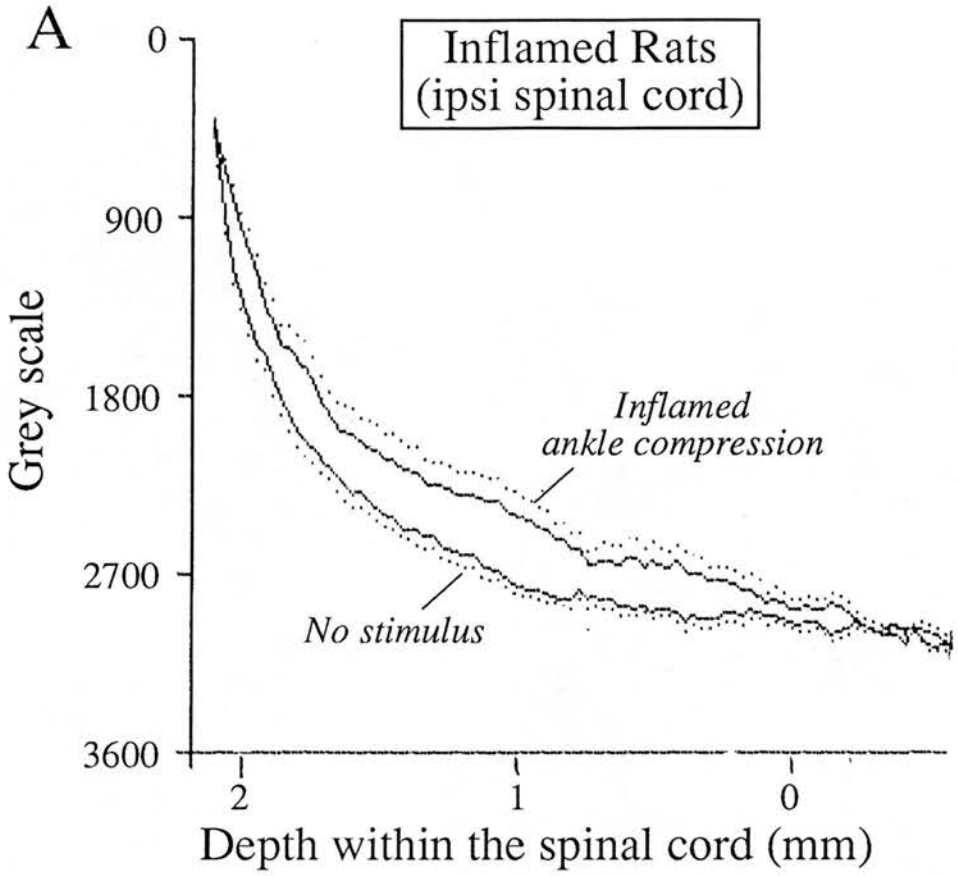
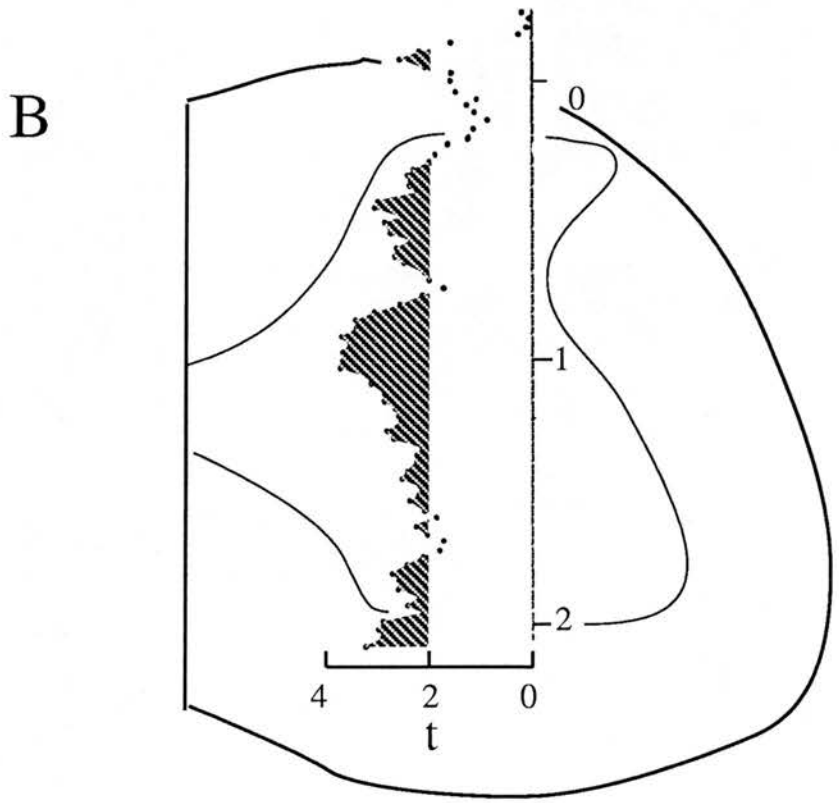

Normal Rats
0
900
1800
2700
3600
Grey scale
Lat ankle compression
No stimulus
2
1
0
Depth within the spinal cord (mm)

FIGURE 16. Release of ir-dynorphin A(1-8) during compression of the inflamed ankle.

(A) The mean image analysis of 30 microprobes present in the ipsilateral side of the spinal cord during the 30 minute period of noxious mechanical stimulation (*inflamed ankle compression*) is displaced above that of microprobes present in the same side of these spinal cords for an identical time period prior to this stimulus (*no stimulus*, n=47). (B) The differences between the two groups of microprobes are plotted with respect to an outline of the spinal cord. The hatched areas indicate where these differences are significant at the $P<0.05$ level.


A
Inflamed Rats
(ipsi spinal cord)
0
900
1800
2700
3600
Grey scale
Inflamed
ankle compression
No stimulus
2
1
0
Depth within the spinal cord (mm)


B
0
1
2
4
2
0
t

(laminae II-V) and the lower ventral horn. No study was made of possible contralateral release of ir-dynorphin(1-8) during compression of the inflamed ankle.

(iii) Animals with developed unilateral ankle inflammation: contralateral side of the spinal cord

Equivalent analysis of the effect of ankle compression on the normal side of animals with unilateral inflammation failed to show any such evoked release of ir-dynorphin A(1-8). Thus, the mean image analysis of microprobes (n=12) present in the side of the spinal cord contralateral to the peripheral ankle inflammation and during lateral compression of the normal ankle displayed no significant differences from that of microprobes (n=28) present in the same side of the spinal cord under basal conditions (not illustrated).

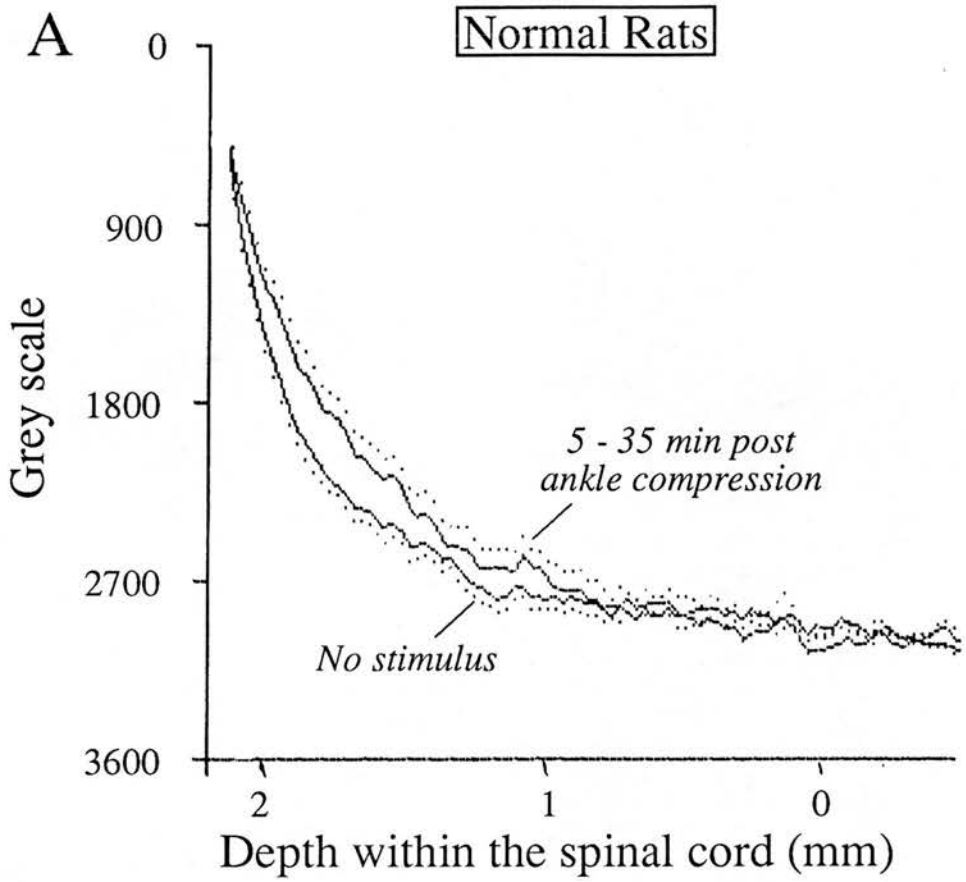
3.2. Microprobes present in the spinal cord following a period of mechanical stimulation

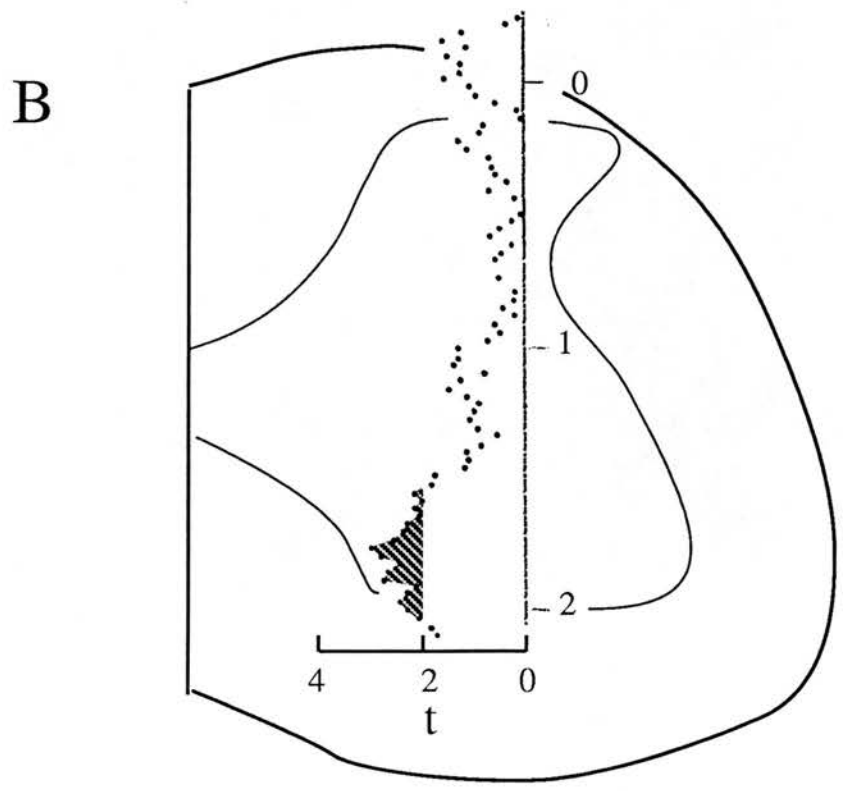
Since there is evidence that some neuropeptides are not rapidly degraded after *in vivo* release in the central nervous system (Duggan et al, 1990; Hope et al, 1990b) it was important to examine the decline of any enhanced presence of extracellular ir-dynorphin A(1-8) after application of a peripheral stimulus. The mean image analyses of microprobes inserted before or during prior mechanical stimulation of the ankle joint region were compared to those of microprobes present for equivalent 30 minute periods, up to one hour and a half after lateral compression of these peripheral tissues.

(i) Normal animals

No increase in the extracellular levels of ir-dynorphin A(1-8) was observed in the spinal cord during mechanical compression of the ankle region of normal rats. However, as illustrated on figure 17, comparisons between the mean image analysis of microprobes inserted prior to the application of lateral compression (n=56) and that of those present in the corresponding 30 minute period post-stimulation (n=21), showed elevated levels of the ir-

FIGURE 17. Release of ir-dynorphin A(1-8) in the spinal cord following a period of ankle compression in normal rats. (A) The mean image analysis of 21 microprobes present in a 30 minute period post ankle compression (*5-35 min post ankle compression*) is displaced above that of microprobes present prior to the application of this mechanical stimulus (*no stimulus*, n=56). (B) The differences between the post ankle compression and no stimulus groups are plotted with respect to an outline of the spinal cord. The hatched areas indicate where these differences are significant at the $P<0.05$ level.


A
Normal Rats
0
900
1800
2700
3600
Grey scale
5 - 35 min post
ankle compression
No stimulus
2
1
0
Depth within the spinal cord (mm)


B
0
1
2
4
2
0
t

dynorphin A(1-8) in the lower ventral horn after the application of ankle compression. No analysis of the levels of ir-dynorphin A(1-8) in the period 40-70 minutes post-compression was made due to insufficient microprobes being present in the spinal cord under such conditions.

(ii) Animals with developed unilateral ankle inflammation: ipsilateral side of the spinal cord

Figure 18 illustrates extracellular ir-dynorphin A(1-8) in the ipsilateral side of the spinal cord before, during and for one hour after the lateral compression of the inflamed ankle. Figure 18B shows that the mean image analysis of microprobes (n=22) present for the first 30 minute period after lateral compression of the inflamed tissues of the ipsilateral ankle joint was displaced above that of microprobes (n=36) present in the same side of these spinal cords prior to such peripheral stimulation. No significant differences however were found between these 'post-compression' microprobes and those present in the spinal cord during this noxious mechanical stimulation (n=28, not illustrated).

The mean image density scan of microprobes (n=14) present in the spinal cord for the period 40-70 minutes post-compression was also found to be significantly elevated above that of microprobes inserted under 'basal' conditions, as illustrated on figure 18C. However, ir-dynorphin A(1-8) levels in the ipsilateral side of the spinal cord had returned to pre-stimulation 'basal' levels after a period of two hours, as no significant difference was found between the mean image density analyses of microprobes (n=8) inserted ipsilaterally in the absence of any prior or concurrent added stimulation and those microprobes (n=8) inserted into this side of the cord again after a period of at least two hours (not illustrated).

(iii) Animals with developed unilateral ankle inflammation: contralateral side of the spinal cord

Equivalent analysis revealed that mechanical manipulation of the normal ankle of animals with unilateral inflammation had not initiated any effect on the levels of ir-dynorphin A(1-8)

FIGURE 18. Persistently raised levels of ir-dynorphin A(1-8) in the spinal cord following a period of compression of the inflamed ankle. For (A), (B) and (C) the no stimulus group represents 36 microprobes present for 30 minutes in the ipsilateral side of the spinal cord of inflamed rats prior to any added stimulation. In (A) the mean image analysis of this group is compared with that of 28 microprobes inserted during compression of the inflamed ankle (*lateral ankle compression*); in (B) the comparison is with the mean image analysis of 22 microprobes present in a 30 minute period after this noxious mechanical stimulus in the same side of these spinal cords (*5-35 min post ankle compression*); in (C) the comparison is with the mean image analysis of 14 microprobes inserted under similar conditions in the same experiments but present in a 30 minute period post ankle compression (*40-70 min post ankle compression*).

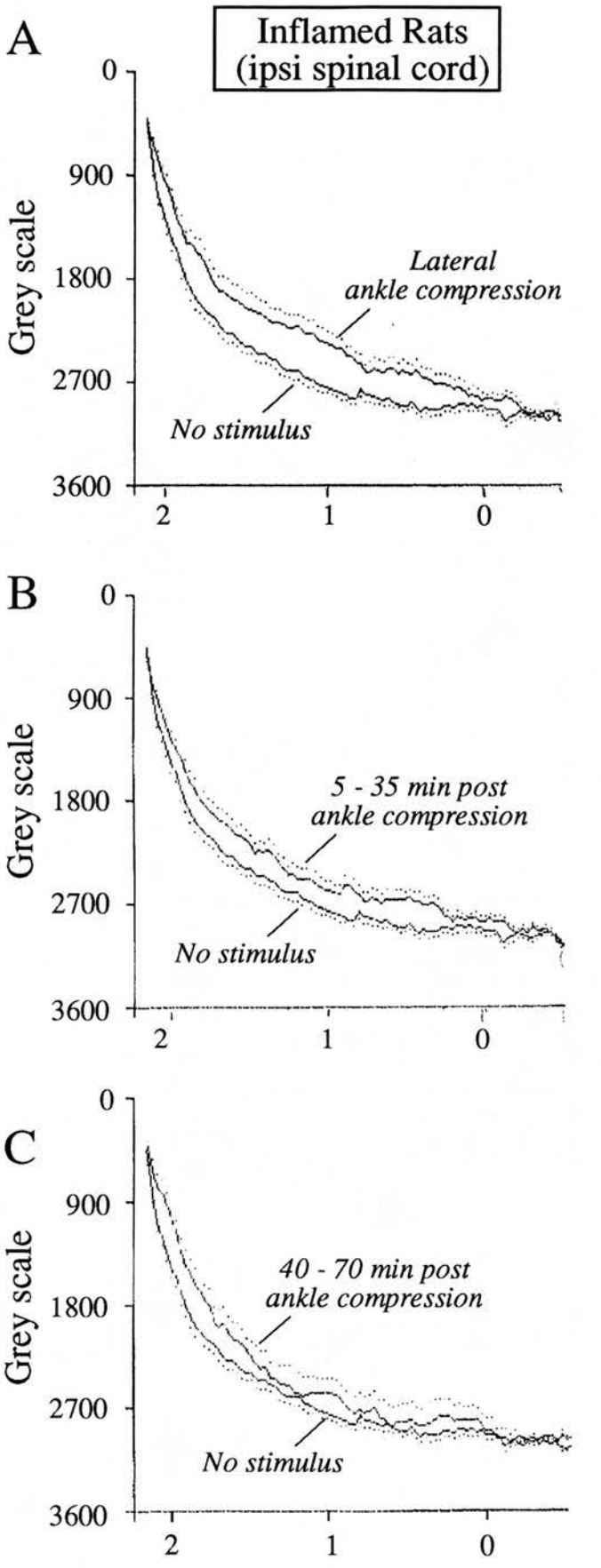
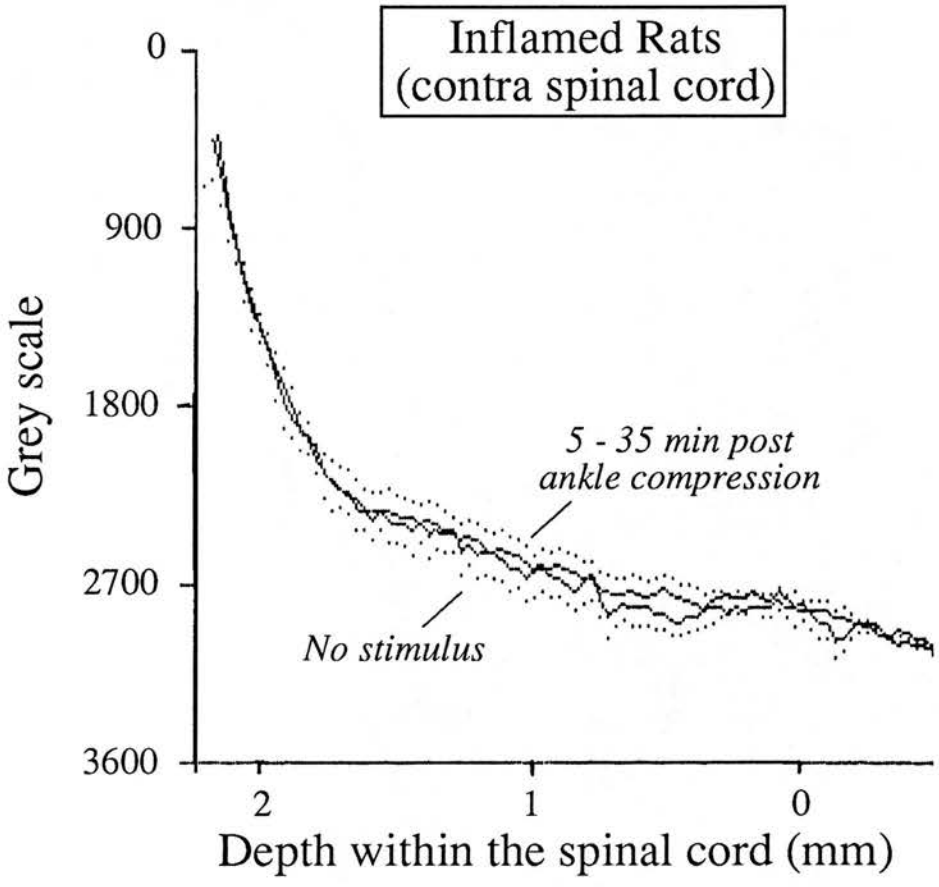

A
Inflamed Rats
(ipsi spinal cord)
Grey scale
0
900
1800
2700
3600
Lateral
ankle compression
No stimulus
2
1
0
B
Grey scale
0
900
1800
2700
3600
5 - 35 min post
ankle compression
No stimulus
2
1
0
C
Grey scale
0
900
1800
2700
3600
40 - 70 min post
ankle compression
No stimulus
2
1
0
Depth within the spinal cord (mm)

FIGURE 19. Failure of compression of the normal ankle to release ir-dynorphin A(1-8) in the contralateral side of the spinal cord in rats with unilateral ankle inflammation. The mean image analysis of the 12 microprobes present in the side of the spinal cord contralateral to the peripheral ankle inflammation for a 30 minute period following lateral compression of the normal ankle (*5-35 min post ankle compression*) is compared to that of microprobes present in the same side of these spinal cords prior to the application of this mechanical stimulation (*no stimulus*, n=20).


Inflamed Rats
(contra spinal cord)
0
900
1800
2700
3600
Grey scale
5 - 35 min post
ankle compression
No stimulus
2
1
0
Depth within the spinal cord (mm)

presence under basal conditions. Thus, as illustrated on figure 19 the mean image analysis of microprobes (n=12) present in the side of the spinal cord contralateral to the peripheral ankle inflammation for the first 30 minute period following lateral compression of the normal ankle displayed no significant differences from that of microprobes (n=20) present in the same side of the spinal cord of these animals prior to application of this ankle compression.

4. THE EFFECT OF FLEXION OF THE ANKLE REGION

4.1. Microprobes present in the spinal cord during stimulus application

Limited data was obtained on the effect of flexion on the basal levels of ir-dynorphin A(1-8) in normal animals and those with unilateral ankle inflammation. That available indicated that flexing the ankles of normal animals (12 microprobes) failed to increase the basal release of ir-dynorphin A(1-8) (9 microprobes) at the spinal cord level in an analogous manner to the application of lateral compression stimuli (not illustrated).

Moreover, in contrast to compression, flexion of the inflamed tissues around the ankles of rats with a developed unilateral inflammation (7 microprobes) failed to increase ir-dynorphin A(1-8) levels (11 microprobes) in the segments of the spinal cord receiving input from the ipsilateral ankles of these animals. Flexing the non-inflamed ankles (4 microprobes) of these animals also had no effect on the basal levels of ir-dynorphin A(1-8) (5 microprobes) present in the contralateral spinal cord (not illustrated).

4.2. Microprobes present in the spinal cord following a period of mechanical stimulation

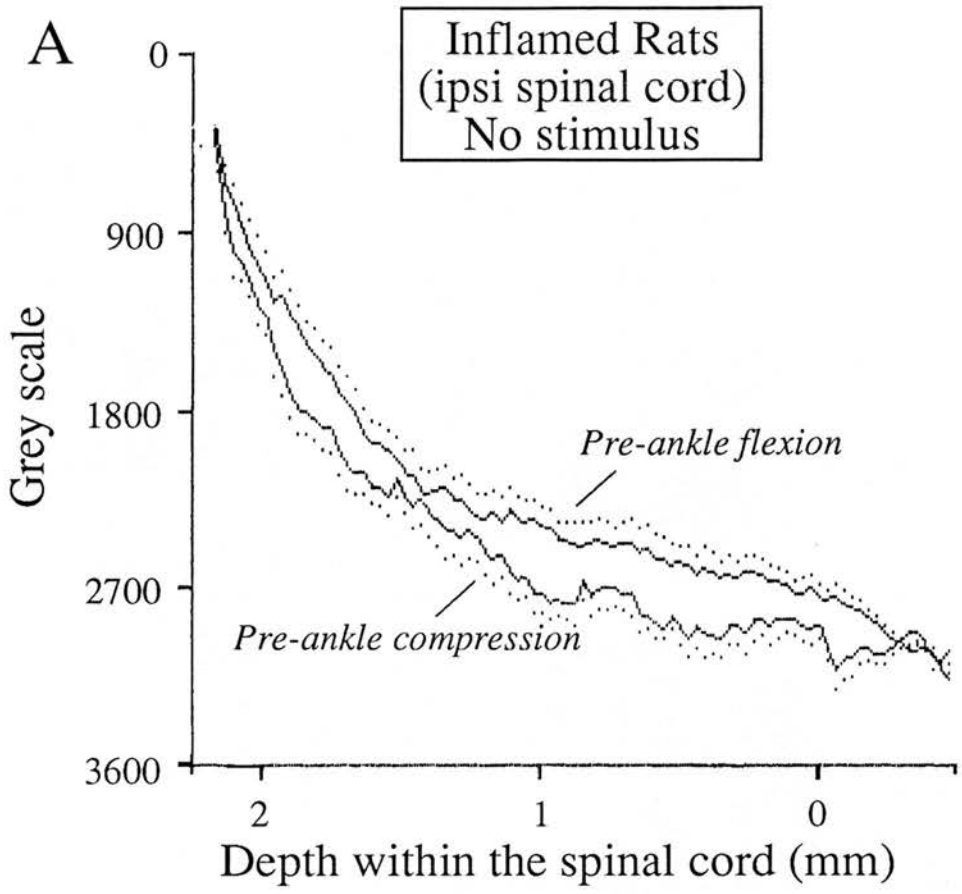
Again limited data was available on the levels of ir-dynorphin A(1-8) in the spinal cord of normal rats and those with unilateral ankle inflammation after the application of flexion stimuli. That available showed no evidence for an increased dynorphin A(1-8) presence.

