

THE EFFECTS OF OPIOIDS ON THE PERIPHERAL TERMINALS
OF RAT AND GUINEA PIG SENSORY NEURONS

by

Steven Blake Ketchum

A thesis submitted to the University of London
for the degree of Doctor of Philosophy

Sandoz Institute for Medical Research

and

Department of Pharmacology

University College London

Gower street

London WC1E 6BT

ProQuest Number: 10609870

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10609870

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Studies *in vivo* and *in vitro* suggest that opioids can modulate nociceptive signals by interacting with receptors on peripheral neurons. We investigated the peripheral actions of mu (μ), delta (δ), and kappa (κ) opioid agonists using an electrophysiological model of inflammatory-type nociception. Dorsal horn convergent neurons were recorded extracellularly in the halothane anesthetized intact adult rat. Subcutaneous injection of formalin into the hindpaw receptive field of these neurons results in two distinct phases of cell firing. Neither morphine, exogenous ligand for the μ receptor, nor the δ agonist Tyr-D-Ser-(tbu)-Gly-Phe-Leu-Thr (DSTBULET) influenced the formalin response when administered peripherally into the paw. The κ -selective ligand U50488H produced a dose-dependent, naloxone-reversible inhibition of both phases of formalin-induced activity which does not result from leakage of the drug into the systemic circulation. Intrathecal administration of μ and δ , but not κ , opioids has previously been shown to inhibit the biphasic formalin response in the adult rat. Our data suggest that different types of opioid receptors may be important in the periphery and spinal cord.

There is some indication that opioid receptor populations are different in adult and neonatal rat spinal cord. For example there are functional μ and κ , but not δ , opioid receptors in an *in vitro* model of nociceptive activity in the neonatal rat spinal cord. We looked at these apparent developmental differences in binding assays in which opioid receptors in the two tissues were characterized by measurements of ligand binding to crude membrane fractions. Results from binding studies agreed well with functional studies, in that δ opioid binding sites were not detected on neonatal rat spinal cord membranes. Levels of κ binding were higher in the neonate than in the adult.

Novel continuous clonal cell lines with some characteristics of nociceptive dorsal root ganglion (DRG) neurons were tested as a potential model system for the action of opioids on primary afferent nerve fibers. Two of the cell lines

expressed δ , but not μ or κ , opioid binding sites. We could not detect effects of δ opioids on potassium currents (as measured by ^{86}Rb efflux) or on the release of substance P-like immunoreactivity (SP-LI). We concluded that these cell lines were not good models for studying opioid action on sensory neurons.

Measurement of SP-LI release from guinea pig cardiac right ventricular slices did provide a useful model to study peripheral actions of opioids. Formalin (0.2%), capsaicin (100 nM-3 μM), and a depolarizing concentration of potassium (100 mM K^+) increased the outflow of SP-LI from heart slices. Agonists at μ , δ , and κ opioid receptors inhibited K^+ -stimulated release and these effects were reversed by naloxone to differing degrees. High concentrations of μ and κ ligands, in their own right, increased the outflow of SP-LI, and these results are compared to previous reports of opioid excitation. Formalin-evoked SP-LI release from heart slices was subject to modulation by opioids. These results agreed well with *in vivo* results, in that SP-LI release evoked by formalin was not inhibited by μ or δ opioid agonists, but was sensitive to blockade by the κ ligand U50488H.

We have demonstrated effects of opioids on the peripheral terminals of sensory neurons in two different models. Peripheral κ , but not μ or δ , receptors were important in modulating formalin-induced effects both *in vivo* and *in vitro*. Central κ receptors, at least in adult rats, have been shown not to influence the formalin response *in vivo* to the same extent as μ and δ receptors. *In vitro*, μ , δ , and κ opioids modulated responses to K^+ depolarization of sensory neurons at the peripheral terminals. These results provide strong evidence that peripheral opioid receptors can modulate nociceptive signals.

ACKNOWLEDGEMENTS

My heartfelt thanks to my supervisors, Dr. I.F. James and Dr. A.H. Dickenson, for their encouragement and invaluable advice during the course of this work.

I am also greatly indebted to Jane Haley for her contributions to our collaborative work and to the helpful discussions which it provoked.

My thanks also to Suki Hothi for her technical assistance, and to Neil Parrott, Tim Morgan, and Dr. Alistair Forbes for help in data processing matters.

I also wish to acknowledge Drs. A. Dray, G.M. Burgess, P.K. Mulderry, J.N. Wood, C.R. Snell, and D. Magnusson, and especially Ann Sullivan, for their many insightful suggestions and stimulating discussions.

I would like to express my gratitude to my parents, to the McNeills, and most of all to Mary, for their reassuring support throughout the writing of this thesis.

All of the work for this thesis was funded by the Sandoz Institute for Medical Research (SIMR). Most of the experiments were carried out in their laboratories. I would like to express my gratitude to Sandoz and to Dr. Humphrey Rang, the director of SIMR, for their generosity and for kindly providing me with the excellent facilities which made this thesis possible.

Some of the work presented in this thesis has been or will be published elsewhere:

James, I.F., Bettaney, J., Perkins, M.N., Ketchum, S.B., & Dray, A. (1990) Opioid receptor ligands in the neonatal rat spinal cord: binding and *in vitro* depression of the nociceptive responses. *Br. J. Pharmacol.* 99: 503-508.

Dray, A., James, I.F., Ketchum, S.B., & Perkins, M.N. (1989) Characterisation of opioid receptors in the neonatal rat spinal cord. *Br. J. Pharmacol.* 96 (Suppl.): 81P.

Dray, A., James, I.F., Ketchum, S.B., & Perkins, M.N. (1989) Opioid receptor types in neonatal rat spinal cord: Ligand binding and inhibition of nociception *in vitro*. In *Advances in the Biosciences, Vol. 75: Progress in Opioid Research* (19th International Narcotics Research Conference; Albi, France, 3-8 July 1988), pp. 65-68. Pergamon Press, Oxford.

James, I.F., Ketchum, S.B., Perkins, M.N., & Dray, A. (1988) Receptor types involved in opioid-induced antinociception in neonatal rat spinal cord *in vitro*. *Soc. Neurosci. Abstr.* 14 (1): 31.

Ketchum, S.B., Hothi, S.K., & James, I.F. (in preparation) Opioid modulation of SP-LI release from the peripheral terminals of sensory neurons in a guinea pig right ventricular slice preparation.

Haley, J.E., Ketchum, S.B., & Dickenson, A.H. (in preparation) Peripheral kappa opioid receptor-mediated antinociception in an electrophysiological model of the formalin response.

CONTENTS

	<u>Page</u>
Title	1
Abstract	2
Acknowledgements	4
Publications	5
Contents	6
<u>CHAPTER 1 - INTRODUCTION</u>	10
1-1	General introduction 11
1-2	Endogenous opioids and neuromodulation 13
1-3	Evidence for multiple types of opioid receptors 19
1-3.1	Historical background 19
1-3.2	Introduction to <i>in vitro</i> evidence for multiplicity of opioid receptors 23
1-3.3	Evidence for separate mu and delta opioid receptor types 26
1-3.4	Evidence for kappa receptors 32
1-3.5	Evidence for sigma receptors 35
1-3.6	Evidence for epsilon receptors 37
1-3.7	Other proposed types of opioid receptors 39
1-3.8	Evidence for subdivision of main types of opioid receptors into different subtypes 42
1-3.9	Summary 47
1-4	Functional roles of opioids 48
1-4.1	Introduction 48
1-4.2	Spinal analgesia 50
1-4.3	Supraspinal analgesia 55
1-4.4	Peripheral mechanisms of opioid analgesia 57
1-5	Introduction to the main text of the thesis 61
<u>CHAPTER 2 - METHODS AND MATERIALS</u>	62
2-1	Opioid binding sites in rat spinal cord homogenates 63
2-1.1	Neonatal rat spinal cord membranes 63
2-1.2	Adult rat spinal cord membranes 65
2-2	<i>In vivo</i> formalin model 66
2-2.1	Extracellular single-unit recording of rat

	dorsal horn convergent neurons	66
2-2.2	Release of substance P-like immunoreactivity (SP-LI) from superfused rat spinal cord <i>in vivo</i>	69
2-3	Radioimmunoassay for SP-LI	70
2-3.1	Iodination of SP	72
2-4	Primary cultures of rat dorsal root ganglion (DRG) sensory neurons	73
2-4.1	Neonatal rat DRG cell cultures	73
2-4.2	Adult rat DRG cell cultures	76
2-4.3	Uptake of Calcium-45 ($^{45}\text{Ca}^{2+}$) into populations of DRG	78
2-4.4	Release of SP-LI from DRG	79
2-5	Neuroblastoma x DRG (ND) hybrid cell lines	80
2-5.1	Hybrid cell tissue culture	80
2-5.2	Opioid binding sites on ND cells: Saturation and competition studies	82
2-5.3	Rubidium-86 ($^{86}\text{Rb}^+$) efflux from populations of ND clonal cells	83
2-5.4	SP-LI content of ND lines	83
2-5.5	Fura 2-AM measurements of intracellular calcium levels ($[\text{Ca}_i^{2+}]$) in ND cells	84
2-6	Guinea pig heart preparation	85
2-6.1	Content of SP-LI in extracts of individual guinea pig heart chambers	85
2-6.2	Opioid binding studies on individual chambers of guinea pig heart	86
2-6.3	Release of SP-LI from pooled slices of guinea pig right ventricle	87
<u>CHAPTER 3 - OPIOID BINDING SITES IN HOMOGENATES</u>		
<u>OF NEONATAL AND ADULT RAT SPINAL CORD</u>		
3-1	Introduction	90
3-2	Results	93
3-2.1	Neonatal rat spinal cord: Saturable binding of opioids	93
3-2.2	Competition binding assays	99
3-2.3	Adult rat spinal cord: Saturation binding studies	102
3-2.4	Effect of neonatal capsaicin treatment on saturable binding of opioids	104
3-3	Discussion	105

<u>CHAPTER 4 - IN VIVO ELECTROPHYSIOLOGY: EFFECTS</u>		
<u>OF PERIPHERALLY ADMINISTERED OPIOIDS</u>		
<u>ON FORMALIN-INDUCED DORSAL HORN</u>		
<u>NEURONAL ACTIVITY</u>		108
4-1	Introduction	109
4-2	Results	116
4-2.1	Prolonged neuronal responses to subcutaneous (s.c.) formalin	116
4-2.2	Peripheral (s.c.) kappa opioid inhibition of formalin response	118
4-2.3	Kappa depression of formalin-evoked activity is naloxone-reversible	121
4-2.4	Lack of kappa effect on electrically-evoked neuronal responses	123
4-2.5	Lack of peripheral mu and delta opioid effects on formalin-induced activity	125
4-2.6	Superfusion studies of exposed spinal cord <i>in vivo</i> : insignificant outflow of SP-LI centrally in response to peripheral formalin stimulation	127
4-3	Discussion	129
<u>CHAPTER 5 - OPIOID EFFECTS ON PRIMARY CULTURES</u>		
<u>OF RAT DORSAL ROOT GANGLION (DRG)</u>		
<u>SENSORY NEURONS</u>		132
5-1	Introduction	133
5-2	Results	136
5-2.1	Potassium (K ⁺)-induced uptake of Calcium-45 (⁴⁵ Ca ²⁺) into populations of rat DRG neurons	136
5-2.2	Inconsistent opioid modulation of K ⁺ -stimulated ⁴⁵ Ca ²⁺ uptake into DRG neurons	141
5-2.3	Inconsistent opioid modulation of K ⁺ -evoked SP-LI release from DRG neurons	143
5-3	Discussion	146
<u>CHAPTER 6 - OPIOID EFFECTS ON NEUROBLASTOMA x DRG</u>		
<u>(ND)CLONAL CELL LINES</u>		149
6-1	Introduction	150
6-2	Results	152
6-2.1	Naloxone-displaceable binding of [³ H]etorphine to ND hybrid cells	152
6-2.2	Opioid sites on ND cells: Saturation binding studies	152
6-2.3	Competitive binding studies	156

6-2.4	Lack of delta opioid modulation of Rubidium-86 ($^{86}\text{Rb}^+$) efflux from populations of ND cells	157
6-2.5	SP-LI content of ND clonal cell lines	159
6-3	Discussion	160
CHAPTER 7 -	<u>OPIOID MODULATION OF SP-LI RELEASE FROM THE PERIPHERAL TERMINALS OF SENSORY NEURONS IN A GUINEA PIG RIGHT VENTRICULAR SLICE PREPARATION</u>	
		163
7-1	Introduction	164
7-2	Results	170
7-2.1	Opioid binding studies on homogenates of individual guinea pig heart chambers	170
7-2.2	Content of SP-LI in extracts of individual guinea pig heart chambers	171
7-2.3	Releasibility of SP-LI from pooled slices of guinea pig right ventricle by potassium (100 mM K^+) depolarization	172
7-2.4	Apparent release of SP-LI by bradykinin explained by interference with the SP radioimmunoassay	174
7-2.5	Capsaicin stimulates release of SP-LI from heart slices and causes desensitization	176
7-2.6	Formalin (0.2%)-evoked outflow of SP-LI	179
7-2.7	Calcium dependence of SP-LI release	181
7-2.8	Noradrenaline modulates K^+ -evoked SP-LI outflow from guinea pig heart slices	182
7-2.9	Naloxone-reversible opioid inhibition of K^+ -stimulated SP-LI release	183
7-2.10	Stimulation of SP-LI outflow by a mu opioid and high concentrations of kappa ligands	187
7-2.11	Opioid effects on formalin-induced outflow of SP-LI	189
7-3	Discussion	190
CHAPTER 8 -	<u>CONCLUSIONS</u>	196
References		203

CHAPTER 1 -
INTRODUCTION

The medical world's fascination with the pharmacological effects of morphine and other opium derivatives (or opiates) greatly predates the Vietnam War, though a resurgence and amplification of scientific interest in opiates followed in its wake. Heightened public awareness of widespread opiate abuse among American G.I.'s led to increased government expenditure on basic opiate addiction research which, in turn, contributed to the growth of this field. Much money and effort, especially within the pharmaceutical industry, has been spent in search of non-narcotic opioid analgesics in which beneficial pain-killing is dissociated from harmful central side effects. Such drugs remain elusive and the unique therapeutic value of narcotic analgesics like morphine has guaranteed their continued use in the clinical control of pain. Despite this, a wealth of information has been gathered over the past two decades to greatly increase our basic understanding of the mechanisms underlying opiate action. Traditionally, opioids have been thought to produce their analgesic effects by interacting with receptors at spinal and supraspinal levels. Investigators, however, are increasingly addressing issues concerning the physiological relevance of opioid mechanisms at peripheral, in addition to central, sites. This thesis presents *in vivo* and *in vitro* evidence supporting a role for opioids in the modulation of nociceptive transmission via receptors located on the peripheral terminals of sensory neurons.

In this introductory chapter I will discuss the discovery of specific binding sites for opioids in neural tissue, the isolation of endogenous ligands for opioid receptors, and the subsequent pharmacological characterization of these peptides. Since much of this thesis will deal with characterizing the receptor types involved in peripheral opioid effects, section 1-3 will survey the literature for evidence supporting the existence of multiple types of opioid receptors. This will be followed by a discussion of the physiological basis of spinal and supraspinal opioid analgesia, pointing out that certain aspects of opioid action at the central endings of sensory nerves (e.g. inhibition of neuropeptide release) are analogous to those proposed for opioid effects at the peripheral endings (section 1-4).

The nomenclature adopted in this thesis is consistent with that outlined by Knapp et al. (1989). As alluded to earlier, "opiate" refers directly to substances produced from the juice of *Papavera somniferum*, the opium poppy, but this definition is sometimes extended to include morphine-like drugs in general. The term "opioid" encompasses all agents that bind to opioid receptors, including the endogenous opioid peptides. Interaction of an "opioid agonist" with its receptor characteristically produces a physiological effect which is antagonized by naloxone in a stereospecific manner. "Opioid antagonists", in contrast, occupy opioid receptors to prevent the binding of agonist ligands without producing tissue effects.

Early observations that morphine could inhibit acetylcholine release from guinea-pig enteric neurones (Paton, 1957; Schaumann, 1957) became the focal point of much research in the 1960s and 1970s as investigators tried to locate prime neurotransmitter targets for the actions of opiates. Analysis of the action of opioids on morphine-sensitive cholinergic junctions in guinea pig ileum (GPI; see Kosterlitz & Waterfield, 1975) and on morphine-sensitive adrenergic junctions in the mouse vas deferens (MVD; Henderson et al., 1972) suggested that these models had good predictive value for the potency of morphine-like drugs. Agonist and antagonist potencies of opioids in modulating electrically-evoked contractions of GPI, for example, correlated well with the affinities of these ligands for stereospecific [³H]naloxone binding sites in brain homogenates (see Kosterlitz & Waterfield, 1975). Rank orders of potency in the GPI bioassay also closely paralleled the relative abilities of these opioids to induce withdrawal in morphine-dependent monkeys and to cause analgesia in humans.

Scientific investigation into the effects of exogenously administered opioids attained new significance after groups in Aberdeen (Hughes, 1975) and Uppsala (Terenius & Wahlstrom, 1975) independently provided evidence for the existence of an endogenous ligand for the opiate receptor. The opioid-like activities of these mediators extracted from rodent or porcine brain were confirmed by testing their abilities to displace [³H]dihydromorphine binding to rat brain (Terenius & Wahlstrom, 1975), or to inhibit responses in the GPI and MVD smooth muscle bioassays (Hughes, 1975). Publication of the structure of the endogenous opiate-like ligands methionine- and leucine-enkephalin (Hughes et al., 1975) was followed by the discoveries of the opiate-like properties of the pituitary factors β -endorphin (Bradbury et al., 1976; Cox et al., 1976) and dynorphin (Goldstein et al., 1981). The complexity of this new-found neurochemical signalling system became further evident with the isolation and identification of a multiplicity of other biologically active opioid peptides (see review by Akil et al., 1984).

Studies on the origin of these opioid peptides led to the discovery that, like other neuroactive peptides, they are derived from the enzymatic cleavage of large molecular weight proteins, or prohormones. Three families of opioid peptides, the endorphins (Nakanishi et al., 1979), enkephalins (Noda et al., 1982), and dynorphins (Kakidani et al., 1982) are well-documented (see Table 1.1) with each family having a separate prohormone precursor that is synthesized with ribosomal participation and stored in vesicular compartments.

Table 1.1. *Correspondence between opioid peptide prohormone precursors, subsequent processing to yield the principal endogenous opioid ligands, and the resulting selectivity for opioid receptor type.*

Endogenous opioid prohormone precursor (opioid peptide family)	Receptor selectivity	Opioid peptide fragment
Proopiomelanocortin (endorphin)	μ delta epsilon	β -endorphin
Proenkephalin (enkephalin)	delta > μ	[Leu]enkephalin [Met]enkephalin [Met]enkephalin-Arg ⁶ -Gly ⁷ -Leu ⁸ [Met]enkephalin-Arg ⁶ -Phe ⁷ Peptide E
Prodynorphin (dynorphin)	kappa delta > μ	α -neo-endorphin β -neo-endorphin Dynorphin A-(1-8) Dynorphin A-(1-17) Dynorphin B-(1-13) [Leu]enkephalin

The various endogenous opioid ligands are selective, to greater or lesser extent, for different opioid receptor types. It is now known that gene products from the endorphin, enkephalin, and dynorphin opioid peptide families bind preferentially to epsilon, delta, and kappa receptors, respectively (Table 1.1). The conspicuous absence of a natural endogenous ligand with high selectivity for the μ opioid receptor has been addressed in recent reports implicating an endogenous role for the nonpeptide morphine. Shortly after Oka et al. (1985) isolated low levels of morphine from toad skin, Goldstein et al. (1985) detected

immunoreactive morphine in extracts of bovine hypothalamus and adrenal gland. Subsequent investigation to determine whether the origin of this morphine was endogenous or exogenous (e.g. introduced through the food chain) revealed that intravenous injection of morphinan biosynthetic precursors caused an increase in levels of codeine and morphine in rat brain, intestine, liver, kidney, and blood (Donnerer et al., 1986). Weitz et al. (1987) have been the first to demonstrate that the complex ring structure of morphine-like compounds can be synthesized from non-morphinan precursors in mammalian tissue. Previously, the intramolecular coupling reaction required for morphine biosynthesis was thought to be restricted to plants such as the opium poppy. The physiological significance of this discovery is still speculative, however, because biosynthesis of morphine-like compounds is detectable in rat liver but not rat brain and bovine adrenal.

Much of the work done to elucidate the pharmacological and physiological relevance of endogenous opioid peptides has focused on their possible roles as neuromodulators in the central and peripheral nervous systems. After processing and packaging, opioids are transported axonally to central and peripheral nerve terminals where they are presumably released in a neurotransmitter-like fashion and gain access to receptors. Deep brain stimulation of periaqueductal areas causing pain relief has been claimed to elevate cerebrospinal fluid (c.s.f.) β endorphin levels (Akil et al., 1978; Hosobuchi et al., 1979) but other studies find no correlation between levels of this endogenous opioid and pain relief (see reviews by Akil et al., 1984; Basbaum & Fields, 1984). Due to the complexities of pain transmission and modulation (see sections 1-3.2 to 1-3.4) a clear role of endogenous opioid peptides in setting the pain threshold is unlikely. Despite this, there is evidence for an involvement of enkephalins and dynorphins in certain pain-related phenomena.

Intra-arterial injections of bradykinin and sciatic nerve stimulation (Yaksh & Elde, 1980) and tooth pulp stimulation (Cesselin et al., 1982) all increase levels of methionine-enkephalin released into c.s.f. Spinal enkephalin (Cesselin et al., 1980) and dynorphin levels (Millan et al., 1986, 1987) are increased in rat models

of chronic arthritic pain. Levels of dynorphin (Iadarola et al., 1988; Millan et al., 1988) and enkephalin (Faccini et al., 1984) biosynthesis at spinal levels are likewise increased in more acute, localized models of inflammation. Noguchi et al. (1989) report that formalin injected into rat hindpaw increases the biosynthesis of enkephalin in the spinal cord.

A neuromodulatory role for endogenous opioid peptides is also indicated by their uneven tissue distribution. Studies have confirmed the localization of endogenous opioids to defined neuronal elements within both the central and peripheral nervous systems. High densities of enkephalin-containing neurons are found in the periaqueductal gray matter (Simantov et al., 1977) and other brain areas associated with pain and analgesia. In the spinal cord the highest concentrations of enkephalinergic (Hokfelt et al., 1977; Simantov et al., 1977) and dynorphinergic fibers (Khachaturian et al., 1982; Cruz & Basbaum, 1985) are present in the most superficial layers of the dorsal horn. Dynorphin has been identified in the terminals of afferent fibers as well as in intrinsic spinal neurons (Botticelli et al., 1981; Cruz & Basbaum, 1985), whereas evidence for the peripheral origins of the enkephalins has not yet been demonstrated (see Hokfelt et al., 1980). Rexed layers I and II, corresponding to the marginal cell zone, and the dorsal layer of the substantia gelatinosa, respectively, receive primary afferent terminals of small unmyelinated C-fibers and thinly myelinated A δ -fibers which are involved in the transmission of specific sensory modalities such as pain and temperature. One of the most likely sites of central opiate analgesic action is here at the point of entrance of nociceptive information into the spinal dorsal horn via small diameter primary sensory neurons. These primary afferent terminals then synapse with second-order neurons that relay sensory information from the periphery to higher centers in the brain-stem and thalamus.

Duggan et al. (1976,1977b) have demonstrated that the iontophoretic administration of morphine into the substantia gelatinosa naloxone-reversibly suppresses the activation of dorsal horn neurons by a peripheral noxious heat stimulus. This suggests that opioid receptors in the substantia gelatinosa (e.g.

lamina II of the spinal dorsal horn) may partly mediate the analgesic effects of opioids. Autoradiographic studies have since demonstrated opioid receptors to be concentrated within the upper laminae of the dorsal horn (Atweh & Kuhar, 1977; Goodman et al., 1980; Herkenham & Pert, 1982), and surgical sectioning of the dorsal roots has shown a proportion of these opioid binding sites to be associated with the central terminals of primary afferent fibers (Lamotte et al., 1976; Ninkovic et al., 1981b,1982).

High concentrations of the undecapeptide substance P, a member of the tachykinin family (Erspamer, 1981), have similarly been demonstrated in neurons terminating in the layers of the spinal cord associated with the processing of primary afferent nociceptive information (Hokfelt et al., 1977). SP-immunoreactive nerves in the spinal cord are, like opioid receptors, susceptible to at least partial depletion by dorsal rhizotomy (Nagy et al., 1983). This evidence, coupled with other findings that substance P-like immunoreactivity (SP-LI) is released from primary afferent nociceptive fibers (Kuraishi et al., 1985a), has implicated SP as a prime candidate for the transmitter of nociceptive information released at the central terminals of small diameter afferent sensory fibers (see reviews by Nicoll et al., 1980; Salt & Hill, 1983). Observations that opioids inhibit the release of SP *in vivo* (Yaksh et al., 1980) and *in vitro* (Jessell & Iversen, 1977) have led to the proposal that opioid modulation of nociceptive transmission may involve direct actions on primary afferent terminals. But suggestions that enkephalinergic axon terminals act presynaptically via axoaxonic synapses on SP-containing fibers in the superficial dorsal horn (Jessell & Iversen, 1977; Mudge et al., 1979) have not been supported by ultrastructural analysis which reveals that there are no close appositions between enkephalin-containing varicosities and primary afferent nerve terminals in the spinal cord (Hunt et al., 1980; Glazer & Basbaum, 1983). Alternatively, presynaptic opioid binding sites may be involved with other endogenous opioids such as dynorphin (Botticelli et al., 1981; Cruz & Basbaum, 1985). It cannot, however, be ruled out that opioids also act postsynaptically (Zieglgansberger & Bayerl, 1976; Zieglgansberger & Tulloch,

Further studies are clearly required to elucidate the exact nature of the direct modulation of spinal SP secretion by opioid receptors at the central terminals of primary afferent sensory neurons. Dale (1935) proposed that mediator substances released from the peripheral and central terminals of sensory neurons would be identical. SP-LI release from the peripheral terminals of sensory nerves has since been demonstrated in dental pulp (Olgart et al., 1977) and, from presumably sensory nerve endings, in a number of other peripheral tissues including canine paw skin (Jonsson et al., 1986), feline knee joint (Yaksh, 1988), and the guinea pig ureter (see Dray et al., 1989b), lung (Saria et al., 1988), and heart (Hoover, 1987; Geppetti et al., 1988). Yaksh (1988) takes this symmetry of mediator release one step further in suggesting that, given the bidirectional axonal transport of substance P (Harmar & Keen, 1982) and opioid binding sites (Young et al., 1980; Laduron, 1984), opioid receptor types involved in modulating nociceptive activity at central terminals will also be present at peripheral sensory nerve endings. In section 1-3.4, evidence for opioid modulation of SP-LI release from the peripheral terminals of sensory nerves will be discussed. Because studies at both the central and peripheral terminals address the varying abilities of different opioid ligands to suppress nociceptive transmission, it is necessary to review the historical evidence for multiple types of opioid receptors in neural tissue.

1-3.1 Historical background

The existence of distinct categories of receptor was first suggested by the work of Dale (1906) on adrenergic receptors, which showed that some of the effects of adrenaline and sympathetic nerve stimulation were antagonized by ergot alkaloids while other responses were unaffected. Later pharmacological work by Ahlquist (1948) conclusively demonstrated that the rank order of potencies of a series of catecholamines fell into two distinct patterns. Based on these results, he postulated the existence of two "types" of adrenoceptors, alpha (α) and beta (β), and later studies using selective antagonists have indicated that further subdivisions, or subtypes, of both α - and β -adrenergic receptors are likely (Lands et al., 1967; Berthelsen & Pettinger, 1977). It has since been shown that receptor heterogeneity, far from being exceptional, is a common pharmacological phenomenon. Multiple types, and in some cases subtypes, of receptors have been proposed for such neurotransmitters as acetylcholine (Bonner, 1989), dopamine (Kebabian & Calne, 1979), γ -aminobutyric acid (GABA; see review by Simmonds, 1983), excitatory amino acids (see review by Watkins, 1984), histamine (Hough & Green, 1984), and serotonin (5-hydroxytryptamine, or 5-HT; Peroutka & Snyder, 1982; Straughan, 1984). Such discoveries have revealed the flexibility and complexity of neurotransmitter action. This complexity has become even more apparent in the past decade which has seen the identification of more than thirty putative peptide neurotransmitters (see Iversen, 1983), with some of these neuropeptide systems involving multiple types of receptors.

The existence of opioid receptors has been accepted ever since Beckett and Casey (1954) established the structural basis for narcotic analgesia. One striking observation was the dramatic difference in analgesic activity between levorphanol and dextrorphan, stereoisomers of the same morphinan compound. Portoghese (1965) suggested the existence of multiple types of opioid receptors to explain how stereochemically dissimilar narcotic analogues, from the morphine, morphinan,

benzomorphan, and phenylpiperidine series of compounds, can have similar analgesic effects. A procedure introduced by Goldstein et al. (1971) provided the first demonstration of specific opioid binding sites. In this study a low percentage of [³H]levorphanol binding to mouse brain homogenates was displaced by unlabelled levorphanol but not by its inactive enantiomer dextrorphan. Subsequent modifications of this technique to reduce the non-specific binding of radioligands to membrane preparations firmly established the presence of stereospecific opioid binding sites in the central nervous system (CNS) of animals (Pert & Snyder, 1973; Simon et al., 1973; Terenius, 1973) and man (Hiller et al., 1973). The subsequent discovery of the enkephalins by Hughes et al. (1975) confirmed the importance of opioid binding sites, and fuelled speculation about the possible existence of a variety of endogenous opioid peptides each potentially exerting its action through a different type of opioid receptor.

Martin and colleagues, in experiments studying the effects of several opiates in chronic spinal dogs, provided the first pharmacological evidence for different types of opioid receptors (Gilbert & Martin, 1976; Martin et al., 1976). Their characterization and nomenclature of multiple receptors derived from findings that morphine, ketocyclazocine, and N-allylnormetazocine (SKF-10,047) exhibited differing profiles of activity on physiological measurements such as pupil size, pulse and respiratory rates, and body temperature. Furthermore, these different opiates were shown to produce different effects upon chronic administration. For example, in dogs made dependent to morphine, neither ketocyclazocine nor SKF-10,047 could suppress withdrawal symptoms, whereas morphine-like drugs could prevent them. On the basis of these results, Martin and his co-workers proposed the existence of three types of opioid receptors which they called mu (μ), kappa (κ), and sigma (σ), for the drugs morphine, ketocyclazocine, and SKF-10,047, respectively.

The use of smooth muscle preparations *in vitro* has greatly aided the further characterization of opioid receptor types. Structure-activity studies in the guinea pig ileum (GPI) and mouse vas deferens (MVD) are based on the potencies of

opioid agonists and antagonists in modulating electrically-evoked contractions (see Kosterlitz & Waterfield, 1975). Lord et al. (1977) showed that enkephalins are more potent than morphine in inhibiting electrically-stimulated contractions of mouse vas deferens (MVD). They called the receptor in MVD delta for deferens. Schulz et al. (1979) described the presence of another type of opioid receptor in rat vas deferens (RVD). This receptor type, named epsilon, was shown to be more sensitive to human beta (β_H)-endorphin than to morphine or enkephalins.

Some questions still remain concerning the existence of ϵ -receptors, and indications are that the sigma receptor is not an opioid receptor but may instead mediate the actions of phencyclidine (PCP)-like compounds via excitatory amino acid receptor interactions (see review by Sonders et al., 1988). Few groups dispute the existence of mu, delta, and kappa opioid receptors (see reviews by Miller, 1982; Wood, 1982; Chang, 1984; Goldstein & James, 1984a; Simon, 1986). This section will focus on current *in vitro* evidence for these types, with proper recognition of other proposed types of opioid receptors. *In vivo* work largely confirms the existence of three main types of opioid receptors, but presentation of this functional evidence will mostly be deferred to section 1-3 and Chapter 4 because *in vivo* interpretation can be complicated by differences in pharmacokinetics and metabolism of different drugs. The different types of opioid receptor are summarized in Table 1.2, though some questions about identification and classification of opioid receptors are certainly not settled and still bear critical discussion (see below).

Table 1.2. Summary of the current prototypical agonists for the multiple types of opioid receptors present in various isolated tissue preparations. References are to reports of the specific ligands. For discussion of the receptors and tissues see the text. Abbreviations: DAGO, [D-Ala²,MePhe⁴,Gly(ol)⁵]enkephalin; DPDPE, [D-Pen², D-Pen⁵] enkephalin; DPLPE, [D-Pen²,L-Pen⁵] enkephalin; U50488H, trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methane sulfonate; U69593, (5_α,7_α,8_β)-(-)-N-methyl-N[7-(1-pyrrolidinyl-1-oxaspiro(4,5)-dec-7-8-yl)]benzeneacetamide; PD117302, (±)-trans-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzo [b]-thiophene-4-acetamide hydrochloride.

Receptor type	Tissue	Ligands	References
mu (μ)	brain GPI MVD	morphine DAGO sufentanil	Handa et al., 1981 Leysen et al., 1983
delta (δ)	brain NG108-15 cells MVD hamster vas deferens	DPDPE DPLPE	Mosberg et al., 1983
kappa (κ)	brain GPI MVD rabbit vas deferens	dynorphin U50488H U69593 PD117302	James, 1986 Piercey et al., 1982 Lahti et al., 1985 Clark et al., 1988
epsilon (ε)	brain ? rat vas deferens	β-endorphin	Schulz et al., 1981b

Five lines of investigation have established that there are multiple types of opioid receptors:

I. Rank potency studies on smooth muscle tissues

The key clue to the existence of different receptor types came from measurements of the rank order of potency for a series of opiate agonists in different smooth muscle bioassays and in competitive binding assays where sites on brain homogenates were labelled with different radioligands. If there is only one type of receptor in all these systems, the rank order of potency should not change. Hence, differences in rank potencies imply different receptor populations. Once such differences have been seen they also provide a basis for measuring selectivity and allow the development of ligands that are selective for the different sites. Synthesis of opioid ligands with increased specificity for only one type of opioid receptor is essential for characterization studies because endogenous opioids are generally poorly selective. The current prototypical agonists for the different receptors are listed in Table 1.2. For comparisons of selectivity at mu, delta, and kappa binding sites see Goldstein and colleagues (James & Goldstein, 1984; Goldstein & Naidu, 1989). The mu receptor is the classical morphine receptor, but both sufentanil and the enkephalin analog [D-Ala²,MePhe⁴,Gly(ol)⁵]enkephalin (DAGO) are more mu-selective than morphine. Enkephalins are generally taken to be delta ligands, but in fact they discriminate poorly between mu and delta receptors. The bridged bis-penicillamine analogs [D-Pen²,D-Pen⁵]enkephalin (DPDPE) and [D-Pen²,L-Pen⁵]enkephalin (DPLPE) are much more selective for delta receptors than the parent peptides. The nonpeptide compounds U50488H, U69593, and PD117302 are highly selective for kappa over mu and delta receptors. Dynorphin and tifluadom are a little less selective, but still clearly kappa ligands. There is as yet no selective ligand for epsilon receptors. β -endorphin certainly binds at epsilon sites but also has good

affinity for mu, delta, and kappa sites. Once identified, selective ligands can be used to study anatomical distribution of the different receptors.

II. Antagonist studies

Different opioid and opiate agonists are differentially sensitive to antagonists in the guinea pig ileum and mouse vas deferens smooth muscle bioassays. The difference is commonly expressed as the antagonist K_e , an estimate of the dissociation constant for the antagonist at the site through which the agonist is producing its effect (see Kosterlitz & Watt, 1968). Different dissociation constants for the same antagonist in competition with different agonists suggests different opioid binding sites.

III. Receptor binding studies on brain homogenates

Opioid binding assays with brain homogenates have also provided important measurements of receptors *in vitro*. Again, rank orders of potency are the important factor in these studies which have analyzed the competition of a variety of unlabelled ligands against different radioligands. Historically, clear demonstration of multiple opioid receptors in binding studies necessitates nearly exclusive labelling of one type or subtype of opioid site (see review by Leslie, 1987). Before relying on a binding assay, however, it is essential to relate properties of the binding sites to activity in appropriate bioassays and establish that the sites do indeed represent relevant receptors.

IV. Protection of opioid binding sites

Activity of different opioids can be selectively inhibited either by induction of tolerance or by alkylation of tissue. The selective effects of these procedures again suggest the existence of different receptor types. A variation on selective alkylation is selective protection from alkylation, where a non-selective alkylating agent is used in the presence of a selective protecting ligand. This leads to inactivation of all sites except the one to which the protector binds.

V. Differential anatomical localization of opioid receptor types

There are differences in anatomical distribution of different receptor types. Though this conclusion requires that sites have already been identified and methods for measuring each site have been developed, it provides strong additional evidence that there are indeed different receptors. It also suggests that different receptors may have different functions.

Evidence from categories I.-V. for mu, delta, kappa, sigma, epsilon, and lambda receptors is presented below.

Lord et al. (1977) used a variety of assay systems in parallel to analyze evidence for non-homogeneous groups of receptors. *In vitro* isolated smooth muscle preparations such as the myenteric plexus-longitudinal muscle preparation of guinea pig ileum (GPI; Paton, 1955) and mouse vas deferens (MVD; Henderson et al., 1972) proved instrumental to discoveries made in these studies.

In these different assays a variety of opiates and opioid peptides were tested for their relative abilities to depress electrically-induced contractions. Lord et al. (1977) found that the GPI was more sensitive to the inhibitory actions of morphine and classical morphine-like compounds than to enkephalins, and thus concluded that the receptor population in GPI resembled Martin's mu opioid receptor. In contrast, they found that enkephalins exhibited greater potency than morphine in MVD and so called this new type of opioid receptor delta (Lord et al., 1976) for deferens.

This evidence for heterogeneous opioid receptors was strongly reinforced by antagonist studies on these same assay systems. In a modification of the method of Arunlakshana and Schild (1959), Lord et al. (1977) measured the effectiveness of the opioid antagonist N-allylnoroxymorphone (naloxone) against a range of opioid peptides and expressed the results in terms of the equilibrium dissociation constant K_e (see also Kosterlitz and Watt, 1968). The antagonist K_e in these experiments is an estimate of the concentration of naloxone which requires a doubling of the concentration of a specified opioid agonist in order to depress smooth muscle contractions to the same extent as in the absence of naloxone. *In vitro*, where it is usually possible to determine the tissue concentration of the antagonist, the K_e can also be expressed as its negative logarithm, or pA_2 value (Furchgott, 1972). In this way, different K_e values for naloxone in competition with different agonists would indicate different populations of receptors. This is what Lord and colleagues (1977) observed, namely that enkephalins were much less sensitive to reversal by naloxone than was morphine in the MVD bioassay. In GPI the enkephalins and morphine were equally antagonized by naloxone,

suggesting that in this tissue the enkephalins were acting through μ -receptors. It is now known that the enkephalins discriminate poorly between mu and delta receptors (James & Goldstein, 1984).

Lord et al. (1977) also found that these receptor types were readily differentiated in binding studies on guinea pig brain homogenates. Their Scatchard analysis of [^3H][Leu]enkephalin binding was non-linear, suggesting the heterogeneous nature of opioid receptors in brain membranes. They provided more compelling evidence for the existence of separate binding sites in reporting that a series of unlabelled opioid peptides competed more effectively for [^3H][Leu]enkephalin binding sites than for sites labelled by [^3H]naloxone, whereas unlabelled morphine was more effective in inhibiting [^3H]naloxone binding. This evidence that the two radioligands label different populations of binding sites agreed well with the results from the GPI and MVD bioassays which indicated heterogeneity of opioid receptor types.

Chang and Cuatrecasas (1979) provided further evidence for the existence of separate μ - and δ -binding sites in receptor binding studies on rat brain homogenates. In these studies they used [^3H]dihydromorphine (DHM) and [^3H]naloxone to label μ -sites selectively and ^{125}I -[D-Ala²,D-Leu⁵]enkephalin (DADLE) as a selective ligand for δ receptors. The synthesis of enkephalin analogues such as DADLE has helped investigators because of their improved stability over the native enkephalins. The authors described non-linearity of Scatchard plots of receptor binding isotherms for ^{125}I -DADLE, thus suggesting heterogeneity of enkephalin binding sites in rat brain. They also reported that the rank order of potencies for morphine and enkephalins in competition for [^3H]DHM or [^3H]naloxone binding sites was different from that in competition for the higher affinity ^{125}I -DADLE sites. The lower affinity ^{125}I -DADLE binding sites reportedly had properties similar to the [^3H]DHM and [^3H]naloxone sites. Chang and Cuatrecasas (1979) thus confirmed the differentiation of morphine, or mu-type, and enkephalin, or delta-type, binding sites as described earlier by Lord and colleagues (1977). Other investigators (Childers et al., 1979; Gillan et al.,

1980; Leslie et al., 1980) have extended the range of radioactively labeled opioid ligands and animal tissues studied, and have further substantiated the proof of separate μ - and δ -opioid receptor populations.

Selective protection experiments have also provided strong evidence for separate mu and delta opioid binding sites. These techniques effectively enrich membrane preparations in a single type of opioid binding site by inactivating other sites. This was useful because it allowed investigators to circumvent technical problems caused by the poor selectivity of radioligands then available. Robson and Kosterlitz (1979) treated guinea pig brain membranes with the non-specific alkylating agent phenoxybenzamine to inactivate binding sites for [³H]DHM and [³H]DADLE irreversibly. In these studies unlabelled DHM was shown to protect [³H]DHM binding sites from inactivation by phenoxybenzamine more effectively than unlabelled DADLE. Conversely, [³H]DADLE sites were better protected by unlabelled DADLE than unlabelled DHM. These results were in excellent agreement with those of Smith and Simon (1980) who carried out similar protection studies using N-ethylmaleimide (NEM) to alkylate opioid binding sites. They found that the irreversible inactivation of [³H]naltrexone binding sites by NEM was prevented more effectively by the unlabelled forms of naltrexone and morphine than by [D-Ala²,Leu]enkephalin and [D-Ala²,Met]enkephalin. Conversely, the enkephalins were more effective than morphine and naltrexone in protecting [³H]DADLE sites.

Site-directed alkylation represents a modification of earlier selective protection techniques, and has been a significant means of investigating different types of opioid receptor in the same tissue. Site-directed agents offer the advantage that, unlike the widespread alkylation obtained with phenoxybenzamine and NEM, they do not interfere non-specifically with pharmacological tissue responses and so can be used in smooth muscle bioassays such as GPI (Chavkin & Goldstein, 1981a; Takemori et al., 1981) and MVD (Ward et al., 1982; Cox & Chavkin, 1983). James and Goldstein (James et al., 1982; Goldstein & James, 1984b; James & Goldstein, 1984) developed a method of enriching tissues in

opioid binding sites using the alkylating opiate β -chlomaltrexamine (β -CNA; Portoghese et al., 1979), a derivative of the antagonist naltrexone. β -CNA will inactivate mu, delta, and kappa opioid binding sites, but enrichment can be obtained by treating membranes in the presence of appropriate protecting ligands. In these studies, sufentanil and DADLE were used to protect selectively mu and delta sites, respectively, from alkylation. Selective inactivation of a single receptor type can be achieved by essentially the same technique. Reagents such as β -funaltrexamine (β -FNA; Portoghese et al., 1980; Ward et al., 1982,1985; Tam & Liu-Chen, 1986) and an isothiocyanate derivative of etonitazene (BIT; Rice et al., 1983) selectively alkylate mu sites. And successful selective inactivation of delta sites (Rice et al., 1983) has been reported with fumaramido oripavine (FAO) and fentanyl isothiocyanate (FIT).

The above evidence for receptor heterogeneity is further supported by tolerance studies, which represent an alternative means of inactivating opioid receptors in isolated tissue preparations or whole animals. A characteristic feature of chronic exposure to opiates is the development of tolerance. Investigators have employed this phenomenon to test the hypothesis that the different types of opioid receptors are functionally independent of one another. The argument being that, if different opioid receptor populations exist, it should be possible to activate a single type of receptor chronically and induce tolerance selectively without affecting pharmacologic activity at other opioid receptor types.

Schulz et al. (1980) induced selective tolerance in mice by implanting osmotic minipumps subcutaneously to infuse the animals chronically with receptor-selective opioid agonists. Vasa deferentia were then removed and set up *in vitro* to compare the electrically evoked twitching responses of opioid-tolerant tissue preparations with control, opioid-naive preparations. The results showed that vasa deferentia from DADLE-infused mice are highly tolerant to DADLE and other delta agonists such as [Leu]enkephalin and [Met]enkephalin, but not to the mu agonists sufentanil, dihydromorphine (DHM), or normorphine. Analogously, they found that preparations from sufentanil-infused mice are tolerant to sufentanil,

and show cross-tolerance to the other mu opioids DHM and normorphine, but lack cross-tolerance to DADLE and the enkephalins. Selective tolerance has also been demonstrated in the guinea pig ileum and in intact animals (Schulz et al., 1981a,c).

This next section describes research which has stemmed from the premise that if the different types of opioid receptors are physically separate entities (e.g. are not interconverting, nor linked by allosteric interactions; see section 1-3.7 for discussion of these topics), then it should be possible to show their differential localization. Chang et al. (1979) and Simon et al. (1980) used *in vitro* receptor binding and competition studies on regional homogenates to show that the distribution of mu and delta binding sites varies between brain regions in the rat. This differential localization of opioid receptors in rat brain has also been confirmed by the more precise techniques of *in vitro* receptor autoradiography (Young & Kuhar, 1979; Herkenham & Pert, 1982). And although similar results have been reported in binding assays on bovine (Ninkovic et al., 1981a) and postmortem human brain (Bonnet et al., 1981) tissue, and in autoradiographic mapping studies of the human central nervous system (Maurer et al., 1983), extensive cross-species comparisons are hindered by a lack of detailed information on opioid receptor distribution in these other species.

The literature is most extensive on distribution studies in the rat, therefore to reduce confusion I will be focusing on this species. Although there is nearly consensus opinion in favor of the differential localization of mu and delta opioid binding sites in rat brain (Goodman et al., 1980; Herkenham & Pert, 1980; Duka et al., 1981; Lewis et al., 1983; Quirion et al., 1983) some inconsistencies are apparent in the literature. Many of these discrepancies have arisen because of the lack of highly selective ligands, and so, to further reduce confusion, I will discuss only several of the latest distribution studies which have used the most selective ligands available.

Using [³H]DAGO and [³H]DPDPE to label mu and delta opioid receptors, respectively, Mansour et al. (1986, 1987) reported distinct differences in the anatomical distributions of mu and delta binding sites. One goal of distribution

studies in general is to provide insight into the possible functional roles of opioid receptors. So that particular attention is often paid to those brain areas typically associated with the processing of nociceptive information, including the thalamus, periaqueductal gray matter, and the medullary raphe nuclei. Mansour et al. (1987) observed high levels of mu binding and correspondingly low levels of delta sites in thalamus, ventral periaqueductal gray, and median raphe of rat brain, and noted this consistency with the possible role of mu receptors in the integration of sensory information (Lewis et al., 1981). The authors reported high levels of delta and low levels of mu sites in caudate putamen, with substantial binding to both sites in cortex and amygdala. Even when the different receptor types were found in the same brain area, there were differences in the precise laminar or regional localization of the two sites. For example, in the frontal parietal cortex, mu sites were concentrated in layers I and IV, while delta binding was densest in layers II, III, V, and VI (Mansour et al., 1987). Similarly, in amygdala, mu binding was found in the lateral, basolateral, and medial nuclear groups, while delta sites were limited to lateral and basolateral nuclei (Mansour et al., 1986).