The apparent lack of effect of flexion may have been partly due to the comparatively large basal levels of ir-dynorphin A(1-8) detected in both the normal and inflamed animals to that found in the rest of the dynorphin study. For example as illustrated on figure 20 the mean image analysis of microprobes (n=11) present under basal conditions in the ipsilateral side of the spinal cord of rats with a unilateral ankle inflammation that were later used to study the effect of ankle flexion were found to be significantly displaced above that of microprobes (n=16) from the same series of experiments that studied the effect of lateral ankle compression.

5. RELEASE OF ir-DYNORPHIN A(1-8) IN THE SPINAL CORD DURING THE DEVELOPMENT OF UNILATERAL INFLAMMATION

In view of the temporal analysis performed by other groups (Draisci and Iadarola, 1989; Iadarola et al, 1988a; b; Iadarola and Draisci, 1990; Lucas et al, 1993; Naranjo et al , 1991) on the expression of the message for prodynorphin, it was of interest to study the release of ir-dynorphin A(1-8) in the spinal cord at an early phase of the inflammatory response. Microprobes present in the spinal cord ipsilateral to the injected ankle for 15 or 30 minutes during the third to the eighth hour after the FCA injections, in the absence of any additional peripheral stimulation, were compared (a total of 79 microprobes; an average of 15 microprobes being inserted for each hourly period). Comparisons were also made to those *in vitro* microprobes that had only been exposed to radiolabelled dynorphin A(1-8) but processed concurrently with those *in vivo* (18 microprobes). Analysis of the data from six experiments provided no evidence that levels of ir-dynorphin A(1-8) were significantly enhanced 3 to 8 hours after the FCA injections. For example, as illustrated on figure 21 the mean image analysis of microprobes (n=19) present in spinal cord ipsilateral to the injected ankle for either 15 or 30 minutes between 7 to 8 hours post-injection, displayed no significant differences from that of microprobes (n=16) present in the same side of the spinal cord 3 to 4 hours earlier.

FIGURE 20. The large basal presence of ir-dynorphin A(1-8) in the animals used to study the effect of ankle flexion compared to that present in animals used in the rest of the dynorphin study. (A) The mean image analysis of 11 microprobes present for 30 minutes in the ipsilateral side of the spinal cord of rats with a unilateral ankle inflammation prior to flexion of the inflamed ankle *(pre-ankle flexion)* is displaced above that of microprobes present in animals belonging to this series under the same basal conditions, but prior to compression of the inflamed ankle (*pre-ankle compression*, n=16). (B) The differences between the two groups of microprobes are plotted with respect to an outline of the spinal cord. The hatched areas indicate where these differences are significant at the $P<0.05$ level.


A
Inflamed Rats
(ipsi spinal cord)
No stimulus
0
900
1800
2700
3600
Grey scale
Pre-ankle flexion
Pre-ankle compression
2
1
0
Depth within the spinal cord (mm)

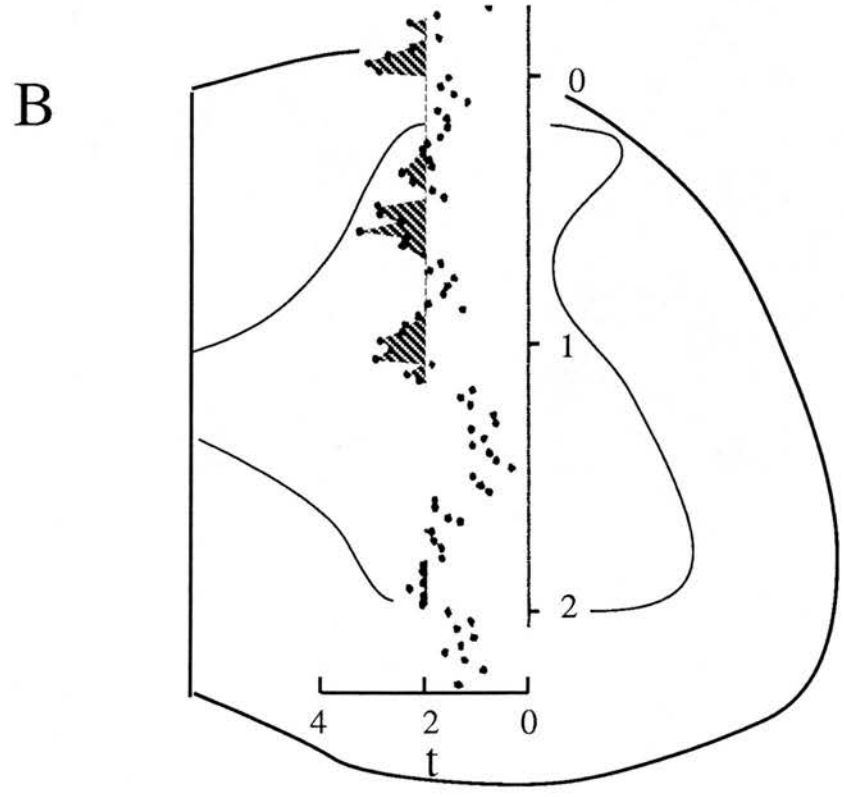
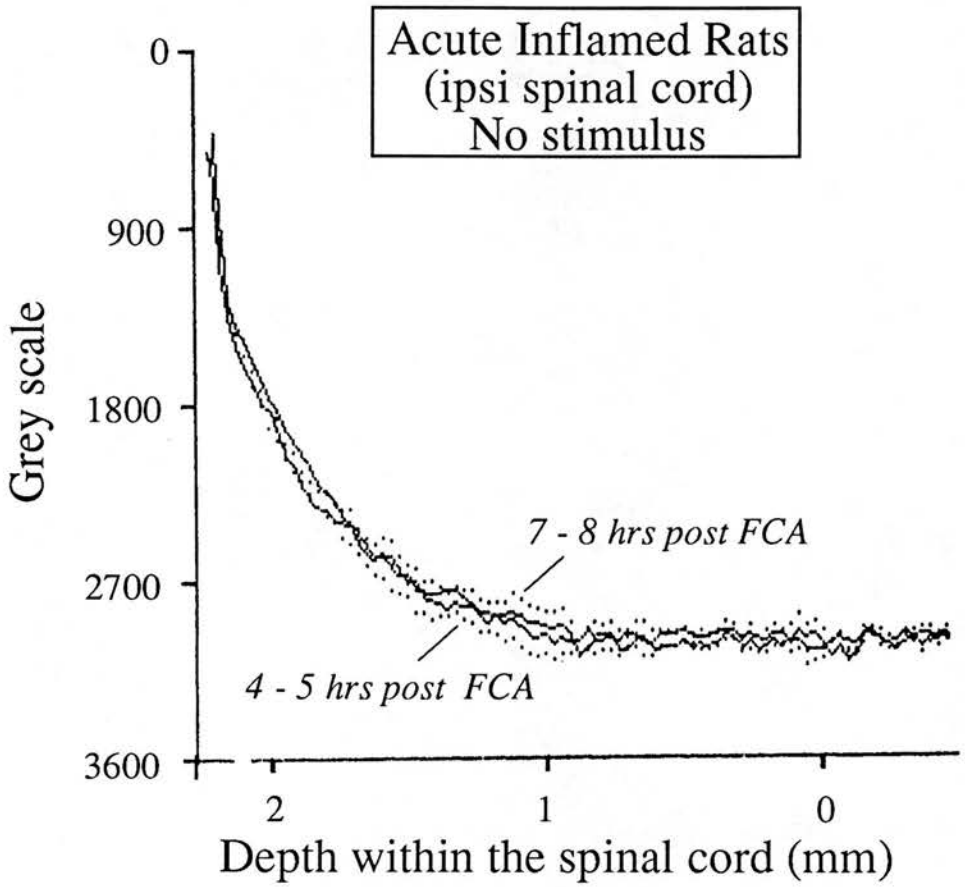

B
0
1
2
4
2
0
t

FIGURE 21. Lack of effect of FCA injections on the basal levels of ir-dynorphin A(1-8) in the ipsilateral spinal cord up to 8 hours post-injection. The mean image analysis of the 19 microprobes present for either 15 or 30 minutes in the side of the spinal cord ipsilateral to the injected ankle between 7 to 8 hours post-injection (*7-8hrs post FCA*) is compared to that of microprobes present in this side of the spinal cord 3 to 4 hours earlier (*4-5hrs post FCA*, n=16).


Acute Inflamed Rats
(ipsi spinal cord)
No stimulus
0
900
1800
2700
3600
Grey scale
7 - 8 hrs post FCA
4 - 5 hrs post FCA
2
1
0
Depth within the spinal cord (mm)

No study was made of the release of ir-dynorphin A(1-8) during the application of mechanical stimuli to the injected ankle under these acute inflammation conditions, that might better mimic the development of the inflammation in the conscious animal over the same time period.

6. SUMMARY OF RESULTS

The present experiments have detected ir-dynorphin A(1-8) in the spinal cord of both normal rats and those with peripheral inflammation but have found significant differences between the two groups. In the absence of any active peripheral stimulus, extracellular ir-dynorphin A(1-8) was found in two areas (lamina I and laminae IV-V) of the dorsal horn of normal rats but occurred throughout the dorsal and ventral horns of both sides of the spinal cord in rats with unilateral ankle inflammation. Differences were also seen when the ankle region was subjected to lateral compression. Ir-dynorphin A(1-8) appeared in the ventral horn not during, but after the period of stimulation in normal animals. With inflamed animals, compression of the inflamed ankle did result in release of ir-dynorphin A(1-8) during the period of stimulus application. This produced elevated levels at three main sites in the spinal grey matter . The largest peak was in the deep dorsal/ upper ventral horn (laminae VI-VII), with further sites of significant release in the mid dorsal horn (laminae II-V) and the lower ventral horn. These levels persisted for at least one hour after the period of stimulation.

4.4 DISCUSSION

The significant results of this study which I will discuss are (a) the presence of ir-dynorphin A(1-8) in the ventral horn region of the lumbar spinal cord both of normal rats and those with a developed peripheral inflammation, (b) the bilateral increases in ir-dynorphin A(1-8) following the development of a unilateral peripheral inflammation, (c) the post-stimulus appearance of ir-dynorphin A(1-8) in the ventral horn of normal rats, (d) the post-stimulus persistence of ir-dynorphin A(1-8) in the ipsilateral side of the spinal cord of rats with a developed unilateral peripheral inflammation.

First however the possible sources of release of dynorphins in the spinal cord of normal rats and those with a developed peripheral inflammation will be considered. The relevant structures include primary afferent fibres, intrinsic neurones of the spinal cord and the spinal terminations of fibres of supraspinal origin.

Localisation of dynorphin in the spinal cord

In normal animals many radioimmunoassay studies have shown higher levels of ir-proDyn derived peptides in the dorsal horn compared to the intermediate or ventral horn zones of the mammalian spinal cord (rat: Botticelli et al, 1981; Zamir et al, 1983 Millan et al, 1984; Pohl et al, 1990. rabbit: Botticelli et al, 1981. human: Przewlocki et al, 1983). Immunocytochemical studies in normal animals have localised ir-proDyn derived peptides mainly in cells and terminals of lamina I-II and lamina IV-VI of the mammalian spinal cord with moderate concentrations in the area around the central canal, lamina X (rat: Vincent et al., 1982; Khatchaturian et al, 1982; Cho and Basbaum, 1988; 1989; Leah et al, 1988; Miller and Seybold, 1987; 1989; Ruda et al., 1988; Weihe et al., 1988; 1989; Nahin et al., 1989; Fallon and Ciofi, 1990; Kajander et al., 1990; Klein et al, 1991. cat: Cruz and Basbaum, 1985; Basbaum et al., 1986; Miller and Seybold, 1987,1989. monkey: Carlton and Hayes, 1989). *In situ* hybridisation studies have also located sites of proDyn synthesis in normal rats

to cells of the superficial and deep dorsal horn (Ruda et al., 1988; Iadarola and Draisci, 1988; Weihe et al., 1989; Noguchi et al, 1991; Przewlocka et al., 1992; Parker et al., 1993; Tolle et al, 1994; Persson et al, 1994). The induction of a peripheral inflammation has been found to increase the spinal content of these ir-proDyn derived peptides in studies in the rat employing radioimmunoassay (Millan et al, 1985; 1986; Iadarola et al, 1988a; b) or immunocytochemical techniques (Millan et al, 1988; Ruda et al, 1988; Nahin et al, 1989; 1992; Takahashi et al, 1988; 1990; Weihe et al, 1988; 1989; Noguchi et al, 1991). This increase in peptide content has been shown to be preceded by an enhancement in the expression of the RNA message for proDyn (Ruda et al, 1988; Weihe et al, 1989; Noguchi et al, 1991; Przewlocka et al 1992; Parker et al, 1993; Persson et al, 1994) by cells in the superficial dorsal horn (laminae I-II) but notably also in deep dorsal horn (laminae IV-VI) neurones. In addition, under these conditions, neurones dorsolateral to the central canal (laminae VI, VII and X) have also been observed to contain high levels of proDyn mRNA (Weihe et al, 1989; Persson et al, 1994).

As indicated in section 1.6.3, the majority of the ir-dynorphin is thought likely to be present in neurones intrinsic to the cord itself. Despite the presence of ir-dynorphin near the sites of termination of primary afferents in the dorsal horn there is little evidence to support a significant presence of dynorphin in primary afferents, particularly in lumbar spinal segments of the rat. Derivatives of proDyn have been demonstrated to exist immunocytochemically in colchicine treated animals in sacral DRG of the cat (Basbaum et al, 1986) and various sensory ganglia of the guinea pig (Weihe et al, 1985; Gibbins et al, 1987), as well as cultured mouse dorsal root ganglia (Sweetnam et al, 1986) although the levels are believed to be very low and no detectable levels of mRNA have been demonstrated. Dorsal rhizotomy had little or no effect on the levels of ir-dynorphin in both the dorsal and ventral horns of the rat spinal cord (Botticelli et al., 1981; Pohl et al., 1990; Klein et al., 1991). Following unilateral multiple dorsal rhizotomy of the cat lumbar and sacral DRG, decreases in the numbers of ir-dynorphin terminals were reported at the sacral but not lumbar level of the spinal cord 10 days later

(Basbaum et al, 1986), and in the rat lumbar spinal cord at the same time period (Tuchscherer and Seybold, 1989). However, these changes may be secondary to the loss of primary afferent neuronal transmission in the spinal cord. Notably no reports of detectable amounts of proDyn mRNA have been observed in rat DRG neurones by either RNA blot or *in situ* hybridisation (as commented upon by Ruda et al, 1995). There is also little evidence to support a significant presence of dynorphin in the spinal terminals of fibres of supraspinal origin (rat: Botticelli et al., 1981; Menetrey and Basbaum, 1987).

Evidence for monosynaptic synapses between dynorphin containing neurones and CGRP/ substance P containing presumed primary afferent fibres has been found at both light microscopic (rat: Takahashi et al, 1988; Cho and Basbaum, 1989) and electron microscopic (rat: Takahashi et al, 1990; Carlton and Hayes, 1989) level. As indicated in section 1.7.3 there is evidence that certain of these dynorphin neurones are the origin of ascending projections to the brain that terminate in the diencephalon (rat: Nahin et al, 1987; Leah et al, 1988) or brainstem (rat: Standaert et al, 1986; Nahin et al 1987; 1989; 1992; Leah et al, 1988). Thus it appears that the cells releasing ir-dynorphin A(1-8) in the present studies were predominantly intrinsic spinal neurones with their cell bodies in the superficial and deep dorsal horn.

Evidence for a ventral horn release of dynorphin

In contrast to the majority of above immunocytochemical studies the present investigations using antibody microprobes have found evidence for a significant presence of ir-dynorphin A(1-8) in fibre terminals in ventral horn. Most of these immunocytochemical studies, however, used cochicine treated animals and hence favoured detecting presence in cell bodies. Hence they do not necessarily give information on sites of release particularly if the relevant neurones have long axons with many branches. Ir-dynorphins have been measured in the ventral horn of the rat spinal cord by radioimmunoassay (Botticelli et al, 1981; Zamir et al, 1983; Pohl et al, 1990) and more recent immunocytochemical studies, notably on

non-colchicine treated animals, have emphasised the finding of ir-dynorphin labelled varicose fibres surrounding both large and intermediate sized motoneurones (Weihe et al, 1989; Kajander et al, 1990; Klein et al, 1991). Klein et al. (1991) found that the rat differs from the cat and primate in this respect. The location of the cell bodies of these dynorphin containing varicosities in the ventral horn were not known but it is probable that they were intrinsic spinal neurones with their cell bodies located more dorsally. Such connections could be inter- and intra-segmental. Klein et al. (1991) also found that electrical stimulation of the small and large diameter fibres of the ipsilateral sciatic nerve (1Hz for 20 minutes) depleted ventral horn ir-dynorphin A(1-8). If such depletion results from prior release then these findings are in accord with some of observations of the present experiments where release in the ventral horn in addition to the dorsal horn was a prominent finding.

In antibody microprobe studies a significant extracellular basal presence of a compound could result from a tonic release from an unknown stimulus, or from a continuous afferent input in nociceptors and other fibres as a result of the surgery required for these preparations. A contribution from cells and fibres ruptured during the introduction of microprobes cannot be excluded although differing basal patterns observed in rats with microprobes bearing antibodies to different peptides makes it unlikely that this is a significant factor (Lang and Hope, 1994; Hope et al., 1994; Schaible et al., 1994). Although the similar basal presence of ir-dynorphin A(1-8) in the dorsal horn of normal and inflamed rats could be the result of surgery, an enhanced afferent input was almost certainly responsible for the increased levels in the ventral horns of inflamed rats, particularly as active manipulations of the inflamed ankle region increased these levels still further. As indicated in section 4.1 a basal presence of ir-dynorphin A(1-17) was detected by Hutchison et al (1990) in the spinal cord of the normal cat using antibody microprobes but the consequences of peripheral inflammation were not examined.

Bilateral increases in dynorphin following the development of a unilateral inflammation

The finding of bilateral increases in ventral horn ir-dynorphin in animals with a peripheral inflammation, when compared with normal animals, was unexpected particularly as most studies of the changes in the mRNA levels for proDyn following unilateral inflammation have found only ipsilateral increases (Iadarola and Draisci, 1988; Iadarola et al., 1988a, b; Ruda et al., 1988; Weihe et al., 1989; Draisci and Iadarola, 1989; Draisci et al., 1991; Parker et al., 1993). Przewlocka et al. (1992) however, observed bilateral increases in the proDyn mRNA in the superficial dorsal horn following unilateral hindlimb inflammation but at relative later stages (5 and 14 days post-injection). This may involve the induction of immediate early genes, such as c-fos, contralaterally. As discussed in section 4.1, there is considerable evidence that the protein products of these genes direct the increased synthesis of proDyn following a spinal input from peripheral nociceptors. However at these later time points, such changes in expression may be correlated with the transfer of inflammation to the contralateral paw, as other groups using similar unilateral models of peripheral inflammation in the rat have shown to occur (Donaldson et al, 1993; Bileviciute et al, 1993, 1994). Although, in the present experiments using the dose of FCA and time course of inflammation described in section 2.3, there was no evidence for such a spread of inflammation to the opposite side. A simple explanation for our finding of bilateral increases in released ir-dynorphin A(1-8) associated with peripheral inflammation is that some of the cells containing this neuropeptide project bilaterally to the ventral horn.

As described in section 1.8.2, the sensitisation of nociceptive primary afferents during the development of a peripheral inflammation, has been shown to produce long term changes in the responsiveness of spinal cord neurones including increases in the spontaneous activity, reduced thresholds and increases in the responsitivity to afferent inputs, as well as expansions of the cutaneous receptive fields beyond the focus of the inflamed tissue to adjacent ipsilateral and even contralateral regions. That contralateral changes may occur is also implied by the

pattern of c-fos protein expression found by some groups following a noxious unilateral input, immunoreactive labelling being evident to a lesser and more limited extent on the contralateral side of the spinal cord, as well as ipsilaterally. (Hunt et al, 1987; Gogas et al, 1991; Leah et al, 1992). Of particular interest to this present study are the findings of Leah et al (1992). This group describe how the expression of c-fos protein in the ipsilateral side of the spinal cord of rats following the application of mechanical or chemical stimulation to the corresponding paw, is significantly increased if either of these noxious stimuli are applied up to an hour and a half before that to the contralateral hindlimb. This effect was seen to persist for at least 12 hours and was not produced if the contralateral stimuli were applied to sites remote from this dermatome, such as the forepaw, indicating both the longevity and specificity of these presumed centrally mediated changes. Thus it is possible that contralateral neurones are similarly sensitised as an inflammation develops unilaterally, in this study as could the spinal neurones in normal animals following the application of noxious mechanical stimuli to the ankle region (see below). This could occur either via supraspinal loops or the direct commissural connections, that have been shown to anatomically exist in the spinal cord (Jacquin et al, 1990).

Post-stimulus appearance of dynorphin

The *de novo* synthesis of c-fos proteins could also explain the delayed increase in ventral horn levels of ir-dynorphin A(1-8) following lateral compression of the ankle of normal rats. This implies a relatively rapid process (30 to 60 minutes) within which the arrival of an input in nociceptors is translated into increased expression of a gene and the release of a neuroactive product of that gene. As discussed section 1.8.3 there is evidence that immediate early genes encoding Fos and related proteins are up-regulated in the spinal cord within 30 minutes of the injection of an inflammatory agent into the rat hindpaw (Draisci and Iadarola, 1989; Hunter et al., 1995). Messenger RNA for proDyn in the spinal cord has been found to be increased within 4 hours of the peripheral injection of an inflammatory agent (Iadarola et al., 1988a, b;

Millan et al., 1988; Iadarola and Draisci, 1988; Draisci et al, 1991; Draisci and Iadarola, 1989; Hunter et al., 1995). The present studies of released ir-dynorphin A(1-8) suggest that the process of synthesis and release is occurring more quickly than can be revealed by indirect methods relating to release, such as mRNA expression and immunocytochemistry. It is thus feasible that lateral compression of the normal rat ankle could induce the expression of such immediate early genes, to result in a delayed appearance of ir-dynorphin A(1-8) post-stimulus in the ventral horn region. This proposal is not invalidated by the finding that no increase in the basal release of ir-dynorphin A(1-8) was detected up to 8 hours after injection of FCA around the ankle. Until inflammation developed this procedure gives only a trivial noxious input (associated with the injection) whereas ankle compression was a severe sustained stimulus.

Although most of the immunocytochemical studies do not refer to a ventral horn expression of the Fos protein following noxious stimulation, some investigators point to a sparse presence (Gogas et al, 1997; Abbadie and Besson, 1992; Abbadie et al, 1992; Chapman et al, 1995a, b; Wei and Zhou, 1995), and again it should be emphasised that c-fos locates cell bodies and not axon terminals where release occurs.

Persistence of ir-dynorphin A(1-8) post-stimulus

The finding of elevated levels of a neuropeptide both during and after a peripheral stimulus has been interpreted in previous microprobe studies as indicating a slow degradation of neuropeptide following release, with the opportunity for diffusion to regions remote from sites of release. For example, *in vitro* neurokinin A appears to be remarkably resistant to enzymes believed to be important in the degradation of substance P (Nyberg et al, 1984; Hooper et al, 1985; 1987; Theodorsan-Norham et al, 1987). Immunoreactive neurokinin A has been shown to persist and spread after release in the cat spinal cord following noxious cutaneous stimulation or the development of peripheral inflammation (Duggan et al, 1990;

Hope et al, 1990b) in contrast to the relatively focal release of substance P detected during the application of the same stimuli (Duggan et al, 1988a; Schaible et al, 1990).

The exact metabolism of dynorphin A(1-8) following release is not known. As indicated in section 1.8.3, during the development of a peripheral inflammation, changes in the activity of certain dynorphin processing enzymes found in the spinal cord and CSF occur (Silberring et al, 1992; Persson et al, 1992a; b). For example, Silberring et al (1992) demonstrated how the activity of the dynorphin converting enzyme in rat spinal cord homogenates which cleaves dynorphin B(1-13) to generate Leu-enkephalin-Arg^6 [or dynorphin A(1-6)] is significantly decreased in the spinal cord on the development of an inflammation in the ipsilateral hindpaw. Although such processes may occur it is also possible that, as with normal animals, lateral compression of the ankle in inflamed animals induced further synthesis and release of proDyn peptides.

Another possible explanation for the apparent persistence of dynorphin A(1-8) could be a technical one, in that the antibody microprobes are binding metabolites of longer derivatives of the prodynorphin molecule that do not bind to the microprobes. However, from present understanding of proDyn processing no such metabolite exists. The dynorphin converting enzyme mentioned above, has a preference in the rat for dynorphin B over dynorphin A or α-neoendorphin derivatives of the prodynorphin precursor (Persson et al, 1989), and details from the manufacturer (Peninsula Laboratories) state that the antibodies raised against the COOH-terminus of the dynorphin A(1-8) peptide used in this present study have negligible zero cross reactivity for the Leu-enkephalin-Arg^6 product. Although it is possible that Leu-enkephalin could be formed from proDyn peptides released by the action of processing enymes such as metalloendopeptidase 24.15, *in vitro* testing performed indicated that the microprobes employed would not interact with such a derivative.

Possible functions of released dynorphin

In this study active manipulation of the inflamed tissues was an adequate stimulus for spinal release of ir-dynorphin. This is an important finding since as reviewed in section 1.8, much of the recent literature has emphasised the hyperexcitability of spinal neurones associated with peripheral inflammation and has directed substantial research towards defining the neurochemical basis of such hyperexcitability.

From a functional viewpoint however, the dynorphins have proven to be enigmatic neuropeptides to study in spinal cord function. As previously outlined in section 1.7, when administered systemically, κ receptor agonists have been demonstrated to depress some spinal reflexes, indeed κ opioid receptors were first proposed from the effects of EKC on spinal reflexes in the chronic spinal dog (Martin et al, 1976). Administered iontophoretically both dynorphin A(1-13) and U50488H have also been described to depress the excitation of spinocervical tract neurones of the cat by noxious peripheral stimuli (Fleetwood Walker et al, 1988, Hope et al, 1990a). However, when applied topically to the spinal cord, dynorphin A and κ receptor ligands have produced variable effects (mainly in the superficial dorsal horn) on neuronal firing with mixed excitatory/ inhibitory actions (Knox and Dickenson, 1987; Sullivan and Dickenson, 1991; Hylden et al, 1991; Dong et al, 1991; Stanfa and Dickenson, 1994). Additionally dynorphins have been reported to antagonise μ mediated antinociception in electrophysiological (Dickenson and Knox, 1987) and behavioural (Tulunay et al, 1981; Schmauss and Herz, 1987) studies.

However, as highlighted in section 1.4, although the dynorphins show some preference for κ opioid receptors, they also display significant affinity for μ and δ sites. There is also evidence for actions of these peptides at non-opioid sites, such as the NMDA receptor (as reviewed by Smith and Lee, 1988; Day et al, 1993; Shukla and Lemaire, 1994), with similar effects being displayed by non-opioid derivatives of dynorphin A, such [des-Tyr^1]dynorphin (2-17) (Walker et al, 1982). Thus, activating or blocking the κ opioid receptor with specific ligands may not reveal the full functional role of released dynorphins and administering

dynorphins in high concentrations may produce effects not present following synaptic release. Notably dynorphin A(1-17) has recently been demonstrated to directly modulate excitatory amino acid induced currents (both NMDA and non-NMDA) in acutely isolated trigeminal (Chen et al, 1995) and spinal dorsal horn (Kolaj et al, 1995) neurones.

Such factors may be responsible for dichotomy of opinion drawn from electrophysiological studies, as to the role of dynorphins at the spinal cord level. From the finding that the topical administration of dynorphin A produced expansions of the receptive fields of the majority superficial dorsal horn neurones in the spinal cord of normal rats, resembling those found under conditions of peripheral inflammation (Hylden et al., 1991), it was proposed that instead of being inhibitory to the hyperalgesia induced by peripheral inflammation, the enhancement of proDyn expression is directly involved in the expression of the hyperalgesic states (Dubner and Ruda, 1992). However, systemic naloxone failed to reverse these receptive field expansions and hence these effects are more suggestive of an action of the dynorphins at non-opioid binding sites. In contrast, Stiller et al (1993) found that the prominent effect of the microiontophoretic administration of the κ receptor antagonist nor-BNI was to enhance the firing of spinal neurones in animals with peripheral inflammation. Significantly firing to manipulation of inflamed peripheral tissues was also enhanced by the κ antagonist suggesting that released dynorphins were acting to inhibit cellular responses.

An inhibitory role of the dynorphins in the expression of pain under conditions of peripheral inflammation is also implied from the findings of behavioural studies that the increased sensitivity of the inflamed paw to noxious pressure can be potentiated by the blockade of κ receptors, with either acute administration of MR2266 (a relatively selective κ antagonist) or long term perfusion with a high dose of naloxone, whilst the antagonism of μ and δ receptors alone is ineffective (Millan et al, 1985, 1986, 1988, 1991). Since these drugs were given systemically a contribution from peripheral opioid receptors that have been observed to become functional in inflamed tissues (Stein et al, 1987) cannot be discounted. Additionally a recent study employing a push-pull cannula, demonstrated how the superfusion

of dynorphin A(1-8) into the dorsal horn of spinalised rats can inhibit the release of ir-substance P following noxious thermal stimulation *in vivo,* which is reversible by norBNI (Zachariou and Goldstein, 1996), a finding again more in favour of the dynorphins acting to moderate, not exacerbate, hyperalgesia in peripheral inflammatory conditions.

My finding of both basal and evoked release of ir-dynorphin A(1-8) in several areas of the spinal cord does suggest an involvement of dynorphins in several aspects of spinal processing. Release in the superficial dorsal horn may be related to antinociception, since these are the areas of the spinal cord where fine sensory fibres that respond to nociceptive stimuli terminate. Similar enhancements of proDyn synthesis have been observed after various other types of intense peripheral stimulus, such as partial injury to peripheral nerves (Kajander et al, 1990; Draisci et al, 1991; Wagner et al, 1993) and dorsal root section (Cho and Basbaum, 1988; Caneletti and Ferri, 1995). An upregulation of proDyn peptides also occurs during chronic stress (Przewlocki et al, 1987) and in late pregnancy/ parturition (Medina et al , 1993)

The prominence of evoked release in the deep dorsal horn together with the ventral horn could reflect an influence on interneurones and motoneurones mediating spinal reflexes and supraspinal control of reflexes. Hindlimb 'flaccid' paralysis figures prominently in early behavioural studies of intrathecally administered dynorphins (Przewlocki et al., 1983; Faden and Jacobs, 1984; Herman and Goldstein, 1985; Stevens and Yaksh, 1986). However, the initial concentrations of the compounds administered by this route are high and the final concentrations at the receptors are unknown. Instead it has been proposed that these naloxone-resistant effects relate more to the role of dynorphins in the pathophysiology of spinal cord trauma and necrosis (Przewlocki et al, 1988; Stewart and Isaac, 1989), via indirect or direct actions involving NMDA receptors (Caudle and Isaac, 1988; Bakashi et al, 1992; Skilling et al, 1992). However, the results presented in this thesis indicate that ir-dynorphin A(1-8) is physiologically released in the ventral in addition to the dorsal horns of the rat by intense peripheral stimulation and hence effects on motor behaviour are likely a normal action of these neuropeptides.

Thus, although it is a common assumption by many investigators to discuss the function of the endogenous opioid peptides at the spinal level primarily with events related to nociception, there is also considerable evidence to suggest that the dynorphins play an important role in motor behaviour. Hence changes in opioid systems, especially proDyn, on the induction of peripheral inflammation are likely to result not only in alterations in neuronal activity related to the transmission of nociceptive information but also to other processes such as those controlling motor output from the spinal cord in these pain states. Inhibition of motor performance is a normal accompaniment of the healing process and this may be an important component of the spinal actions of dynorphins as inflammation develops peripherally.

CHAPTER 5: Studies of the Spinal Release of Cholecystokinin - Effect of Acute Morphine Administration

5.1 INTRODUCTION

It has been proposed that stimulation of opioid receptors may trigger a progressive compensatory increase in the activity of CCK containing neurones in the spinal cord (as discussed in section 1.9), with implications for not only the endogenous modulation of the analgesic effects of opioids but also the development of tolerant states following the prolonged administration of opiates. However, only a few studies have been performed to investigate these CCK-opioid interactions directly, and these have produced a confusing array of data. Hence, despite considerable research little is known about the level at which this interaction occurs and the mechanisms involved.

No consistent effect has been observed on the synthesis of CCK in the CNS, following acute or chronic opiate administration. Whereas early investigators reported alterations in the levels of ir-CCK determined by radioimmunoassay in certain brain regions (Morley et al, 1986; Faris et al, 1986) and in the spinal cord (Watkins et al, 1985b) following morphine, later studies found no evidence for such changes (Rosen and Brodin, 1989; Pohl et al, 1992). Alone these ir-CCK levels are difficult to interpret with regard to the activity of CCK releasing neurones, since parallel activation of the synthesis and release of CCK might well result in unchanged tissue levels of ir-CCK. Rises in CCK levels could reflect inhibition of release. However, studies of proCCK mRNA levels that provide a better index of the synthesis state of the peptide have likewise given mixed results, with some evidence for increased synthesis of CCK in certain brain regions following acute and chronic morphine administration (Zhou et al, 1992; Ding and Bayer; 1993; Pu et al, 1994), including the spinal cord (Ding and Bayer; 1993), though some investigators find no such effects (Pohl et al, 1992). Although not a complete study (only the cerebral cortex was investigated) no changes in the CCK-B receptor binding characteristics (both Kd and Bmax) have been shown to occur in morphine induced tolerant and abstinence states (Welin et al, 1994). Thus, overall the evidence available, although limited, suggests that the mechanism by which CCK-opioid

interact at the spinal cord level may involve an increased activity of CCK releasing neurones.

The findings of studies measuring the spinal release of ir-CCK into a surface perfusate when opioids have been given topically or systemically, are equally far from uniform, and complex opioid receptor interactions have been proposed to account for the release patterns observed. *In vivo,* a naloxone reversible increase in ir-CCK levels has been observed in spinal perfusates following the administration of analgesic doses of morphine either intrathecally (1μ M; Tang et al, 1984) or subcutaneously (5mg/ kg; Zhou et al, 1993b), this effect declining as morphine administration continues (Pu et al, 1994). Similar effects were found following acute and chronic periods of electroacupuncture (Zhou et al, 1993a; Sun et al, 1995). However, by contrast the acute administration of the selective μ opioid receptor agonist DAMGO (1μM) has been found to inhibit ir-CCK release into perfusate evoked either by potassium or by direct high threshold stimulation of the rat sciatic nerve, in a naloxone reversible fashion (Rodriguez and Sacristan, 1989).

In vitro investigations by Benoliel and co-workers (1991, 1994b) using perfused slices of the rat dorsal horn appear to confirm these *in vivo* findings, describing increased ir-CCK release with 10μM morphine added to the perfusate and an inhibition of the potassium evoked ir-CCK release with 0.1 to 10μM DAMGO, although the effect of morphine was found not to be reversed by naloxone but by the selective δ antagonist ICI154129. Some explanation of this apparent dichotomy was proposed from further studies analysing the effect of selective δ and κ agonists on ir-CCK release. In contrast to the monophasic inhibitory influences of DAMGO on CCK release, a concentration dependent biphasic effect was observed with the selective δ agonist DTLET, a decrease in ir-CCK release being observed at low concentrations (0.01 to 3μM), but an increase in ir-CCK release at a high concentration (10μM). Since both effects were blocked by selective δ antagonists naltrindole and ICI154129, it was proposed that these concentration dependent effects could result from actions on differing receptors or receptor subtypes, as well as different neuronal populations (Benoliel et al, 1991; 1994b). Similarly low doses of morphine (0.01 to 0.1μM) were found to exert inhibitory effects on

ir-CCK release, and like DAMGO, these were reversed by naloxone. Adding to the complexity, the κ agonist, U50488H was found to produce an inhibitory influence only at a high concentration (10μM), at lower concentrations (1μM) appearing instead to prevent the negative influence of 10μM DAMGO on peptide release. In contrast, no interaction was apparent between δ and κ agonists and effects on CCK release. Hence this group have proposed that, since 10μM morphine likely stimulates all the opioid receptors, the overall effect observed is that of the δ receptor, the antagonistic effects of κ versus μ receptor stimulation negating each other (Benoliel et al, 1991, 1994b). A preliminary study by these investigators on the effect of opioid receptor agonists on the spinal ir-CCK release in morphine tolerant rats, found that under these conditions only the excitatory influence of opioids on CCK releasing spinal neurones was observed (Benoliel et al, 1994a). However, such spinal perfusion studies are unable to localise sites of release within the spinal cord and the dorsal horn *in vitro* preparation of Benoliel and co-workers can at best determine release from the limited neuronal systems remaining operative in this part of the spinal cord.

Hence, when I commenced my studies there was a need for further study to investigate the interaction between opioid and CCK synthesising neurones at the spinal cord level. Thus, in the experiments described in this thesis I employed microprobes bearing immobilised antibodies to CCK to study the release of ir-CCK in the spinal cord of normal rats under both basal conditions and following acute morphine administration, with a view to study the phenomenon in animals chronically treated with morphine. However, due to the failure of the CCK microprobes to continue to perform satisfactorily as assessed by *in vitro* testing I was unable to perform such experiments. This was due to a change in the competence of the antiserum purchased from Peninsula Laboratories. Although several other commercial sources of CCK antiserum and radioactive peptide were investigated, attempts to resume this study were not successful (see appendix A.II for details). The data that will now be presented was from experiments performed using only the antiserum shown to be competent.

5.2 MATERIALS AND METHODS

1. MICROPROBE PREPARATION

Antibody microprobes were prepared as previously described (section 3.2), using a polyclonal antiserum (Peninsula Laboratories) that had been raised in rabbits against the COOH-terminus of desulphated CCK 26-33 [CCK-8(NS)]. Data supplied by Peninsula Laboratories indicated that this antiserum displayed 100% cross reactivity with porcine CCK-33, caerulein and human gastrin; 78% reactivity with sulphated CCK 26-33 [CCK-8(S)]; 63% reactivity with CCK 27-33; 14% reactivity with CCK 30-33 [CCK-4]; negligible cross reactivity (<0.1%) with human pancreatic polypeptide and zero cross reactivity with vasoactive intestinal peptide (human, porcine rat). No information was available concerning the affinity of this antibody for Bolton-Hunter CCK-8(S).

2. EXPERIMENTAL PROTOCOL

A total of 10 male Wistar rats (weight range 400 to 460g; mean ± s.e.m. = 417 ± 2g) were used in this study.

Anaesthesia was induced, cannulae inserted and the lumbar spinal cord exposed, as outlined in sections 2.2.3 and 2.5. All microprobes were inserted 2.25mm into the dorsal spinal cord and remained *in situ* for either 5, 15 or 30 minutes. During these periods no peripheral stimuli were applied, allowing the basal presence of ir-CCK to be examined in these animals for modification by morphine and naloxone. To increase the likelihood of detecting spinal release of CCK with acute administration of morphine:

(a) a total dose of 25mg/kg Morphine HCl (25mg/ml in Ringers' solution) was administered intravenously in divided doses over a two hour period, followed immediately by

(b) a single intravenous injection of 1mg/kg Naloxone HCl (10mg/ml in Ringers' solution), that was repeated for a second time 30 minutes later to block the action of morphine at opioid receptors (as described in detail in section 2.4).

As this study formed the pilot of a proposed series of experiments investigating the effect of acute and chronic morphine administration (see appendix AII), during the administration of these drugs all microprobes were inserted into the spinal cord for the maximum period used (30 minutes). Blood pressure, breathing rate and end tidal CO_2 were monitored throughout. Unfortunately due to the limited experiments that were able to be performed using the competent antiserum, no study was made of the effect of naloxone administration alone on the basal levels of ir-CCK prior to morphine administration.

After removal from the spinal cord, microprobes were washed and incubated for approximately 24 hours in [^{125}I]Bolton-Hunter CCK-8(S) [Amersham International] and prepared for analysis as previously described (sections 3.4 and 3.5).

3. *In vitro* tests

Prior to use and throughout use *in vivo*, the sensitivity of the prepared antibody microprobes were tested as outlined in section 3.3.

5.3 RESULTS

A total of 138 microprobes coated with antibodies to CCK were inserted into the rat spinal cord and form the basis of this analysis. An additional 400 microprobes were used for *in vitro* sensitivity tests.

1. *IN VITRO* TESTS

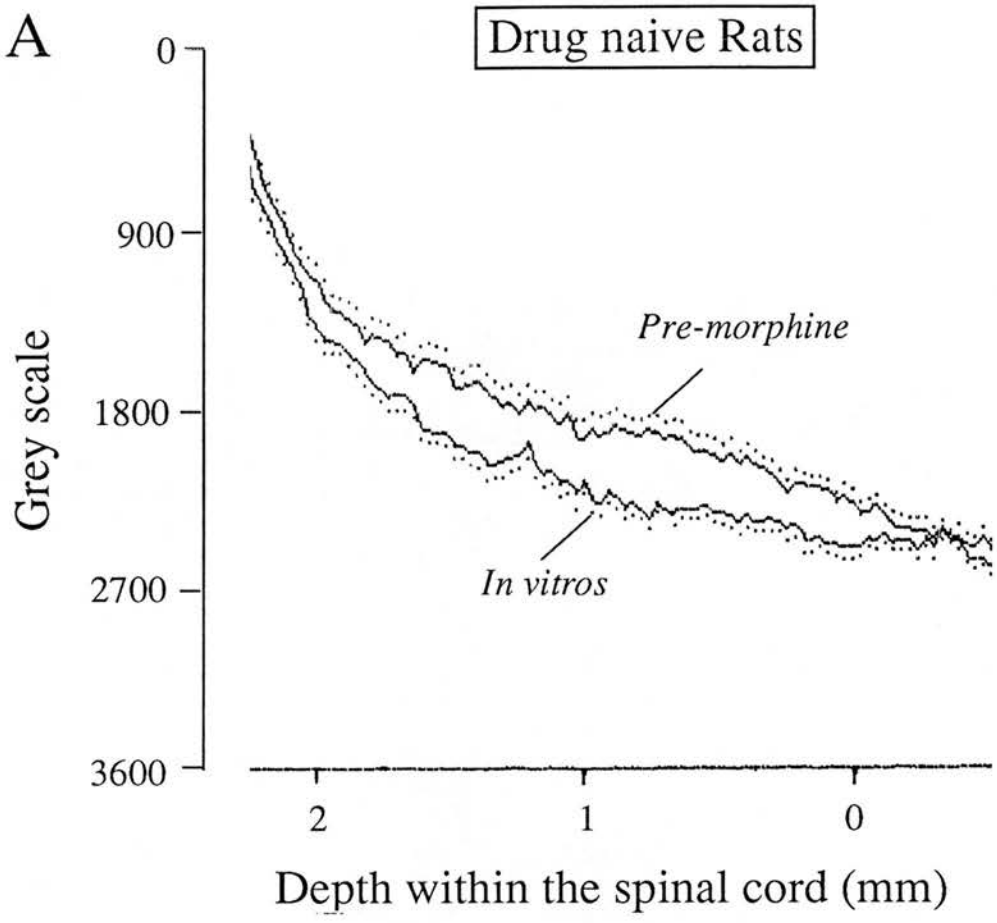
The counts of total radioactivity of microprobe tips indicated that over 10% of the total radioactivity in which they had been incubated bound to the microprobes. *In vitro* tests indicated that CCK-8(NS) suppressed such binding on average by 88%, whilst that of CCK-8(S) resulted on average in a 69% suppression. Hence, it can be assumed that the non-specific binding for these microprobes accounts for less than 20% of the total binding. No evidence for a significant cross reactivity of the antisera with CGRP was found.

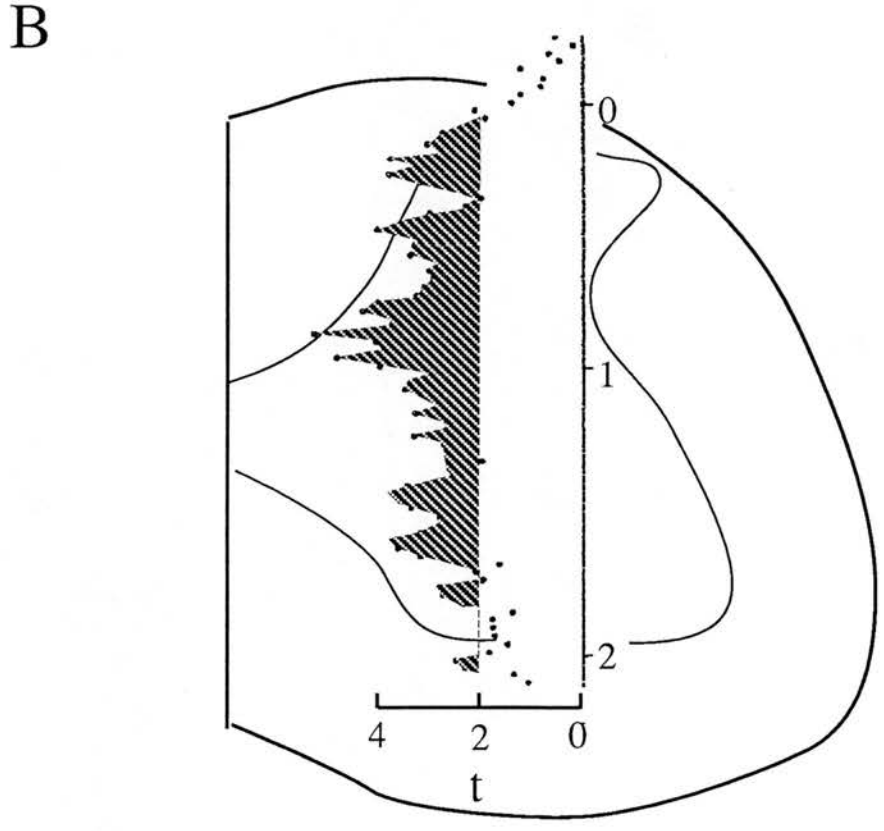
2. BASAL LEVELS OF ir-CCK

As previously described (section 4.3.2), a basal presence of a neuropeptide at a particular site in the nervous system is inferred by differences between the mean image analysis of microprobes not inserted into the nervous system but simply incubated in the radiolabelled ligand and that of microprobes placed in the nervous system for a comparable time but in the absence of any active stimulus. Using microprobes that had been present in the spinal cord for either 5, 15 or 30 minutes, such comparisons indicated that ir-CCK was present in the rat spinal cord under these 'basal' conditions. Since during the administration of the drugs, all the microprobes were inserted into the spinal cord for 30 minutes, only the data derived from these microprobes present will be presented below.

Figure 22A compares the mean image density scan of microprobes (n=28) present in both sides of the spinal cord of normal (drug naive) rats for periods of 30 minutes and that of the *in vitro* microprobes (n=32) that had not been inserted into the spinal cord but simply

FIGURE 22. Basal presence of ir-CCK in the spinal cord of normal (drug naive) rats. (A) The mean image analyses of two groups of microprobes are plotted with respect to length: those present in the spinal cord of normal (drug naive) animals for 30 minutes in the absence of any peripheral stimulation (*pre-morphine*, n=28) and those which are not inserted into the spinal cord but simply incubated in [^{125}I]Bolton-Hunter CCK-8(S) (*in vitros*, n=32). With each mean image analysis the mean grey scale was determined in 30μm intervals and a line joins these points. At each analysis point the standard error mean 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 error of the differences of means at each analysis point in the mean image analyses shown in (A), is related to an outline of the spinal cord at the area sampled. The hatched areas indicate the sites where these differences are significant at the $P<0.05$ level.


A
Drug naive Rats
0
900
1800
2700
3600
Grey scale
Pre-morphine
In vitros
2
1
0
Depth within the spinal cord (mm)


B
0
1
2
4
2
0
t

incubated in [^{125}I]Bolton-Hunter CCK-8(S) and processed concurrently with those used *in vivo*. Figure 22B plots in 30μm intervals the 't' statistics derived from the differences between the mean image analyses of the two groups. The hatched area indicates where these differences are significant at the $P<0.05$ level (i.e. $t>2$). This is approximately at nearly all sites from 0.1 to 2.1mm from the dorsal surface of the spinal cord with three main zones. The largest peak was in the mid to deep dorsal/ upper ventral horn (laminae III-VII), with further major sites in the superficial dorsal horn (lamina I-II) and the mid/ lower ventral horn. Thus, in normal rats extracellular ir-CCK was present in both the dorsal and ventral horns.

3. THE EFFECT OF ACUTE MORPHINE ADMINISTRATION ON BASAL LEVELS OF ir-CCK

The acute administration of morphine (25mg/ kg) over a two hour period failed to alter the presence of ir-CCK found under basal conditions in the rat spinal cord. As illustrated on figure 23 no significant differences were found between the mean image analyses of all the microprobes (n=28) present in the spinal cord over the two hours within which morphine was administered, and that of the microprobes (n=16) present under basal conditions in 7 animals.

4. THE EFFECT OF NALOXONE ADMINISTERED AFTER MORPHINE ON THE SPINAL RELEASE OF ir-CCK

Despite the surprising lack of effect of morphine, subsequent injections of naloxone evoked a spinal release of ir-CCK. This is illustrated in figure 24A in which the mean image analysis of all the microprobes (n=12) present in the spinal cord within two 30 minute periods of naloxone administration can be seen to be displaced above that of microprobes (n=12) present over the prior two hour period of morphine administration in these same group of animals. Since only isolated points attain significance there is no hatching of the 't' plot of figure 24B. This preliminary result indicates that the levels of ir-CCK were

FIGURE 23. Lack of effect of acute morphine administration on basal levels of ir-CCK. The mean image analysis of the 28 microprobes present in the spinal cord for 30 minute periods over two hours of morphine adminstration (*during morphine*) is compared to that of microprobes present under basal conditions in the same animals (*pre-morphine*, n=16).