Martin and colleagues (Gilbert & Martin, 1976; Martin et al., 1976) postulated the existence of the kappa opioid receptor based on *in vivo* studies with ketocyclazocine, a benzomorphan-type opiate. Another member of this class of opiates, ethylketocyclazocine (EKC), has been used as the prototypical kappa ligand for most of the early *in vitro* work on kappa receptors. Initially, binding experiments which attempted to label [³H]EKC sites on rat brain membranes did not produce evidence for a separate kappa binding site (Chang et al., 1980; Hiller & Simon, 1980; Pasternak, 1980). With hindsight, these early studies were unsuccessful probably because of the low concentration of kappa receptors in rat brain (Harris & Sethy, 1980) and the relatively poor selectivity of EKC, which also binds to mu and delta sites (Romer et al., 1980; James & Goldstein, 1984.)

Kosterlitz et al. (1981) were able to demonstrate kappa-like binding sites in guinea pig brain, a tissue relatively rich in kappa opioid receptors. Their Scatchard analysis showed high- and low-affinity sites for the saturable stereospecific binding of [³H]EKC to brain homogenates, and they differentiated [³H]EKC sites from [³H]dihydromorphone (DHM) sites in competition assays. For example, the mu ligand DAGO had high affinity for [³H]DHM sites but competed poorly for [³H]EKC sites. Kosterlitz and his colleagues also showed that the putative kappa agonists EKC, bremazocine, and MR 2034 ((-)-(1R,5R,9R,2"S)-5,9-dimethyl-2-tetrahydrofurfuryl-2'-hydroxy-6,7-benzomorphan) differentiated poorly between [³H]DHM and [³H]EKC sites, thus confirming other reports that these unlabelled kappa ligands have high affinities for both mu and kappa binding sites in brain homogenates of guinea pig and rat (Chang et al., 1980; Harris & Sethy, 1980; Hiller & Simon, 1980; Romer et al., 1980; Snyder & Goodman, 1980). They estimated that about 30% of the opioid sites in guinea pig brain are kappa-like.

Evidence for the existence of opioid receptors in rat brain which show a preference for kappa-type benzomorphans has also been obtained. Wood et al. (1981) compared the abilities of 26 opiates and opioid peptides to compete for [³H]EKC, [³H]DHM, and [³H]DADLE binding sites in rat brain and found

evidence for labelling of kappa-like sites by [³H]EKC. Chang et al. (1981) used saturating concentrations of morphiceptin and DADLE to block binding of [³H]diprenorphine to mu and delta binding sites, respectively, on rat brain membranes. They found that the remaining population of sites had relatively low affinities for mu and delta ligands compared to kappa ligands. Here the technique of blocking cross-reacting sites unmasked the binding of [³H]diprenorphine to kappa-like sites. Gillan and Kosterlitz (1982) used this blocking technique to unmask binding of [³H]bremazocine to kappa sites in rat brain. They used DAGO to block mu sites and DADLE to block delta sites. The blocking technique has also been used to study sigma receptors.

Binding sites for [³H]EKC in guinea pig brain can be inactivated by alkylation with phenoxybenzamine and protected from inactivation with EKC, which is a more potent protector than either DHM or DADLE. EKC was not selective in these experiments and also protected [³H]DHM and [³H]DADLE sites from phenoxybenzamine (Kosterlitz et al., 1981). The site-directed alkylating agent β -CNA can also be used to inactivate opioid binding sites in guinea pig brain. Dynorphin A protected high affinity sites for [³H]EKC without protecting [³H]DHM and [³H]DADLE sites. The sites protected by dynorphin had kappa-like properties in competition binding assays (James et al., 1982).

In the GPI, the naloxone K_e for a series of benzomorphans is higher than for normorphine (Hutchinson et al., 1975). Since enkephalins act at mu receptors in GPI (Lord et al., 1977), it is unlikely that delta receptors are active in this tissue. The benzomorphan receptors, therefore, are different from mu and delta types, and Lord et al. (1977) suggest that they correspond to kappa receptors.

As in the binding assay, opioid receptors in smooth muscle bioassays can be inactivated with β -CNA. In GPI dynorphin A-(1-13) selectively protects EKC activity without protecting activity of normorphine or [Leu]enkephalin (Chavkin et al., 1982; Cox & Chavkin, 1983). Inactivation of kappa receptors can also be achieved by selective induction of tolerance. For example, vasa deferentia from mice tolerant to ketocyclazocine were cross-tolerant to other kappa agonists, but

not mu or delta agonists (Wuster et al., 1981). Vasa made tolerant to dynorphin were tolerant to EKC and to peptides that act through kappa receptors, but were not tolerant to peptides that activate delta receptors (Schulz et al., 1982).

The rabbit vas deferens is insensitive to morphine, enkephalins, β -endorphin and SKF-10,047, but agonists with some activity at kappa receptors (for example EKC, ketocyclazocine, and bremazocine) are active. This has led to the suggestion that the rabbit vas deferens contains only the kappa type of opioid receptor (Oka et al., 1981). Indeed the rabbit vas deferens has been used to assess kappa activity of several opioid agonists (Oka et al., 1982; Hayes & Kelly, 1985; Kosterlitz et al., 1986).

The distribution of kappa receptors in rat brain is different from mu and delta receptors, with relatively high levels in amygdala, caudate putamen, hypothalamus, and median eminence (Mansour et al., 1986,1987). There is a high density of kappa receptors in deep layers of cerebral cortex in guinea pig brain, where there are some delta but very few mu sites (Goodman & Snyder, 1982a,b). Opioid receptors in guinea pig cerebellum are mostly kappa (Robson et al., 1984), and in rat pituitary are exclusively kappa (Herkenham et al., 1986).

Two patterns emerged from early competition binding experiments with putative sigma opioid receptors in mammalian brain membranes (see reviews by Quirion et al., 1987; Sonders et al., 1988). In one pattern the sites had high affinity for benzomorphan opiates (cyclazocine, pentazocine, ketocyclazocine) and haloperidol, and low affinity for phencyclidine (PCP) and N-ethyl-1-phenylcyclohexylamine (PCE). In the other pattern the sites had high affinity for cyclazocine, PCP, and PCE and low affinity for pentazocine, ketocyclazocine, and haloperidol compared to SKF 10,047. This difference was confirmed in a later study by Sircar et al. (1986), who found at least two sites for (+)-[³H]SKF 10,047 in rat brain. The lower affinity, more numerous site was similar to the first pattern in competition assays. The higher affinity sites corresponded to the second pattern. Sircar et al. (1986) suggested that the low affinity site for (+)-SKF 10,047 is the sigma opioid receptor and is responsible for the psychotomimetic effects of sigma opiates.

This low affinity (+)-SKF 10,047 binding site, previously termed the common PCP/ σ receptor, is now known as the PCP receptor (see review by Sonders et al., 1988). The high affinity site is now called the haloperidol-sensitive sigma receptor. The reported differences in neuroanatomical distribution of these two sites in rodent brain provides the strongest evidence that they are distinct entities (Largent et al., 1986; Sircar et al., 1986). Recent characterization of these sites has been aided by the development of selective radioligands for the two binding sites. The most recent autoradiographic studies have labelled the haloperidol-sensitive sigma receptors with [³H](+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ([³H](+)-3-PPP; Largent et al., 1986) and [³H]1,3-di-ortho-tolyl guanidine ([³H]DTG; Weber et al., 1986; McLean & Weber, 1988) and PCP sites with the PCP congener [³H]-1-[1-(2-thienyl)cyclohexyl]piperidine ([³H]TCP; Vignon et al., 1986).

However, most ligands show considerable crossreactivity, which is a major reason why it is still unclear which site mediates the psychotomimetic effects of

benzomorphan opiates and PCP. *In vivo* pharmacological characterization of the sigma site has yet to be clearly demonstrated, and it remains unclear whether sigma receptors are physiologically relevant in mediating responses to exogenously administered ligands and their putative endogenous counterparts (Su et al., 1986; Contreras et al., 1987). Sonders et al. (1988) direct attention to recent studies which suggest that the N-methyl-D-aspartate (NMDA) subtype of excitatory amino acid receptors may mediate some of the behavioral effects of PCP and sigma opiates. PCP binding sites are reportedly linked with the NMDA receptor-channel complex, though there may be multiple subtypes of PCP binding sites in rat brain associated with different structures (Itzhak, 1989; Vignon et al., 1989).

Neither the haloperidol-sensitive sigma site nor the PCP site are strictly opioid receptors because of the very low affinity for naloxone, and the relationship of these sites to the σ opioid receptor first proposed by Martin et al. (1976) has not yet been established.

Lord et al. (1977) described distinct mu and delta opioid receptor populations in GPI and MVD, respectively, based on results showing that the rank potencies of different opioids to inhibit electrically-induced contractions varied between the two tissues. A number of groups have applied this same methodology to studies on rat vas deferens (RVD), and have gathered evidence for the existence of another type of receptor, called epsilon (ϵ), which is selective for β -endorphin (Lemaire et al., 1978a,b; Schulz et al., 1979). Although morphine can antagonize the actions of β -endorphin in RVD (Huidobro-Toro et al., 1982), Wuster et al. (1978,1980) have reported that the potencies of morphine, enkephalins, and other opiates are a hundred- to a thousand-fold lower in RVD than in GPI and MVD. Analysis of the structural requirements for activity of β -endorphin and its fragments has revealed that, whereas the long-chained peptide β -endorphin (sequence 1-31) and long fragments (i.e. sequence 1-23) exhibit a similar potency in all three bioassays, shorter fragments lose activity in the RVD as compared to MVD and GPI (Schulz et al., 1981b). Studies on the interactions of different agonists present at the same time in rat vas preparations also suggest that β -endorphin interacts at receptors different from those activated by DADLE or mu agonists (Garzon et al., 1985). Hence, rat vas deferens contains mu receptors, but also receptors that prefer β -endorphin over any other class of opiates or opioids.

Cross-tolerance studies have been carried out on RVD, and provide further evidence for a separate population of receptors in this tissue which are highly selective for β -endorphin. Schulz et al. (1981b) have shown that vasa deferentia from rats chronically infused with etorphine are cross-tolerant to the mu agonist sufentanil, but not to β -endorphin.

Though there are several studies of β -endorphin binding (Akil et al., 1980; Ferrara & Li, 1980; Hammonds et al., 1981b,1982; Toogood et al., 1986) there is no conclusive evidence for epsilon receptors from binding experiments. This is probably because β -endorphin binds with high affinity to other types of opioid receptor, and there is as yet no selective ligand for epsilon sites. There is,

however, some indication that β -endorphin binds to sites in rat brain which are distinct from mu and delta sites, based on differential effects of divalent cations (Hazum et al., 1979a,b; Law et al., 1979b), different regional distribution (Akil et al., 1980; Goodman et al., 1983), and structure-activity relations (Hammonds et al., 1984). In other studies, NG108-15 cells are reported to express β -endorphin binding sites that are not completely blocked by enkephalins or morphine (Hammonds et al., 1981a). Binding sites in mouse brain appear to be different from sites in rat vas deferens (Lin-Shiau & Li, 1984), a tissue shown above to be rich in epsilon receptors. Chang et al. (1981) found benzomorphan binding sites in rat brain which they suggest correspond to epsilon receptors because of their ligand selectivity in competition assays (Chang et al., 1984). Autoradiographic measurement of the distribution of these sites in rat brain was different from that of mu, delta, or kappa sites and similar to that of sites for β -endorphin (Crain et al., 1985).

In summary, there is some evidence for the existence of epsilon receptors, the most convincing of which comes from in vitro bioassays with rat vas deferens (RVD). However several groups working with this tissue have suggested that the presence of a novel (epsilon) opioid receptor type need not be postulated to explain the differing potencies of β -endorphin and mu-selective agonists (e.g. morphine) in inhibiting electrically-evoked contractions of RVD (Smith & Rance, 1983; Sheehan et al., 1988). Sheehan et al. (1988) have proposed that both morphine and β -endorphin act via mu-type receptors, but that RVD is characterized by a low effective receptor reserve. They suggest that this issue of 'spare receptors' can explain how the lower efficacy of morphine (e.g. in comparison to β -endorphin) results in morphine's behaviour as a competitive antagonist in RVD.

I. Lambda receptors

Evidence for the existence of lambda opioid receptors comes from a series of binding experiments performed by Sadee and colleagues (Grevel & Sadee, 1983; Rosenbaum et al., 1984; Grevel et al., 1985) who found a high affinity site for [³H]naloxone which had low affinity for diprenorphine. Diprenorphine was then used to block binding of [³H]naloxone to mu sites and the properties of the remaining sites were studied. Both etorphine and sufentanil, which have higher affinity than naloxone for the mu site, had much lower affinity than naloxone for these diprenorphine-insensitive sites. Hence the [³H]naloxone was not labelling mu receptors under these conditions. Similarly, delta receptors were not being labelled since both [Met] and [Leu]enkephalin had very low affinity for the labelled sites. They also ruled out labelling of kappa, sigma, and epsilon receptors because of the low affinities of EKC and dynorphin, SKF-10,047 and phencyclidine, and β-endorphin, respectively. This new type of binding sites, now called lambda receptors, was found to be selective for 4,5-epoxy morphinans such as naloxone, nalorphine, naltrexone, and morphine. Anatomical distribution of the lambda sites was different from mu sites, notably in cerebellum where levels of lambda sites were relatively high compared to mu sites, and in midbrain and thalamus where levels of mu sites were higher than lambda (Grevel & Sadee, 1983).

Perhaps one reason that other groups have not reported lambda-like binding sites is that they are particularly labile. They can be labelled *in vivo* (Rosenbaum et al., 1984; Grevel et al., 1985), but *in vitro* lose affinity for naloxone if membranes are washed during the isolation procedure (Grevel & Sadee, 1983; Grevel et al., 1985).

So far the evidence for lambda receptors comes only from these binding experiments. It remains to be seen if these sites really are functional receptors and which, if any, of the effects of opiates they control.

II. Allosteric interactions and interconverting receptors

Several groups have suggested that there are functional and physical interactions between the different types of opioid receptors. There is an extensive literature base on this subject, so I will pick several reports as examples of arguments for such interactions.

Delta agonists, when given *in vivo* at doses that do not themselves produce any effect, enhance morphine-induced analgesia (Lee et al., 1980b; Barrett & Vaught, 1982). In contrast, dynorphin, presumably acting at kappa receptors, antagonizes the actions of morphine in functional assays of analgesia (Tulunay et al., 1981) and respiration (Woo et al., 1983). The mechanism of these effects is unknown and may well involve interactions between different neuronal systems. There have been suggestions, however, that there is a direct physical interaction between receptors.

Bowen et al. (1981) have proposed a model involving two types of anatomically distinct binding sites for [³H]DADLE, called type 1 and type 2. Their type 2 receptors correspond roughly to delta sites. The type 1 receptors are suggested to convert between mu and delta conformations depending on concentrations of guanosine 5'-triphosphate (GTP), sodium, and divalent cations. This model of 'interconverting receptors' is not widely accepted because of the demonstration of distinct mu and delta binding sites by selective protection (Robson & Kosterlitz, 1979; Smith & Simon, 1980; James et al., 1982), and by reports of their discrete anatomical distributions (Mansour et al., 1986,1987).

A second model involves allosteric interactions between mu and delta sites (Rothman & Westfall, 1982a,b; Rothman et al., 1985). In this model there are again two types of delta site, one of which is linked to mu sites, the other of which is independent of other receptor types. The allosterically linked site inhibits binding to mu receptors by a non-competitive mechanism. There is some evidence from binding assays for allosteric linkages in brain membranes (Rothman & Westfall, 1982a,b; Rothman et al., 1985), but it is not accepted universally. For example, Barrett and Vaught (1983) designed experiments specifically to test the

allosteric model and found no evidence for interactions between mu and delta sites. Allosteric interactions should have been easier to detect than in other studies, which mainly focused on subtle differences in binding parameters, because the authors' predictions for the gross shape of binding curves were different for models of allosteric and independent sites.

In any case there are problems with the allosteric model as a mechanism for the functional interaction between opioid receptor types. Firstly, it is difficult to explain enhancement of morphine effects *in vivo* by delta ligands when *in vitro* the effect is to inhibit binding at mu receptors. Secondly, detailed studies of the anatomical distribution of the different types of receptors show different patterns of localization for mu and delta sites. Even when these sites are found in the same brain region, the precise distribution within that region is different (Mansour et al., 1986,1987). This is not consistent with widespread allosteric interactions. It therefore remains to be established whether there are such links between receptor types and, if so, then the functional significance of allosteric interactions needs to be identified.

I. μ_1 and μ_2 subtypes

There have been some suggestions that the main types of opioid receptors can be further divided into different subtypes. The subdivision that has attracted most attention is probably that proposed by Pasternak and his colleagues, who claim to have identified two subtypes of mu receptor, which they call μ_1 and μ_2 (Pasternak & Wood, 1986), based on studies with the irreversible opiate ligand naloxazone. The μ_2 receptor is reported to be the most abundant and corresponds to the well established mu receptor discussed above. There are relatively few μ_1 receptors and they supposedly have high affinity for most opioid ligands. In Pasternak's model, μ_1 receptors mediate the analgesic effects of opioids and opiates (Pasternak et al., 1980a,b; Zhang & Pasternak, 1981a,b). There are many papers presenting evidence for μ_1 sites and discussing their role in analgesia (see review by Pasternak and Wood, 1986), but the model depends on a few binding experiments that need close inspection. These crucial binding studies were interpreted as follows.

Naloxazone, an analogue of naloxone, inhibits irreversibly high affinity binding of [3 H]naloxone, [3 H]DHM, [3 H][D-Ala²,Met⁵]enkephalinamide, [3 H]EKC, and [3 H]SKF-10,047 to rat brain membranes as judged by Scatchard plots and non-linear least squares analysis of binding isotherms (Pasternak, 1980; Pasternak et al., 1980a,b; Pasternak et al., 1981). Competition curves are biphasic, and naloxazone inhibits high affinity binding whichever combination of radioligand and competing ligand is used (Pasternak, 1980; Hazum et al., 1981; Wolozin & Pasternak, 1981). Since naloxazone inhibits only high affinity binding of each radioligand (from the Scatchard plots), and since the competition experiments show that high affinity sites for competing ligands are also blocked by naloxazone, then it is argued that the high affinity sites for the radioligand and the competing ligand must be the same. Furthermore, since the above is true for all combinations of radioligand and competing ligand tested, all the ligands must

share the common high affinity site.

This argument requires that naloxazone be absolutely specific for high affinity sites. If a proportion of low affinity sites for the radioligand were blocked then one could argue that these low affinity radioligand sites correspond to the high affinity sites for competing ligand that are blocked in the competition assays. If there is any doubt about the specificity of naloxazone in binding assays, its specificity in assays of analgesia must also be considered doubtful.

Demonstration that naloxazone is specific for high affinity sites depends on very good estimates of site numbers (B_{\max}) before and after naloxazone treatment. None of the data presented as Scatchard plots in the papers cited allow such good estimates. In many cases the estimates of B_{\max} depend on unreasonable extrapolations from too few data points on a small part of the binding curve (see Klotz, 1982 for a discussion on estimating numbers of sites). The fact that in some cases computers have been used to fit the data to binding models does not make the parameter estimates more valid.

There are disagreements between results obtained with naloxazone and those obtained with other methods. For example, Chang and Cuatrecasas (1979) found two binding sites with affinities of 0.8 nM and 6 nM for ^{125}I -DADLE on rat brain membranes. They clearly identified the higher affinity site as an enkephalin (delta) receptor. Hazum et al. (1981) also found two sites (K_d 0.5 nM and 5.2 nM) for the same ligand, but claim that the higher affinity site is a μ_1 receptor. In a study where Barrett and Vaught (1983) designed their competition experiments so that different receptor models (i.e. independent sites, allosteric interaction, common high affinity site) would give clear qualitative differences in the behavior of the curves, they found no evidence for μ_1 sites. In contrast, computer fitting of binding experiments has produced models that incorporate a μ_1 site (Lutz et al., 1984, 1985; Toll et al., 1984).

The evidence for μ_1 sites is not yet conclusive. Unless these sites can be convincingly demonstrated in relatively simple binding experiments, the assumptions implicit in the studies of the distribution (Goodman et al., 1985;

Moskowitz & Goodman, 1985), ontogeny (Zhang & Pasternak, 1981a), and biological role (Pasternak & Wood, 1986) of putative μ_1 receptors are suspect.

There is other evidence for subtypes of mu receptor not necessarily related to Pasternak's μ_1 and μ_2 receptors. In binding experiments with rat brain membranes, β -FNA (supposedly a selective blocker of mu receptors) only partially blocked binding of [3 H]DHM and [3 H]oxymorphone, both of which are supposedly selective for mu receptors (Rothman et al., 1983). In another study, Stautner et al. (1982) found that [3 H]sufentanil labelled more binding sites in rat brain than did [3 H]DHM, and there were differences in anatomical distribution and modulation of binding by Na^+ and GTP of sites for the two ligands. Both of these studies led to the suggestion of mu-subtypes in rat brain.

Takemori and Portoghese (1985) found β -FNA insensitive sites in GPI which they classified as a subtype of mu receptor based on affinity for naloxone. Sayre et al. (1984) found differential effects of β -FNA on GPI and MVD. They suggested that the mu receptor populations were different in these two tissues. Cross tolerance studies in GPI and MVD suggested to Schulz and his colleagues that there are subtypes of mu and kappa receptors (Schulz & Wuster, 1981; Wuster et al., 1983). They made preparations that were tolerant to one type of agonist selective for mu, delta, or kappa receptors and looked at potency shifts for other agonists. The argument is that those agonists that became less potent would act through the same receptor as the agonist used to induce tolerance. Agonists whose potency did not change act through other receptor types. Furthermore, the argument continues, if potency of agonists with the same receptor preference shifts by different degrees, then there must be subtypes of that receptor. However, different degrees of shifts in agonist potency can also be explained in terms of differing intrinsic efficacies (see Christie et al., 1988). Chronic opioid treatment may thus reduce the number of opioid receptors present in the membrane and/or impair the receptor-effector coupling such that agonists having low intrinsic efficacy (e.g. morphine) may not depress electrically-evoked smooth muscle contractions to the same extent as more efficacious agonists. Ligands of higher efficacy would thus be less affected by the decreased receptor reserve and would be able to produce more pronounced inhibitory effects via activation of the remaining receptors.

II. kappa₁ and kappa₂ subtypes

Subtypes of kappa receptors have been suggested based on results from binding studies on membranes prepared from human brain (Pfeiffer et al., 1981) and guinea pig spinal cord (Attali et al., 1982). These studies labelled kappa sites with [³H]diprenorphine, and [³H]etorphine and [³H]ethylketocyclazocine (EKC), respectively. Binding experiments in general depend heavily on finding radioligands that do not cross-react with other receptor types. The absolute specificity of the radioligands was not demonstrated convincingly in either of these studies.

In particular, the observed heterogeneity of spinal [³H]EKC binding sites upon which Attali et al. (1982) formed the basis for their separation of kappa binding into DADLE-sensitive (κ_1) and DADLE-insensitive (κ_2) components can partly be explained by the nonselective labelling of kappa sites and by the additional labelling of mu and delta receptors. Suppression of binding to these additional sites with unlabelled ligands, however, still suggested the presence of subtypes of kappa binding sites characterized by differing affinity profiles for a range of opiates and opioids.

Zukin et al. (1988) have recently described a high affinity κ_1 site predominating in guinea pig brain membranes, and a low affinity κ_2 site which predominates in homogenates of rat brain. These studies, in which the binding of [³H]EKC to non-kappa sites was blocked with unlabelled mu and delta ligands, tested the abilities of synthetic kappa-selective agonists to displace blocked [³H]EKC binding from sites on brain membranes. The rank orders of potency of these kappa ligands in the two tissues suggested that the κ_1 sites (guinea pig brain) have lower affinity for the benzeneacetamide U50488H and do not bind U69593 even at high concentrations. Zukin et al. (1988) therefore called these the U69593-sensitive (κ_1) and U69593-insensitive (κ_2) sites.

But as Traynor describes (1989), functional studies *in vivo* and on isolated tissue preparations *in vitro* have neither confirmed nor disproved conclusively this

binding data which apparently suggests subtypes of kappa receptors. One factor hindering the resolution of this issue is the lack, until relatively recently, of selective kappa antagonists.

In summary, there are intriguing hints that the established types of opioid receptor may be subdivided, but as yet there is no conclusive demonstration that this is the case.

It is now well established that there are at least three and probably four different opioid receptor types called mu, delta, kappa, and epsilon. Much is known concerning the relationships between the endogenous opioid peptides and opioid receptors in radioligand binding assays and *in vitro* pharmacologic assays. For example, dynorphin A is selective for kappa receptors in binding to brain membranes, in the guinea pig ileum (GPI), and in the mouse vas deferens (MVD) bioassay. Enkephalins are somewhat selective for delta receptors in MVD and binding experiments. β -endorphin is associated with epsilon receptors. None of these peptides, however, is totally selective. All will interact with more than one receptor type. For example, β -endorphin also binds to mu and delta sites, and enkephalins bind to mu in addition to delta receptors. Characterization studies of the multiple opioid receptors have benefitted from the synthesis of ligands with increased selectivity for single types of opioid sites.

Though sigma receptors clearly exist, they are probably not opioid in nature. Lambda receptors and subtypes of opioid receptors have yet to be conclusively demonstrated.

1-4.1 Introduction

I now wish to address the functional aspects of the opioids in central processes, putting this thesis into the context of what is known about opioid effects outside of the peripheral nervous system. The framework of this discussion will be to focus on the effects of morphine, exogenous ligand for the mu receptor, bringing in other opioid ligands as appropriate. Morphine produces analgesia by actions at two main sites in the central nervous system (see reviews by Duggan & North, 1984; Besson & Chaouch, 1987). One is a direct action on the spinal cord, and the second is an action on the spinal cord but via descending controls of brain stem origins. Additional actions to thalamic and cortical levels may occur but, since the ascending input to the latter areas depends on spinal mechanisms, the first two actions are likely to be of paramount importance.

A few notes introducing the neuronal basis of nociceptive transmission may be appropriate (see review by Besson & Chaouch, 1987). Afferent nerve fibers, which conduct information from the periphery to the central nervous system, can be classified on the basis of such factors as diameter, conduction velocity, and the type of stimuli to which the fiber responds. Sensory fibers can thus be divided into three groups: A β or large myelinated fibers conveying mainly tactile information; C or unmyelinated fibers carrying mainly noxious messages; and A δ or small myelinated fibers conveying mixed messages.

The substantia gelatinosa is the translucent zone of the spinal dorsal horn (laminae I and II) where most opioid peptides and receptors are found and where C fibers terminate. This has been taken as evidence for the localization of opioid receptors on the presynaptic endings of primary afferents terminating within this area of the dorsal horn, though the possibilities of alternative sites of spinal opioid action will be addressed in section 1-4.2. The dendrites of dorsal horn convergent neurons are also found in the superficial spinal laminae. Convergent neurons, which respond to both innocuous and noxious stimuli from skin, muscle, and

viscera, have been used extensively for the electrophysiological examination of spinal and supraspinal mechanisms of opioid analgesia.

The ability of epidural and intrathecal opioids (e.g. morphine and diamorphine) to relieve pain in humans (see Bromage, 1989) shows the clinical relevance of the spinal action of opioids. The wealth of information yielded from animal studies into spinal mechanisms of opioid analgesia is too great to be discussed here (see reviews by Yaksh & Noueihed, 1985; Besson & Chaouch, 1987), and therefore only a few recent findings indicative of a direct spinal action of opioids will be considered.

Le Bars et al. (1975) have shown that intravenous (i.v.) administration of morphine (2 mg/kg) results in a rapid depression of the spontaneous activity of convergent neurons in the cat spinal cord responding to A and C fiber inputs and to tactile and noxious stimuli. In the rat, i.v. morphine (3 mg/kg) strongly inhibits the responses of convergent neurons to noxious radiant heat (Le Bars et al., 1979c). However all neurons are not influenced in the same manner; the responses to low intensity stimuli are not altered, while the responses of the same cells to nociceptive stimuli are greatly reduced. These inhibitory effects of morphine on spontaneous and evoked activities of dorsal horn cells are specific as demonstrated by their reversal by naloxone, the opioid receptor antagonist (Le Bars et al., 1975,1979c).

Jurna & Grossman (1976) demonstrated the ability of i.v. morphine (at doses as low as 0.5 mg/kg) to naloxone-reversibly reduce the responsiveness of ascending axons of the cat spinal cord to electrical stimulation of the sural nerve at A δ and C fiber intensities. Comparing morphine depression of evoked activity in decerebrate cats with and without sectioning of the spinal cord, they concluded that spinal, as well as supraspinal, effects contributed to this analgesic action of morphine. This is also suggested by experiments in the spinal rat which show that systemic morphine strongly depresses electrically-evoked A δ - and C- but not A β -fiber discharges in ascending axons (Jurna & Heinz, 1979) and dorsal horn convergent neurons in a naloxone-sensitive manner (Le Bars et al., 1979c). The demonstration by Le Bars et al. (1979c) of a significant dose-response relationship

for morphine depression of neuronal activity is an additional argument for the pharmacological nature of the inhibitory effect.

A dose-dependent, naloxone-reversible depression of C fiber evoked activity in rat convergent neurons following intrathecal morphine has recently been described (Dickenson & Sullivan, 1986). Again this effect was selective, with A β responses being little altered. This differential opioid effect on the responses of convergent neurons to the stimulation of various fiber types suggest that morphine does not act directly on the convergent neuron but rather on elements pre-synaptic to the recorded cell. The hypothesis of additional mechanisms of action, including post-synaptic actions cannot however be totally excluded and may be important with high doses (Zieglgansberger & Bayerl, 1976; Zieglgansberger & Tulloch, 1979; Yoshimura & North, 1983).

At the spinal level opioid receptors are located predominantly in the most superficial laminae of the dorsal horn; the marginal zone of Waldeyer (lamina I) and the substantia gelatinosa (lamina II). The convergent neurons are located mainly in the dorsal horn, the most numerous being around lamina V but they can be found in most laminae. It seems from anatomical evidence that these deep cells send dendrites towards the superficial laminae of the dorsal horn (Fitzgerald, 1989). In addition, the majority of C fiber terminals and the dendrites of the convergent neurons are found largely in the superficial zones of the dorsal horn. The work of Duggan and colleagues (reviewed by Duggan & North, 1984) involved use of microelectrophoresis to demonstrate that the major site of action of morphine is in the superficial zones.

This group followed the firing rate of convergent neurons and observed that morphine ejected near the cell body of the neuron has little effect, but that ejection of morphine into the substantia gelatinosa causes an inhibition of the neuronal responses to noxious stimuli without altering responses to innocuous stimuli (Duggan et al., 1976, 1977b). The inhibitory effects of morphine were long lasting and could be reversed by electrophoretic application of naloxone in the substantia gelatinosa or by low doses of i.v. naloxone. This type of effect is probably very

important in the general mechanism of morphine analgesia since the inhibitory effects of systemic morphine on C fiber response can be reversed by iontophoresis of naloxone into the substantia gelatinosa (Johnson & Duggan, 1981).

Several mechanisms have been considered for the mechanism of action of morphine in the substantia gelatinosa, with an action of morphine on the fine primary afferent fibers by a presynaptic mechanism attracting most attention. Biochemical studies support this hypothesis; the finding that a dorsal rhizotomy is accompanied by a drop in the number of specific binding sites for opiates in the dorsal horn suggests that a number of opioid receptors are situated on primary afferents (Lamotte et al., 1976).

The suppressive effects of morphine on nociceptive transmission have been compared to those of endogenous opioids. The iontophoresis of methionine (met)-enkephalin into substantia gelatinosa produces an equally selective inhibition of the responses of convergent neurons to a peripheral noxious heat stimulus (Duggan et al., 1976). However the action of met-enkephalin seems more widespread since ejection close to the cell bodies of deep dorsal horn neurons is also effective (Duggan et al., 1977a; Zieglansberger & Tulloch, 1979). Enkephalinergic terminals have been observed on the soma and dendrites of dorsal horn cells projecting in the spinothalamic tract (Ruda, 1982). Although the superficial dorsal horn contains high levels of met-enkephalin and leucine-enkephalin, these opioids are also found in lamina V. Thus the above results with enkephalin administration near the somata of dorsal horn neurons may indicate a postsynaptic mechanism for this opioid modulation of nociceptive activity.

Recent electrophysiological studies have compared the effects of intrathecal morphine with those of enkephalin analogues with varying affinities for the mu and delta opioid receptors. Morphine, methadone, and DAGO, all with high selectivity for the mu receptor, can produce complete inhibition of the C fiber-evoked activity upon intrathecal administration (Dickenson & Sullivan, 1986; Dickenson et al., 1987; McQuay et al., 1989). The dose-response curve for this

effect is steep. Intrathecal delta agonists also produce selective effects over a similar dose range (Dickenson et al., 1987; Sullivan et al., 1989). Both groups of ligands used in these studies produce effects antagonized by intrathecal naloxone.

In contrast, dynorphin and stable synthetic analogues with kappa receptor selectivity produced both dose-dependent inhibitions and excitations of convergent neurons when administered intrathecally, the mean effect being little overall change in activity of the population of cells (Knox & Dickenson, 1987). Thus, while morphine and enkephalin-like compounds produce inhibitory effects which differ only quantitatively, kappa agonists produce more complex effects which do not have any clear relationship to analgesia. Confirmation of these conclusions arises from behavioral studies in rats and primates, mostly from the group of Yaksh and Noueihed (1985). Here intrathecal administration of morphine, delta ligands and other morphine-like opiates produce marked antinociception in a variety of behavioral tests. Kappa agonists seem to produce little effect on responses to cutaneous stimuli but may become effective when the test employed uses visceral stimulation. This agrees with experiments showing that the noxious cutaneous stimulus-evoked release of substance P from afferent fibers in the spinal cord is reduced by mu and delta but not kappa agonists (Hirota et al., 1985).

Several groups have suggested that the action of kappa agonists may be dependent on the intensity of the stimulus (Parsons & Headley, 1989a,b; Parsons et al., 1989; Millan, 1990). For example, kappa agonists have been shown to be more potent in modulating the tail-withdrawal response to low as compared to moderate or high intensity heat stimuli in the rat (Millan, 1989).

To summarize this section, a clear direct spinal action of morphine has been documented in many animal studies and is manifest by a selective inhibition of activity transmitted into the spinal cord by fine afferent nociceptive fibers. The effects of spinally applied mu receptor opioids are shared by delta receptor agonists in both electrophysiological and behavioral studies, although quantitative differences may exist. Kappa opioids produce less obvious effects. The site of action of opioids is probably predominantly presynaptically on the terminals of nociceptive fibers.

Many of the animal studies described in this section have examined the spinal effects of systemic or intrathecal opioids on electrically-evoked neuronal activity. Chapter 4 of this thesis describes the use of a more prolonged noxious stimulus, e.g. the subcutaneous injection of formalin into the rat hindpaw, to evoke dorsal horn neuronal activity. In the formalin model, the ability of the mu receptor peptide DAGO to inhibit cell responses following intrathecal administration is increased greatly if the opioid is applied to the spinal cord before injection of the formalin (Dickenson & Sullivan, 1987b). This may well have relevance to the effectiveness of opioid pretreatment in reducing post operative pain in man (Bach et al., 1988; McQuay et al., 1988; Wall, 1988).

The supraspinal sites of opioid action are much less well understood than the spinal sites. It is clear that morphine can act within the periaqueductal gray matter of the midbrain and at more caudal sites within the medial brain stem (e.g. nucleus raphe magnus) to produce analgesia (see review by Basbaum & Fields, 1984; Yaksh et al., 1988). There may also be actions within the thalamic areas and at cortical levels (see Yaksh et al., 1988). The latter has not been studied directly, mostly due to ignorance of cortical pain mechanisms but there is a differential distribution of mu, delta, and kappa receptors within the laminae of the sensory cortex. Thus the ability of intraventricular morphine to relieve pain in man and to elicit analgesia in animals is presumed to be due to actions at one or more of these sites (Yaksh et al., 1988).

It is generally held that opioid receptors in the periaqueductal gray matter, particularly in ventral zones and more caudally within the raphe nuclei of the brain stem, are involved in the supraspinal effects of morphine (see Dickenson & Le Bars, 1987). Thus microinjection of opioids into these zones produces a naloxone-reversible analgesia and, conversely, systemic morphine analgesia is reduced by either destruction of, or naloxone injection into, these areas (Yaksh et al., 1976; Lewis & Gebhart, 1977; Dickenson et al., 1979; Azami et al., 1982).

The periaqueductal gray (PAG) projects to the nucleus raphe magnus (NRM) of the brain stem which in turn sends massive descending projections, mainly using serotonin (5-hydroxytryptamine, or 5HT) as transmitter, onto the spinal cord (see Bowker et al., 1988). Interference with this spinal serotonergic activity alters the antinociceptive effects produced by PAG morphine in the rat (Yaksh, 1979). A second descending system is a noradrenergic pathway which may also be involved in opioid analgesia (see Proudfit, 1988).

The action of morphine on the PAG and NRM is apparent at low doses, and it is likely that the opioid reduces the descending inhibitory controls of brain stem origin which are triggered by noxious inputs and modulate the transmission of

nociceptive messages at the spinal level (Le Bars et al., 1981a).

Enkephalins produce analgesia when administered into the NRM and adjacent zones, and a transient effect is also seen on i.c.v. injection (Hollt et al., 1982). Dynorphin 1-13 and synthetic kappa agonists, by contrast to spinal administration, produce analgesia without other overt effects following i.c.v. injection (Tiseo et al., 1988; Millan et al., 1989).

Although the above may indicate that delta and kappa, as well as mu, opioid receptors mediate supraspinal analgesia, the involvement of the delta receptor is not yet confirmed. The ability of a variety of enkephalin analogues to elicit analgesia in the rat following i.c.v. injection correlates in a linear fashion with their affinity for the mu receptor in binding assays on brain membranes (see Chaillet et al., 1984). Within the PAG there are moderate to dense levels of mu and kappa opioid binding sites but very few delta sites (Mansour et al., 1987). It may then be that the enkephalins produce their supraspinal actions by interactions with the mu receptor for which they have low affinity. This would contrast with the clear role of the delta receptor at spinal levels.

To summarize this section, whereas morphine and other agonists at the mu receptor have clear supraspinal sites of action in producing analgesia, particularly at midbrain and brainstem sites, the influence of other opioids is as yet unclear. Although the zones where microinjection of morphine produces analgesia are reasonably well mapped, the final mechanisms underlying these effects are less well understood. However an interaction between opiates and descending inhibitory control, probably serotonergic and noradrenergic projections onto the spinal cord, is likely as are effects on processing of pain messages in the forebrain.

The vast body of knowledge on opioid antinociception at spinal and supraspinal levels reflects the fact that, for many years, opioids were traditionally viewed to exert their effects solely via receptors in the central nervous system. This emphasis, however, has changed recently through the use of models involving inflammatory pain states.

The peripheral terminals of small diameter primary afferent nerve fibers are increasingly seen to be functionally and morphologically similar to central terminals in the dorsal horn of the spinal cord. Substance P (SP), synthesized in the cell bodies of dorsal root ganglia, is transported both peripherally and centrally (Harmar & Keen, 1982), with evidence that SP in both the central (Otsuka & Konishi, 1976; Duggan & Hendry, 1986; Go & Yaksh, 1987) and peripheral (Brodin et al., 1981; White & Helme, 1985) terminals exist within pools releasible by depolarization. Opioid binding sites are also transported in both peripheral and central directions in primary afferent fibers (Young et al., 1980; Laduron, 1984), and opioid receptors have been shown to modulate the peripheral (Brodin et al., 1983; Lembeck & Donnerer, 1985; Yaksh, 1988) and central (Jessell & Iversen, 1977; Yaksh et al., 1980) release of SP from small diameter afferents.

As mentioned in section 1-2, several lines of evidence indicate that SP may convey afferent information via release at the central terminals of sensory fibers. Activation of sensory endings may also cause nerve impulses to travel antidromically to cause the local release of SP and other mediators which may then exert local tissue effects (see review by Holzer, 1988). Bayliss (1901) and Bruce (1913) first recognized that the antidromic stimulation of afferent nerve fibers results in a neurogenic inflammatory response characterized by plasma protein extravasation and vasodilatation. Since these discoveries, several peptides localized to unmyelinated afferents have been shown to play a role in neurogenic inflammation, with much attention focused on substance P. For example, intravenous infusion of SP causes vasodilatation and increases in vascular permeability in a variety of tissues (Saria et al., 1983) including skin, gut, and

respiratory tract. SP antagonists (Holmdahl et al., 1981; Rosell et al., 1981; Lembeck et al., 1982; Couture & Cuello, 1984) reduce these effects as does capsaicin (Jancso et al., 1967; Gamse et al., 1980), a neurotoxin which produces selective degeneration of small diameter afferent neurons. Blockade of nervous conduction by tetrodotoxin (Szolcsanyi, 1984) or local anesthetics (Jancso et al., 1968) has been shown not to inhibit capsaicin-induced neurogenic inflammation in the skin of rat paw. This suggests that the peripheral terminals of primary afferent sensory neurons can be stimulated directly to produce local effects via neuropeptide release independently of action potential generation.

For example, the role of SP in heat-induced oedema formation (Saria, 1984; Yonehara et al., 1987) has been studied extensively. Opioids have been shown to inhibit heat stimulus-evoked release of SP into the subcutaneous space and to modulate the formation of thermal oedema (Yonehara et al., 1988), suggesting that opioid receptors on the peripheral endings of small diameter afferents may be involved in the regulation of inflammatory responses. A variety of other studies also suggest a peripheral site of action of opiates. Opioids have been shown to inhibit the release of substance P from peripheral terminals of knee joint sensory nerves (Yaksh, 1988), to inhibit spontaneous discharges of fine afferent fibers innervating inflamed tissue (Russell et al., 1987), and to inhibit vasodilatation and increases in vascular permeability evoked by antidromic stimulation of primary afferent fibers (Bartho & Szolcsanyi, 1981; Lembeck et al., 1982; Lembeck & Donnerer, 1985; Russell et al., 1985). Lembeck et al. (1982) argued that the effects of opiates on peripheral sensory nerve endings are pharmacologically, but not physiologically, significant, though behavioral studies have suggested otherwise.

Investigators have shown that peripheral (intra-dermal) administration of low doses of morphine reduces evoked nociceptive responses in a model of prostaglandin hyperalgesia (Ferreira & Nakamura, 1979; Ferreira, 1983). Local injections of morphine reverse PGE₂ induced hyperalgesia in rat paw. An opioid receptor is involved in this effect since naloxone is an antagonist, and since

levorphanol is active but its enantiomer dextrorphan (which does not activate opioid receptors) is not (Ferreira et al., 1982). The relevant receptors are probably in the periphery because the enkephalin analogue [D-Ala²,D-Leu⁵]enkephalin, which does not cross the blood-brain barrier is also active when injected into the paw (Ferreira & Nakamura, 1979).

Other groups have found that opiate antagonists which are presumed not to cross the blood-brain barrier (because they are quaternary amines and positively charged) reverse morphine-induced analgesia (Mickley et al., 1985; Shibata et al., 1986a). There has been a suggestion that opiates can differentially affect different components of formalin pain response. When formalin is injected into the paw of a mouse there are two phases to the response, an early phase which may be mediated by direct stimulation of primary afferents and a later phase, which may be a consequence of an inflammatory reaction (Shibata et al., 1986b). Morphine, dynorphin and enkephalin analogues injected into the paw all inhibited the early response. Dynorphin and the enkephalin analogues inhibited the later response, whereas morphine caused hyperalgesia in this second phase. Again the evidence that peripheral receptors are involved is that all these opioid effects (including the hyperalgesia) were reversed or blocked by an antagonist that supposedly does not penetrate the CNS (Shibata et al., 1986a).

Evidence has also accumulated to suggest that peripheral opioid receptors mediate antinociception in other inflammatory conditions (Joris et al., 1987; Stein et al., 1988, 1989).

In summary, the inhibitory actions of opioids on neurogenic inflammation may result from their inhibition of the release of SP-LI and other neurotransmitters from the peripheral endings of sensory nerves. This point of view is based on the fact that opioid peptides do not inhibit the effects of exogenous SP in a peripheral tissue preparation (Smith & Buchan, 1984). However, it is unclear whether endogenous opioid peptides would modulate neuropeptide release after release from sensory nerve terminals or would exert their actions as circulating hormones.

The main theme of this thesis is the action of opioids on peripheral

terminals of sensory neurons. We have examined the possible role of opioid receptors on these peripheral terminals in analgesia by measuring how responses of convergent neurons in the spinal cord to inflammatory stimuli are modulated by peripherally administered opioids. We have also measured the relative abilities of a series of opioid ligands to influence evoked release of SP-LI in several preparations of peripheral sensory nerve endings.

Protocols for experimental techniques and materials used in this project are detailed in Chapter 2. Experimental results are presented in Chapters 3 to 7. The layout of each of these chapters will be a short introduction reviewing the relevant literature and stating the main questions to be addressed. This will be followed by a results section and then these results will be discussed. Thus each of the results chapters will more or less be entities unto themselves, with the final chapter tying together the main points from each of the results chapters.

Although the major theme of this thesis is peripheral opioid effects, a potentially useful *in vitro* model currently in use at the Sandoz Institute is the neonatal rat spinal cord with attached tail (Yanagisawa & Otsuka, 1984; Yanagisawa et al., 1985). Results in this preparation indicate that delta opioids perfused onto the spinal cord do not depress responses to nociceptive stimuli, while *in vivo* models show that intrathecal application of delta ligands inhibits neuronal responses to such stimuli in the adult rat (Sullivan et al., 1989). In Chapter 3 we have addressed these differences by looking at opioid binding to neonatal and adult rat spinal cord membranes. Receptor-type in the neonate is not well-characterized, so we were interested in whether the lack of delta activity corresponded to an absence of delta opioid binding sites. Chapter 4 investigates whether peripherally administered opioids can inhibit formalin-induced neuronal firing in the dorsal horn. This work complements previous work looking at central effects of opioids in this electrophysiological model.

Since it is often easier to use functionally relevant models *in vitro*, we turned to the biochemical study of opioid effects on primary cultures of dorsal root ganglion (DRG) neurons (Chapter 5), and later on neuroblastoma x DRG (ND) hybrid cell lines (Chapter 6). In Chapter 6 we assess whether ND cells are useful models for opioid effects on nociceptive sensory neurons. Chapter 7 describes a guinea pig heart slice preparation in which we investigated opioid modulation of substance P release from the peripheral terminals of sensory neurons.

CHAPTER 2 -
METHODS AND MATERIALS

2-1.1 Neonatal rat spinal cord membranes

As described in Dray et al. (1989a), newborn or one day old Sprague-Dawley rats (Charles River) were killed by cervical dislocation, decapitated, and spinal cords removed carefully. Spinal cords were then homogenized (Kinematica Polytron, setting 6 for 10 sec) in 10 volumes of 50 mM Tris-HCl (Tris) buffer, pH 7.4. A crude membrane fraction was isolated by centrifuging the homogenate at 17000g for 20 min. Membranes were resuspended in Total Hanks balanced salt solution (composition mM: NaCl 136.9, Na₂HPO₄ 0.34, MgSO₄ 0.81, KH₂PO₄ 0.44, KCl 5.37, CaCl₂ 1.26, glucose 5.55), buffered to pH 7.4 with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and left on ice for 30 min. The Na⁺ in the Total Hanks buffer increases the rate of dissociation of endogenous opioid ligands from opioid receptors (Simantov et al., 1976; Childers et al., 1979). The membranes were then centrifuged as above and washed twice in Tris buffer. The resulting pellet was finally resuspended in Tris to give approximately 0.3 mg protein/ml. The binding assay mixture contained 250 µl of this preparation plus appropriate radioligands (and unlabelled ligands where necessary) in a total volume of 500 µl of Tris buffer. Samples were incubated at room temperature for 60 min, and assays terminated by filtering (Brandel 48-well cell harvester, Gaithersburg, Maryland) through Whatman GF/C glass fiber filters and washing three times with 5 ml of ice-cold Tris buffer. The filters were counted in 5 ml scintillation cocktail (Beckman CP) for 5 min on a liquid scintillation counter (Beckman model no. LS-1801, Palo Alto, CA). All measurements were made in triplicate.