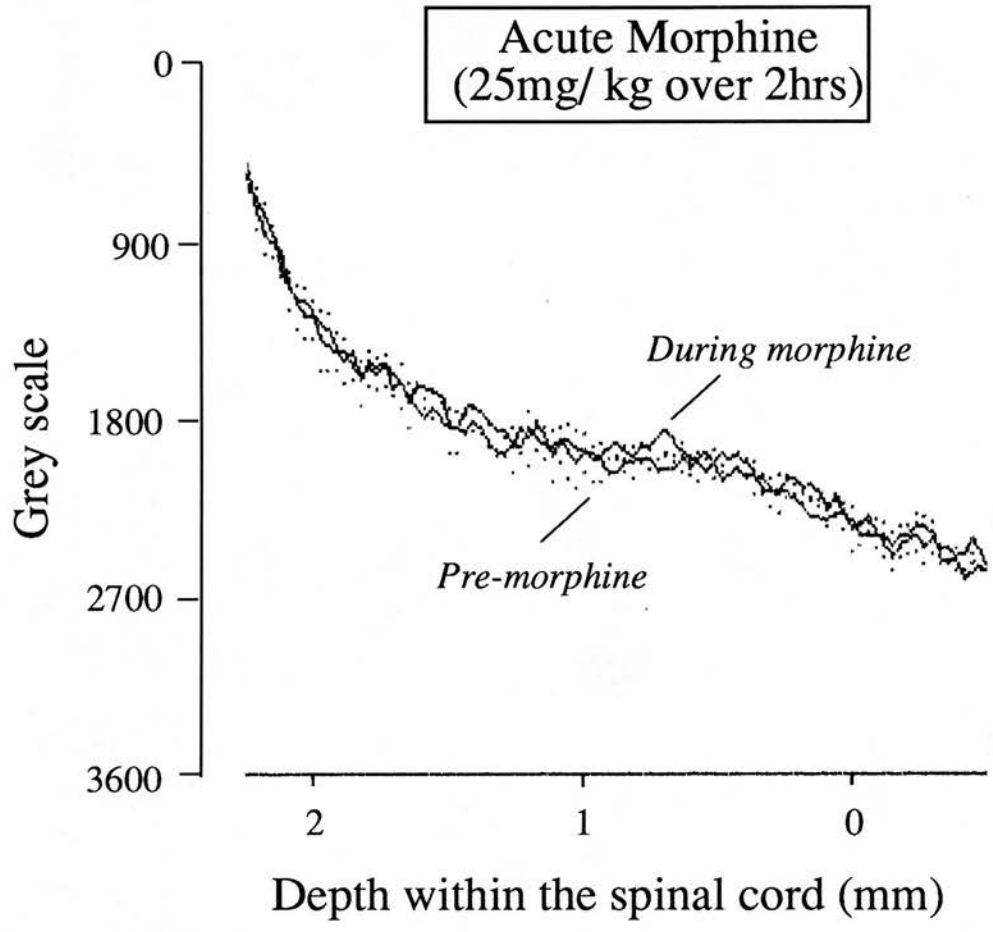
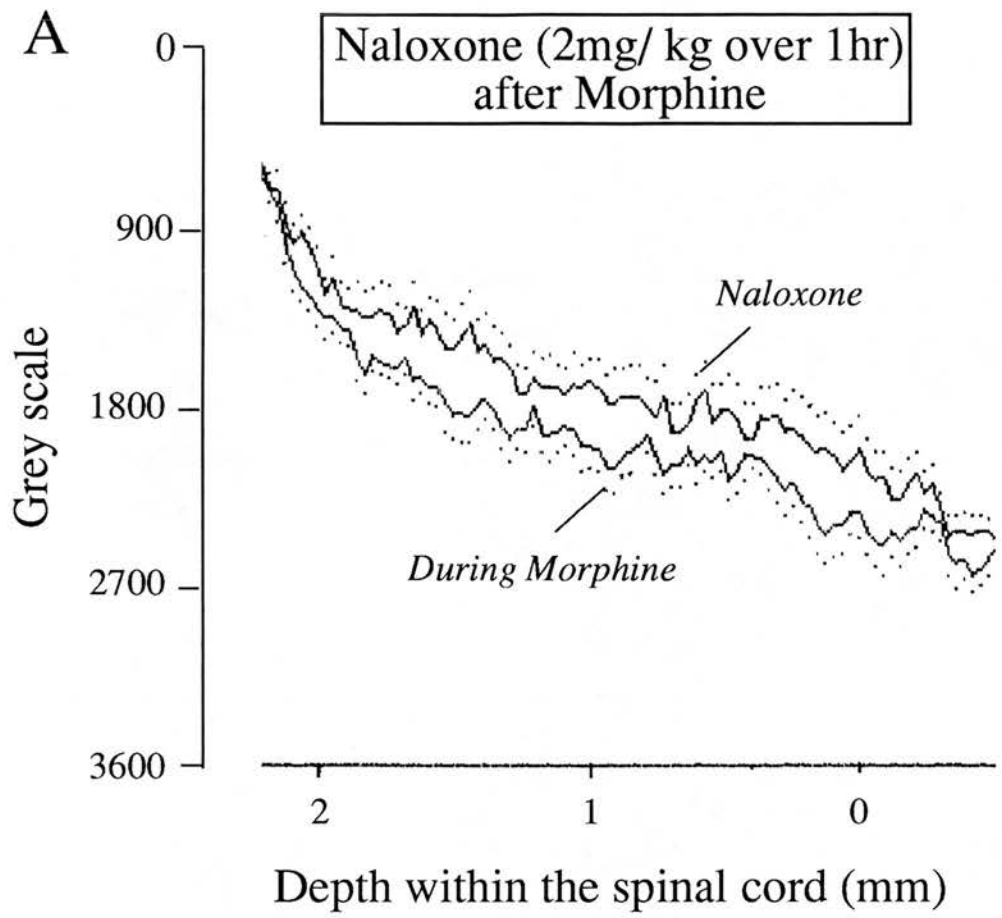
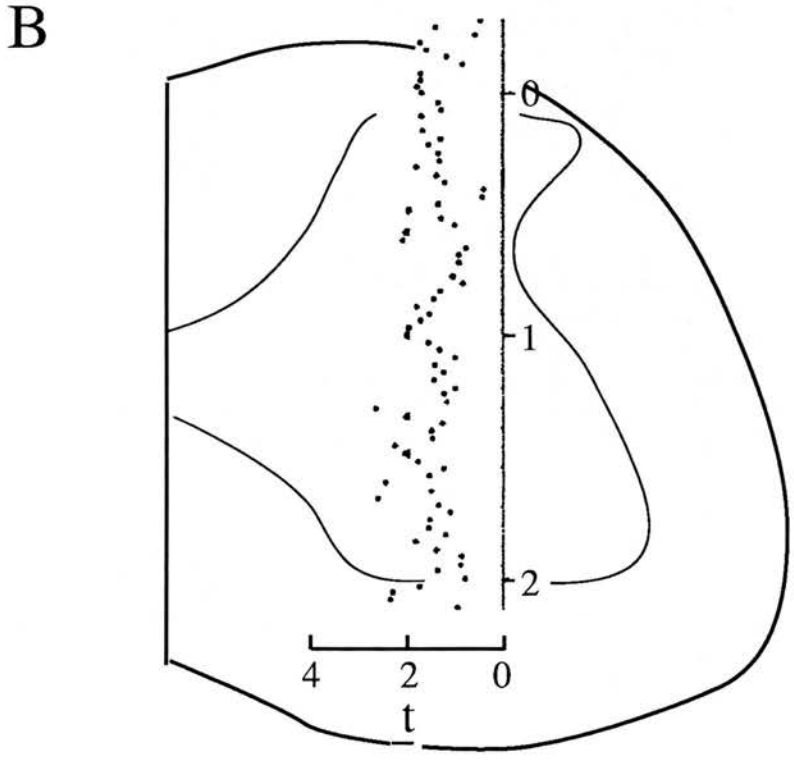

Acute Morphine
(25mg/ kg over 2hrs)
0
900
1800
2700
3600
Grey scale
During morphine
Pre-morphine
2
1
0
Depth within the spinal cord (mm)

FIGURE 24. Release of ir-CCK on naloxone administration following morphine.

(A) The mean image analysis of 12 microprobes present in the spinal cord during the two consecutive 30 minute periods of naloxone administration *(naloxone)* is displaced above that of microprobes present over the prior two hour period of morphine administration in these same group of animals (*during morphine,* n=12). (B) The differences between the two groups of microprobes are plotted with respect to depth in the spinal cord. Since only isolated points attain significance at the $P< 0.05$ level, there is no cross hatching as in other figures.


A
Naloxone (2mg/ kg over 1hr)
after Morphine
0
900
1800
2700
3600
Grey scale
Naloxone
During Morphine
2
1
0
Depth within the spinal cord (mm)


B
0
1
2
4
2
0
t

significantly elevated in some areas of the mid/ deep dorsal horn and ventral horn. The data however, was only collected from only 3 animals. As previously indicated no study was made of the effect of naloxone administration on the basal levels of ir-CCK prior to morphine administration.

5. RECOVERY FROM THE EFFECT OF NALOXONE ADMINISTERED AFTER MORPHINE

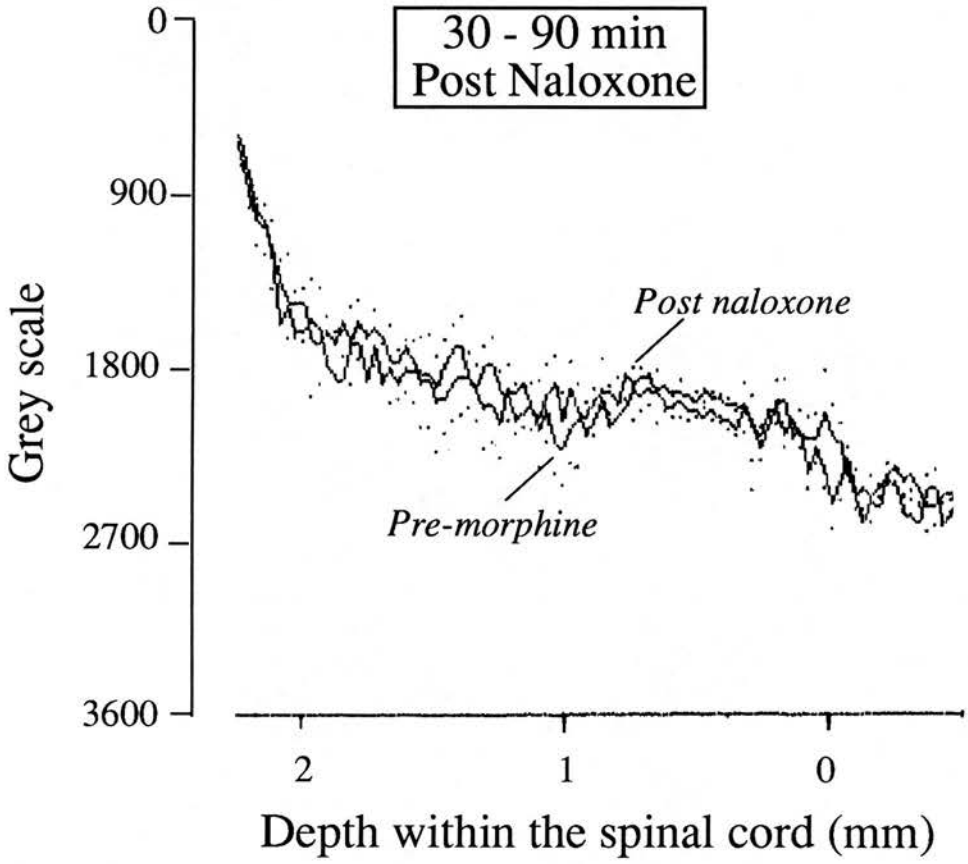
The evidence for naloxone administration producing a significant increase in the release of ir-CCK in the rat spinal cord following acute morphine administration would be improved by higher numbers of microprobes. However, the mean image analysis of microprobes (n=9) present in the spinal cord for the two consecutive 30 minute periods immediately following those of naloxone administration, can be seen in figure 25 to be nearly completely coincidental to that of microprobes (n=7) present under basal conditions in this same group of animals.

This observation of recovery strengthens the case that naloxone evoked a release of ir-CCK in the spinal cords of rats previously exposed to acute doses of morphine. The briefness of the effect (i.e. less than 30 minutes) perhaps reflects the relatively short half-life of naloxone relative to that of morphine.

6. SUMMARY OF RESULTS

The present experiments have detected an extensive presence of ir-CCK in normal (drug naive) rats involving both the dorsal and ventral horns. The largest peak was in the mid to deep dorsal/ upper ventral horn (laminae III-VII) with further major sites inthe superficial dorsal horn (laminae I-II) and the mid/ lower ventral horn. The acute administration of high doses of morphine intravenously (25mg/ kg over two hours) failed to alter this basal presence of ir-CCK. However, preliminary data suggests that subsequent injections of naloxone (1mg/ kg) evoked release of ir-CCK in some areas of the ventral horn. This effect is short lived, with a return to basal levels occurring in less than 30 minutes.

FIGURE 25. Recovery from the effect of naloxone administered after morphine. The mean image analysis of the 9 microprobes present in the spinal cord for the two consecutive 30 minute periods immediately following those of naloxone administration (*post naloxone*, n=9) is compared to that of microprobes present in under basal conditions in the same animals (*pre-morphine*, n=7).


30 - 90 min
Post Naloxone
0
900
1800
2700
3600
Grey scale
Post naloxone
Pre-morphine
2
1
0
Depth within the spinal cord (mm)

5.4 DISCUSSION

This study has only examined CCK release following acute morphine administration and whether CCK release is enhanced or reduced during the development of tolerance to exogenously administered opiates was not investigated. Never the less I shall attempt to explain why the release of ir-CCK was increased only when naloxone followed morphine administration and place this within a hypothesis of CCK as an 'anti-opioid' substance. First, however, the distribution of possible CCK releasing neurones and CCK receptors in the spinal cord of the normal animal will be considered and this distribution compared to that found in the present experiments.

Localisation of CCK and CCK receptors in the spinal cord

Cholecystokinin immunoreactivity has been described to be present to some extent at all levels of the spinal cord, appearing to be concentrated in fibre terminals in lamina I and II, and the region around the central canal, lamina X, forming an arc around the lateral edge of the substantia gelatinosa into laminae III, IV and V (rat: Larsson and Rehfeld, 1979; Loren et al, 1979; Vanderhaeghen et al, 1980, 1982; Jansco et al, 1981; Stengaard-Pederson and Larsson, 1981c; Gibson et al, 1981; Schroeder et al, 1983; Conrath-Verrier et al, 1984; Fuji et al, 1985; Tuscherer et al, 1987; Nahin et al, 1987; Hokfelt et al, 1988; Leah et al, 1988. cat: Maderdrut et al, 1982. guinea-pig: Stengaard-Pederson and Larsson 1981. man: Chung et al, 1989. monkey: La Motte et al, 1988). Cell body staining though rare has also been observed more distinctly in some studies in lamina III-V, lamina VII and lamina X of the spinal cord, notably to a greater extent at the lumbosacral level (Gibson et al, 1981; Vanderhaeghen et al, 1982; Schroeder et al, 1983; Conrath-Verrier et al, 1984; Fuji et al, 1985; Nahin et al, 1987; Leah et al, 1988; Hokfelt et al, 1988; La Motte et al, 1988). This has been confirmed by recent *in situ* hybridisation studies (rat: Cortes et al, 1990; Abelson and Miceyvich, 1991; Schiffman et al, 1991. guinea-pig: Cortes et al, 1990).

Early immunocytochemical studies pointed to the existence of CCK in primary afferent neurones (rat: Lundberg et al, 1978; Dalsgaard et al, 1982; Otten and Lorez, 1983; Tuchscherer and Seybold, 1985. guinea-pig: Lundberg et al, 1978; Larsson and Rehfeld, 1979; Lindh et al, 1988. cat: Leah et al, 1985; Dockray et al, 1981), which appeared to be supported by the reduction in ir-CCK found in the superficial laminae of the dorsal horn following neonatal capsaicin treatment (rat: Jansco et al, 1981; Gibson et al, 1982; Priestly et al, 1982; Schultzburg et al, 1982; Micevych et al, 1983; Conrath-Verrier et al, 1984) and dorsal rhizotomy (cat: Maderdrut et al, 1982). Support was also provided by the apparent similarity in staining with peptides known to be predominantly located in primary afferents, such as substance P (rat: Gibson et al, 1981; Jansco et al, 1981), with some evidence for the coexistence of these peptides in spinal ganglia neurones (rat: Dalsgaard et al, 1982; Otten and Lorez, 1983; Tuchscherer and Seybold, 1985; Tuchscherer et al, 1987. cat: Leah et al, 1985. guinea-pig: Gibbins et al, 1987), and their apparent parallel reduction in the dorsal horn following neonatal capsaicin treatment (Jansco et al, 1981; Tuchscherer et al, 1987).

Some investigators, however have found no such reductions in ir-CCK in the dorsal horn following neonatal capsaicin or rhizotomy treatments when examined in the rat by radioimmunoassay (Marley et al, 1982; Schultzburg et al, 1982; Gibson et al, 1982; Zouaoui et al, 1990; Pohl et al, 1990). Such discrepancies are thought likely to reflect differences in the type of immunoreactive material detected by radioimmunoassay and immunocytochemistry. Since radioimmunoassays are usually performed with the antiserum very dilute, only those immunoglobulins from a polyclonal antisera with the greatest affinity for a given antigen (in this case CCK-8) are likely to be involved compared to immunocytochemistry, in which a range of immunoglobulins are probably involved (Schultzberg et al, 1982; Zouaoui et al, 1990). The additional material localised in the immunocytochemical studies although likely related in sequence to CCK could belong to a different family of peptides. In fact it has been proposed by Hokfelt and co-workers (Ju et al, 1986, 1987a; Williams et al , 1987; Hokfelt et al, 1988) that the ir-CCK in primary afferents

could simply reflect a cross reactivity of the COOH-terminally directed antisera commonly employed with CGRP, that has been demonstrated to be present in many primary sensory neurones and fibres in the superficial dorsal horn (Rosenfeld et al, 1983; Gibson et al, 1984). These investigators found primary afferents to only demonstrate immunoreactivity for CCK only when COOH-terminally directed antisera were used, not polyclonal or monoclonal antisera directed against any other sequence of CCK molecule, and that all the sensory ganglion cells displaying such immunoreactivity also stained positive for CGRP. Although CCK-8 and CGRP-37 exhibit only limited structural homology, sharing a similar COOH-terminal sequence, Gly-X-X-X-Phe-NH_2, this very low degree of identity in crucial positions may be sufficient to cause cross-reactivity. Ju et al (1986) found that the staining for ir-CCK was almost completely abolished by pre-absorption with 10^{-5}M CGRP, although it was found that concentrations up to 10^{-4}M CCK-8 did not influence staining for ir-CGRP. Orazzo et al (1993) though have since shown that CGRP antisera do react with the COOH-terminus of CCK. Thus, CCK and CGRP may share similar antigenic determinants and the capsaicin induced disappearance of ir-CCK from primary sensory neurones could reflect depletion of CGRP, rather than CCK itself. However, it must be noted that the concentration of CGRP required to block the CCK staining is very high and it is not certain how this relates to the situation *in vivo*. Also Zouaoui et al (1990) found on examination by high pressure liquid chromatography that the ir-CCK material from rat dorsal horn depleted by neonatal capsaicin treatment was distinct from both genuine CCK and CGRP.

On the whole this evidence along with recent failure of *in situ* hybridisation studies to localise the CCK precursor mRNA within the DRG of the rat (Cortes et al, 1990; Seroogy et al, 1990; Schiffman et al, 1991; Ghilardi et al, 1992) makes the presence of CCK in primary afferents in the rat very unlikely. However, it should be noted that although minimal ir-CCK has been observed in the DRG of normal rats, these levels were considerably increased after treatment with nerve growth factor (Otten and Lorez, 1983). Likewise the levels of proCCK mRNA have been found to be low in the DRG of normal rats but considerably increased

between 2 to 3 weeks after lesioning the sciatic nerve (Verge et al, 1993; Xu et al, 1993). Thus, it remains possible that a very small population of primary sensory neurones may synthesise CCK peptides at a very low rate, that is increased by peripheral axotomy. Doubts as to the presence of CCK in primary afferents has so far been confined to the rat. A prominent expression of ir-CCK (Lindh et al, 1988; Gibbins et al, 1987) and proCCK mRNA (Cortes et al, 1990; Seroogy et al, 1990) has been found in the DRG of the guinea pig, as well as that of the monkey (Verge et al, 1993). It is suggested that perhaps species differences may exist in the level at which CCK participates in the sensory processing in the normal animal, with CCK in the rat only showing an involvement after an intensive peripheral insult, such as nerve injury (Verge et al, 1993). There is also evidence for a CCK presence in motoneurones, though whether this immunoreactivity represents genuine CCK has likewise been debated by Hokfelt and co-workers (Ju et al, 1986; Hokfelt et al; 1988). However, other investigators have found it to be present by immunocytochemistry under normal conditions (rat: Schroder et al, 1983) and 24 hours following ligation of the sciatic nerve (rat: Cortes et al, 1991), as well as by *in situ* hybridisation (rat: Abelson and Micevych, 1991; Schiffman et al, 1991; Cortes et al, 1990. guinea-pig: Cortes et al, 1990).

Whilst it is likely that the majority of CCK is located in neurones that are intrinsic to the spinal cord, there is evidence that some may form ascending pathways, particularly those located in cell bodies around the central canal, in lamina VII and X (Zouaoui et al, 1991), with an immunoreactive presence being described in neurones of the spinoreticular tract (Nahin, 1987; Leah et al, 1988), the spinomesencephalic tract (Leah et al, 1988), the spinosolitary tract (Leah et al, 1988) and the spinothalamic tract (Ju et al, 1987b; Leah et al, 1988). There is also considerable evidence that some of the ir-CCK found at the spinal level may be of a supraspinal origin, with transections of the rat spinal cord at the upper cervical but not the lower cervical or thoracic level shown to result in accumulations of ir-CCK in the rostral side only (Fuji et al, 1985). Projections descending from the periaqueductal grey (rat: Skirboll et al, 1983), the midbrain nucleus of Edinger Westphal (cat: Maciewiez et al, 1984),

and the raphe nuclei (rat: Mantyh and Hunt, 1984) have all been described to contain ir-CCK. The results of a more recent study of the effect of thoracic transection on ir-CCK suggests that some of these descending fibres may project to lamina II of the dorsal horn (Zouaoui et al, 1991).

Detectable levels of [^{125}I]-CCK binding have been found in all laminae of the mammalian spinal cord, with the highest density of binding in lamina I and II of the dorsal horn (Van Dijik et al, 1984; Hill et al, 1988; Hill and Woodruff, 1990; Ghilardi et al, 1992). The origin of at least a substantial proportion of these CCK receptors appears to be the primary afferent neurone itself, since DRG contain moderate densities of receptor sites and neonatal capsain treatment results in a significant reduction of the density of CCK binding sites in several laminae (Ghilardi et al, 1992). However as previously indicated (section 1.9.1) species differences have been shown to exist. The use of selective CCK-A and CCK-B receptor antagonists have demonstrated that whereas the CCK-B is the prominent receptor subtype in rat and rabbit spinal cord, that in the monkey cord is the CCK-A receptor despite (like the rat and rabbit) the CCK-B receptor being the more prominent on DRG neurones (Hill et al, 1988; Hill and Woodruff, 1990; Ghilardi et al, 1992).

In agreement with these anatomical studies the present study has revealed an extensive presence of ir-CCK in the normal (drug naive) rat. Despite the poor specificity (i.e. high degree of cross reactivity) of the polyclonal antiserum employed for the other derivatives of precholecystokinin plus gastrin and caerulein, this immunoreactive material is likely to represent sulphated CCK-8, since as indicated in section 1.9.1 it is this form of CCK that predominates in the vertebrate CNS including the rat spinal cord and gastrin exists in only significant quantities in the neurohypophysis. As previously considered for the spinal release of dynorphin (section 4.4), little can be determined about the physiology of a neuropeptide from its basal presence, since the contributions from the anaesthetic and surgical preparation are unknown. Under such conditions release is best equated with a significant increase in the extracellular levels of a peptide following a defined stimulus. Since more detailed

investigations were unable to be carried out, the relative contribution to this basal presence from primary afferent fibres, intrinsic neurones and the terminations of fibres of supraspinal origin are unknown. Due to the extensive nature of the basal levels of ir-CCK found, an input from descending fibres is probable and similarly a contribution from primary afferents cannot be discounted. To this purpose it would obviously be of interest to determine the effect of nerve stimulation at both Aβ and Aδ/ C fibre intensity on this ir-CCK presence, as well as the effect of spinal transection at the upper thoracic segments.