[³H][D-Ala²,MePhe⁴, Gly(ol)⁵]enkephalin ([³H]DAGO; 41 Ci/mmol, Amersham International plc), [³H][D-Pen²,D-Pen⁵]enkephalin ([³H]DPDPE; 31 Ci/mmol, Amersham), and [³H]U69593 (see below for chemical formula; 60 Ci/mmol, New England Nuclear) were used as selective mu, delta, and kappa radioligands, respectively. [³H]Ethylketocyclazocine ([³H]EKC; 24 Ci/mmol,

New England Nuclear) was also used to label kappa receptors after its binding to mu sites was blocked with 100 nM unlabelled DAGO. Non-saturable binding was measured in the presence of 10 μ M naloxone. Parameters for the saturable binding of opioid ligands were estimated by non-linear regression on the binding isotherm, assuming a single type of non-interacting binding sites. Hill slopes are from linear regression on Hill plots.

Receptor type was determined in competition experiments between 1 nM of each of these radioligands and unlabelled mu (DAGO), delta (DPDPE), and kappa (U50488H, U69593, PD117302, nor-binaltorphimine) selective ligands. IC_{50} values (the concentration of competing ligand required to reduce saturable binding of the radioligand by 50%) and Hill slopes were estimated by linear regression on Hill plots of the competition data, and are presented as means \pm standard errors of means (SEM).

Unlabelled DAGO and DPDPE were from Peninsula Labs, San Carlos, CA. U50488H (trans-(\pm)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl] benzene-acetamide methane sulfonate), and U69593 (($5_{\alpha},7_{\alpha},8_{\beta}$)-(-)-N-methyl-N[7-(1-pyrrolidinyl-1-oxaspiro(4,5)-dec-7-yl)]benzeneacetamide) were from UpJohn, Kalamazoo, Michigan. PD117302 ((\pm)-trans-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl] benzo[b]-thiophene-4- acetamide hydrochloride) was a gift from Dr. J. Hunter, Parke-Davis Research Unit, Cambridge. Nor-binaltorphimine was from Research Biochemicals Inc. Naloxone hydrochloride was from Sigma.

2-1.2 Adult rat spinal cord membranes

Adult male Sprague-Dawley rats (200-250g, older than 2 months; Charles River) were killed by suffocation in excess carbon dioxide, decapitated, and the spinal columns removed. For each binding assay the spinal cords from 4 animals, control or capsaicin-treated (see protocol below), were exposed carefully using bone-cutting scissors, removed, and pooled in ice-cold 50 mM Tris-HCl (Tris, pH 7.4). Spinal cords were then homogenized (Kinematica Polytron, setting 6 for 10 sec) in 10 volumes of Tris, and centrifugation of the homogenate at 17000g for 20 min resulted in a crude membrane fraction. Membranes were resuspended in Total Hanks balanced salt solution buffered to pH 7.4 with 10 mM HEPES, and incubated on ice for 30 min to allow dissociation of endogenous opioids. The membranes were then centrifuged as above, resuspended in Tris, and centrifuged again. The resulting membrane preparation was resuspended in Tris to give approximately 0.3-0.5 mg of protein/ml. The setup of the binding assay, statistical methods for estimations of binding parameters, and materials used were exactly the same as for the neonates (see section 2-1.1).

For the capsaicin pre-treatment experiments, male Sprague Dawley rats received subcutaneous injections (in the back of the neck) with 10 μ l of 25 mg/ml capsaicin (synthetic, 98% pure, from Sigma) on the second and third days after birth, and 20 μ l of this same capsaicin dose (approximately 50 mg per kg body weight) on the fourth day. A group of control litter-mates was treated with vehicle, which consisted of 10% ethanol-10% Tween 80 (v/v) in 0.9% (w/v) sterile saline, according to the same schedule.

2-2.1 Extracellular single-unit recording of rat dorsal horn convergent neurons

Using the protocol developed by Dickenson & Sullivan (1987a,b), adult male Sprague-Dawley rats (200-250g; University College London animal house) were anesthetized with halothane (in a gaseous mixture of 66% N₂O and 33% O₂) in a small perspex box and then maintained on 1.5-2% halothane (delivered through a nose cone) for subsequent surgery. During surgery and throughout the experiment, the animal's body temperature was maintained automatically at 36.5-37°C by feedback control of a rectal thermal probe to a heating pad (Animal Blanket Control Unit, Harvard). Following cannulation of the trachea the rat was mounted in a stereotaxic frame and a laminectomy performed, exposing the L₁-L₃ segments of the lumbar dorsal horn.

In this procedure, fat and connective tissue were removed from the surface of the spinal cord. This included removal of the dura mater but not the pia mater. The spinal cord was held rigid by clamps supporting the vertebrae rostral and caudal to the exposed spinal cord. After surgery, halothane anesthesia was reduced to 1% which was sufficient to produce complete areflexia in the animal and maintain it in good physiological condition for the duration of the experiment. Anesthetic delivery was maintained using a closed system consisting of a polythene Y-piece, with one end connected to the halothane dispenser and the other vented outside through rubber tubing.

A tungsten electrode was lowered into the dorsal horn by means of a SCAT microdrive (Digitimer) which measured the depth of the electrode tip in the dorsal horn. Differential extracellular recording was then carried (using a Neurolog NL 100 headstage), with the voltage signal being amplified (via Neurolog models NL 104 and NL 106), filtered (Neurolog NL 125), and fed into the window discriminator of a spike trigger (Neurolog NL 200) whose potentiometer gated only those spikes with an amplitude that crossed a pre-set threshold. Each gated spike resulted in the generation of a brightening pulse which, when fed into the second channel of the storage oscilloscope (Tektronix 5013N), appeared as an intense 'dot' on each of the gated action potentials. This ability to discriminate visually the single unit activity of a dorsal horn convergent neuron from the background multiple unit recording was complemented by audio discrimination. The filtered signal described above was fed into a speaker by way of an audio-amplifier (Neurolog NL 120). When the tip of the tungsten electrode was positioned near a neuron, the signal from that cell was distinct (e.g. stood out) from the background neuronal activity. A cell found by these methods of discrimination was then characterized as described below. Neurons in both deep (>500 microns) and superficial (<250 microns) laminae, but not in the substantia gelatinosa (250-500 microns), were used in experiments. Extracellular recordings were made of single dorsal neurons that responded to both innocuous (brush and/or prod) and noxious (heat, pinch) stimuli applied to the ipsilateral receptive fields in the toe region of the hindpaw. After characterization of the neuron by means of natural stimuli, C and A β fiber-evoked responses to transcutaneous electrical stimulation

were measured by post-stimulus time histogram (PSTH) recordings. Sixteen electrical pulses (0.5 Hz, 2 msec wide) were applied via needles inserted into the center of the receptive field, providing a constant reproducible test stimulus for the experiment. Stimulation was applied at 3 times the threshold current for C fiber activation and a PSTH was constructed using an averaging module (Neurolog 750) and displayed on a chart-recorder. The resulting PSTH allowed for the C fiber-evoked neuronal response to be separated, by latency and threshold, from the A β fiber-evoked response and quantified. Next, A β fiber-evoked responses were measured separately at 3 times the threshold for A β fiber activation.

Then the spontaneous activity of the neuron was recorded for 10 min. Typically the convergent neurons studied in this preparation had no or very low levels of spontaneous activity and any cell showing a rate higher than 20 spikes min⁻¹ was not used for these experiments. Formalin (5%, prepared in saline from 37% formaldehyde, in a 50 μ l volume) was then injected subcutaneously (s.c.) into the receptive field and the neuronal firing response monitored for 60 min.

Subcutaneous injection of formalin into the receptive field generates two phases of neuronal activity in cells (Dickenson & Sullivan, 1987a,b). Peak 1 (all activity in the first 10 min) occurred immediately following injection and subsided within several minutes. Peak 2 (all activity in the remaining 50 min) developed between 20-40 min following injection of formalin and persisted for up to an hour. The total spike count in peak 1 and peak 2 was taken in each experimental series and compared to the controls where 50 μ l distilled water (dH₂O) or saline, as appropriate, was administered in place of 50 μ l of the opioid compounds.

U50488H and Tyr-D-Ser-(tbu)-Gly-Phe-Leu-Thr (DSTBULET) were dissolved in dH₂O, while morphine and naloxone were made up in saline. The opioids (all at 100 μ g in a 50 μ l volume) and the dH₂O or saline were injected into the same area of the receptive field as formalin, except for naloxone which was injected either intraplantar (100 μ g in a 100 μ l volume) or intraperitoneal (given according to weight, approx. 0.4 ml). Opioids were administered 10 min before formalin to give it time to interact with opioid receptors prior to the onset of this

long-lasting nociceptive stimulus.

Morphine sulfate and naloxone hydrochloride were purchased from Sigma. DSTBULET was the kind gift of Dr. B.P. Roques, and U504884 was from UpJohn (Kalamazoo, Michigan).

Statistical analysis utilized the Mann-Whitney U-test (2-sided for opioid dose response, 1-sided for naloxone reversal). The Mann-Whitney nonparametric analysis tests the hypothesis that the medians of two independent groups are equal (see Bruning & Kintz, 1977). All formalin response values quoted are means \pm S.E.M., but median values have been calculated and were always found to fall within the limits of the mean. Experimental data has also been analyzed by Student's t-test and t-tests gave similar results to the Mann-Whitney U-test.

2-2.2 Release of substance P-like immunoreactivity (SP-LI)
from superfused rat spinal cord *in vivo*

Surgical procedure was the same as described in section 2-2.1, except that urethane (initial dose was given by body weight, approx. 0.4 ml of a 25% solution [wt/vol] in saline; and topped up with an additional 0.5 ml if necessary) was used as the anesthetic in most experiments. Halothane (1%) anesthesia was also employed in these studies. After surgery, an agar well (2%, wt/vol, in artificial cerebrospinal fluid [CSF, composition mM: NaCl 138.6, KCl 3.35, CaCl₂ 1.26, MgCl₂ 1.16, NaHCO₃ 21.0, NaHPO₄ 0.58, glucose 10.0]) was built up around the exposed segments of the lumbar dorsal horn, using agar-saturated cotton to help reinforce the sides of the well. The agar well was filled with 200 μ l CSF (37°C, gassed with 95% O₂/CO₂), inlet/outlet pipes from the peristaltic pump positioned, and the exposed spinal cord superfused (100 μ l/min) with artificial CSF (37°C, gassed with 95% O₂/CO₂) for 30 min before the start of the experiment. After this accommodation period, basal release of substance P-like immunoreactivity (SP-LI) was measured for 30 min by collecting 10 min fractions of the superfusate onto acetic acid (0.1 M final concentration at 25 degrees Celsius).

Activation of central terminals by stimuli of a variety of modalities was then assessed by measuring SP-LI in 10 min fractions. Subcutaneous injection of formalin (5% in a 50 μ l volume) into the rat hindpaw was the main stimulus in these studies. Other cutaneous stimuli tested include noxious pinch (continuous pinching of single hindtoe for 5 min with forceps), noxious heat (70°C soldering iron adjacent to, but not touching, hindtoe for 2 min), and transcutaneous electrical stimulation (as described in section 2-2.1, with 2 min of 2 msec pulses at 15 volts/0.45 Hz administered by a Digitimer Ltd. isolated stimulator, model DS2). In some experiments central terminals were stimulated by including either depolarizing concentrations of potassium (60 mM, prepared by substituting KCl for NaCl isosmotically) or capsaicin (1 μ M) in the CSF superfusion buffer. All fractions were frozen immediately at -70°C, freeze dried (Edwards modulyo), and the lyophilizates assayed for SP-LI (see section 2-3).

2-3 Radioimmunoassay for SP-LI

This protocol is a modification of that described by McGregor (1982) for measuring substance P-like immunoreactivity (SP-LI). All freeze-dried samples were reconstituted in 150 μ l of a potassium phosphate (0.06 M, pH 7.4) assay buffer (containing 0.05 M NaH_2PO_4 , 0.01 M KH_2PO_4 , 0.01 M EDTA, 0.01 M NaN_3 , and 0.3% bovine serum albumin [BSA]) with 0.1 M (final concn) acetic acid added to facilitate the dissolution of the lyophilizates. The bacteriostat sodium azide (NaN_3) helps to prevent bacterial contamination, while BSA prevents binding of peptide to plastics. After reconstitution, samples were incubated with ^{125}I -SP (in-house iodination, see protocol below), anti-SP antibody SP 3 (this antiserum, raised from rabbit, was purchased from Prof. Stephen Bloom, Hammersmith Hospital), and assay buffer (to a total volume of 400 μ l) for 48 hr at 4°C. Incubation of the radioimmunoassay (RIA) at low temperature improves the sensitivity of the assay by helping to minimize proteolytic degradation and evaporation, and increasing the avidity of antibodies (McGregor, 1982). Standard curves were generated by assaying serial dilutions of synthetic SP standards (Peninsula) that were diluted in the appropriate test buffer. Standards were set up in duplicate to increase precision and detect random errors.

After the incubation period 200 μ l buffer containing charcoal (10 mg per ml) and dextran (clinical grade, avg. MW = 78000, Sigma; added to charcoal suspension at 10% {wt/wt} of the charcoal) was added to the assay tubes and bound and free antigen separated by centrifugation at 2800g for 25 min at 4°C (the entire separation procedure was carried out at 4°C). After centrifugation the supernatant (antibody-bound ^{125}I -SP) was separated from the carbon pellet (free ^{125}I -SP) with a Pasteur pipette and supernatants counted for 10 min on a Crystal multi-well detector gamma system (United Technologies Packard). Initially, both the free and antibody-bound ^{125}I -SP fractions were counted to reduce error in calculation of % antibody-bound ^{125}I -SP, but results were not affected significantly by measuring supernatants alone (see Sarson, 1982 for a discussion of RIA

statistical methods). Using antibody SP 3, the sensitivity of the assay was 0.5 fmol per tube. Intra- and inter-assay variation was 4% and 7%, respectively.

None of the physiological salt solutions (Krebs, CSF, Hanks) used, nor any of the drugs tested (e.g. capsaicin and opioid ligands) interfered with binding of the iodinated tracer to the antibody with the exception of bradykinin. We found that bradykinin only cross-reacted with the SP 3 antibody 1:30000, but after lyophilization, perfusion buffers containing bradykinin concentrations as low as 100 nM effectively inhibited binding to the antibody (see Chapter 7, section 7-2.4, for presentation and discussion of these results).

Antibodies in the SP 3 antiserum are directed towards the carboxy (C) terminus of the undecapeptide SP. McGregor & Bloom (1983) have reported that the SP 3 antibody does not cross-react significantly (<0.05%) with such common mammalian peptides as bombesin, neurotensin, somatostatin, and leu- and met-enkephalin, although it does cross-react with the tachykinins eleodoisin (5%) and physalaemin (15%) which share the common C-terminal sequence Phe-X-Gly-Leu-Met-NH₂. The C-terminal fragments SP₂₋₁₁, SP₃₋₁₁, SP₄₋₁₁, SP₅₋₁₁, and SP₆₋₁₁ have been found to displace the binding of iodinated tracer to antibody SP 3 almost as well as SP₁₋₁₁, while minimal cross-reactivity was seen with the shorter fragment SP₇₋₁₁ and the N-terminal 1-9 fragment (McGregor & Bloom, 1983) and with the tachykinins neurokinin A (NKA) and neurokinin B (NKB; Ghatei & Bloom, personal communications). Analysis of SP-LI release samples by high performance liquid chromatography (HPLC) has not been done to identify the immunoreactive species in our studies, however several investigators have shown that the concentrations of SP fragments in neural tissue are insignificant compared to authentic SP (Kream et al., 1985; Sakurada et al., 1985).

Mild oxidative iodination of SP, to reduce the risk of oxidating SP at the C-terminal methionine, was achieved by using Chloramine T in a method based on that described by Hunter and Greenwood (1962). A solution containing 7.5 nmol (10 µg) of the tyrosine analogue [Tyr⁸]SP in 50 µl of 0.3 M (pH 7.5) potassium phosphate buffer was mixed with 0.4 nmol (1 mCi) Na-¹²⁵I (Amersham IMS 30) in a 10 µl volume. The reaction was started by adding 26 nmol (7.5 µg) Chloramine T in 25 µl phosphate buffer and terminated 10 sec later by the addition of 138 nmol (26 µg) sodium metabisulphite in 100 µl phosphate buffer. Radiolabelled SP was separated from unreacted radioiodide by reverse phase high performance liquid chromatography (HPLC) using a Waters HPLC system and Waters octadecasilyl silica (C-18) column, particle size 5 microns, eluted with a linear gradient of 10-40% acetonitrile/water mixtures containing 0.2% by volume trifluoroacetic acid (TFA). Immediately after the addition of sodium metabisulphite, the iodination reaction mixture was diluted out to the maximum injection volume (250 µl) with water containing 0.2% TFA and then injected onto the HPLC column which was pre-equilibrated with 10% acetonitrile at a flow rate of 1 ml/min. Fractions were collected every two minutes and [¹²⁵I] radioactivity monitored with a gamma radiation monitor (Mini-instruments, Ltd., series 900) to detect the major peak(s) of reaction product. Individual fractions were incubated with the SP 3 antibody to assess the antigenicity of the radiolabelled SP. Tracer aliquots (250 µl) were frozen at -20°C and found to be stable for 2-3 months.

2-4 Primary cultures of rat dorsal root ganglion (DRG)
sensory neurons

2-4.1 Neonatal rat DRG cell cultures

Newborn Sprague-Dawley rats (Charles River) were killed by cervical dislocation, decapitated, and spinal cords removed and discarded. DRG from all spinal levels were then removed under sterile conditions and collected in Ham's F-14 nutrient medium (F14) supplemented with 1.176 g/L sodium bicarbonate, 1 mM glutamine, 100 µg/ml penicillin, and 100 Units/ml streptomycin. Ganglia were dissociated in a series of steps (at 37°C), beginning with a 35 min incubation in 0.125% (wt/vol) collagenase to dissociate the collagen component of the connective tissue intracellular matrix. Ganglia were washed in F14, then incubated for a further 30 min in 0.25% (wt/vol) trypsin to break cell-cell interactions. Ganglia were then washed twice in F14 supplemented with 10% (vol/vol) heat-inactivated horse serum (F14HS). Further dissociation of DRG was achieved by adding the following solution (0.25 ml volumes) to each ml of the resuspended ganglia and incubating for 30 min: Deoxyribonuclease 5'-oligonucleotidohydrolase (DNase), 0.4 mg/ml; soya bean trypsin inhibitor, 0.55 mg/ml; and MgSO₄, 5 mM in F14. DNase cleaves genetic material which, after being released from cells lysed by enzymatic dissociation, tends to promote reaggregation, thus impairing further breakdown of cell-cell interactions. A specific inhibitor of trypsin is included to block the action of any trypsin left over from the previous dissociation step. A single-cell suspension was readily obtained by trituration of the treated ganglia by 6-8 passages through the tip of a fire-polished, siliconized Pasteur pipette. The resulting cell suspension was then filtered through 90 micron nylon mesh to selectively filter out cellular debris, large clumps of neurons, and pieces of tissue which were not properly dissociated. Cells were then spun down (200g for 5 min), and resuspended in F14HS. The yield of neurons was approximately 60000 per animal.

To purify the preparation, cells were pre-plated onto 60 mm dishes

(Nunclon) coated previously with poly-D-L-ornithine (PORN, 500 $\mu\text{g/ml}$) and incubated at 37°C, 97% air:3% CO₂ for 90 min. This pre-plating process takes advantage of the fact that non-neuronal cells stick down more quickly and securely than neurons. Loosely attached cells were removed by gentle agitation, spun down and resuspended in F14HS supplemented with 200 ng/ml nerve growth factor (NGF), 10⁻⁵M cytosine- β -D-arabinofuranoside (Ara-C), and 30% C6-glioma-conditioned medium (vol/vol, 2 days on a confluent monolayer). NGF is essential for the survival of neonatal cells and enhances the formation of neural processes in cultures of dissociated ganglionic cells. Ara-C is used to kill non-neuronal cells by blocking the S-phase of the cell cycle, thereby inhibiting DNA synthesis. Conditioned medium contains essential nutrients and an NGF-like growth factor (Monard et al., 1975,1977; Barde et al., 1978) which may be necessary for the survival and growth of primary cultures of sensory neurons. After this pre-plating period the number of non-neuronal cells is reduced to a large extent but some do remain in culture.

Cells were plated at a density of 1000 neurons/well onto 60 well Terasaki plates (Nunclon) or at 20-30 x 10³ cells/well onto 4 well plastic dishes (13 mm, LIP) previously coated with 500 $\mu\text{g/ml}$ PORN and laminin (12 $\mu\text{g/well}$ for Terasakis, 5 $\mu\text{g/ml}$ for 4 well dishes) and placed in a 37°C incubator with 3% CO₂. PORN improves the growth and attachment of cells because the positively charged residue of this peptide attracts the negatively charged sialic (or neuraminic) acid groups found in nerve cell proteins. Laminin is a large (MW = 10⁶ daltons) extracellular matrix glycoprotein which stimulates the attachment of epithelial and endothelial cells (Johansson et al., 1981), perhaps by anchoring cells to type IV collagen present in basement membranes (Leivo et al., 1980). Together PORN and laminin provide a good substrate for neuronal cultures. The cells were fed 24 hours later with the final resuspension medium, and every 2 to 4 days up until the time of experimentation.

Tissue culture materials were obtained from Gibco, except for F14 (Imperial Laboratories), collagenase (from *Clostridium histolyticum*,

Boehringer-Mannheim), trypsin (Worthington Biochemicals), DNase and trypsin inhibitor (Sigma), laminin (Bethesda Research Laboratories), and nerve growth factor, which was a generous gift of Dr. R.M. Lindsay.

Adult Sprague-Dawley rats (>200g, 3-4 months old) were killed by suffocation in excess carbon dioxide, decapitated, and the spinal columns removed. These were pinned dorsal-side up and spinal cords exposed with bone-cutting scissors. DRG (lumbar, cervical, and thoracic; 40-45 per animal) with attached roots were then dissected aseptically and pooled in Ham's F-14 nutrient medium supplemented with 10% (vol/vol) heat-inactivated horse serum (F14HS). Ganglia were freed carefully of roots and connective tissue sheaths before being dissociated enzymatically at 37°C in a series of steps (Lindsay, 1988; Wood et.al., 1988; see section 2-4.1 for explanation of these steps). Cleaned ganglia in F14HS were treated for with 0.125% collagenase for 90 min, followed by a further 90 min incubation in fresh collagenase-containing F14HS. The ganglia were then washed three times in F-14 medium, digested with 0.25% trypsin for 30 min with F14HS, and finally taken up in 2 ml of F14HS containing DNase (80 µg/ml) and soybean trypsin inhibitor (100 µg/ml).