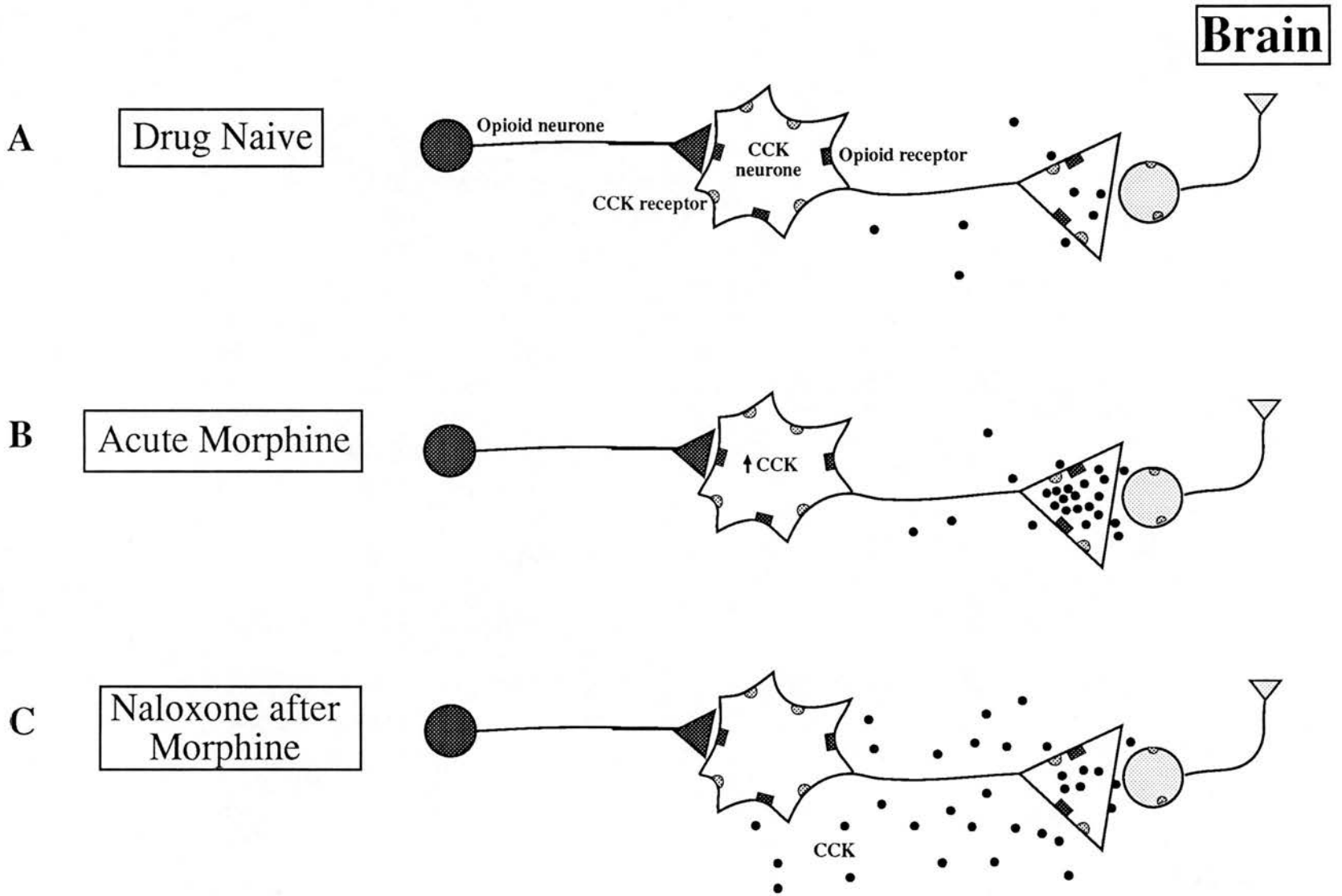
An hypothesis of CCK acting as an 'anti-opioid' in the spinal cord

As outlined in section 5.1, both increases and decreases in the basal levels of ir-CCK have been observed in a surface perfusate following the acute administration of opioids to spinal tissue. This inconsistency is puzzling. The present study found no effect on ir-CCK detected with the administration of relatively high doses of morphine. However, the finding that subsequent injections of naloxone did evoke a spinal release of ir-CCK does suggest that some of the CCK releasing neurones are under opioid control. Unfortunately no study of the effect of naloxone administered alone on the basal levels of ir-CCK was performed which limits any interpretation of the present findings. Since as discussed in section 1.9, CCK antagonists have been observed to facilitate opioid mediated analgesia acutely, it is likely that in the normal (drug naive) animal CCK is being tonically released to affect opioid receptor binding. Whilst it is possible that morphine did have an effect in reducing the tonic release of CCK, the high basal levels of ir-CCK in the present study may have made such an action difficult to detect. High basal levels of CCK in normal rats was proposed by Stanfa and Dickenson (1993) as an explanation for the inability of exogenous CCK-8 to reduce the effect of morphine on C fibre evoked responses in dorsal neurones, as other investigators had previously shown (Wiesenfeld-Hallin and Duranti, 1987; Kellstein et al, 1991). Recent behavioural studies have suggested that release of endogenous CCK is influenced by the environment to which animal exposed. As previously indicated it has been suggested that

CCK is released in 'safe' situations to limit the analgesic actions of endogenous opioids released in contrast by environmental cues in aversive or dangerous situations (Wiertelak et al, 1992; 1994). However, confusingly it has also been reported that CCK receptor antagonists do not enhance morphine antinociception in rats in 'familiar' situation but do so in animals exposed to a novel environment, suggesting that CCK release is associated with 'stress' (Lavigne et al, 1992). Although these results are conflicting, they highlight the plasticity of the CCK systems. It is thus possible that such factors may play a role in the basal levels of ir-CCK in the anaesthetised and surgically prepared animals used in these present experiments and such levels may contribute to the lack of effect of morphine observed on basal ir-CCK levels. An alternative explanation, however, for the ineffectiveness of the high doses of morphine administered could be of a more pharmacological nature, due to antagonism at non-opioid receptor sites by its metabolite morphine-3-glucuronide. Recent studies in Sprague-Dawley rats have provided evidence that the ratio of this metabolite to morphine in blood plasma (Smith and Smith, 1995) and cortical extracellular fluid (Barjavel et al, 1995) is highly inversely correlated with the level of antinociception achievable. However, whether the build-up of morphine-3-glucuronide in the spinal cord of the Wistar rats used in the present study was significant compared to the levels of morphine over the two hour time period involved, and played a factor in the lack of effect of the high doses of morphine observed on the basal levels of ir-CCK, requires further investigation.

Considering both the findings of this present study and those discussed in sections 1.9 and 5.1, an hypothesis by which CCK may interact with exogenously administered opiates (and endogenously released opioids) in the spinal cord as an 'anti-opioid' and contributes to the development of opioid tolerance is presented below (**in bold**), along with supporting evidence where this is available from the work of other groups. This hypothesis is outlined schematically in figure 26(A, B, C).

FIGURE 26. Schematic representation of an hypothesis of CCK acting as an 'anti-opioid' in the spinal cord. In (A), (B) and (C) opioid releasing neurones are illustrated as contacting neurones capable of synthesising CCK. These CCK releasing neurones display both opioid and CCK receptor sites. In the spinal cord of the normal (drug naive) animal, CCK shows some tonic release (A). On opioid receptor activation (as in the present study following the acute administration of morphine) an upregulation of CCK synthesis is proposed (B). However, this is only observed when the opioid receptor effect is partially removed (as in the present study following the administration of naloxone and, it is predicted, as tolerance to opiate effect develops), as illustrated in (C). This proposed hypothesis is described in detail in the text of section 4.4


Brain
A
Drug Naive
Opioid neurone
CCK neurone
Opioid receptor
CCK receptor
B
Acute Morphine
CCK
C
Naloxone after Morphine
CCK

It is proposed that:

(1) Some central neurones have the ability to synthesise and release CCK in response to opioid receptor occupancy. Some evidence for this exists at the level of the hypothalamic neurohypophyseal axis where CCK has been described to be colocalised with oxytocin within the magnocellular neurosecretory neurones of the hypothalamus (Vanderhaeghen et al, 1981). These neurones are subject to regulation by opioids at the level of cell bodies and at the terminals in neural lobe to reduce the release of oxytocin into the blood (as recently reviewed by Russel et al, 1995).

(2) These neurones possess receptors for CCK along with opioid receptors on their cell bodies and axon terminals (as illustrated on figure 26A). As previously indicated there is evidence that both CCK receptors (Ghilardi et al, 1992; Zhang et al, 1993) and opioid receptors (La Motte et al, 1976; Gamse et al, 1979; Fields et al, 1980; Besse et al; 1990) are present on dorsal root ganglion cells. Notably a recent study involving whole-cell patch-clamp recordings of acutely isolated rat dorsal root ganglion neurones demonstrated that the depressive effect produced by the selective μ agonist, ohmefentanyl, on voltage gated Ca^{2+} currents, could be readily reversed by CCK-8 through CCK-B receptors located on the same neurone, and that this effect was not mediated by a reciprocal action on the same Ca^{2+} currents (Liu et al, 1995), although CCK may mobilise intracellular stores of Ca^{2+} (Wang et al, 1992). It has also been proposed that CCK and opioid receptors may belong to the same complex (Hudson et al, 1992).

(3) With acute morphine administration (as illustrated on figure 26B), CCK synthesis is increased but is not released while the acute action of the opiate (inhibition of firing) is undiminished (no tolerance). As indicated in the introduction to this section, although there have been limited investigations of CCK synthesis under such conditions there is some evidence to support such an effect (Zhou et al, 1992; Ding and Bayer, 1993; Pu et al, 1994).

(4) Only when the opioid receptor-mediated inhibitory influence is partially removed will this CCK be released (figure 26C). As in the present study where the administration of naloxone is seen to result in the release of CCK.

(5) Released CCK can access opioid receptors on both axon terminals and cell bodies, having little effect per se, but acting as a specific 'anti-opioid' compound to reduce the effect of opioid receptors. As outlined in section 1.9 this may occur either by an uncoupling of the opioid receptor-effector systems ('desensitisation') or by the internalisation of the opioid receptors themselves ('down- regulation'). Whilst early studies reported that CCK-8 did not inhibit opioid binding directly (Stengaard Pedersen and Larsson, 1981a), more recent reports have indicated that CCK-8 does appear to affect the binding characteristics of opioid receptors in neonatal (Johnson et al, 1987), and adult rat brain (Wang et al, 1989) indirectly via actions on CCK receptors. Another binding study found that whilst CCK-8 was very effective in suppressing the binding of [^{3}H]-DAMGO to μ receptors and [^{3}H]U69593 to κ receptors, no effect was observed on the binding of [^{3}H]DPDPE to δ receptors in the rat brain (Wang and Han, 1990). Similarly electrophysiological (Magnuson et al, 1990) and behavioural (Wang et al, 1990) investigations have determined that CCK-8 preferentially antagonises the analgesia mediated by μ and κ opioid receptors respectively, but not that of δ receptors. Since a remarkable lack of cross tolerance has been demonstrated among μ, δ and κ receptor agonists, such findings may partly explain the results of Benoliel and co-workers (1991; 1994a; b), that imply a significant role for the δ receptor in the morphine induced release of ir-CCK at the spinal cord level. Such effects may also be mediated by allosteric interactions between opioid and CCK receptors in which the conformational state of an opioid receptor could be modified, as proposed from the finding of a recent study in rat hippocampal slices (Miller and Lupica, 1994).

From such an hypothesis it would be predicted:

(6) With chronic morphine administration, as tolerance develops, small amounts of CCK will be released, which by slightly removing the opiate inhibition of release will act as a positive feed-back process to eventually result in complete tolerance.

It should be noted, however, that such an hypothesis could not be proposed if the morphine administered was found to be ineffective at the opioid receptors due to antagonism by a significant build-up of its morphine-3-glucuronide metabolite or if naloxone alone was found to have an effect on ir-CCK basal levels in the absence of prior morphine administration. Further studies are required.

Overall the findings from behavioural investigations into role of CCK in opioid analgesia have supported an involvement of CCK releasing neurones in the development of tolerant states only (as discussed in section 1.9), with no evidence for a role in either the development or expression of physical dependence. This was also confirmed more recently by Pournaghash and Riley (1991), who demonstrated the failure of even high doses of CCK (up to 40ng/ kg) to precipitate withdrawal in morphine dependent rats as naloxone (10mg/ kg) was able to do. Thus, CCK studies imply that the mechanisms that underlie tolerance and dependence states are not identical and may be differently regulated depending on the system and species studied. It is possible that the involvement of other anti-opioid peptides, for example neuropeptide FF. (Malin et al, 1990a; b) may play more of a defined role in the development of physical dependence upon opiates. To this purpose it would be obviously be of interest to study the release of ir-CCK under conditions of tolerance to and dependence upon opiates.

As to the potential therapeutic use of CCK receptor antagonists. Whilst there is evidence that CCK-opioid interactions occur in humans, proglumide enhancing the analgesia induced by morphine (Price et al, 1985; Lavigne et al, 1989), studies in rats have indicated that compensatory alterations may develop during chronic CCK blockade which may limit the

clinical effectiveness of CCK antagonists as useful adjuvants in the management of chronic pain (Kellstein and Mayer, 1990), the long term administration of proglumide and lorglumide appearing to result in a loss of facilitation (after 8 days) or a reduction (at 22 days) of morphine antinociception.

Although not dealt with in the context of this thesis, it has been proposed that adaptive changes in the functional activity of spinal CCK systems are responsible for alterations in the potency of morphine at the spinal level observed in chronic pain states (Stanfa et al, 1994). Namely enhanced spinal levels of CCK may be responsible for opiate insensitivity of neuropathic pain observed in animal models (Xu et al, 1993) and in the clinic (Arner and Meyerson et al, 1985; Portenoy et al, 1991), whilst reduced spinal levels may explain the observed enhancement of the inhibitory effects of intrathecal morphine on the C fibre evoked responses of dorsal horn neurones in animals with a peripheral inflammation (Stanfa and Dickenson, 1993). Further studies however, are required to determine the basal release of CCK in these different pain states.

CONCLUDING REMARKS

Following the discovery of the endogenous opioid peptides, it was anticipated that many new insights into the understanding of opiate drug action would be gained, ultimately resulting in the development of new analgesics with enhanced potency but reduced side effects, including a lower abuse potential. However, despite considerable research and accumulating knowledge of the opioid peptides and their actions, especially at the cellular level (as reviewed in the introduction of this thesis), the isolation of the opioid peptides has not resulted in effective improvements to opiate therapy. Indeed the significant advances to opiate therapy in recent years have been pharmacokinetic in nature, involving the synthesis of opiate drugs with a range of duration of actions, the development of patient controlled analgesia, and the widespread use of opiates by epidural and intrathecal routes either intraoperatively or for the treatment of chronic pain. This may be a reflection of our lack of understanding of the physiology of opioid peptides in chronic pain states.

An example of this is the dichotomy of opinion which has evolved in recent years as to the role of the endogenous opioids, in particular the dynorphins, in the hyperexcitabilty of spinal cord neurones following the development of a peripheral inflammation. This has resulted in an apparent shift in emphasis from viewing the opioid peptides as inhibitory to the changes induced in the spinal cord under such conditions, exerting a modulatory influence on the processing of nociceptive information, to one in which a direct contribution to the formation of the hyperalgesic states is implied (Dubner and Ruda, 1992). These two opposing views need further data to come to a complete overview of how nociceptive information is processed centrally under inflammatory conditions, and the role the opioid peptides play within these evolving processes. My studies clearly show that within the time period studied, dynorphins are released when inflamed peripheral tissues are manipulated. Since this release is widespread involving both dorsal and ventral horns, an involvement of these opioid peptides in several aspects of spinal function is implied. From my reading of the literature and from my

results I have proposed that the released dynorphin exerts an inhibitory role on the transmission of nociceptive information under normal and inflamed conditions, and also likely plays a role in motor function, perhaps setting the level at which simple reflexes are operative at the spinal cord level.

The opioid field has grown increasingly complex with multiple opioid peptides exerting their effects via multiple opioid (and possibly non-opioid) receptors to impart a number of short and long term effects on neuronal function. Additionally the opioid systems are known to be widely distributed throughout the CNS and to influence many functions other than those pertaining to the processing of nociceptive information. Although in no instance is a full description of a central 'opioid event' available, knowledge of opioid events could be considerably improved by further studies aimed at determining the opioid receptor sites functionally active in response to a particular stimulus and which opioid peptides mediate these effects. To this end further antibody microprobe studies (or other release studies capable of significant spatial localisation) and electrophysiological studies involving the administration of selective opioid receptor antagonists, are imperative. The recent cloning of the opioid receptors has significantly added to the range anatomical and behavioural techniques by which the functions of the opioid systems can be investigated and these are likely to provide further insight into the functions of the opioid systems at the spinal level.

From a more holistic viewpoint the opioid systems have been proposed to be important in the modulation of basic survival instincts involving reward and the reinforcement of behaviours, such as food and water intake, and sexual activity (Terenius, 1992). Acute pain is also recognised as being essential for survival enabling suitable avoidance/ protective responses to be learnt and autonomic responses activated, hence preventing excessive tissue damage. Acute pain, however, is not a protean benefit, since the disruption to motor performance produced by pain may prevent an adequate escape from a threatening environment. The interplay of a hypersensitive pain system and an inhibited pain system ('stress-induced analgesia'), indicates the need to regulate the central processing of nociceptive

information. It is not surprising therefore that the opioid systems themselves also appear to be highly regulated. Cholecystokinin has been proposed to impart such a role in the certain CNS systems, thus acting as an 'anti-opioid' peptide. My studies perhaps indicate an association between CCK and opioid systems, with the suggestion perhaps of an increased availability of this neuropeptide for release at the spinal level after only an acute exposure to an opiate drug. However, additional experiments investigating the effect of naloxone alone and lower doses of morphine on ir-CCK basal levels need to be performed to strengthen the evidence for such an interpretation. Based on the plethora of studies performed, there is strong evidence to support such a release of CCK in the development of tolerant states to exogenously administered opioids. To this end, as previously indicated, it would of considerable interest to extend the studies described in this thesis to determine the release of CCK in opiate tolerant and dependent states. Additionally since it has been proposed that the spinal levels of CCK may be instrumental in the creation of opioid insensitive (neuropathic) and hypersensitive (inflammatory) pain states (Stanfer et al, 1994), it would also be informative to use the antibody microprobe technique to examine CCK release in animal models involving nerve damage or tissue injury.

APPENDIX: Technical Considerations of the Antibody Microprobe Technique

A.I GENERAL

Although (as outlined in section 1.10) the antibody microprobe technique has been successfully employed for the detection of many neuropeptides in the CNS, this has not been true for all neuropeptides studied. In recent years in our laboratory problems have been encountered during the *in vitro* testing of galanin, endothelin, dynorphin A, met-enkephalin and as indicated in section 5 of this thesis, cholecystokinin. Some of these problems have been overcome allowing the studies to proceed successfully, however in other instances the problem has appeared to be more of a fundamental nature, particularly with the competency of the antiserum purchased, and have not been resolved. Problems have presented as: (a) a failure of the immobilised antibodies to bind adequate amounts of the radiolabelled peptide ('tracer'); (b) poor sensitivity (characterised by the least concentration of unlabelled peptide able to suppress binding of tracer to immobilised antibodies)/ and or specificity (characterised as extent of cross reactivity of immobilised antibodies with peptides other than that used to raise the antiserum) of microprobes for the neuropeptide being studied; and (c) inadequate suppression of tracer binding to microprobes by a high concentration (i.e. 10^{-5}M) of unlabelled peptide.

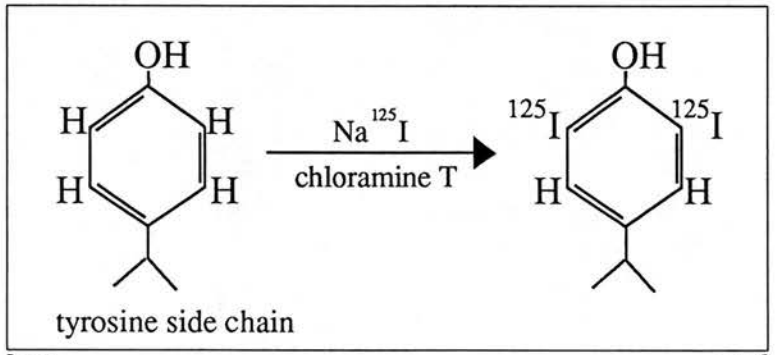
There are many possible explanations why antibody microprobes can fail to perform satisfactorily on *in vitro* testing (described in section 3.3). Some of these will be now outlined, and then considered more specifically in appendix A.II in relation to the problems encountered with CCK microprobes.

(i) Problems with the attachment of the radiolabel to the peptide

Two approaches are commonly employed to label peptides with the radioisotope ^{125}I. In one the iodide is oxidised, for example by chloramine T (Greenwood et al, 1963), to form iodine, which substitutes onto the aromatic side-chain of tyrosine residues of the peptide (as illustrated on figure 27A for [^{125}I]CCK-8(desulphated)). At low levels of specific activity

FIGURE 27. Different methods used for iodination of CCK-8. (A) [^{125}I]CCK-8 (desulphated) is prepared by the iodination of tyrosine residue on NH_2-terminal of the peptide. This is achieved as illustrated (in box) by the substitution of ^{125}I onto the aromatic side-chain of tyrosine in the presence of the oxidising agent, chloramine T. (B) [^{125}I]CCK-8 (sulphated) is prepared by the conjugation of the NH_2-terminal of CCK-8 with the iodinated Bolton-Hunter reagent [N-succinimidyl 3-(4-hydroxy 5-[^{125}I]idophenyl)propionate]. Adapted from Chard (1987).

A



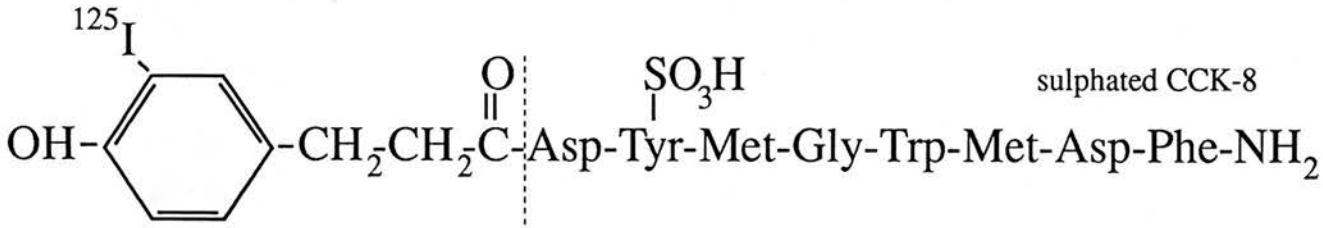
^{125}I

desulphated CCK-8

Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2

B

Bolton-Hunter reagent



sulphated CCK-8

(defined as the radioactivity in Curies [Ci] per unit mass or mole of ligand) the majority of substitutions are single, only at higher levels is diiodotyrosine formed. The other approach involves the conjugation of the peptide to a molecule that can be labelled with iodine, such as the Bolton-Hunter reagent [N-succinimidyl 3-(4-hydroxyphenyl)propionate] (Bolton and Hunter, 1973), which includes a phenol group capable of iodination, and a carboxyl group which can be directly coupled to free amino groups on lysyl or NH_2-terminal residues of the peptide (as illustrated on figure 27B for [^{125}I]CCK-8(sulphated)). Which approach is chosen is often dependent on the availability of suitable tyrosine residues (if any present) on the neuropeptide of interest, namely tyrosine residues that if iodinated will not alter the binding characteristics of the peptide. The specific activities of the iodinated peptides purchased for the purpose of the studies described in this thesis were in the range of 1000 to 2000 Ci/ mmole.

The occurrence of low '0M' microprobe counts (i.e. those microprobes not exposed to any concentration of unlabelled peptide solution but simply incubated in the radiolabelled peptide) could be due to poor iodination of the peptide purchased, or a rapid dissociation of the radiolabel from the peptide after purchase ('deiodination'), or damage to the peptide structure (for example, by the iodination process itself). To accurately determine the iodination status of the tracer purchased and assess the integrity of the peptide structure, requires some degree of chemical assessment of the components of a sample of the tracer after separation for example by gel filtration chromatography. This has not been pursued to any great extent in our laboratory.

It is of note that for the purpose of the antibody microprobe technique, low counts alone do not pose an irreconcilable problem, as long as the microprobes display a satisfactorily level of sensitivity and specificity *in vitro* for the particular neuropeptide being investigated. The main drawback is the length of time in which the microprobes must be placed next to an X-ray film to obtain satisfactory autoradiographic images (perhaps more than a month) which obviously slows down the progress of a study.

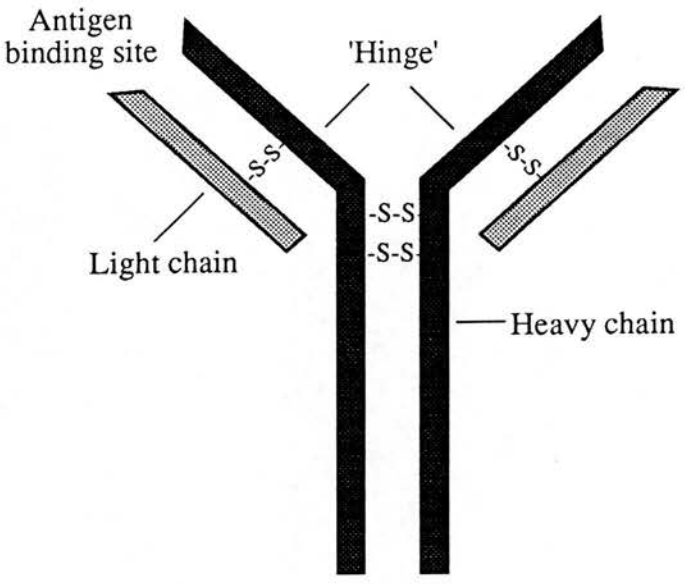
(ii) Problems with the binding of the radiolabelled peptide to the immobilised antibodies

IgG molecules are the most abundant immunoglobulins found in the serum of a number of species including rabbits, in which most polyclonal antisera to neuropeptides are raised by artificial immunisation. The structure of an IgG molecule is illustrated in figure 28A. As indicated in section 3.2.2 the process of immobilisation of these antibodies to the siloxane coated microprobes involves the coupling of the Fc region (illustrated in figure 28B) of the IgG molecules in a polyclonal antiserum via glutaraldehyde/ protein A. It is possible for IgG molecules raised to certain neuropeptides that this coupling may cause alterations in the antigen binding site or affect the ability of the antigen binding domain to interact with particular conformations of the peptide, perhaps by limiting movement of the 'hinge' region of the immunoglobulins. Different coupling methods may be overcome some of these problems, but this has still to be investigated fully. Such problems may present themselves as background or low '0M' microprobe counts resulting from either a complete failure or poor ability of the immobilised antibody to bind the radiolabelled peptide.