The ganglia were then triturated by 6-8 passages through the tip of a fire-polished, siliconized Pasteur pipette. The yield of phase-bright neurons was consistently between 1.5 and 2.0 x 10⁵ from 40-45 ganglia. Neuronal enrichment was achieved by plating the dissociated ganglia from one adult in two 60 mm poly-D-L-ornithine (PORN) pre-coated culture dishes (Nunclon) overnight at 37°C in a 97% air:3% CO₂ environment. After this pre-plating process (approx. 15-20 hr), the non-neuronal cells were attached firmly to the dish, while most of the neurons were only weakly adherent. Most dead cells and axonal and myelin debris were discarded by careful aspiration of the culture medium. The attached neurons were then dislodged selectively by gently delivering a stream of culture medium from a constricted Pasteur pipette. The neurons from two dishes were collected in a total of 12 ml of F14HS in a conical tube. Centrifugation of the cell suspension at 100g for 5 min so that viable neurons were lightly pelleted resulted in further neuronal enrichment, leaving myelin debris, dead cells, and small non-neuronal

cells in suspension. Cells were then plated onto Terasakis (500 cells/well) or 4 well dishes (10-15 x 10³ cells/well) previously coated with PORN (500 µg/ml) and laminin (5 µg/ml), and maintained at 37°C, 97% air:3% CO₂ in F14HS (5 ml for Terasakis, 2 ml for 4 well dishes). Conditioned medium and NGF are reported to be unnecessary for the survival of primary cultures of adult DRG cells (Lindsay, 1988) although recent studies indicate that NGF regulates levels of the neuropeptides SP and CGRP in cultured adult DRG neurons (Lindsay et al., 1989).

Adult DRG cells were fed with this final resuspension medium every 2 to 4 days up until the time of experimentation.

Terasaki plates of cultured neonatal and adult rat DRG neurons (approx. 1000 neurons/well; 6-10 days post-plating) were equilibrated with four washes of Ca^{2+} - and Mg^{2+} -free Hanks' balanced salt solution (HBSS, pH 7.4) buffered with 10 mM HEPES. The solution in each well was then replaced with the same buffer containing 10 $\mu\text{Ci/ml}$ $^{45}\text{Ca}^{2+}$ (60 Ci/mmol; Amersham), in the presence and absence of drugs for 10 min at room temperature. Test solutions used in these experiments included: 5.4-50 mM KCl; 3 μM capsaicin; 0.3 μM (+) Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate), Miles Pharmaceuticals, USA; 10 nM-300 μM nifedipine; 100 nM-1 mM cadmium chloride; 1 μM -10 mM cobalt chloride; 0.1 nM-1 μM dynorphin B; 0.1 nM-30 μM dynorphin A or dynorphin A(2-17); and 10 μM U50488H, Upjohn. The assay was stopped by washing the plates six times in HEPES-buffered total (Ca^{2+} - and Mg^{2+} -containing) HBSS, followed by evaporation of residual buffer in the wells at 60°C for 30 min. $^{45}\text{Ca}^{2+}$ was then extracted by adding 10 μl of 0.1% sodium dodecyl sulfate to each well, leaving for 30 min, and transferring well contents to scintillation vials. Samples were counted in 2 ml of Beckman CP scintillant for 5 min.

Some calcium accumulation assays were carried out in sodium-free buffer containing isosmotic sucrose. All reagents were obtained from Sigma unless otherwise indicated. Dynorphin peptides were purchased from Peninsula Ltd. Experiments using the light-sensitive dihydropyridine compound Bay K 8644 were conducted in a darkened laboratory.

Neonatal and adult rat DRG cell cultures were studied 6-10 days after plating onto 4 well plastic dishes. Prior to experimentation, the cells were washed free of growth medium and placed in a pH 7.4 control potassium solution (composition mM: NaCl 118.5, KCl 4.7, CaCl₂ 2.5, MgCl₂ 2.6, KH₂PO₄ 1.2, NaOH 12.5, Hepes 25) for 30 min. High K⁺ solutions were prepared by substituting KCl for NaCl isosmotically. All subsequent manipulations were done on dry blocks (Techne) warmed to 37°C. At the end of the incubation period the bathing solution was removed, 50 µl of test solution added to each well, and plates incubated for 5 min. SP-LI release samples (50 µl) were then collected onto acetic acid (0.1 M, final concn), and frozen at -70°C. The cells were extracted with 1M acetic acid. SP-LI was measured by direct radioimmunoassay (see protocol in section 2-3) of the incubation solutions and cell extracts.

Test solutions used in these experiments included: 5.4-100 mM KCl; 100 nM capsaicin (Sigma); 0.1-1 µM Bay K 8644 (Miles Pharmaceuticals, USA); 10 nM-10 µM bradykinin (purchased from Cambridge Research Biochemicals); 100 nM-100 µM U50488H (UpJohn); 3-300 nM DAGO (Peninsula); 1 µM morphine sulfate (Sigma); 1 µM DADLE or DPDPE, (Peninsula). When examining opioid effects on SP-LI release, cells were exposed to the opioid ligands for 5 min prior to and throughout the 5 min K⁺ stimulus.

2-5.1 Hybrid cell tissue culture

Hybrid cells stored in liquid nitrogen were thawed rapidly in a 37°C water bath. After gentle resuspension of the cell solution, the hybrids were transferred to the bottom of a centrifuge tube containing Dulbecco's Modified Eagles Medium (DMEM, with sodium pyruvate and 1000 mg/L glucose) to which was added 20% (vol/vol) myoclone foetal calf serum (FCS, heat inactivated and mycoplasma/virus screened), 1000 units of penicillin/streptomycin, and 2 mM L-glutamine. The cells were then spun down for 5 min at 200g, and the pellet resuspended in 1 ml DMEM + 20% FCS. The hybrids were transferred to the bottom of a 50 cm² flask (Nunclon) containing 8 ml of this same medium, and stored in a 37°C incubator at 5% CO₂. The cells were fed after 24 hr, passaged after 72 hr, and subsequently grown in 80 cm² plastic culture flasks (Nunclon) containing 25 ml of DMEM + 10% FCS. Flasks of cells were then passaged every 2 to 4 days, depending on cell density, in order to protect the cells from exposure to an acidic environment. All tissue culture materials were from Gibco.

The passage number (p) is an approximation of the number of generations which hybrid cells have spent in culture, and is increased by one with each subcultivation. A well-defined window of passage numbers (p.5-p.20) was maintained consistently in order to improve reproducibility of results, and to safeguard against the effects of chromosome shedding and fluctuating protein concentration seen with increasing passage number (Hamprecht, 1977). This subcultivation, or splitting, process involved pouring off the growth medium which contained cell debris and hybrid cells in suspension. Those hybrid cells adhering to the bottom of the flask were harvested by adding fresh medium and hitting the side of the flask. These cells were then spun down at 200g for 5 min, and the pellet resuspended. Cell clumps were broken up by gentle trituration of the hybrids through a 19 gauge needle (Becton Dickinson), and the resulting uniform cell solution transferred to a new culture flask.

The maintenance of a rigid passage window required the frequent freezing down of cells at low passage number in order to maintain sufficient stocks of hybrids. Prior to freezing the cells all stock solutions were chilled to 4°C and all cryotubes (Nunclon) to -20°C. After harvesting the cells from culture flasks, the cells were spun down and resuspended in 1 ml of DMEM + 20% FCS + 10% DMSO. The cells were then aliquoted into cryotubes, stored overnight at -70°C, and transferred onto liquid nitrogen the following day.

For some experiments hybrid cells were differentiated with either low serum (1% foetal calf serum), retinoic acid (1 µM, from Sigma), dibutyl cyclic adenosine monophosphate (dbcAMP, 1 mM; Sigma), nerve growth factor (200 ng/ml; kindly supplied by Dr. R.M. Lindsay), or a cocktail of these. This process of differentiation induced visible morphological changes such as increased neurite outgrowth in ND hybrids. Differentiation also causes alterations in the expression of neuronal surface markers and various functions characteristic of sensory neurons (Jessell et al., 1984; Wood et al., 1990).

2-5.2 Opioid binding sites on ND cells: Saturation and competition studies

After being harvested, the non-differentiated ND hybrid cells were spun down at 200g for 5 min. The pellet was washed twice with 5 ml of 50 mM Tris-HCl/320 mM sucrose, pH 7.4. Resuspension of the final pellet was in this Tris/sucrose buffer and either a saturation or competition binding assay protocol followed. For the saturation studies the amount of [³H]etorphine (46 Ci/mmol, Amersham) added to cells was varied (0.1-5 nM). Receptor type was determined in competition experiments between 1 nM [³H]etorphine and unlabelled DAGO (10 nM to 10 μM), or DPLPE (10 nM to 10 μM), or U50488H (0.03 to 100 nM). All measurements were made in triplicate. Samples were incubated at room temperature for 60 min, filtered through Whatman GF/C glass fiber filters with 5 ml Tris/Sucrose buffer, followed by two 5 ml washes. The filters were then placed in scintillation vials and counted for 5 min.

Approximations of the affinity (K_d) of the etorphine radioligand for opioid receptors and the maximum number of binding sites (B_{max}) were yielded from transformations of the saturation data using the plot suggested by Scatchard (1949) and by non-linear regression on the binding isotherms. Transformation of the Scatchard plot results by the method of Hill (1910) provided evidence concerning the number of classes of opioid binding sites involved.

2-5.3 Rubidium-86 ($^{86}\text{Rb}^+$) efflux from populations of ND clonal cells

This protocol, essentially as described by Wood et al. (1988), is a modification of $^{86}\text{Rb}^+$ efflux assays previously reported by Amer & Stallcup (1981) and Tomazawa et al. (1985). Terasaki plates of differentiated or non-differentiated ND hybrid cells (approx. 1000 cells/well) were incubated for 2 hr (at 37°C, 5% CO_2) in growth medium (F14HS plus NGF; see section for 2-4.1 for details) containing 2 $\mu\text{Ci/ml}$ $^{86}\text{Rb}^+$ (approx. 300 mCi/mmol; Amersham International plc). All subsequent manipulations were carried out on dri-blocks warmed to 37°C. After this incubation the plates were washed four times, followed by three further washes at 1 min intervals with HEPES-buffered Dulbecco's Modified Eagle's Medium (DMEM). The DMEM on the plates (9 ml) was then changed at 1 min intervals, collecting each sample in a scintillation vial. After measuring background levels of $^{86}\text{Rb}^+$ efflux over the first seven collections, DMEM containing capsaicin (1 μM), high K^+ (50 mM), or the delta opioid DPDPE (1 μM) was added for two consecutive washes, followed by five further washes with drug-free DMEM. The counts remaining in the cells were measured after dissolution in 0.1% sodium dodecyl sulfate. Radioactivity was measured in a liquid scintillation counter without added scintillant. The rate of $^{86}\text{Rb}^+$ efflux was expressed as a rate constant by calculating the amount of $^{86}\text{Rb}^+$ released in each 1 min sample as a fraction of the amount of $^{86}\text{Rb}^+$ present in the cells at the beginning of the collection period.

2-5.4 SP-LI content of ND lines

Differentiated or non-differentiated ND hybrid cells were harvested, spun at 200g for 5 min, and extracted in acetic acid (0.1 M final concn) by boiling in a water bath for 1 hr. All extract samples were centrifuged as above, decanted, frozen at -70°C, and lyophilized. Samples were then assayed for SP-LI as described in section 2-3.

2-5.5 Fura 2-AM measurements of intracellular calcium levels ([Ca_i²⁺]) in ND cells

Differentiated or non-differentiated ND hybrid cells were harvested with DMEM + 10% FCS and spun at 200g for 5 min. The pellet was resuspended in 10 ml of the above solution and triturated through a 19 gauge needle to break up clumps of cells. A 1 ml aliquot of this cell suspension was then placed into a conical tube (10 ml, Sterilin) and loaded with 20 µl of a 1 mM stock (for a 20 µM final concn) of the membrane permeant acetoxymethyl (AM) ester form of the fluorescent calcium chelator Fura 2 [2-(6-(bis(carboxymethyl)amino)-5-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)ethoxy-2-benzofuranyl)-5-oxazolecarboxylic acid; purchased from Molecular Probes, Inc., Eugene, Oregon). These cells were gassed (95% O₂/CO₂) and incubated at 37°C for 20-30 min to achieve loading of the cells with Fura 2-AM. After this incubation period, loaded cells were spun at 600g for 5 min, washed with 10 ml Total Hanks balanced salt solution (buffered to pH 7.4 with 10 mM Hepes), spun as before, and resuspended in 4 ml Total Hanks. Fluorescence (emission wavelength = 504 nm; exciting wavelength = 338 nm) was monitored on a Perkin-Elmer (LS-5) luminescence spectrometer. Calculations of intracellular calcium ([Ca²⁺_i]) before and after stimulation by bradykinin (BK, 1 µM; purchased from Cambridge Research Biochemicals) were based on the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}_i] = K_d ((F - F_{min}) / (F_{max} - F)), \text{ where:}$$

F = fluorescence after administration of 1 µM BK

F_{min} = fluorescence after 10 mM MnCl₂ added

F_{max} = fluorescence after 1 µM ionomycin added

and K_d = 224 nM.

2-6 Guinea pig heart preparation

2-6.1 Content of SP-LI in extracts of individual guinea pig heart chambers

Dunkin-Hartley guinea pigs (250-350g; Interfauna UK, Ltd.) were killed by cervical dislocation, and hearts separated into the four chambers in ice-cold 0.32 M sucrose, 50 mM Tris-HCl buffer (pH 7.4). Chambers were then weighed separately after shaking off excess moisture, and homogenized using a Kinematica Polytron (setting 6 for 20 sec) in 10 volumes of ice-cold distilled H₂O (i.e. 1 g tissue/ 10 ml dH₂O). Each chamber homogenate was then separated into two aliquots of equal volume to carry out tissue extractions and protein determinations. For extraction of SP-LI, one half of the homogenate sample was boiled in 0.1 M acetic acid (final concn) for 30 min, spun at 45000g for 30 min, the supernatant decanted and assayed directly by radioimmunoassay (RIA; see section 2-3). Freeze-drying the supernatant gave precisely the same measurements of tissue SP-LI as by direct RIA.

Sodium hydroxide (1 M final concentration) was added to the remaining half of the homogenate sample and incubated at room temperature for 2 hr. Protein determination was by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Absorbances were read on a Perkin-Elmer (Lambda 5 UV/VIS) spectrophotometer.

2-6.2 Opioid binding studies on individual chambers
of guinea pig heart

Hearts were removed immediately after killing albino guinea pigs (250-350g; Dunkin-Hartley strain, Interfauna UK, Ltd.) by cervical dislocation, washed in ice-cold 0.32 M sucrose, 50 mM Tris-HCl buffer (pH 7.4), and separated into the right and left atria and the right and left ventricles. Each chamber of the guinea pig heart was homogenized in at least 40 volumes of ice-cold 50 mM Tris-HCl buffer using a Kinematica Polytron (setting 6 for 20 sec), and a crude membrane fraction isolated by centrifuging the homogenate at 45000g for 20 min. Membranes were resuspended in Tris-HCl (Tris), centrifuged as above, and washed twice in Tris buffer. The resulting pellet was resuspended in Tris to give approximately 6 mg heart tissue (original wet weight) per ml. In a modified version of the protocol used by Kruminis et al. (1985) to detect opioid binding sites in rat heart, the binding assay mixture contained 500 μ l of this membrane preparation (approximately 0.15 mg protein/ml) plus appropriate radioligands and unlabelled opioid ligands in a total volume of 1 ml of Tris buffer. The binding mixture was allowed to incubate for 60 min at 25°C before the assay was terminated by filtration through Whatman GF/C glass fiber filters, followed by three rapid washes with 5 ml of ice-cold Tris buffer. In these assays 1-10 nM [3 H]DAGO, [3 H]DPDPE, [3 H]U69593, or [3 H]naloxone was challenged with either 10 μ M unlabelled naloxone or 1 μ M unlabelled DAGO, DPDPE, or U69593. All measurements were made in triplicate.

Stocks of tritiated DAGO, DPDPE, and U69593 were identical to those used in binding studies on spinal cord membranes (see section 2-1.1 for the specific activities and suppliers of these radioligands). Tritiated naloxone (60 Ci/mmol) was purchased from Amersham. Unlabelled DAGO and DPDPE were from Peninsula Labs, unlabelled naloxone hydrochloride from Sigma, and unlabelled U69593 from UpJohn.

2-6.3 Release of SP-LI from pooled slices of guinea pig
right ventricle

Dunkin-Hartley guinea pigs (250-350g) were killed by cervical dislocation, hearts rapidly removed and dissected (by light microscopy) into individual chambers at 37°C in an oxygenated (95% O₂/CO₂) physiological (Krebs) salt solution (composition mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgCl₂ 0.54, NaH₂PO₄ 1.06, NaHCO₃ 24.5, glucose 10.0). Whole hearts or chambers were then chopped into 700 micron slices on a McIlwain tissue chopper, and slices equilibrated in oxygenated Krebs for 30 min (with gentle agitation every 5 min). Heart slices were placed onto a Whatman GF/C filter in the bottom of a perspex perfusion chamber. High potassium (100 mM KCl) solutions were prepared by substituting KCl for NaCl iso-osmotically. (In preliminary experiments lower e.g. 60 mM concentrations of KCl were used to stimulate heart slices but, as described in section 7-2.3, only 100 mM KCl always produced SP-LI release from guinea pig cardiac right ventricular tissue.) Drugs (e.g. capsaicin, formalin, and opioids) dissolved in oxygenated Krebs were administered by peristaltic pump (LKB), and fractions collected each minute onto acetic acid (for a 0.1 M final concn.). Fractions of heart perfusate were frozen at -70°C, freeze-dried, and SP-LI detected by radioimmunoassay (RIA; see section 2-3 for protocol).

At the end of each experiment, acidified heart slices (0.1 M acetic acid) were homogenized (Polytron, setting 6 for 20 sec), boiled for 30 min, and centrifuged at 45000g for 30 min. After centrifugation the supernatant was decanted and assayed directly for SP-LI. The SP-LI released in each 1 min fraction was then expressed as a percentage of the total SP-LI content at the beginning of the respective collection period.

The first stimulus in these experiments was always 100 mM KCl and was used as an internal control. The effects of mu (DAGO), delta (DADLE), and kappa (U50488H, U69593) opioids on SP-LI release from guinea pig right ventricular slices was then quantified against a subsequent KCl, capsaicin (100 nM-3 µM), or formalin (0.2%) stimulus. Opioids were applied 3 min before and throughout the 3 min stimulation of heart tissue. Areas under the two stimulus peaks were calculated, and the second peak expressed as a percentage of the first.

All peak ratios were then normalized to control experiments in which heart slices were exposed to two KCl stimuli.

Opioid agonist IC_{50} values were determined by plotting percentage inhibition of the KCl control response against opioid concentration.

CHAPTER 3 -
OPIOID BINDING SITES IN HOMOGENATES
OF NEONATAL AND ADULT RAT SPINAL CORD

A growing body of evidence suggests that the spinal cord is a major locus for the analgesic actions of opiates (see reviews by Akil et al., 1984; Basbaum & Fields, 1984). *In vivo* studies on adult mammalian spinal cord support the involvement of mu (Schmauss & Yaksh, 1984) and delta (Porreca et al., 1984; Rodriguez et al., 1986) opioid receptors in spinal antinociception, though evidence for the involvement of kappa opioid receptors is less clear. Kappa ligands are active in some functional tests (Schmauss & Yaksh, 1984; Porreca et al., 1987) but inactive in others (Schmauss & Yaksh, 1984; Leighton et al., 1988). This chapter deals with binding of opioids to sites in spinal cord from neonatal and adult rats. Our interest in neonatal spinal cord comes from attempts to set up *in vitro* a relevant functional model for antinociception. With adult spinal cord we wanted firstly to compare the population of receptors to that found in neonates and secondly to determine which receptors were expressed on the central terminals of afferent C-fibres.

A preparation from the neonatal rat has been developed in which the isolated spinal cord with attached tail can be maintained for prolonged periods *in vitro* (Yanagisawa & Otsuka, 1984; Yanagisawa et al., 1985). In this preparation, the activation of polymodal nociceptors in the tail can be measured by recording the depolarization produced in a spinal ventral root. Nociceptive mechanisms are therefore amenable to analysis *in vitro*.

Several groups have reported that the nociceptive reflex in this isolated spinal cord-tail preparation is sensitive to opioids. Micromolar concentrations of morphine (Dray & Perkins, 1987; Otsuka & Yanagisawa, 1988), met-enkephalin (Yanagisawa & Otsuka, 1984), and dynorphin (Otsuka & Yanagisawa, 1988) have been shown to depress ventral root depolarizations, though the specific opioid receptors involved were not studied extensively. We have shown that there are functional mu and kappa opioid receptors in neonatal spinal cord, and that activation of either of these receptors depresses the ventral root response evoked by peripheral noxious heat or chemical stimuli (Dray et al., 1989a; James et al.,

1990). Results presented here make up the binding component of studies reported elsewhere (Dray et al., 1989a; James et al., 1990).

Pharmacological and physiological evidence indicates that multiple types of opioid receptors are present in the adult mammalian spinal cord (see review by Yaksh & Noueihed, 1985). Opioid receptors are concentrated within laminae I and II of the adult mammalian spinal dorsal horn (Atweh & Kuhar, 1977; Goodman et al., 1980; Herkenham & Pert, 1982); that is within the laminae which receive the terminations of small diameter myelinated and unmyelinated primary afferent nerve fibers (Lamotte, 1977; Ralston & Ralston, 1979). Surgical sectioning (rhizotomy) of the dorsal roots results in a 40-50% reduction of opioid binding in the upper laminae of the dorsal horn (LaMotte et al., 1976; Ninkovic et al., 1981b,1982), suggesting that opioid receptors are associated, at least in part, with the terminals of primary afferent fibers where they modulate directly nociceptive transmission. Dorsal rhizotomy also depletes spinal cord levels of the neuropeptide substance P (Nagy et al., 1983), a biochemical marker for a subpopulation of primary sensory neurons and a putative nociceptive transmitter whose release *in vitro* (Jessell & Iversen, 1977) and *in vivo* (Yaksh et al., 1980) is inhibited by opioids. Treatment of the neonatal rat with the neurotoxin capsaicin produces depletions of SP similar to that observed with dorsal rhizotomy (Nagy et al., 1983). Jancso et al. (1977) first described the degeneration of primary afferent neurons after neonatal capsaicin treatment, and subsequent morphological studies have elaborated on the relative destruction of small diameter myelinated and unmyelinated fibers from dorsal roots (Lawson, 1981; Nagy et al., 1981). A consistent observation of such morphological studies is the nearly complete (up to 90%) loss of unmyelinated C-fibers in dorsal roots with capsaicin doses of 50 mg/kg and higher (see review by Lawson, 1987).

Receptor binding studies on homogenates of adult rat spinal cord indicate the presence of mu, delta, and kappa opioid binding sites (Traynor et al., 1982; Czlonkowski et al., 1983; Traynor & Rance, 1984; Traynor & Wood, 1987). Several of these studies have used radiolabelled forms of non-selective opioid

ligands such as diprenorphine and bremazocine, so that one goal of this project was to assay for opioid binding sites in adult spinal tissue with [³H]DAGO, [³H]DPDPE, and [³H]U69593, ligands with high selectivity for mu, delta, and kappa receptors, respectively. Results from these binding studies on adult rat spinal cord are compared with our results from neonatal tissue (sections 3-2.1 and 3-2.2).

Given our interest in opioid modulation of nociceptive transmission at primary afferent terminals, we also investigated whether destruction of C-fibers affected parameters of opioid binding to membranes of adult rat spinal cord. Several groups have demonstrated previously that opioid receptors are, at least in part, located presynaptically on capsaicin-sensitive primary afferent fibers projecting within the dorsal horn of the rat spinal cord. These investigators, however, have observed capsaicin-induced reductions (40-60%) in the binding of opioids to the dorsal zone of the rat spinal cord again using non-selective radioligands such as [³H]naloxone (Nagy et al., 1980; Daval et al., 1987; Hamon et al., 1989), [³H]diprenorphine (Gamse et al., 1979), and [³H]bremazocine (Daval et al., 1987; Hamon et al., 1989). For our studies, therefore, we assayed the binding of the more selective ligands [³H]DAGO, [³H]DPDPE, blocked [³H]EKC, and [³H]U69593 to spinal cord homogenates from adult rats treated neonatally with capsaicin (50 mg per kg body weight; see section 2-1.2 of Methods and Materials for protocol). Binding to spinal cord membranes from capsaicin-treated animals was compared to that in tissue from a vehicle control group of litter-mates.