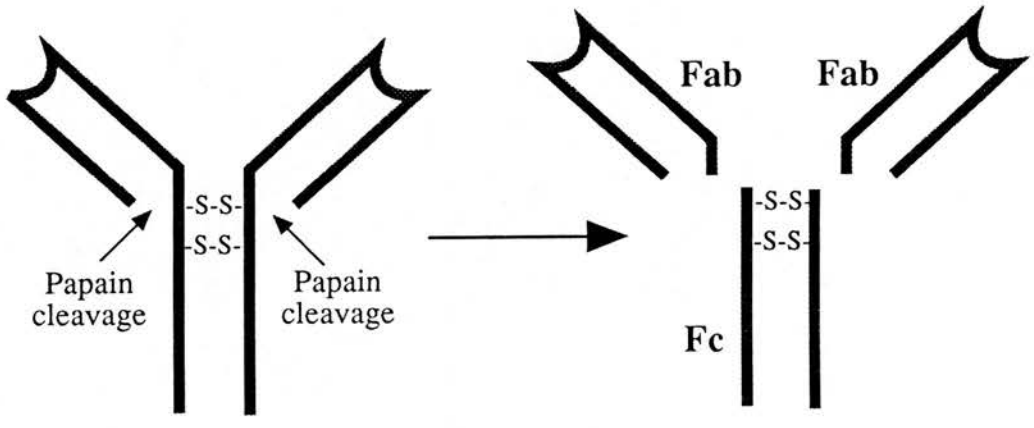
To check that the IgG molecules have not been seriously damaged in the antigen binding domain during the manufacturing process, the prepared microprobes can be incubated in capillaries containing radiolabelled $F(ab)_2$ fragments of antibodies directed against Fc region of the IgG molecules of the host species (usually rabbit) in which the polyclonal antisera had been raised (as illustrated on figure 28C). These microprobes can then be processed as described in section 3.3 for *in vitro* testing, the ability or not to detect radioactivity on these probes determining whether antibodies capable of binding $F(ab)_2$ fragments are actually bound to the microprobes and how uniformly bound. It is also possible that the iodination of the peptide itself may place a conformational constraint on the peptide and limit the extent to which it may interact with the IgG binding sites, as well as possibly causing a steric hindrance to binding due to the position of the radiolabel on the peptide. If the tracer is not suspected of being damaged or poorly iodinated, then the performance of a radioimmunoassay may be informative of the competency of the 'free' (i.e. non-immobilised) antiserum.

FIGURE 28. The structure of the IgG molecule. (A) This consists of four peptide chains linked by disulphide bonds; two 'heavy' and two 'light' chains. (B) Limited enzyme digestion by the protease papain, splits the IgG molecule into three domains; two of which are identical and form the arms of the 'Y' (known as 'Fab' fragments as contain the antigen binding sites), the third domain forming the base of the 'Y' and the effector region of the antibody (known as the 'Fc' fragment because it can be crystallised). The region between the Fab and Fc fragments is known as the 'hinge', since it allows lateral and rotational movement of the two antigen binding domains to enable them to interact with a large number of different antigen conformations. (C) Treatment with the protease pepsin can be used to release the two antigen binding domains still bound together, a fragment known as $F(ab)_2$. Adapted from Harlow and Lane (1988).

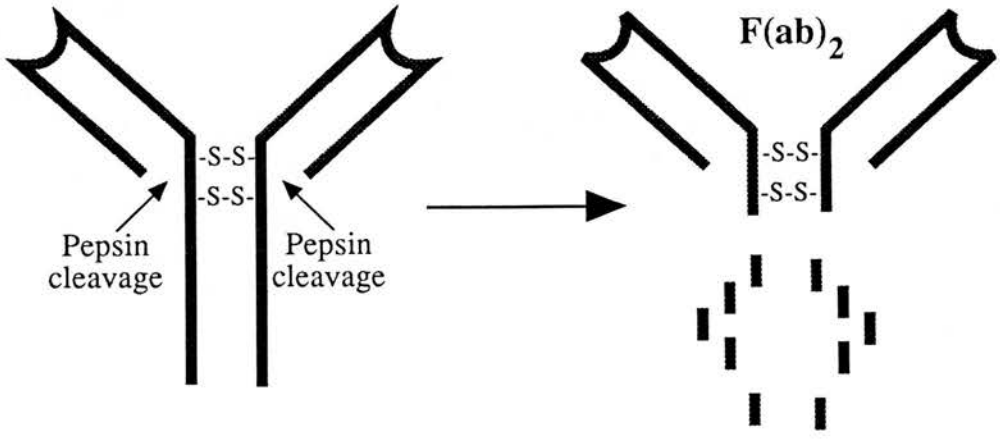
A


Antigen
binding site
'Hinge'
-S-S-
-S-S-
-S-S-
-S-S-
Light chain
Heavy chain

B


-S-S-
-S-S-
Papain
cleavage
Papain
cleavage
Fab
Fab
-S-S-
-S-S-
Fc

C


-S-S-
-S-S-
Pepsin
cleavage
Pepsin
cleavage
F(ab)2
-S-S-
-S-S-

(iii) Problems with the competency of the polyclonal antiserum

In the course of the immune response to a particular injected neuropeptide, large numbers of IgG molecules are produced with slightly variable antigen binding site regions, some of which fit the neuropeptide very closely and thus have a high affinity, others less closely and are of a lower affinity. It is suspected that often the reason for the failure of antibodies to perform adequately as part of antibody microprobes may be due to a high proportion of the immunoglobulins in particular batch of antiserum purchased being of a low affinity nature, with great variability between different batches (of different lot number) of antiserum being sometimes encountered. Microprobes do not allow simultaneous competition between ligands for binding to available sites but rather the unlabelled (or endogenous peptide) is allowed first access and the radiolabelled peptide follows. For this to succeed it is important that the antibody have a high affinity, such that the radiolabelled peptide does not displace the previously bound unlabelled form. This may be indicated by the 'titre' of the antiserum purchased or IC_{50} value (although this information is not always provided by the manufacturer). Such problems may present as low sensitivity and or/specificity of the prepared microprobes for the neuropeptide being studied, as well as poor suppression of the binding of the tracer to microprobes by the unlabelled peptide. Although the use of monoclonal antibodies or more NH_2-terminally directed antisera could provide the answer to such problems, monoclonal antibodies are not available for most neuropeptides and the use of NH_2-terminally directed antisera is often limited by the availability of COOH-terminally radiolabelled peptides.

A high percentage of low affinity antibodies may not necessarily affect the performance of the polyclonal antiserum in radioimmunoassay and immunocytochemical procedures for which they have been usually prepared, since both the labelled and unlabelled forms of the peptide are allowed to compete. Many procedures to improve sensitivity and specificity of a polyclonal antiserum are not applicable under conditions of antibody microprobe technique,

which involves immobilisation of IgG molecules and incubation times limited by their ultimate use *in vivo*.

(iv) Problems with non-specific binding

With certain peptides (both labelled and unlabelled) it has been suspected that losses may occur due to the binding of the peptide to the inner surfaces of glass microcapillaries, effectively lowering their concentrations in the capillary. Sigmacote treatment of these surfaces appears to have limited this problem but it is important to reassess the possibility that this may be occurring when a problem with low '0M' microprobe counts or poor suppression of tracer binding by the unlabelled peptide is found. Non-specific binding sites on the microprobe surface to which the radiolabelled peptide could bind is minimised by using an excess of a general binding agent, such as bovine serum albumin (BSA), in the tracer solution. Although for the purpose of antibody microprobe studies BSA has commonly been used, other binding agents such as gelatin and casein, have sometimes proved more effective (as will be discussed in A.II), perhaps causing less steric hindrance to peptide-antibody interactions. The use of such substances also serves to reduce the effect of any contamination in the tracer solution.

A.II CHOLECYSTOKININ *IN VITRO* ASSAY

As indicated in section 5.1 the original intentions of the cholecystokinin study were not completely fulfilled due to the failure of the CCK microprobes to continue to perform satisfactorily, as assessed by *in vitro* testing. The following account chronicles the 'history' of the CCK *in vitros* performed, indicating the problems encountered and offering explanations where possible. In total a 1091 microprobes were employed!

(i) Failure of immobilised antibodies from Amersham antiserum to bind [^{125}I]Bolton-Hunter CCK-8(sulphated).

The first antibodies for CCK tested *in vitro* were purchased from Amersham International [N1591; Lot no. 11445]. The polyclonal antisera had been raised in rabbits against the COOH-terminus of sulphated CCK 26-33 [CCK-8(S)] covalently conjugated to BSA for use in radioimmunoassay (RIA). An iodinated form of CCK was also purchased from Amersham International. This was [^{125}I]Bolton-Hunter CCK-8(S), prepared by the conjugation of the NH_2-terminal of the sulphated form of CCK-8 with iodinated Bolton-Hunter reagent, as illustrated in figure 27B. Table 6 displays the results of an *in vitro* assay on microprobes prepared using this source of antibody and tracer and following the protocol described in section 3.3. The counts (cor.cpm) obtained for these microprobes were at background level. This was not due to a failure to immobilise the antibodies onto the microprobes since high counts were obtained when anti-rabbit ^{125}I-IgG $F(ab)_2$ fragments were incubated with these same microprobes, as also illustrated on table 6. Instead, it appeared that the radiolabelled tracer had not bound to the immobilised antibody.

TABLE 6. The failure of immobilised antibodies from Amersham CCK(RIA) antiserum to bind [^{125}I]Bolton-Hunter CCK-8(sulphated). The counts (cor.cpm) obtained for microprobes prepared using this antiserum were at background level. This is in contrast to those obtained following the incubation of the microprobes in anti-rabbit ^{125}I-IgG F(ab)$_2$ fragments.

CCK-8 *IN VITRO* [19/ 1/ 95] - AMERSHAM CCK (RIA) ANTISERUM

PROBE TYPE [CCK]	COR.CPM [^{125}I]BH CCK-8 (S)	COR.CPM Anti-rabbit ^{125}I-IgG F(ab)$_2$
0M	19.3	1992.9
	10.2	2155.6
	22.4	2156.7
	24.4	1937.9
	21.4	2036.6
CCK-8 (NS) 10^{-7}M	20.6	2361.0
	13.4	2221.0
	12.4	2347.6
	21.6	2732.7
	72.1	2159.2
CCK-8 (NS) 10^{-5}M	11.2	2016.3
	19.3	2086.5
	15.3	2622.6
	-10.2	2355.0
	5.1	2485.2
CCK-8 (S) 10^{-7}M	8.0	2168.0
	9.0	2425.0
	15.0	2473.0
	-3.0	1894.0
	-3.0	2139.0
CCK-8 (S) 10^{-5}M	4.2	2358.0
	22.9	2253.0
	16.6	2984.3
	4.2	2272.8
	8.3	2401.6

(ii) Success obtained using microprobes prepared with Peninsula antiserum.

The next source of antibodies were purchased from Peninsula Laboratories [RAS 7181; Lot no. 005790]. This polyclonal antisera that had been raised in rabbits against the COOH-terminus of desulphated CCK 26-33 [CCK-8(NS)], again for use in radioimmunoassay. On *in vitro testing,* microprobes prepared with this antiserum consistently displayed high counts for the unsuppressed binding of the tracer to the microprobes, and good suppression of tracer binding by the unlabelled peptide. An example of the results of such an *in vitro* assay performed, is illustrated on table 7A. Since in this assay the same [^{125}I]Bolton-Hunter CCK-8(S) tracer was employed as for those employing the Amersham antiserum, it can be concluded that the tracer being used was competent and that any problems were likely due to the antiserum employed (at least when immobilised onto the microprobes).

(iii) BSA vs. gelatin conditions tested.

As indicated in A.I(iv) BSA (bovine serum albumin) is usually present in the tracer solution to occupy any non-specific binding sites on the microprobe surface and to reduce the effect of any contamination in the tracer solution. However, other general binding agents have been tried such as gelatin and casein. Notably casein is presently being employed instead of BSA in a microprobe study of the release of neuropeptide Y in the rat spinal cord by Mark et al.

In the information supplied with the antiserum from Amersham it was stated that gelatin should be used in the radioimmunoassay buffer in preference to BSA since:

"spurious results may be obtained with certain preparations of BSA".

Hence, *in vitro* assays were performed to determine whether gelatin had any effect on the failure of immobolised antibodies from Amersham International to bind [^{125}I]Bolton-Hunter CCK-8(S), as described in (i).

TABLE 7. Success and then failure of microprobes prepared with Peninsula antisera. The results of *in vitro* assays testing the unsuppressed ('0M') and suppressed (unlabelled sulphated and desulphated CCK-8) binding of [^{125}I]Bolton-Hunter CCK-8(sulphated) to microprobes prepared with Peninsula antisera. (A) Microprobes bearing immunoglobulins from RIA-1 antiserum show high counts (cor.cpm) for the unsuppressed binding of the tracer to the microprobes, and good suppression of tracer binding by 10^{-6}M CCK-8 (sulphated and desulphated). (B) The counts obtained for the '0M' microprobes in comparison using the RIA-2 antiserum are low, and are not dramatically suppressed by prior incubation in unlabelled peptide.

A CCK-8 *IN VITRO* [26/ 1/ 95] - PENINSULA CCK (RIA-1) ANTISERUM

PROBE TYPE [CCK]	COR.CPM [^{125}I]BH CCK-8 (S)	COR.CPM MEAN±S.E.M.
0M	1090.5	
	1180.1	
	1102.7	1025.3 ± 60.7
	1035.6	
	712.1	
	1030.7	
CCK-8 (NS) 10^{-6}M	87.3	
	100.8	94.0 ± 14.5
	128.8	(↓91%)
	59.0	
CCK-8 (S) 10^{-6}M	358.3	
	330.5	
	336.7	322.8 ± 12.9
	286.2	(↓69%)
	351.1	
	274.2	

B CCK-8 *IN VITROS* -[25/ 5/ 95] AND [5/ 6/ 95]

	PENINSULA CCK (RIA-1) ANTISERUM [25/ 5/ 95]		PENINSULA CCK (RIA-2) ANTISERUM [5/ 6/ 95]	
PROBE TYPE [CCK]	COR.CPM [^{125}I]BH CCK-8 (S)	COR.CPM MEAN±S.E.M.	COR.CPM [^{125}I]BH CCK-8 (S)	COR.CPM MEAN±S.E.M.
0M	2237.4		533.9	
	1893.4		345.4	
	2170.4	1958.9 ± 156.5	410.9	399.5 ± 43.8
	2191.6		459.9	
	1301.8		247.2	
CCK-8 (S) 10^{-7}M	601.0		201.6	
	777.0		279.5	
	386.7	619.2 ± 62.1	364.2	296.5 ± 31.5
	578.4	(↓68%)	340.7	(↓26%)
	754.0			
CCK-8 (S) 10^{-5}M	246.7		330.9	
	439.5		238.6	
	374.7	442.0 ± 84.0	216.4	270.5 ± 25.6
	707.2	(↓77%)	348.1	(↓32%)
			218.5	

As can be seen from table 8, the finding of such an *in vitro* assay revealed that although when gelatin was used in the PBS-azide solution to make up the tracer, the counts obtained for '0M' microprobes bearing Amersham antibodies were slightly higher than background, poor suppression of these counts was obtained by the unlabelled peptide. This was also true for microprobes bearing Peninsula antibodies. The reason for this is unknown.

(iv) Failure of the CCK microprobes to continue to perform satisfactorily.

A replacement batch of antiserum from Peninsula Laboratories failed to perform satisfactorily on *in vitro* testing. This was of a different lot number [021294-1]. Table 7B compares the findings from *in vitros* employing the same batch of radiolabelled ligand but these different lot numbers of Peninsula antisera. Unfortunately the remainder of the initial batch (RIA-1) of antiserum was exhausted before the second batch (RIA-2) arrived, and so a direct comparison between the antiserum in the same assay could not be made. It can be seen that the counts obtained for the '0M' microprobes using the RIA-2 antiserum are low, and are not dramatically suppressed by prior incubation in unlabelled peptide. This is in comparison to those microprobes employing the RIA-1 antisera, which show high counts and good suppression under comparable conditions. Since the replacement antiserum had an IC_{50} of 38 pg/ ml, compared to the previous one of 3.4 pg/ ml, it was possible that this antiserum contained a higher proportion of low affinity immunoglobulins, binding both the unlabelled and radiolabelled peptide poorly, and this was responsible for its failure to perform satisfactorily by the antibody microprobe technique.

Attempts were made to obtain another batch of CCK antiserum from Peninsula Laboratories of the same original lot number, but since this was not possible other lot numbers of CCK antiserum available were tested. This consisted of another polyclonal antiserum prepared for use in radioimmunoassays [RAS 7181; Lot no. 020187-1], and one prepared for use in immunocytochemistry (ICC) [RAS 7181N; Lot. no G019531-1] all raised in under the same conditions as RIA-1 and RIA-2. Neither antiserum however, performed satisfactorily

TABLE 8. Comparison of the use of BSA vs. gelatin in radiolabelled peptide solution. The results of an *in vitro* assay to determine whether gelatin in the tracer solution instead of BSA had any effect on the failure of immobolised antibodies from Amersham to bind [^{125}I]Bolton-Hunter CCK-8(sulphated) are shown. Although the counts (cor.cpm) obtained for '0M' microprobes radioactive emission were slightly higher than that of background, poor suppression of these counts was obtained by incubation in 10^{-7}M sulphated CCK-8. This was also true for microprobes bearing Peninsula antibodies.

CCK-8 *IN VITRO* [24/ 4/ 95] - AMERSHAM CCK (RIA) ANTISERUM
- PENINSULA CCK (RIA-1) ANTISERUM

PROBE TYPE	0M		CCK-8 (S) 10^{-7}M	
	COR.CPM [^{125}I]BH CCK-8 (S)	COR.CPM MEAN ± S.E.M.	COR.CPM [^{125}I]BH CCK-8 (S)	COR.CPM MEAN ± S.E.M.
AMERSHAM ANTISERUM/ BSA	-6.1		10.3	
	-7.1	BACKGROUND	10.3	18.5 ± 5.3
	9.2		17.5	
	-6.1		36.0	
	27.8			
PENINSULA ANTISERUM/ BSA	969.5		393.3	
	800.6	691.3 ± 70.3	344.9	320.5 ± 23.6
	424.2		336.6	(↓54%)
	571.5		289.8	
	652.8		237.9	
	729.0			
AMERSHAM ANTISERUM/ GELATIN	100.8		101.0	
	179.7	100.3 ± 20.7	34.0	87.1 ± 34.0
	42.6		25.0	(↓13%)
	153.7		45.8	
	65.0		229.9	
	60.0			
PENINSULA ANTISERUM/ GELATIN	926.6		1159.0	
	518.3	1293.6 ± 228.3	954.0	1097.5 ± 92.8
	1970.0		826.0	(↓15%)
	1501.0		1439.5	
	1552.0		1108.9	

on *in vitro* testing. The ICC antiserum did show higher unsuppressed counts than the RIA antisera but there was poor suppression of the binding of the tracer by the unlabelled peptide and great 'scatter' amongst the counts obtained. This is illustrated on table 9. No improvement was found by using gelatin or casein in the tracer buffer.

(v) Investigations into the lack of competency of replacement batches of Peninsula antiserum.

As indicated in appendix AI there are many possible explanations why antibody microprobes can fail to perform satisfactorily on *in vitro* testing. Although the competency of the replacement batches of Peninsula antisera was suspected, it was tested whether:

a) The radiolabelled peptide was binding non-specifically (either to the inner surfaces of the glass microcapillaries or to non-antibody sites on the microprobes). This was investigated by counting the radioactive emission of the capillaries following incubation of the microprobes in tracer made up with BSA, gelatin or casein. No evidence for tracer binding to the inner surfaces of the microcapillaries was found. That the radiolabelled peptide was also not binding to the microprobe surface, which would result in an apparent poor suppression by unlabelled peptide, was revealed by the failure of the tracer to bind microprobes in the absence of immobilised antibodies, and the failure of non-related peptides such as substance P and CGRP to cause any suppression of binding.

b) The presence of the iodinated Bolton-Hunter reagent on the NH_2-terminus of CCK-8(S) was interfering with the binding of the radiolabelled peptide to the immobilised antibodies. Although the antiserum purchased from Peninsula Laboratories (as stated in section 5.2.1) displayed only 14% cross reactivity with CCK-4, it is possible that the antibodies could be recognising more the NH_2-terminus of CCK-8. Thus, so if the antiserum contained a higher proportion of low affinity immunoglobulins this could greatly effect the ability of the tracer to bind to the immobilised antibodies and to apparently suppress the binding of the unlabelled peptide. To test this an iodinated form of CCK was purchased

TABLE 9. Investigations into the lack of competency of replacement batches of Peninsula antiserum. The results of an *in vitro* assay testing the unsuppressed ('0M') and suppressed (10^{-6}M sulphated and desulphated CCK-8) binding of [^{125}I]Bolton-Hunter CCK-8(sulphated) to microprobes prepared with either RIA-2 or ICC Peninsula antisera. Neither antiserum performed satisfactorily.

CCK-8 *IN VITRO* [22/ 6/ 95] - PENINSULA CCK (ICC) ANTISERUM

	A PENINSULA CCK (RIA-2) ANTISERUM		**B** PENINSULA CCK (ICC) ANTISERUM	
PROBE TYPE [CCK]	COR.CPM [^{125}I]BH CCK-8 (S)	COR.CPM MEAN±S.E.M.	COR.CPM [^{125}I]BH CCK-8 (S)	COR.CPM MEAN±S.E.M.
0M	486.4		1182.9	
	293.2		1038.8	
	364.2	374.4 ± 47.7	1854.0	1228.9 ± 157.2
	500.9		1272.0	
	227.2		796.7	
CCK-8 (NS) 10^{-6}M	480.0		1009.0	
	244.0		894.7	
	161.8	293.0 ± 58.4	663.1	706.9 ± 95.5
	286.3	(↓22%)	479.3	(↓42%)
			488.6	
CCK-8 (S) 10^{-6}M	376.1		755.1	
	324.7		548.8	
	264.8	273.5 ± 38.3	994.9	798.6 ± 65.7
	281.4	(↓27%)	863.2	(↓32%)
	120.6		831.2	

from Peninsula Laboratories prepared by the direct substitution of radioisotope ^{125}I onto the aromatic side-chain of the Tyr^2 residue of desulphated CCK-8, as illustrated in figure 27B. However, no improvement of the unsuppressed or suppressed binding of the tracer to any of the antisera purchased from Peninsula was evident by *in vitro* testing.

That these antisera likely contained a high proportion of low affinity immunoglobulins and this was the main reason for their poor performance on *in vitro* testing was supported by the finding that if the microprobes were incubated in the concentration of unlabelled peptide for longer periods of time more satisfactory suppression of tracer binding was achieved, as shown in table 10 using the Peninsula ICC antisera and [^{125}I]CCK-8(NS) tracer.

(vi) Other sources of antiserum tested.

As well as the antisera already stated, other sources of antiserum selective for the COOH-terminal of cholecystokinin were purchased and tested *in vitro* as described. These were from Biogenesis Ltd., UK [2050-0309; Lot no. 950726C] and Affinity Research Products Ltd., UK [CA 1127; Lot no. Z00049]. Both had been raised in rabbits against the COOH-terminus of sulphated CCK-8 covalently conjugated to BSA prepared for use in radioimmunoassay. When these antibodies were immobilised onto microprobes however, like those purchased from Amersham International, they failed to bind either the Amersham or Peninsula radiolabelled tracer.

(vii) Findings from radioimmunoassays.

Although the antibody microprobes technique follows the same basic principles of that of the radioimmunoassay technique, as indicated in appendix A.I, there are differences, and hence it is useful to check the performance of an antiserum by conventional radioimmunoassay. This was done for the antisera described in this appendix for CCK following the 'double-antibody' separation procedure outlined by Peninsula Laboratories. In this the antibody-radiolabelled peptide complex is precipitated by an antibody directed

TABLE 10. Investigation of prolonged incubation times on the success of unlabelled CCK to suppress tracer binding. Increasing the contact desulphated CCK-8 with microprobes bearing immobolised antibodies from Peninsula ICC antisera resulted in a more satisfactory suppression of [^{125}I]CCK-8(desulphated) binding.

CCK-8 *IN VITRO* [21/ 8/ 95] - PENINSULA CCK (ICC) ANTISERUM

PROBE TYPE [CCK-8(NS)] + INCUBATION TIME	COR.CPM [^{125}I]CCK-8 (NS)	COR.CPM MEAN ± S.E.M
0M	761.5	
	848.8	756 ± 30.3
	731.5	
	682.0	
10^{-7}M 30 MIN. INCUBATION	921.1	
	610.8	620.7 ± 86.0
	459.9	(↓18%)
	729.1	
	382.4	
10^{-7}M 60 MIN. INCUBATION	296.9	
	443.9	282.4 ± 50.9
	87.5	(↓63%)
	280.5	
	303.1	
10^{-5}M 30 MIN. INCUBATION	250.8	
	134.5	308.7 ± 48.7
	329.9	(↓59%)
	376.1	
	452.0	
10^{-5}M 60 MIN. INCUBATION	161.5	
	111.5	136.4 ± 19.4
	75.5	(↓82%)
	202.6	
	130.9	

against the bound antibody (in this instance those present in normal rabbit serum), the 'second' antibody being specific to the immunoglobulins of the species in which the bound 'first' antibody was raised (in this instance those present in goat 'anti-rabbit' IgG serum).

It was found that the antisera purchased from Peninsula appeared to perform satisfactorily under these conditions with good suppression of either tracer binding with a 10^{-8}M solution of the unlabelled peptide (both sulphated and desulphated). Interestingly, the antibodies present within the RIA antisera purchased from Amersham International, Biogenesis Ltd. and Affinity Research Products Ltd., although failing to bind the tracer purchased from Peninsula, [^{125}I]CCK-8(NS), did bind that from Amersham, [^{125}I]Bolton-Hunter CCK-8(S), as indicated in table 11. This is curious since such a distinction had not been found when these antibodies had been immobilised to microprobes.