3-2.1 Neonatal rat spinal cord: Saturable binding of opioids

In these binding experiments mu, delta, and kappa opioid binding sites were labelled with [³H][D-Ala²,MePhe⁴,Gly(ol)⁵]enkephalin ([³H]DAGO), [³H][D-Pen²,D-Pen⁵]enkephalin ([³H]DPDPE), and the benzeneacetamide [³H]U69593 (see section 2-1.1 of the Methods and Materials for complete structure), respectively (see Table 1.2). Binding to kappa sites was also determined with [³H]ethylketocyclazocine ([³H]EKC), a less selective ligand which is known to have high affinity for mu receptors (Kosterlitz et al., 1981). Therefore when using [³H]EKC, its binding to mu sites was blocked with a high concentration (100 nM) of unlabelled DAGO. This is referred to below as blocked [³H]EKC. [³H]DPDPE was used as the delta ligand in the binding assays because DPLPE, which our group has used in the functional assays (Dray et al., 1989a; James et al., 1990), is not available in radiolabelled form. Newborn or one day old rats were killed by cervical dislocation, spinal cords removed, and crude membrane fractions prepared (as described in section 2-1.1 of the Methods and Materials). Saturation and competition binding studies were carried out in triplicate, in 50 mM Tris-HCl (Tris), pH 7.4 at 25°C for 60 min. Specific binding to neonatal rat spinal cord membranes was defined as the difference between total binding, measured in the absence of naloxone, and non-specific binding, in the presence of 10 μM unlabelled naloxone.

Specific binding to delta sites could not be detected in neonatal rat spinal cord using the selective ligand [³H]DPDPE. Binding isotherms for [³H]DAGO, [³H]EKC, and blocked [³H]EKC, displayed in Figure 3.1, reveal that the specific binding of these radioligands to spinal cord membranes is saturable. The top two curves of this figure suggest that there are similar levels of opioid binding sites for [³H]EKC and [³H]DAGO, with possibly more sites labelled by [³H]EKC. Blocking [³H]EKC interactions at mu receptors with 100 nM unlabelled DAGO (bottom curve of Figure 3.1) demonstrates that binding at mu sites accounts for

most of the [3 H]EKC binding. The remaining blocked [3 H]EKC probably represent binding at kappa sites.

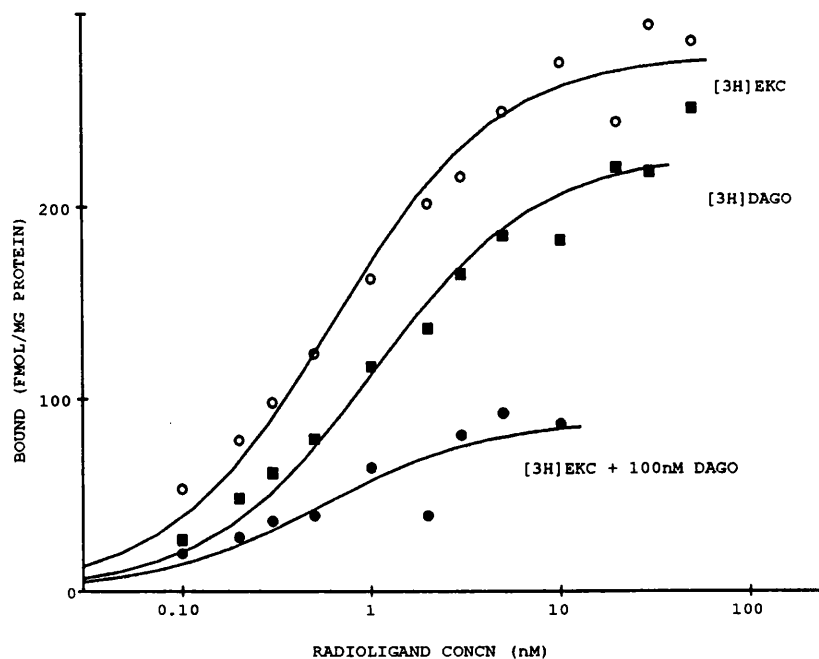


Figure 3.1. Results of a series of studies on the saturable binding of opioid radioligands to neonatal rat spinal cord homogenates. In these studies saturable binding was at least 70% of total binding (with the levels of nonspecific binding of each radioligand therefore representing no greater than 30% of total binding to the membrane preparation). Each point represents the mean from four to six experiments. Error bars have been left out of this graph, and several others in this chapter, to avoid cluttering the plots. Means and standard errors (SEM) for the relevant binding parameters generated in these saturation studies are reported in Table 3.1.

This was confirmed by experiments looking at the competition of unlabelled mu (DAGO), delta (DPDPE), and kappa (U50488H) selective ligands for [3 H]EKC and blocked [3 H]EKC binding sites on spinal cord membranes from neonatal rat. Figure 3.2 shows that DAGO competes more effectively for [3 H]EKC sites than either DPDPE or U50488H, though even at the highest concentration tested (100 nM) DAGO does not completely displace the radioligand. After blocking the binding of [3 H]EKC to mu sites with 100 nM unlabelled DAGO, the kappa-selective ligand U50488H displaces nearly all of the remaining binding sites (Figure 3.3). Neither DPDPE nor DAGO competed significantly for the binding of blocked [3 H]EKC.

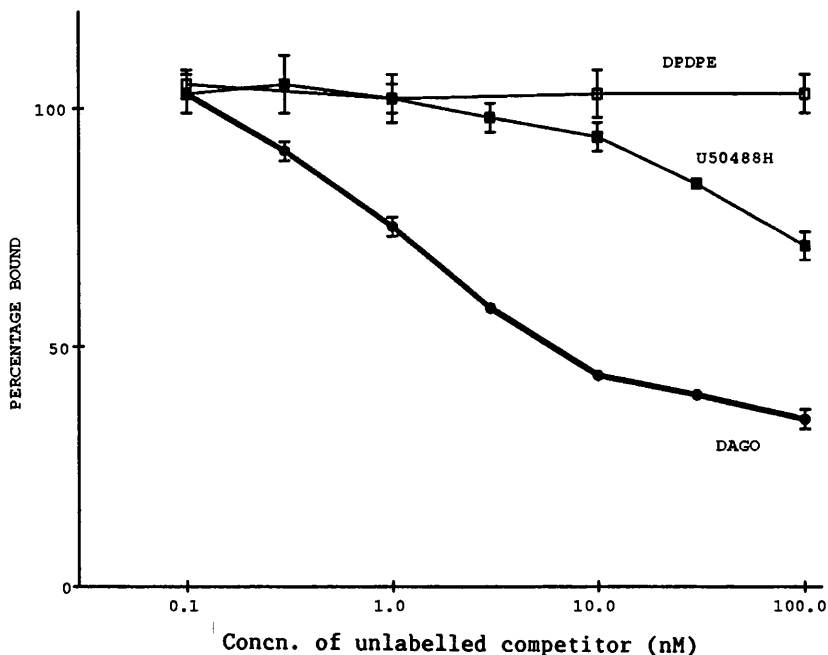


Figure 3.2. Summarized results of a series of competition experiments between 1 nM [³H]EKC and unlabelled DAGO, DPDPE, or U50488H (ligands highly selective for μ , δ , and κ opioid receptors, respectively) for binding sites on spinal cord membranes from neonatal rats. Data for this figure, and Figure 3.3, are means \pm SEM from three to six experiments.

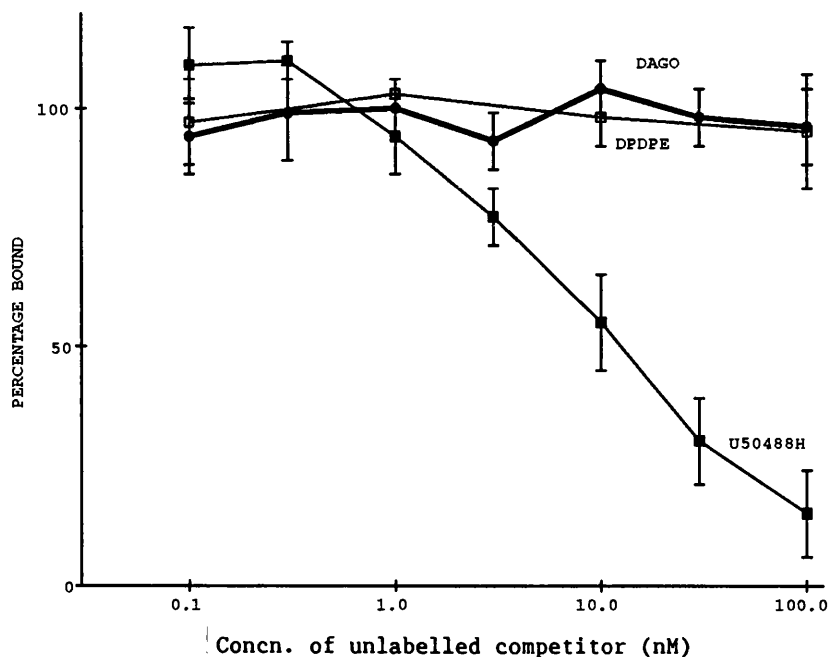


Figure 3.3. Competition curves demonstrating the effect of blocking [³H]EKC binding to mu sites (with 100 nM unlabelled DAGO) on the relative abilities of unlabelled DAGO, DPDPE, or U50488H to compete with 1 nM [³H]EKC for opioid binding sites on homogenates of neonatal rat spinal cord.

Estimates of the affinities (K_d) of radioligands for their respective receptors, and the maximum number of binding sites (B_{max}) labelled by each radioligand were obtained by nonlinear regression on the binding isotherm. These parameters, reported in Table 3.1, indicate the presence of high affinity binding sites for [3 H]DAGO, [3 H]EKC, and blocked [3 H]EKC on neonatal rat spinal cord homogenates. Figure 3.4 shows that transformation of the [3 H]DAGO binding data yields a linear Scatchard plot, consistent with this radioligand labelling a single class of binding site. The Hill plot transformation of the [3 H]DAGO binding results gives a line of approximately unity slope (Figure 3.5), again consistent with [3 H]DAGO labelling a single type of opioid binding site.

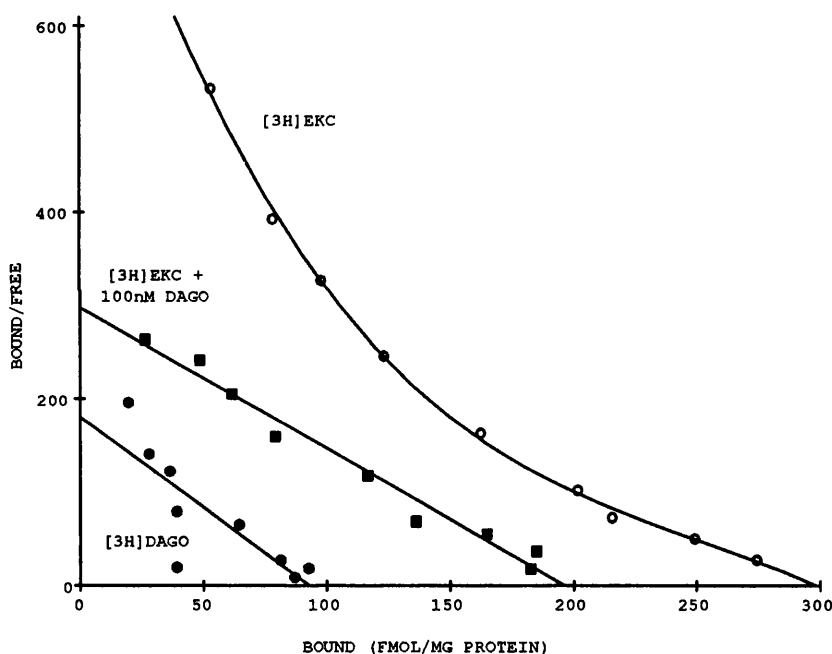


Figure 3.4. *Scatchard plot transformations of the saturation binding data presented in Figure 3.1. Units (on the vertical axis) for the amount of specific radioligand bound are fmol/mg protein, with the levels of free radioligand expressed as a nanomolar (nM) concentration.*

Although the Hill slope for [3 H]EKC was approximately unity (Figure 3.5), the curvilinear relationship of the Scatchard plot for [3 H]EKC (Figure 3.4) suggests that this radioligand is binding to more than one type of opioid receptor.

This emphasizes the dangers of relying too heavily on unity Hill slopes and linear Scatchards as assurances of site homogeneity (see Goldstein & James, 1983). Similarly, although data for the specific binding of [³H]EKC to nominally kappa sites (blocked [³H]EKC) reveals a linear Scatchard relationship (Figure 3.4), the Hill plot has a slope significantly less than unity (Figure 3.5). This perhaps indicates that [³H]EKC labels more than one type of binding site even after blocking its interactions at mu sites with an excess of unlabelled DAGO.

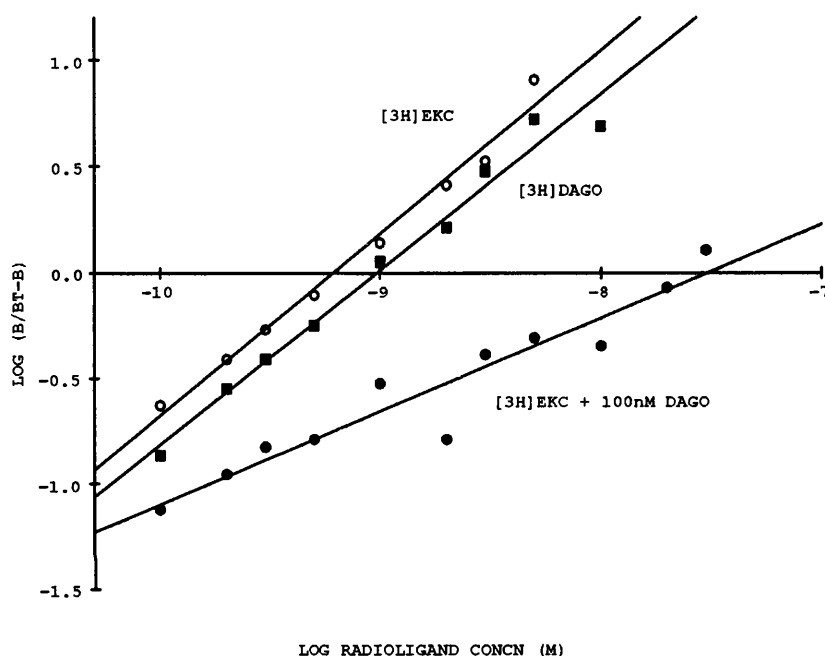


Figure 3.5. Hill plots of binding data from Figure 3.1. Abbreviations on the vertical axis stand for the amount of specific radioligand binding (B ; in fmol/mg protein), and the maximum number of specific binding sites (B_T , better known as B_{max} ; in fmol/mg protein).

We examined this further in a series of experiments using the more selective kappa ligand [³H]U69593 to label kappa binding sites. Figure 3.6 shows that, in these studies, blocked [³H]EKC labelled approximately twice as many saturable kappa binding sites as did [³H]U69593. Analysis of the saturation binding data for [³H]U69593 yielded a linear Scatchard (plot not shown) and a Hill slope close to unity (Table 3.1). The nature of these kappa opioid binding sites on neonatal rat spinal cord membranes was investigated more closely in competitive binding studies.

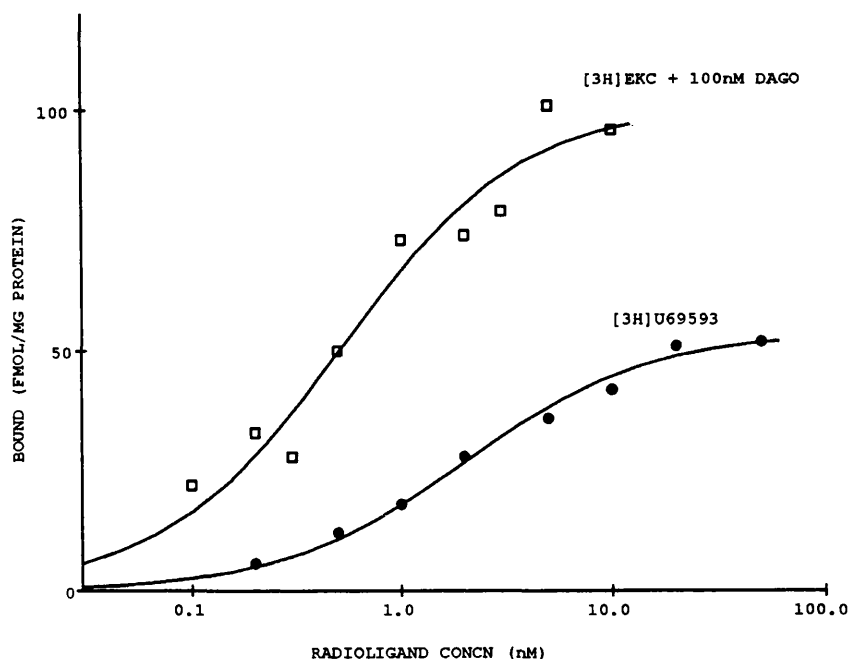


Figure 3.6. Results of a series of experiments comparing the saturable binding of [³H]U69593 and blocked [³H]EKC to kappa sites on spinal cord membranes from neonatal rats. Each data point represents the mean from four to six experiments.

Table 3.1. Parameters for binding of opioid ligands to membranes of neonatal rat spinal cord. Saturable binding was measured as described in section 2-1.1 of the Methods and Materials chapter. Parameters were estimated by non-linear regression on the binding isotherm, assuming a single type of non-interacting binding sites. Hill slopes are from linear regression on Hill plots. *Binding of [³H]EKC was measured in the presence of 100 nM unlabelled DAGO, to prevent interaction at mu sites. The K_d and B_{max} values listed below for binding experiments with unblocked [³H]EKC have been calculated from the lower affinity component of the non-linear Scatchard plot for unblocked [³H]EKC presented in Figure 3.4. Data are means \pm SEM from four to six experiments.

Radioligand	K_d (nM)	B_{max} (fmol/mg protein)	Hill slope
[³ H]DAGO	0.94 ± 0.06	220 ± 40	0.92 ± 0.03
[³ H]U69593	2.3 ± 0.60	55 ± 6	0.92 ± 0.03
[³ H]EKC*	0.80 ± 0.19	110 ± 20	0.72 ± 0.07
[³ H]EKC	0.66 ± 0.08	280 ± 50	0.91 ± 0.03
[³ H]DPDPE	not detectable	-----	-----

3-2.2 Neonatal rat spinal cord: Competition binding assays

The selectivity of [³H]DAGO, [³H]U69593, and blocked [³H]EKC was investigated in competition experiments using a range of unlabelled receptor-selective ligands. DAGO (Handa et al., 1981) was used to compete for mu sites. Competition at kappa sites involved the use of the kappa-selective agonists U50488H (VonVoigtlander et al., 1983), U69593 (Lahti et al., 1985), and PD117302 (Clark et al., 1988), and the selective kappa antagonist nor-binaltorphimine (nor-BNI; Birch et al., 1987; Portoghese et al., 1987). DPDPE was used as a selective delta ligand. In these competition assays, radiolabelled ligands were used at a concentration of 1nM.

As shown in Table 3.2.A, unlabelled DAGO and naloxone were much more potent than either the delta ligand DPDPE or the kappa ligands in competition for [³H]DAGO sites. Dray et al. (1989a) reported that DPLPE, which has a very similar affinity and selectivity to DPDPE (James & Goldstein, 1984), may exert its weak antinociceptive activity via the mu receptor. This is consistent with the low, but measureable, affinity of DPDPE in competing for [³H]DAGO sites in neonatal spinal cord.

The kappa ligands U50488H, U69593, PD117302, and nor-BNI competed for [³H]U69593 sites much more effectively than either DAGO or DPDPE. Notably there were no large differences in affinity for these sites between U50488H and the other kappa ligands. The antagonist nor-BNI had approximately 100-fold higher affinity for [³H]U69593 sites than for [³H]DAGO sites, confirming previous reports of its selectivity (Takemori et al., 1988).

Once binding to mu receptors had been blocked with a high concentration of unlabelled DAGO, neither DAGO nor DPDPE competed for the remaining [³H]EKC binding sites. The affinities of kappa ligands for sites labelled with blocked [³H]EKC were generally lower than affinities for [³H]U69593 binding sites. As in the competition assays for [³H]U69593 binding sites, U50488H was as

potent as U69593 and PD117302 in competing for blocked [³H]EKC binding sites. Naloxone showed significantly lower affinity for sites labelled with blocked [³H]EKC than for [³H]U69593 sites. Using the equation described by Cheng & Prusoff (1973) to express naloxone's IC₅₀ value (= 3.5 ± 0.1 nM, from Table 3.2) in terms of a K_I value, it may be seen that naloxone has an anomalously high affinity (K_I= 2.5 nM) for kappa sites compared to previous reports (e.g. naloxone K_I values 5-15 times greater than ours have been reported by: Hutchinson et al., 1975; Lord et al., 1977; Kosterlitz et al., 1981; James & Goldstein, 1984). One speculative explanation is that there exists a subtype of kappa opioid receptor (e.g. a U69593-sensitive site) that binds naloxone with a higher affinity (e.g. than sites labelled by [³H]EKC), but we have no evidence for this and more experiments would need to be performed to test properly this hypothesis. Further commentary on the possible heterogeneity of kappa sites in spinal cord will be postponed until the discussion portion (section 3-2.5) of this chapter.

In summary, saturation and competition studies indicate the presence of specific high affinity binding sites for selective mu ([³H]DAGO) and kappa ([³H]U69593, blocked [³H]EKC) radioligands on neonatal rat spinalcord membranes. Presentation of results from a functional correlate of antinociception in vitro (neonatal rat isolated spinal cord with attached tail) which compare favorably to this binding data will be postponed to section 3-2.5. Next, as a prelude to Chapter 4 which examines the peripheral effects of opioids on nociceptive activity in the adult rat spinal dorsal horn, I will present data on the binding of opioids to sites in homogenates of adult rat spinal cord.

Table 3.2. Competition for opioid binding sites on neonatal rat spinal cord membranes. IC_{50} values (the concentration of competing ligand required to reduce saturable binding of the radioligand by 50%) and Hill slopes were estimated by linear regression on Hill plots of the competition data, and are reported as means \pm SEM from 3 to 6 experiments. *Measurements with [3H]EKC were made in the presence of 100 nM unlabelled DAGO to prevent interaction at mu sites. In the competition assays, saturable binding was at least 70% of total binding.

Competitor	Radioligand		
	[3H]DAGO	[3H]U69593	[3H]EKC*
A. IC_{50} values (nM)			
DAGO	1.0 \pm 0.21	400 \pm 12	> 1000
U50488H	> 1000	0.86 \pm 0.06	18 \pm 4.4
U69593	> 1000	0.82 \pm 0.46	24 \pm 10
PD117302	900 \pm 52	0.49 \pm 0.04	31 \pm 10
nor-BNI	78 \pm 7.0	0.78 \pm 0.10	5.8 \pm 2.2
naloxone	2.1 \pm 0.07	3.5 \pm 0.10	120 \pm 46
DPDPE	620 \pm 14	> 1000	> 1000
B. Hill Slopes			
DAGO	0.92 \pm 0.03	0.88 \pm 0.11	-----
U50488H	-----	1.0 \pm 0.04	0.86 \pm 0.08
U69593	-----	0.87 \pm 0.08	0.63 \pm 0.07
PD117302	-----	0.98 \pm 0.10	0.79 \pm 0.04
nor-BNI	1.0 \pm 0.03	0.98 \pm 0.15	0.49 \pm 0.10
naloxone	0.88 \pm 0.01	0.96 \pm 0.17	1.0 \pm 0.09
DPDPE	1.2 \pm 0.10	-----	-----

Homogenates of adult rat spinal cord, as in membranes prepared from neonates, displayed saturable mu and kappa binding sites (Figure 3.7 and Table 3.3). Blocked [³H]EKC was found to label a greater number of binding sites in adult cord than [³H]U69593, similar to the results from neonatal studies. This is in agreement with several other reports of opioid sites on adult rat spinal cord using non-selective benzomorphan-type ligands to label kappa sites (Lahti et al., 1985; Nock et al., 1988). The main difference in binding characteristics seen at these disparate developmental timepoints is that adults do have measureable levels of delta binding sites (Table 3.3 as compared to Table 3.1).