(viii) Future studies

It is possible that sources of CCK antiserum could be found that will perform satisfactorily when immobilised onto the microprobes, or that the manufacturers of the antiserum indicated in this appendix may produce antiserum containing a greater proportion of high affinity antibodies to CCK-8. Alternatively the use of gastrin antibodies should be considered, since such antibodies will bind equally the COOH-terminus of CCK-8, and as indicated in section 1.9, gastrin itself displays a very limited distribution in the CNS.

TABLE 11. *In vitro* testing of antisera by radioimmunoassay. Comparison made between a radioimmunoassay performed using (A) [^{125}I]CCK-8(desulphated) and (B) [^{125}I]Bolton-Hunter CCK-8(sulphated), in both instances 10^{-6}M desulphated CCK-8 was used to suppress tracer binding.

RADIOIMMUNOASSAYS - [13/ 9/ 95] AND [6/12/95]

	PENINSULA [^{125}I]CCK-8 (NS) [13/ 9/ 95] n=4		AMERSHAM [^{125}I]BHCCK-8 (S) [6/ 12/ 95] n=4	
ANTISERUM	**0M**	**CCK-8 (NS) 10^{-8}M**	**0M**	**CCK-8 (NS) 10^{-8}M**
PENINSULA (RIA-2)	689.2 ± 13.9	268.2 ± 31.9 (↓ 62%)	758.1 ± 116.6	266.5 ± 16.4 (↓ 65%)
PENINSULA (ICC)	4644.2 ± 115.6	1593.7 ± 66.1 (↓ 66%)	8782.0 ± 38.0	1250.3 ± 40.7 (↓ 86%)
AMERSHAM (RIA)	202.6 ± 18.2	230.9 ± 18.5 (↓ 0%)	640.1 ± 53.3	248.4 ± 10.9 (↓ 61%)
BIOGENESIS (RIA)	194.7 ± 6.0	214.6 ± 13.1 (↓ 0%)	1164.6 ± 80.0	182.3 ± 38.5 (↓ 84%)
AFFINITY (RIA)	221.5 ± 16.6	189.1 ± 11.6 (↓ 15%)	1338.9 ± 49.8	269.8 ± 14.1 (↓ 80%)

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PUBLICATIONS

The following publications were all derived from experimental work with which I was involved during my postgraduate years on a MRC Studentship:

"Afferent volley patterns and the spinal release of immunoreactive substance P in the dorsal horn of the anaesthetized spinal cat." Duggan A.W., Riley R.C., Mark M.A., MacMillan S.J.A. and Schaible H.-G. *Neuroscience 65 (1995) 849-858*

"Peripheral inflammation and dynorphin release in the rat spinal cord." Riley R.C., Zhao Z.Q. and Duggan A.W. *Analgesia 1 (1995) 687-690*

"Spinal release of immunoreactive dynorphin A(1-8) with the development of peripheral inflammation in the rat." Riley R.C., Zhao Z.Q. and Duggan A.W. *Brain Research 710 (1996) 131-142*

"Studies of the release of immunoreactive galanin and dynorphin A(1-8) in the spinal cord of the rat." Duggan A.W. and Riley R.C. *Progress in Brain Research: Towards the Neurobiology of Chronic Pain [Ed. Carli G. and Zimmermann M.] (in press)*

"The burst-like firing of spinal neurones in rats with peripheral inflammation is reduced by an antagonist of NMDA." Grubb B.D., Riley R.C., Hope P.J., Pubols L., and Duggan A.W. *Neuroscience(in press)*

COMMUNICATIONS

In addition the following communications to learned societies were made:

Oral:	February 1994	Physiological Society meeting [The University of Bristol] "Release of immunoreactive dynorphin A(1-8) in the spinal cord of rats with a unilateral ankle inflammation"
Poster:	November 1995	25th Annual Meeting of the Society for Neuroscience [San Diego] "Measurement of cholecystokinin release in the rat spinal cord using antibody microprobes"
	September 1995	"Pain Mechanisms and Management" meeting [Pain Research Institute, Liverpool] "Changes in the spinal release of the inhibitory peptides dynorphin and galanin, with the development of peripheral inflammation - studies with antibody microprobes"
	July 1995	XXVI International Narcotics Research Conference meeting [University of St. Andrews] "Spinal release of immunoreactive dynorphin A(1-8) with the development of peripheral inflammation in the rat"

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Research report

Spinal release of immunoreactive dynorphin $A_{(1-8)}$ with the development of peripheral inflammation in the rat

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Research report

Spinal release of immunoreactive dynorphin $A_{(1-8)}$ with the development of peripheral inflammation in the rat

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Abstract

Microprobes bearing immobilised antibodies to dynorphin $A_{(1-8)}$ were used to study the basal and evoked release of this prodynorphin derived peptide in the spinal cord of urethane anaesthetised normal rats and those with a peripheral inflammation. In the absence of any active peripheral stimulus the antibody microprobes detected immunoreactive (ir)-dynorphin $A_{(1-8)}$ in two areas (lamina I and laminae IV–V) in the dorsal horn of the spinal cord of normal rats. With the development of unilateral ankle inflammation over 3 to 5 days following subcutaneous injections of Freund's complete adjuvant, a basal presence of ir-dynorphin $A_{(1-8)}$ was found in both the dorsal and ventral horn regions of both sides of the spinal cord. Lateral compression of the ankles of the normal animals did not release ir-dynorphin $A_{(1-8)}$ during the period of stimulation, but this neuropeptide was detected in increased amounts in the ventral horn following the stimulus. By contrast, compression of inflamed ankles produced elevated levels of ir-dynorphin $A_{(1-8)}$ during the period of stimulus application at three major sites in the ipsilateral spinal grey matter. The largest peak was in the deep dorsal horn/upper ventral horn (laminae VI–VII), with further sites of significant release in the mid dorsal horn (laminae II–V) and the lower ventral horn. The observation that ir-dynorphin $A_{(1-8)}$ is physiologically released in the ventral and deep dorsal in addition to the superficial dorsal horn of the rat suggests an involvement of dynorphins in several aspects of spinal function.

Keywords: Dynorphin $A_{(1-8)}$; Peripheral inflammation; Spinal cord; Release; Antibody microprobe

1. Introduction

When inflammation develops peripherally, alterations occur in the synthesis and release of neuropeptides in the spinal cord. Such release has been studied directly with a variety of techniques and an enhancement has been shown for compounds contained within primary afferent fibres such as substance P (SP) [18,57,65], neurokinin A [26], and calcitonin gene related peptide (CGRP) [18,66]. These studies have revealed significant differences in the timing of the enhanced release of these neuropeptides as inflammation develops, suggesting that there are evolving processes influencing the spinal processing of nociceptive information.

There are no reports of altered spinal release of the prodynorphin derived peptides as inflammation develops peripherally and this is a significant omission, as it is this family of neuropeptides which shows the greatest changes in synthesis and spinal content with inflammation. Such increases have occurred with dynorphin $A_{(1-8)}$ (prodynorphin 209–216) [34,35,51,52,63], dynorphin $A_{(1-17)}$ (prodynorphin 209–225) [44,46,47,79,80] and α-neoendorphin (prodynorphin 175–184) [61]. Preceding these rises in neuropeptide levels, the messenger RNA levels for prodynorphin in spinal neurones have also been found to be increased [12,13,25,34–36,58,61,63,79].

An increased RNA message for prodynorphin could simply be a response to prior release. It has been proposed, however, that the immediate stimulus to elevated levels of dynorphins is increased transcription of the prodynorphin gene through cellular immediate early gene products, such as c-fos. There is a close temporal link between the increases in c-fos and messenger RNA levels for prodynorphin observed in the spinal cord of the rat on the induction of a peripheral inflammation [12,42,53] and the co-localisation of c-fos and the prodynorphin message within single neurons of the spinal cord [42,53,56]. Since the gene for c-fos is expressed by many neurones of the spinal cord in

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response to the arrival of impulses in nociceptive primary afferents [1,7,17,19,20,28,33,41,55,56], it is possible that the increased synthesis of dynorphins is a primary response to this input and not a homeostatic secondary response following prior release. Supporting this proposal is the finding of synapses between dynorphin containing spinal neurones and CGRP and SP containing presumed primary afferent fibres [4,9,73,74], and more recently from the demonstration that intrathecal pretreatment with an antisense nucleotide to c-fos mRNA synthesis inhibits the increase in the synthesis of prodynorphin by spinal neurones following peripheral inflammation [29].

Such considerations indicate the need to determine the adequate stimuli producing a spinal release of dynorphins under in vivo conditions both in normal animals and in those with peripheral inflammation. Specifically, are dynorphins tonically released in both normal and inflamed animals and is such release increased or decreased by manipulation of the inflamed tissues? The present experiments have employed microprobes bearing immobilised antibodies to dynorphin $A_{(1-8)}$ to study the release of immunoreactive (ir)-dynorphin $A_{(1-8)}$ in the spinal cord of normal rats and those with a unilateral ankle inflammation. We chose dynorphin $A_{(1-8)}$ since there is evidence that this peptide is the major end product of the processing of prodynorphin in several areas of the central nervous system, including the spinal cord [72,77], and it is this form of the dynorphin molecule which has been most extensively studied in relation to peripheral inflammation [34,35,51,52,63].

2. Materials and methods

2.1. Microprobe preparation

Antibody microprobes for the detection of ir-dynorphin $A_{(1-8)}$ were prepared following the technique previously described [14,15]. Briefly, conventional glass micropipettes were heat sealed at both ends and incubated for up to 24 h in a 10% solution of γ-aminopropyltriethoxysilane in toluene. This procedure produced a fine, granular siloxane polymer with free amino groups on the outer surface of each microprobe to which protein A (Sigma Chemical, UK) was immobilised with glutaraldehyde. Protein A, is a staphylococcal derived protein which binds to the Fc region of some subclasses of IgG antibodies and this property was utilised to bind immunoglobulins present in a polyclonal antiserum raised in rabbits against the C terminus of dynorphin $A_{(1-8)}$ (Peninsula Laboratories Europe UK), to microprobes.

2.2. Animal preparation

A total of 35 male Wistar rats (weight range 250 to 422 g; Charles River, UK) were used in this study.

2.2.1. Induction of peripheral inflammation

Eighteen animals received 4 subcutaneous injections of Freund's complete adjuvant (FCA, Sigma Chemical, UK, 1.0 mg/ml, total volume of 0.15 ml) around one ankle joint, either under diethyl ether or halothane anaesthesia, 3–5 days before being used in an experiment. Throughout this time the animals were monitored for the development of any secondary swellings at other peripheral sites, and the circumferences of both ankles were measured on the day of the experiment. These injections resulted in a swelling of the ankle region, from an average diameter in normal animals of 2.7 cm to 4.0 cm. Previous histological studies by other groups [22] have shown that the inflammation resulting from this procedure involves both the joint and periarticular tissues (connective tissue, tendons and overlying skin). Injected animals showed little or no apparent discomfort, gait impairment or weight alteration. An additional six rats received FCA injections on the morning of the experiment, to allow study of the release of ir-dynorphin $A_{(1-8)}$ at an early phase of the inflammatory response.

2.2.2. Surgical procedure

Anaesthesia was induced by a single intraperitoneal (i.p.) injection of 1 g/kg urethane (25% solution). Cannulae were then 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 monitoring blood pressure and ensuring that the corneal blink and hindpaw withdrawal reflexes remained absent. Further injections of urethane were given i.p. when necessary to maintain a satisfactory level of anaesthesia. Blood oxygenation was assisted by directing a gentle jet of humidified oxygen towards the opening of the tracheal cannula. The animals body temperature was monitored with a rectal probe and maintained between 36–38°C using a controlled heating system.

An extended laminectomy was made at vertebral levels T_{12}–L_2 to expose the dura mater and the lumbar spinal cord (spinal segments L_1–L_6) receiving input from the ankle joint area. The animal was supported in a metal frame and six swan neck clamps were positioned under alternate mammillary processes of the exposed vertebrae. A solid agar pool was formed at the site of the laminectomy into which a window over the lumbar spinal segments was made. 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. Any excess fluid was removed by suction at an edge of the opening. Although continuous irrigation minimised the collection of inflammatory exudates on the surface of the cord, it was often necessary to remove any small accumulations of fibrinous material from pia openings with sterile fine forceps and hand controlled suction.

2.3. Experimental protocol

Before microprobes were inserted into the spinal cord, the suitability of potential penetration sites was examined by recording (with a 4 M NaCl filled microelectrode) the excitatory responses of neurones in the deep dorsal horn (approximately 0.6 to 1.0 mm from the dorsal surface of the spinal cord) to light mechanical cutaneous stimuli. In order to permit microprobe entry it was often necessary to remove the pia mater at these sites using sterile fine forceps. Microprobes were broken back to give tip diameters of approximately 10 μm and some were filled with Pontamine sky blue 2% (w/v) in 1.2 M sodium acetate solution to allow easy visualisation of the tips for accurate placement in the cord. Pairs of labelled microprobes were introduced into the same side of the spinal cord to a depth of 2.25 mm from the dorsal surface using two stepping motor driven micromanipulators. Initially, microprobes were kept in the cord for 5, 15 or 30 min but as preliminary analysis indicated that the probability of binding any dynorphin $A_{(1-8)}$ increased with length of time in the spinal cord, all later experiments employed incubation times of 30 min. During these periods either no stimulus was applied or the ankle joint was mechanically manipulated.

Since the most likely stimulus producing pain from inflamed skin, joints or tendons is that resulting either from direct physical contact with an environmental object or movement of the damaged tissues, the ankle joint region was only subjected to mechanical stimulation. A typical protocol was 3 min of mechanical stimulation followed by 2 min of no stimulation. In the initial experiments the inflamed tissues were compressed laterally, with a modified strong spring clip. In later experiments this stimulus was quantified by the application of a pneumatic compression device incorporating a strain gauge for measuring applied force and employing electronic control of the times of application. Typically, a force of 14.7 N was applied, the surface area of the compression device contacting the rat ankle being 20 mm^2.

Although inflammation was induced unilaterally, pairs of microprobes were inserted into both sides of the spinal cord, comparable stimuli being applied to the inflamed and uninflamed hind limbs. With each side of the spinal cord, periods of no stimulation usually preceded those of mechanical stimulation to the ipsilateral ankle region but also often followed them. Due to uncertainty on the persistence of any possibly released ir-dynorphin following peripheral stimulation, it was usual to perform periods of peripheral stimulation (and associated periods of no stimulation) only twice, once on each side of the spinal cord. In six experiments, however, the chosen protocol was carried out more than once on one side of the cord and a time of at least 2 h elapsed between periods of stimulation.

At the end of each experiment Pontamine sky blue was ejected iontophoretically at several defined sites in the spinal cord. The cords were then removed, fixed and sectioned to determine the location of the resultant dye spots. These data were essential to allow locations on the probes to be related to positions within the spinal cord.

2.4. Treatment of microprobes and data analysis

Upon removal from the spinal cord, microprobes were washed for 15 min in ice-cold phosphate-buffered saline (PBS) containing 0.1% Tween-80 (Pierce and Warner Ltd, UK) and then incubated for approximately 24 h at 6°C in capillaries (treated with Sigmacote to prevent binding of peptides to glass) containing [^{125}I]dynorphin $A_{(1-8)}$ (porcine; Peninsula, UK) diluted in a PBS azide solution containing bovine serum albumin (0.1%). The final dilution of the radiolabelled peptide resulted in approximately 2000 counts min^{-1} μl^{-1}. After a final 15 min wash in ice cold PBS Tween-80 (0.1%) solution, the distal portions of the microprobes were broken off, mounted on a sheet of paper and placed in a cassette next to monoemulsion X-ray film (Kodak NMB) for 3–5 days. At least two films were derived from each experiment. The resultant microprobe autoradiographs were analysed with a computer based image analysis system employing an Imaging Technology PC Vision Plus frame grabber board operating in a DCS 286e (AT based) computer, as previously described [23]. 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 resolution of the analysis system used, this gave a 10 μm interval between each integration. However, since this was beyond the biological resolution of the microprobe method (see Duggan [14]), the average of three successive integrals was taken to give a final plotted 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 and stimulus conditions for that particular microprobe, were stored in a computer file. Regions of bound endogenous ir-dynorphin $A_{(1-8)}$ 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 program was then used to retrieve defined groups of these microprobes and produce 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. Differences between groups were determined, the significance of the differences at each of the 30 μm analysis points being estimated using the Student's *t*-test. Release was equated with increased extracellular levels produced by a stimulus. It should be noted that with mean image analyses of results from several microprobes, the location of sites of release is less precise than with individual microprobes. Although all microprobes were inserted 2.25 mm into the spinal cord,

the relationship of spinal laminae to distance to the tip will vary slightly between animals and with distance from the midline within any one animal.

2.5. In vitro tests

The antiserum used was polyclonal and directed against the C-terminus of dynorphin $A_{(1-8)}$ (porcine). The manufacturer stated that this antibody had negligible cross reactivity (< 0.01%) for the prodynorphin derived peptides prodynorphin (209–240), dynorphin $A_{(1-17)}$, dynorphin $A_{(1-13)}$, dynorphin $B_{(1-13)}$, α-/β-neoendorphin and zero cross-reactivity for other tested opioid peptides, e.g., Leu/Met enkephalins. In parallel with the present experiments, the sensitivity of the prepared antibody microprobes was regularly assessed by incubating a small number either directly in [^{125}I]dynorphin $A_{(1-8)}$ or a range of concentrations (10^{-5} to 10^{-9} M) of unlabelled dynorphin $A_{(1-8)}$ (Peninsula) in vitro at 37°C for 30 min prior to the incubation in a solution of [^{125}I]dynorphin $A_{(1-8)}$ (porcine) for approximately 24 h at 6°C. Following washing in PBS/Tween-80, the distal portion of these microprobes were broken off, mounted on small pieces of cardboard and placed in tubes for processing by a 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 dynorphin $A_{(1-8)}$ (porcine) suppressed such binding by greater than 55%, with a 10^{-5} M solution 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. Information supplied by the manufacturer on the specificity of the antiserum was confirmed on microprobes by demonstrating negligible or zero suppression of binding of [^{125}I]dynorphin $A_{(1-8)}$ by a range of concentrations of the peptides dynorphin $A_{(1-17)}$, dynorphin $A_{(1-13)}$ and Leu-enkephalin (Peninsula).

3. Results

A total of 496 microprobes coated with antibodies to dynorphin $A_{(1-8)}$ inserted into the rat spinal cord form the basis of this analysis. An additional 572 microprobes were used for in vitro sensitivity tests.

3.1. Basal levels of immunoreactive (ir) dynorphin $A_{(1-8)}$

Since the primary aim of this study was to study the release of ir-dynorphin $A_{(1-8)}$ in animals with a developed peripheral inflammation, it was necessary to make comparisons between normal rats and those with unilateral ankle inflammation. In the case of the latter group, the release of ir-dynorphin $A_{(1-8)}$ was examined in both sides of the spinal cord.

A basal presence of a neuropeptide at a particular site in the nervous system is inferred by differences between the mean image analysis of microprobes not inserted into the nervous system but simply incubated in the radiolabelled ligand, and that of microprobes placed in the relevant area for a comparable time but in the absence of any active stimulus. Such comparisons indicated that ir-dynorphin $A_{(1-8)}$ was present in the spinal cord of normal rats and both sides of the spinal cord of those with developed unilateral ankle inflammation under these 'basal' conditions. As microprobes present in the spinal cord for 5 or 15 min failed to detect ir-dynorphin$_{(1-8)}$ consistently, the data presented below were derived from microprobes present for 30 min in the spinal cord only.

3.1.1. Normal animals

Fig. 1A compares the mean image density scan of microprobes ($n = 77$) present in both sides of the spinal cord of normal rats for periods of 30 min and prior to any mechanical stimulation of the corresponding ankle regions, with that of in vitro microprobes ($n = 98$) that had only been exposed to radiolabelled dynorphin $A_{(1-8)}$ but processed concurrently with those used in vivo. Fig. 1B plots in 30 μm intervals the 't' statistics derived from the differences between the mean image analyses of the two groups. The hatched area indicates where these differences are significant at the $P < 0.05$ level ($t > 2$) and this is approximately from 0.5 to 1.0 mm from the dorsal surface of the spinal cord. Thus, in normal animals extracellular ir-dynorphin $A_{(1-8)}$ was present in zones of both the superficial and deep dorsal horn approximating to laminae I and IV–V.

3.1.2. Animals with developed unilateral ankle inflammation – ipsilateral side of the spinal cord

A more extensive basal presence of ir-dynorphin $A_{(1-8)}$ was found in the ipsilateral side of the spinal cord (58 microprobes) in animals with unilateral ankle inflammation than in normal animals. When compared with in vitro microprobes (not inserted into animals) significant levels of ir-dynorphin $A_{(1-8)}$ were found from the dorsal surface of the spinal cord down to 2.0 mm from the dorsal surface (not illustrated). This approximates to the whole of the dorsal horn and ventral horn. Fig. 2 illustrates differences between the mean image analysis of microprobes in this group and that of microprobes present in the spinal cord of normal animals but without added stimulation.

This shows comparable basal levels of ir-dynorphin $A_{(1-8)}$ in the dorsal horn of normal and inflamed animals but significantly greater levels of this neuropeptide at many sites in the ventral horn of rats with peripheral inflammation.

3.1.3. Animals with developed unilateral ankle inflammation – contralateral side of the spinal cord

The basal extracellular levels of ir-dynorphin $A_{(1-8)}$ in the contralateral side (34 microprobes) of the spinal cords of animals with developed ankle inflammation were found to be comparable to those present in the ipsilateral side under these basal conditions. Fig. 3 illustrates the nearly completely coincident mean image analyses of these two groups of microprobes.

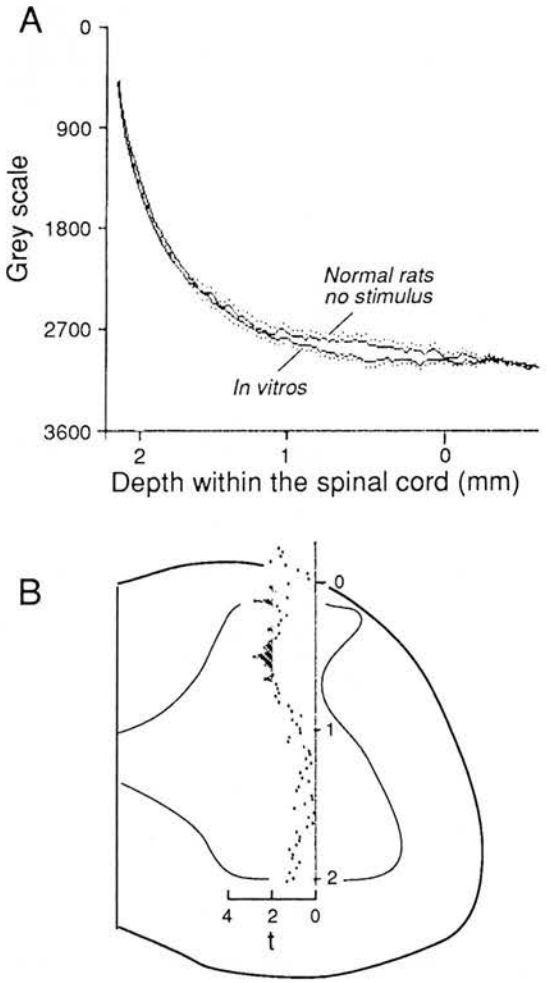

Fig. 1. Basal presence of ir-dynorphin $A_{(1-8)}$ in the spinal cord of normal rats. A: the mean image analyses of two groups of microprobes are plotted with respect to length: those present in the spinal cord of normal animals for 30 min in the absence of any peripheral stimulation (normal rats, no stimulus, $n = 77$) and those which are not inserted into the spinal cord but simply incubated in [^{125}I]dynorphin $A_{(1-8)}$ (in vitros, $n = 98$). With each mean image analysis the mean grey scale was determined in 30 μm intervals, a line joining these points. At each analysis point the standard error 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 error of the differences of means at each analysis point in the mean image analyses shown in A, is related to an outline of the spinal cord at the area sampled. The hatched areas indicate the sites where these differences are significant at the $P < 0.05$ level.

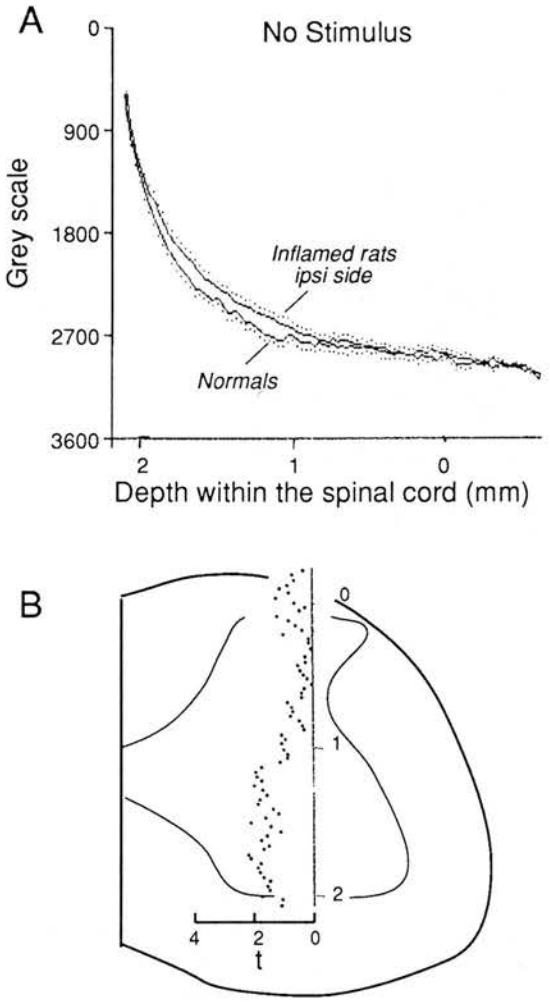

Fig. 2. Effect of peripheral inflammation on the basal levels of ir-dynorphin $A_{(1-8)}$ in the ipsilateral side of the spinal cord. A: the mean image analysis of 58 microprobes present for 30 min in the ipsilateral side of the spinal cord in rats with a unilateral ankle inflammation in the absence of any added stimulation (inflamed rats, ipsilateral side) is compared to that of microprobes present under similar basal conditions in normal rats (normals, $n = 77$). B: the differences between the two groups of microprobes are plotted with respect to depth in the spinal cord. Since only isolated points attain significance at the $P < 0.05$ level, there is no hatching as in Fig. 1.

3.2. The effect of lateral compression of the ankle region

3.2.1. Microprobes present in the spinal cord during stimulus application

Although significant basal levels of ir-dynorphin $A_{(1-8)}$ were present in both normal rats and those with unilateral ankle inflammation, these animals differed markedly in their responses to lateral compression of the ankle.

3.2.1.1. Normal animals. The application of lateral compression to the ankle joint and surrounding tissue of normal rats failed to increase extracellular levels of ir-dynorphin $A_{(1-8)}$ during the period of stimulus application. No significant differences were found between the mean image density scans of microprobes ($n = 29$) present in the

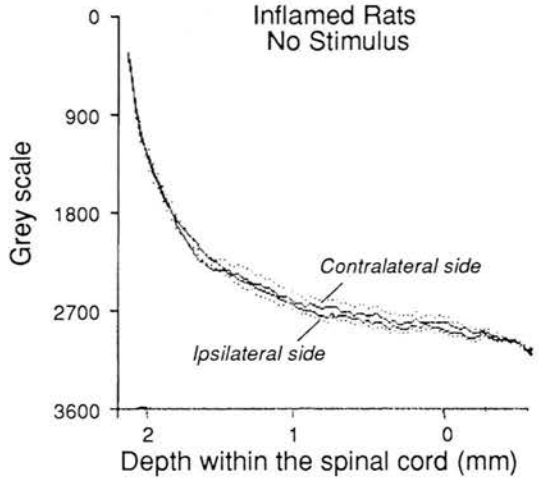

Fig. 3. Comparable basal presence of ir-dynorphin $A_{(1-8)}$ in both sides of the spinal cord of rats with unilateral inflammation. This figure compares the mean image analysis of microprobes present for 30 min in the ipsilateral side of the spinal cord of rats with unilateral ankle inflammation (ipsilateral side, $n = 58$) to that of microprobes present in the contralateral side of the same animals in the absence of any added stimulation (contralateral side, $n = 34$).

ipsilateral spinal cord during the application of the mechanical stimuli and those microprobes ($n = 56$) present in the same animals in the absence of any prior stimulation, as illustrated in Fig. 4.

3.2.1.2. Animals with developed unilateral ankle inflammation – ipsilateral side of the spinal cord. Lateral compression of the ankle with a developed inflammation, significantly increased the extracellular levels of ir-dynorphin $A_{(1-8)}$ above those found under basal conditions in the ipsilateral side of the spinal cord. This is illustrated in Fig.

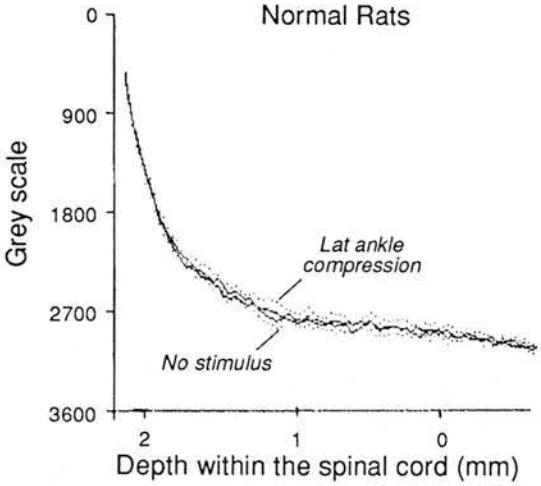

Fig. 4. Failure to release ir-dynorphin $A_{(1-8)}$ during a period of ankle compression in normal animals. The mean image analyses of the 29 microprobes present in the spinal cord for 30 min during the application of the mechanical stimuli (lat. ankle compression, $n = 29$) is compared to that of microprobes present in the same animals in the absence of any prior stimulation (no stimulus, $n = 56$).

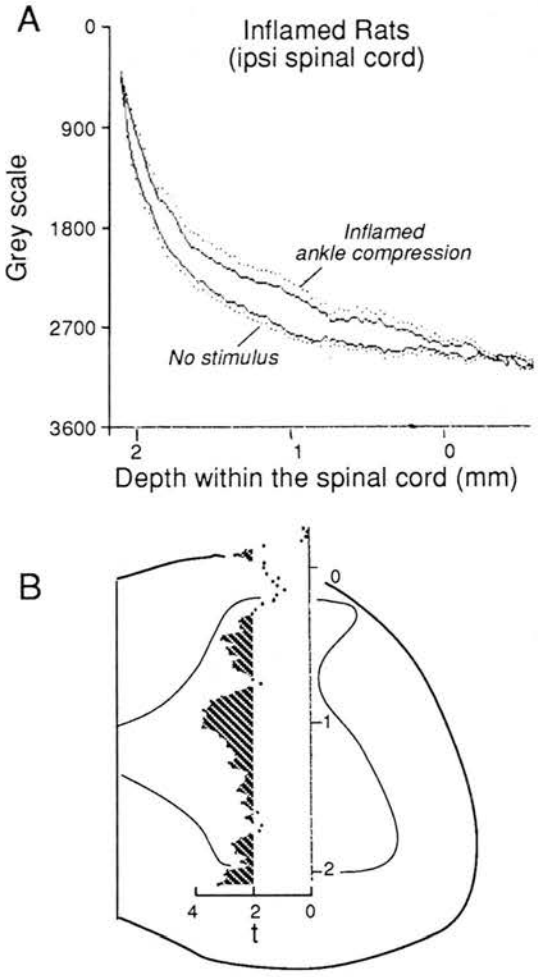

Fig. 5. Release of ir-dynorphin $A_{(1-8)}$ during compression of the inflamed ankle. A: the mean image analysis of 30 microprobes present in the ipsilateral side of the spinal cord during the 30 min period of noxious mechanical stimulation (inflamed ankle compression) is displaced above that of microprobes present in the same side of these spinal cords for an identical time period prior to this stimulus (no stimulus, $n = 47$). B: the differences between the two groups of microprobes are plotted with respect to a diagram of the spinal cord. The hatched areas indicate where these differences are significant at the $P < 0.05$ level.

5A in which the mean image analysis of microprobes ($n = 30$) present in the ipsilateral side of the spinal cord during the 30 min period of noxious mechanical stimulation can be seen to be displaced above that of microprobes ($n = 47$) present for an identical time period prior to this stimulus in the same animals, but in the absence of any added peripheral stimulation. These enhanced extracellular levels of ir-dynorphin $A_{(1-8)}$ were found to be statistically significant at the $P < 0.05$ level at nearly all sites sampled from 0.25 mm to 2.2 mm from the dorsal surface of the spinal cord. As Fig. 5B shows, there were three main sites of release. The largest peak was in the deep dorsal/upper ventral horn (laminae VI–VII), with further major sites in the mid dorsal horn (laminae II–V) and the lower ventral horn. No study was made of possible contralateral release

of ir-dynorphin$_{(1-8)}$ during compression of the inflamed ankle.

3.2.1.3. Animals with developed unilateral ankle inflammation – contralateral side of the spinal cord. Equivalent analysis of the effect of ankle compression on the normal side of animals with unilateral inflammation failed to show any such evoked release of ir-dynorphin A$_{(1-8)}$. Thus, the mean image analysis of microprobes ($n = 12$) present in the side of the spinal cord contralateral to the peripheral ankle inflammation and during lateral compression of the normal ankle displayed no significant differences from that of microprobes ($n = 28$) present in the same side of the spinal cord under basal conditions (not illustrated).

3.2.2. Microprobes present in the spinal cord after the application of mechanical stimuli

Since there is evidence that some neuropeptides are not rapidly degraded after release in the central nervous system [16,26], it was important to examine the decline of any enhanced presence of extracellular ir-dynorphin A$_{(1-8)}$ after application of a peripheral stimulus. The mean image analyses of microprobes inserted before or during prior mechanical stimulation of the ankle joint region were compared to those of microprobes present for equivalent 30 min periods, up to 1.5 h after lateral compression of these peripheral tissues.

3.2.2.1. Normal animals. No increase in the extracellular levels of ir-dynorphin A$_{(1-8)}$ was observed in the spinal cord during mechanical compression of the ankle region of normal rats. However, as illustrated in Fig. 6, comparisons between the mean image analysis of microprobes inserted prior to the application of lateral compression ($n = 55$) and that of those present in the corresponding 30 min period post-stimulation ($n = 21$), showed elevated levels of the ir-dynorphin A$_{(1-8)}$ in the lower ventral horn after the application of ankle compression.

3.2.2.2. Animals with developed unilateral ankle inflammation – ipsilateral side of the spinal cord. Fig. 7 illustrates extracellular ir-dynorphin A$_{(1-8)}$ in the ipsilateral side of the spinal cord before, during and for 1 h after the lateral compression of the inflamed ankle. Fig. 7B shows that the mean image analysis of microprobes ($n = 21$) present for the first 30 min period after lateral compression of the inflamed tissues of the ipsilateral ankle joint was displaced above that of microprobes ($n = 36$) present in the same side of these spinal cords prior to such peripheral stimulation. No significant differences however were found between these 'post-compression' microprobes and those present in the spinal cord during this noxious mechanical stimulation ($n = 28$, not illustrated).

The mean image density scans of microprobes ($n = 14$) present in the spinal cord for the period 35–65 min post-compression was also found to be significantly elevated above those placed under 'basal' conditions, as illustrated in Fig. 7C. However, ir-dynorphin A$_{(1-8)}$ levels in the ipsilateral side of the spinal cord had returned to pre-stimulation 'basal' levels after a period of 2 h, as no significant difference was found between the mean image density analyses of microprobes ($n = 8$) inserted ipsilaterally in the absence of any prior or concurrent added stimulation and those microprobes ($n = 8$) inserted into this side of the cord again after a period of at least 2 h (not illustrated).

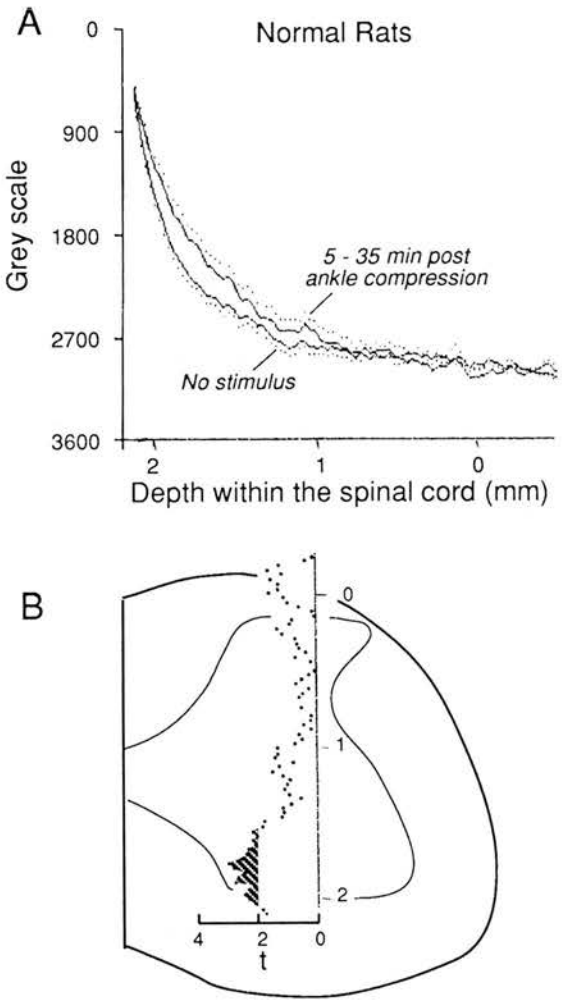

Fig. 6. Release of ir-dynorphin A$_{(1-8)}$ in the spinal cord following ankle compression in normal rats. A: the mean image analysis of 21 microprobes present in the 30 min period post ankle compression (5–35 min post ankle compression) is displaced above that of microprobes present prior to the application of this mechanical stimulus (no stimulus, $n = 55$). B: the differences between the post ankle compression and no stimulus groups are plotted with respect to a diagram of the spinal cord. The hatched areas indicate where these differences are significant at the $P < 0.05$ level.

3.2.2.3. Animals with developed unilateral ankle inflammation – contralateral side of the spinal cord. Equivalent analysis revealed that mechanical manipulation of the normal ankle of animals with unilateral inflammation had not

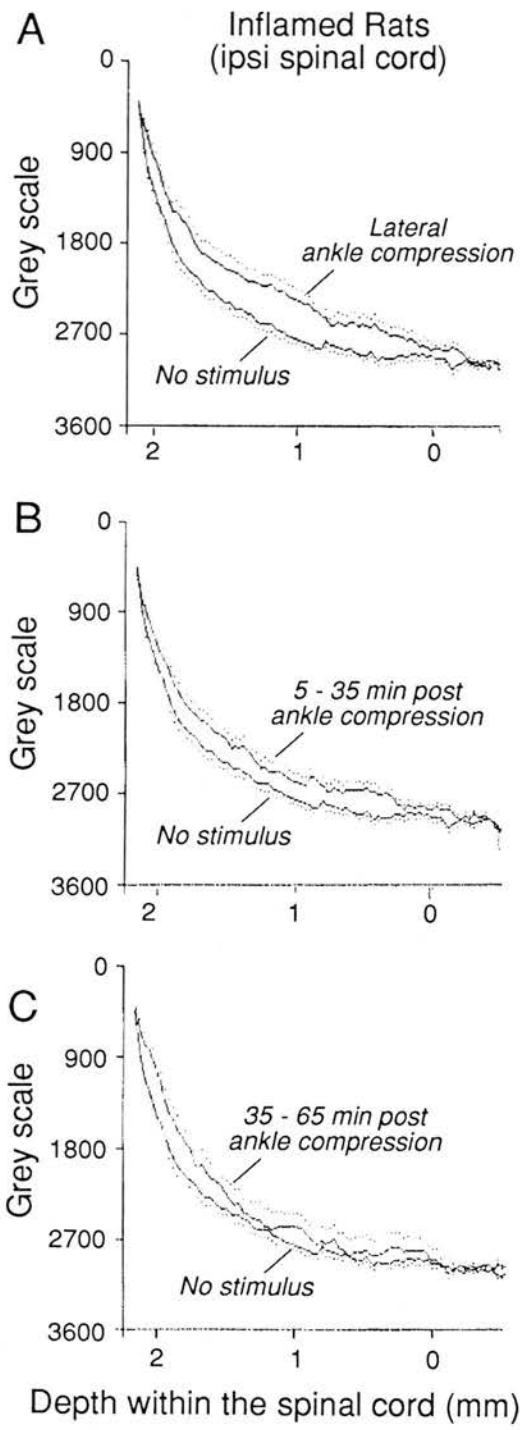

Fig. 7. Persistently raised levels of ir-dynorphin $A_{(1-8)}$ in the spinal cord following compression of the inflamed ankle. For A, B and C the no-stimulus group represents 36 microprobes present for 30 min in the ipsilateral side of the spinal cord of inflamed rats prior to any added stimulation. In A, the mean image analysis of this group is compared with that of 28 microprobes inserted during compression of the inflamed ankle (lateral ankle compression); in B, the comparison is with the mean image analysis of 21 microprobes present in the first 30 min period after this noxious mechanical stimulus in the same side of these spinal cords (5–35 min post ankle compression); in C, the comparison is with the mean image analysis of 14 microprobes inserted under similar conditions in the same experiments but present in the second 30 min period post ankle compression (35–65 min post ankle compression).

initiated any effect on the levels of ir-dynorphin $A_{(1-8)}$ presence under basal conditions. Thus, the mean image analysis of microprobes ($n = 10$) present in the side of the spinal cord contralateral to the peripheral ankle inflammation for the first 30 min period following lateral compression of the normal ankle displayed no significant differences from that of microprobes ($n = 20$) present in the same side of the spinal cord of these animals prior to application of this ankle compression.

3.3. Release of ir-dynorphin $A_{(1-8)}$ in the spinal cord during the development of unilateral ankle inflammation

In view of the temporal analysis performed by other groups [12,34–36,42,53] on the expression of the message for prodynorphin, it was of interest to study the release of ir-dynorphin $A_{(1-8)}$ in the spinal cord at an early phase of the inflammatory response. Microprobes present in the spinal cord for 15 or 30 min during the third to the eighth hour after the FCA injections, in the absence of any additional peripheral stimulation were compared (a total of 79 microprobes; an average of 15 microprobes being inserted for each hourly period). Comparisons were also made to those in vitro microprobes that had only been exposed to radiolabelled dynorphin $A_{(1-8)}$ but processed concurrently with those in vivo (18 microprobes). Analysis of the data from six experiments provided no evidence that levels of ir-dynorphin $A_{(1-8)}$ were significantly enhanced 3–8 h after the FCA injections.

4. Discussion

The present experiments have detected ir-dynorphin $A_{(1-8)}$ in both normal rats and those with peripheral inflammation, but have found significant differences between the two groups. In the absence of any active peripheral stimulus, extracellular ir-dynorphin $A_{(1-8)}$ was found in the dorsal horn of normal rats but occurred throughout the dorsal and ventral horns of both sides of the spinal cord in rats with unilateral ankle inflammation. Differences were also seen when the ankle region was subjected to lateral compression. Ir-dynorphin $A_{(1-8)}$ appeared in the ventral horn not during, but after the period of compression in normal animals. With inflamed animals, compression of the inflamed ankle did result in release of ir-dynorphin during the period of stimulus application. This produced elevated levels at three main sites in the spinal grey matter. The largest peak was in the deep dorsal/upper ventral horn, with further sites of significant release in the mid dorsal horn and the lower ventral horn.

In order to consider the possible significance of our findings, it is first necessary to consider the possible sources of release of dynorphins in the spinal cord of normal rats and those with peripheral inflammation. The relevant structures include primary afferent fibres, intrinsic

neurones of the spinal cord and the spinal terminations of fibres of supraspinal origin. In normal animals many radioimmunoassay studies have shown higher levels of ir-prodynorphin derived peptides in the dorsal horn compared to the intermediate or ventral horn zones of the mammalian spinal cord (rat [3,45,60,81], rabbit [3], human [62]). Immunocytochemical studies in normal animals have localised ir-prodynorphin derived peptides mainly in cells and terminals of lamina I–II and lamina IV–VI of the mammalian spinal cord with moderate concentrations in lamina X (rat [8,9,37,38,48,49,52,63,76,79,80], cat [2,11,48,49]). It should be noted that most of these studies have used colchicine treated animals and hence favour detecting presence in cell bodies. Moreover, studies of mRNA for prodynorphin only locate sites of synthesis within cell bodies, and in normal rats these have been found mainly in the superficial and deep dorsal horn [36,58,59,61,63,79]. The induction of a peripheral inflammation has been found to increase the spinal content of these ir-prodynorphin derived peptides in studies in the rat employing radioimmunoassay [34,46,47,63] and immunocytochemical techniques [44,51,52,63,73,74,79,80]. This increase in peptide content has been shown to be preceded by an enhancement in the expression of the RNA message for prodynorphin [58,59,61,63,79] by cells in the superficial dorsal horn (laminae I–II), but notably also in deep dorsal horn (laminae IV–VI) neurons. In addition, under these conditions, neurons dorsolateral to the central canal (laminae VI, VII and X) have also been observed to contain high levels of prodynorphin mRNA [59,79].

Despite the presence of ir-dynorphin near the sites of termination of primary afferents in the dorsal horn, there is little evidence to support a significant presence of dynorphin in primary afferents, particularly in lumbar segments [2,72,78]. Dorsal rhizotomy has had little or no effect on the levels of ir-dynorphin in both the dorsal and ventral horns of the spinal cord [3,38,60]. There is also little evidence to support a significant presence of dynorphin in the spinal terminals of fibres of supraspinal origin [3,43]. Thus it appears that the cells releasing ir-dynorphin$_{(1-8)}$ in the present studies were predominantly intrinsic spinal neurones with their cell bodies in the superficial and deep dorsal horn. It needs to be emphasised, however, that where dynorphins are released will depend on the projections of these dynorphin-containing cells.

It has been suggested that some dynorphin-containing spinal neurones project to supraspinal areas [40,50–52,69]. More recent immunocytochemical studies, notably on non-colchicine treated animals, however, have emphasised the finding of ir-dynorphin labelled varicose fibres surrounding both large and intermediate sized motoneurones [37,38,79]. Klein et al. [38] found that the rat differed from the cat and primate in this respect. The location of the cell bodies of these dynorphin containing varicosities in the ventral horn were not known, but it is probable that they were intrinsic spinal neurones with their cell bodies located more dorsally. Such connections could be inter- and intra-segmental. Klein et al. [38] also found that electrical stimulation of the small and large diameter fibres of the ipsilateral sciatic nerve (1 Hz for 20 min) depleted ventral horn ir-dynorphin $A_{(1-8)}$. If such depletion results from prior release then these findings are in accord with some observations of the present experiments, where release in the ventral horn in addition to the dorsal horn was a prominent finding.

In antibody microprobe studies, a significant basal presence of a compound could result from a tonic release from an unknown stimulus, or from a continuous afferent input in nociceptors and other fibres as a result of the surgery required for these preparations. A contribution from cells and fibres ruptured during the introduction of microprobes cannot be excluded, although differing basal patterns observed in rats with microprobes bearing antibodies to differing peptides makes it unlikely that this is a significant factor [27,39,66]. Although the similar basal presence of ir-dynorphin $A_{(1-8)}$ in the dorsal horn of normal and inflamed rats could be the result of surgery, an enhanced afferent input was almost certainly responsible for the increased levels in the ventral horns of inflamed rats, particularly as active manipulations of the inflamed ankle region increased these levels still further. In the normal cat a basal presence of ir-dynorphin $A_{(1-17)}$ was detected by Hutchison et al. [30] using antibody microprobes, but the consequences of peripheral inflammation were not examined. The finding of bilateral increases in ventral horn ir-dynorphin in animals with a peripheral inflammation, when compared with normal animals, was unexpected, particularly as most studies of the changes in the messenger RNA levels for prodynorphin following unilateral inflammation have found only ipsilateral increases [12,13,34–36,58,63,79]. Przewlocka et al. [61], however, observed bilateral increases in the prodynorphin mRNA in the superficial dorsal horn following unilateral hindlimb inflammation but at relative later stages (5 and 14 days post-injection). An alternative explanation for our finding of bilateral increases in released ir-dynorphin $A_{(1-8)}$ associated with peripheral inflammation is that some of the cells containing this neuropeptide project bilaterally to the ventral horn.

There is evidence that immediate early gene products, such as c-fos, are responsible for the increased synthesis of prodynorphin following a spinal input from peripheral nociceptors (see Section 1). The delayed increase in ventral horn levels of ir-dynorphin $A_{(1-8)}$ following lateral compression of the ankle of normal rats may have resulted from such a mechanism. This implies a relatively rapid process (30 to 60 min) within which the arrival of an input in nociceptors is translated into increased expression of a gene and the release of a neuroactive product of that gene. Messenger RNA blot analysis has revealed a rapid and pronounced elevation in immediate early genes encoding c-fos and related proteins in the spinal cord within 30 min

of the injection of an inflammatory agent into the rat hindpaw [12,28,29]. Messenger RNA for prodynorphin in the spinal cord has been found to be increased within 4 h of the peripheral injection of an inflammatory agent [12,13,29,34,35,44]. Our studies of released ir-dynorphin $A_{(1-8)}$ suggest that the process of synthesis and release is occurring more quickly than can be revealed by indirect methods relating to release, such as mRNA expression and immunocytochemistry. Our finding of elevated levels of a neuropeptide both during and after a peripheral stimulus we would normally interpret as indicating a slow degradation following release, with the opportunity for diffusion to regions remote from sites of release [16,64]. This may still occur with dynorphin $A_{(1-8)}$, but it is possible that lateral compression of the inflamed ankles induced further synthesis and release of this neuropeptide.

Active manipulation of the inflamed tissues was an adequate stimulus for spinal release of ir-dynorphin in the present experiments. This is an important finding, since much of the recent literature emphasises the hyperexcitability of spinal neurones associated with peripheral inflammation [21,24,31,54,67,68] and has directed much research towards defining the neurochemical basis of such hyperexcitability. Although the effects of topically administered dynorphins led Hylden et al. [32] to propose these compounds as producing hyperexcitability of spinal neurones, Stiller et al. [71] found that the κ opiate antagonist nor-binaltorphamine enhanced the firing of spinal neurones in animals with peripheral inflammation. Significantly, firing to manipulation of inflamed peripheral tissues was also enhanced by the κ antagonist, suggesting that released dynorphins were acting to inhibit cellular responses. It appears that when inflammation develops peripherally, a complex response occurs in the spinal cord that involves both inhibitory [5,55,70] and excitatory neuroactive compounds [6,10,16,65,75].

Our finding of both basal and evoked release of ir-dynorphin$_{(1-8)}$ in several areas of the spinal cord does suggest an involvement of dynorphins in several aspects of spinal processing. Release in the superficial dorsal horn may be related to antinociception. The prominence of evoked release in the deep dorsal horn together with the ventral horn could reflect an influence on interneurones and motoneurones mediating spinal reflexes and supraspinal control of reflexes. Thus effects on motor behaviour, perhaps related to immobility and healing, are possible.

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