Our results indicate that there were approximately 3-4 fold lower levels of spinal [³H]DAGO sites in adult as compared to neonate. We found roughly 6-7 fold fewer [³H]U69593 sites in adults compared to neonates. Allerton et al. (1989a,b) have recently described similar differences in levels of [³H]DAGO and [³H]U69593 sites in 9-16 day old rats as compared to adults.

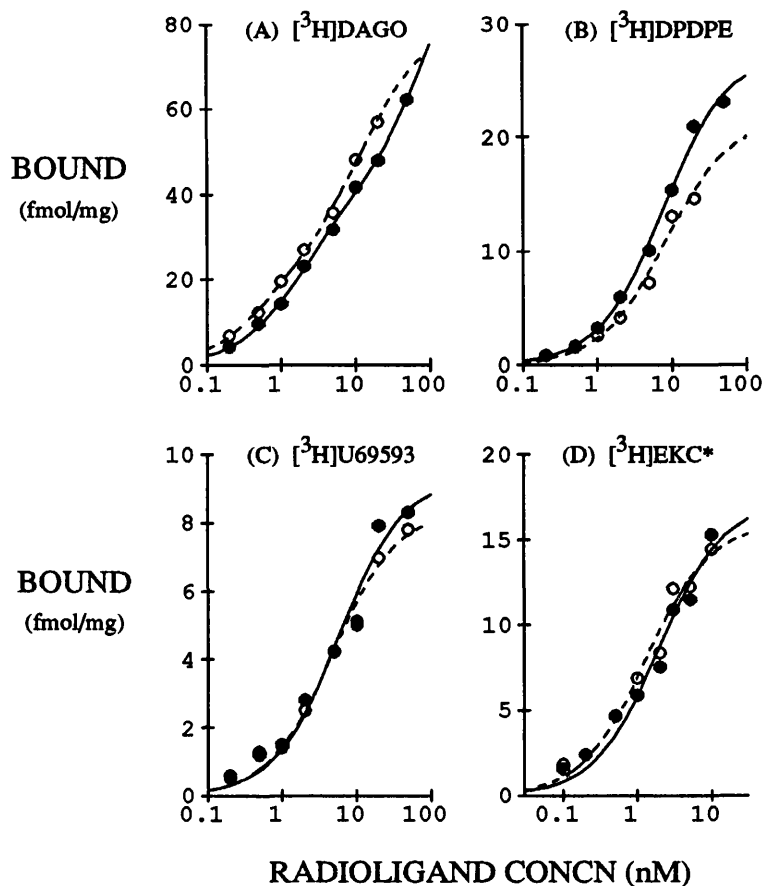


Figure 3.7. Binding isotherms from saturation studies on spinal cord membranes prepared from control (unfilled circles connected with dashed line) and capsaicin-treated (filled circles connected with solid line) adult rats.

Table 3.3. Parameters for binding of opioid ligands to membranes of adult rat spinal cord ($n=3$ for both control and capsaicin-treated rats).

Radioligand	CONTROL		CAPSAICIN-TREATED	
	K_d (nM)	B_{max} (fmol/mg)	K_d (nM)	B_{max} (fmol/mg)
[3H]DAGO	2.5 ± 0.3	61 ± 2	4.1 ± 0.7	62 ± 3
[3H]DPDPE	8.2 ± 2.4	22 ± 3	7.6 ± 1.2	27 ± 1
[3H]U69593	4.9 ± 0.8	8.4 ± 0.5	5.7 ± 1.2	9.3 ± 0.7
[3H]EKC (blocked)	1.3 ± 0.3	16 ± 1.2	1.9 ± 0.4	17 ± 1.2

3-2.4 Adult rat spinal cord: Effect of neonatal capsaicin treatment on saturable binding of opioids

As indicated in Table 3.3, neither the affinities (K_d) nor the binding capacities (B_{max}) of the radioligands to adult rat spinal cord homogenates were altered significantly by neonatal capsaicin treatment. Lawson (1987) highlights the varying extent of C-fiber destruction by neonatal capsaicin treatment, even within the same group. This leads to the possibility that significant levels of primary afferent fibers with opioid binding sites remain after our protocol. In future studies it would be desirable to confirm the effectiveness of the capsaicin treatment either by morphological estimation of the extent of sensory fiber loss or by comparison of spinal SP-LI content in capsaicin-treated versus vehicle control rats. Alternatively, assuming that this capsaicin protocol substantially destroys sensory fibers, then these results suggest that a number of opioid binding sites in adult rat spinal cord are located elsewhere, either on postsynaptic elements or on capsaicin-insensitive sensory fibers.

Our studies on binding to membranes of neonatal rat spinal cord have shown that there are no binding sites for delta-selective ligands, there are mu sites labelled by [³H]DAGO, and kappa sites which can be labelled with [³H]U69593 and [³H]EKC. Hill slopes for [³H]DAGO and [³H]U69593 binding were unity, consistent with single binding sites for both of these radioligands. In the absence of blocking ligands, [³H]EKC binds predominantly to mu receptors. In the presence of unlabelled DAGO, so that binding to mu receptors can be blocked, [³H]EKC sites are still heterogeneous as judged by Hill slopes of binding isotherms and from competition assays.

Zukin et al. (1988) have described subtypes of kappa (κ) receptors in rat and guinea pig brain based on heterogeneity of [³H]EKC binding. In their model, κ_1 sites bind PD117302, U69593, and U50488H with reasonable affinities. They report that κ_2 binding sites have lower affinities than κ_1 sites for U50488H and PD117302, and do not bind U69593 even at high concentrations (up to 10 μ M). [³H]U69593 sites that we measure in neonatal rat spinal cord homogenates could correspond to the κ_1 receptors proposed by Zukin and colleagues (1988). The additional kappa-like binding with [³H]EKC is unlikely to correspond to Zukin's κ_2 receptors because although the competition assays showed that U69593 had a relatively low affinity for these sites, so too did U50488H and PD117302. Heterogeneity of blocked [³H]EKC binding sites may represent heterogeneity of kappa receptors but it can be difficult to interpret results from experiments with non-selective radioligands such as [³H]EKC even after blocking interactions at other sites (Traynor, 1989).

Mu and kappa receptors have been found in binding studies with neonatal rat brain (see review by McDowell & Kitchen, 1987), but previous descriptions of the presence of opioid binding sites on neonatal rat spinal cord have not involved extensive determinations of receptor type with selective radioligands and competition assays (Kirby, 1981; Kirby & Mattio, 1982). In agreement with

previous studies (Kirby, 1981; Kirby & Mattio, 1982), our data shows that levels of opioid binding to spinal cord are higher in neonatal rat than in the adult. It must be clarified that this apparent developmental decrease in the number of opioid sites, rather than reflecting an actual ontogenetic loss of opioid receptors, probably indicates that levels of certain sites plateau early on in development (see McDowell & Kitchen, 1987). Kirby (1981) reported that binding of [³H]diprenorphine ([³H]DPN) to spinal cord homogenates is detectable beginning at embryonic day 16, or approximately 6 days before birth. In these experiments, the specific binding of [³H]DPN, which lacks agonist activity and has high affinity for each of the opioid receptor types, reached a peak on the sixth day postnatally, with adult levels of binding reported at 15 days postnatally. Similarly, the specific binding of [³H]naloxone to rat spinal cord has been reported to peak at day 6 after birth and then level off to adult values at day 15 postnatally (Kirby & Mattio, 1982). Furthermore, as is generally acknowledged in studies of receptor ontogeny, there is a statistical tendency for estimates of the maximum number of binding sites (B_{max}) at later developmental stages to be lowered when results are corrected for tissue protein concentration (Kitchen et al., 1990).

Our opioid binding studies, in which delta sites were present on spinal cord membranes prepared from adult but not newborn rat, agree well with functional studies on these two age groups. Dray and colleagues report that functional mu and kappa, but not delta, opioid receptors are involved in the modulation of nociception in neonatal rat spinal cord (Dray et al., 1989a; James et al., 1990). In the adult rat, however, spinal mechanisms are sensitive to delta opioids (Sullivan et al., 1989).

Allerton et al. (1989b) have also reported a correlation between the ontogeny of opioid binding sites and function. In comparative studies on adult and 9-16 day old rats, they have detected a five-fold higher density of kappa opioid receptors in spinal cord membranes prepared from the young rat. Allerton and colleagues (1989a,b) have shown that in the 9-16 day old rat, as in the neonatal rat (Dray et al., 1989a; James et al., 1990), the kappa ligand U69593 can

naloxone-reversibly depress nociceptive activity in an *in vitro* spinal cord preparation. This contrasts to results suggesting that intrathecal kappa opioids do not depress electrically-evoked activity in the adult rat spinal cord (Knox & Dickenson, 1987; Leighton et al., 1988).

The differential antinociceptive effects of spinally-applied opioids observed with these three age groups may be due to the time course of development and/or axoplasmic transport of opioid receptors to central terminals. Opioid receptors are known to be transported peripherally as well as centrally (Young et al., 1980; Laduron, 1984), and the next experimental chapter addresses evidence for the functional relevance of opioid sites in the periphery.

CHAPTER 4 -
IN VIVO ELECTROPHYSIOLOGY:
EFFECTS OF PERIPHERALLY ADMINISTERED OPIOIDS
ON FORMALIN-INDUCED DORSAL HORN NEURONAL
ACTIVITY

In vivo investigation of opioid modulation of nociception has focused mainly on the effects of centrally-acting opiates in a variety of behavioral models developed to quantify the pain felt by animals subjected to acute short-lasting stimuli. Antinociceptive models such as the tail-flick, hot-plate, abdominal constriction, and paw pressure tests have proven valuable in the study of mechanisms of pain and in the testing of potential analgesics. Different types of pain (i.e. noxious thermal, chemical, mechanical) may be transmitted and processed in different ways in the central nervous system (Dennis & Melzack, 1979). Therefore these techniques are commonly used in combination to generate *in vivo* pharmacological profiles of opiate analgesics. There are numerous reports that these models of antinociception differ markedly in their sensitivity to opioid receptor agonists (Tyers, 1980; Upton et al., 1982; Hayes et al., 1987; Shaw et al., 1988). Tyers (1980) attempted to explain these differences by postulating the existence of at least two neuronal pathways which transmit heat and non-heat nociceptive information separately and possess different classes of opioid receptors. Later investigators have re-examined this hypothesis and reported that these differential sensitivities are largely attributable to the relative efficacies of opioid agonists (Hayes et al., 1987) and to different stimulus intensities employed in the various antinociceptive models (Shaw et al., 1988).

Dubuisson and Dennis (1977) recognized the usefulness of the aforementioned models in analgesic assessment, but also discussed drawbacks inherent to these methods. They emphasized the difficulties of using transient pain stimuli (i.e. brief exposures to heat or skin deformation) to assess the onset and duration of analgesics and questioned the relevance of extrapolating results obtained with measures of short-lasting pain to states of clinical pain which are generally more long-lasting or tonic in nature. Following these lines of argument, Dubuisson and Dennis (1977) introduced a formalin test, in which the subcutaneous (s.c.) injection of a small volume of dilute formaldehyde (formalin) solution into cat and rat hindpaw was found to produce a prolonged noxious

stimulus. A variety of species including mice (Hunnskaar et al., 1985; Shibata et al., 1989) and monkey (Alreja et al., 1984) have been rated for their behavioral responses to s.c. injection of formalin. This chemical stimulus produces two characteristic phases of pain (Dubuisson & Dennis, 1977; Hunnskaar et al., 1985): an early short-lived response and a later longer-lasting response, with each phase possibly representing different nociceptive mechanisms.

Much of what is known about the formalin model has been gleaned from behavioral studies, but, increasingly, investigative emphasis has diversified to examine more closely the nociceptive mechanisms underlying the formalin-induced behavioral effects. Likely focal points of investigation are the various classes of neurons residing in the dorsal horn of the spinal cord. This is because nociceptive information from peripheral sites of tissue injury is known to be transmitted via fine sensory nerve fibers, C and A δ primary afferents, which impinge centrally in the dorsal horn. One class of neurons which comprises a large proportion of cells in the dorsal horn, and receives C- and A β -fiber inputs (Price & Wagman, 1970; Price et al., 1971; Gregor & Zimmermann, 1972), has been shown to respond to a range of nociceptive stimuli including strong activation by intra-arterial administration of the pain-producing peptide bradykinin (Besson et al., 1972). Le Bars et al. (1986) refer to these cells as convergent, thus emphasizing their characteristic convergence of excitatory and inhibitory influences as reported by previous groups (Wall, 1960,1967; Mendell, 1966; Iggo, 1974; Zimmermann, 1977). Given that a proportion of these cells project into higher central (i.e. spinothalamic, spinoreticular, spinocervical) pathways (Willis, 1982), convergent dorsal horn neurons may play a critical role in the transfer of sensory information from nociceptors to the spinal cord and brain.

Convergent cells are found deep in the spinal dorsal horn in lamina V (nomenclature of Rexed, 1954; see Wall, 1967), though they are also located in all other spinal laminae including more superficially in laminae I and II (Handwerker et al., 1975; Cervero et al., 1976; Price et al., 1979). Dickenson and co-workers at University College London (UCL) have reported the effects of intrathecal opiates

on the responses of deep and superficial dorsal horn convergent neurons to acute transcutaneous electrical stimulation of A- and C-fibers (Dickenson & Sullivan, 1986; Dickenson et al., 1987; Knox & Dickenson, 1987). More recently they have developed an electrophysiological correlate of the formalin model to investigate the effects of this noxious chemical on the activity of these cells (Dickenson & Sullivan, 1987a,b). In these experiments, an adult male Sprague-Dawley rat (200-250g) is anesthetized with halothane and the L₁-L₃ segments of the lumbar spinal cord exposed. This region is concerned with the processing of nociceptive information from the hindlimbs. A microelectrode is then lowered into the dorsal horn and the activity of a single dorsal horn neuron is monitored extracellularly. Prior to recording, each cell must respond to noxious heat, innocuous brushing, and transcutaneous electrical stimulation, and also have no or very low spontaneous activity. Once a cell satisfying all of these criteria is located, formalin (50 µl of a 5% solution in saline) is injected s.c. into the hindpaw and the firing rate of the neuron is recorded for 60 min (see section 2-2.1 of Methods and Materials for full protocol). This preparation was found to yield a biphasic neuronal firing response (Figure 4.1), with the times of onset and the relative durations of the phases correlating well with behavioral studies (Dickenson & Sullivan, 1987b). The UCL group now uses this model to study the pharmacology of formalin-induced activity in convergent cells, including the effects of receptor-selective opioids on this response.

Previous investigators using the formalin test to assess opiate^{ant}nociception in behavioral studies have all pretreated animals with opiates before injection of formalin, and have noted marked reductions in formalin-induced pain with this pretreatment protocol (Dubuisson & Dennis, 1977; Alreja et al., 1984; Shibata et al., 1989). Dickenson and Sullivan (1987b) found that intrathecal application of the potent mu opioid [D-Ala²,MePhe⁴ Gly(ol)⁵]enkephalin (DAGO) was more effective in modulating the biphasic excitation of dorsal horn neurons by formalin when administered before, as opposed to after, formalin injection. They suggested that the diminished ability of DAGO to affect the second peak when administered

post-formalin was an indication that the phasic activity of the first peak induces changes in neural systems leading to the second, tonic phase of neuronal firing. This is in agreement with Woolf and colleagues who, in studies on flexion reflex activity in the rat, have shown that a brief nociceptive input causes a prolonged increase in central excitability (Woolf, 1983; Cook et al., 1986) which, once established, is less susceptible to opioid inhibition (Woolf & Wall, 1986). Subsequent studies with formalin have addressed issues concerning this evidence that the observed effects of s.c. formalin may involve central changes accompanying peripheral activation of nociceptors.

Formalin injection does not result in visible signs of oedema until after the normal time course (60 min) of experimentation (e.g. by 2 hr post-formalin injection), however a number of investigators have implicated the involvement of inflammatory mechanisms in the formalin response. Haley et al. (1989) have provided electrophysiological evidence for a role of bradykinin in formalin-induced nociception. In these studies an antagonist at the B₂ subtype of bradykinin receptor greatly inhibited the second peak of the formalin response while having no effect on the first phase. The fact that the second peak was not abolished completely in these experiments was taken as indirect evidence suggesting the possible involvement of other inflammatory mediators such as histamine, serotonin, and prostaglandins. Shibata et al. (1989) have reported that bradykinin may be important in the manifestation of the first and second phase responses, though they concluded that the actions of bradykinin were mediated through the bradykinin B₁ receptor. There is, however, some doubt concerning the selectivity profiles of the antagonists used in these studies. Shibata et al. (1989) have also seen inhibition of the second phase upon treatment with compound 48/80, a mast cell depletor, and with inhibitors of prostaglandin synthesis, thus further implicating the participation of inflammatory mediators in the formalin response.

The tachykinin substance P (SP) has been implicated as a transmitter in fine nociceptive fibers in many studies using local or systemic administration of the

neurotoxin capsaicin to yield relatively selective depletion of SP-containing unmyelinated afferents (see reviews by Pernow, 1983; Salt & Hill, 1983). SP has also been reported to stimulate the release of histamine (Foreman & Jordan, 1983), to induce plasma extravasation (Saria et al., 1983), and to contribute to leukotriene- and prostaglandin-mediated hypersensitivity (see Payan et al., 1984), thus firmly establishing the role of SP in neurogenic inflammation. Shibata et al. (1989) have observed inhibition of the first, but not the second, phase of the formalin response after local capsaicin treatment of sciatic nerve. This group suggests that formalin activation of fine nociceptive fibers causes local and distant release of SP via axon reflexes.

With all these complex events going on simultaneously, central processing of the nociceptive information is likely to involve an array of excitatory and inhibitory influences at various levels in the dorsal horn of the spinal cord. Exemplifying this complexity, Dickenson and Sullivan (1987a) found that tactile segmental stimulation (brushing applied to the adjacent hairy skin of the thigh) and noxious pinch of the nose, tail, and paws produced profound inhibitions of formalin-evoked neuronal activity. These results were comparable to inhibitions produced by natural mechanical and thermal, and transcutaneous electrical stimuli in similar studies investigating the effects of such diffuse noxious inhibitory controls (DNIC) on convergent dorsal horn neurons (Le Bars et al., 1979a,b). DNIC modulation of afferent input in the spinal cord is believed to involve the activation of descending inhibitory serotonergic pathways (Le Bars & Villanueva, 1988).

Under normal conditions nociceptive C and A δ afferent nerve fibers display low levels of spontaneous activity, but following sensitization by inflammatory mediators possess lower nociceptive thresholds and increased levels of spontaneous firing. Heapy et al. (1987) have provided electrophysiological evidence that injection of dilute formalin solutions (1%) into rat hindpaw receptive fields evokes an immediate and intense biphasic stimulation of C- but not A δ -fibers. This is consistent with the findings of Dickenson and Sullivan (1987a)

who have reported that dorsal horn convergent neurons activated only by innocuous stimuli, and therefore without C-fiber input, fail to respond to formalin.

Further evidence that an ongoing peripheral input via the sensory terminals of C-fibers during the second peak is necessary to evoke excitation of dorsal horn cells comes from findings that s.c., but not intravenous, administration of the local anesthetic lignocaine (2%) during the second phase can abolish the formalin response (Dickenson & Sullivan, 1987a). Recently it has been suggested that the first phase is also subject to local anesthetic blockade (Haley et al., 1990). Collectively, these results imply the necessity for peripheral inputs in the manifestation of both phases, though Dickenson and Sullivan (1987a) suggest that this does not exclude the possibility that central changes may result in additional excitation independently thus leading to amplification of the original peripheral input.

These early experiments, then, highlight the involvement of C-fibers in the generation of the formalin response, but do not implicate the involvement of specific neurotransmitter systems. Many substances have been proposed as neurotransmitters released at the spinal cord level by nociceptive afferents, including excitatory amino acids, vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), somatostatin, ATP, and substance P (SP). It has been suggested, for example, that N-methyl-D-aspartate (NMDA) mediated events may augment the nociceptive responses of dorsal horn convergent neurons to formalin (Haley et al., 1990). Most of the attention, however, has focused on SP, which has fulfilled many of the criteria necessary to establish its role in the transmission of nociceptive information (see reviews by Nicoll et al., 1980; Salt & Hill, 1983). In the spinal dorsal horn SP is found predominantly in laminae I and II, and can be released by cutaneous stimuli of a variety of modalities including noxious chemical, mechanical, thermal, and electrical stimulation (Duggan & Hendry, 1986; Duggan et al., 1987; Go & Yaksh, 1987; Duggan et al., 1988). This central release of SP-LI is also subject to modulation by systemically or spinally administered opioid agonists in a naloxone-sensitive manner (Kuraishi et al., 1983;

More to our interests, subcutaneous injection of formalin into the rat hindpaw has been shown to increase the amount of immunoreactive SP in the upper two laminae of the dorsal horn within 60 min (Kantner et al., 1985), the normal time frame of behavioural and electrophysiological experiments using formalin as a noxious stimulus. They suggest that this build up of SP-LI in the dorsal horn may reflect both increased synthesis and release of SP (Kantner et al., 1986). Recent evidence from this group shows that morphine pretreatment (s.c. into the dorsal aspect of the neck) increases the levels of SP-LI built up in the dorsal horn during formalin-induced nociception, and that this effect is reversed by naloxone (McCarson & Goldstein, 1989). It has been proposed that this increase may be due to morphine inhibition of SP-LI release.

We have therefore, in later experiments, investigated whether peripheral opioid modulation of the formalin response, measured centrally as decreased dorsal horn convergent neuronal activity, results in a change in SP-LI release centrally.

To summarize this section, the precise mechanisms by which formalin acts remain unclear. The characteristic biphasic response seen with this noxious chemical nociceptive stimulus, however, may result from both direct activation of nociceptors and later-developing inflammatory processes. This explanation is based partially on reports that different classes of analgesics, including opiates, vary in their relative abilities to influence the early and late phases of the formalin response (Dubuisson & Dennis, 1977; Hunskaar et al., 1985; Hunskaar & Hole, 1987; Shibata et al., 1989). Dickenson and Sullivan have established that intrathecal application of mu (Dickenson & Sullivan, 1987b) and delta (Sullivan et al., 1989), but not kappa (except at high concentrations; personal communications) opioid ligands dose-dependently inhibit both phases of the formalin response. Jane Haley and I have studied peripheral injection of these same receptor-selective opioid agonists and found a different profile from that seen centrally.

4-2.1 Prolonged neuronal responses to subcutaneous (s.c.) formalin

A total of 126 single unit extracellular recordings were made of dorsal horn convergent neurons receiving both A β - and C-fiber afferent inputs from receptive fields in the hindpaws of 52 rats. Single convergent neurons were located with the aid of visual (storage oscilloscope) and audio (speaker) equipment (for details of the single unit recording discrimination see section 2-2.1 of the Methods and Materials). Neurons were separated into two distinct populations in terms of depth from the surface of the dorsal horn. Superficial cells (0-250 μ m) and deep cells (>500 μ m) could not be distinguished from each other by their neuronal responses and responded similarly to opiates. A third, intermediate group of neurons (250-500 μ m) in substantia gelatinosa was not included in these studies because of previous reports that substantia gelatinosa cells often exhibit excitatory responses to intrathecal opiates (Dickenson & Sullivan, 1986; Sullivan et al., 1989).

An acute burst of activity is seen immediately after s.c. formalin injection and lasts about ten minutes (Figure 4.1). This response cannot be attributed to the injection procedure since saline alone produces little neuronal activity. This initial phase is followed by a return to background level of activity, and then at 20 min after injection a second tonic period of excitation begins. The second peak of neuronal activity displays a more gradual onset than the first peak. Although the maximum level of the firing rate is lower, the duration of this second phase is longer, with significant levels of neuronal firing still occurring at the end of the 60 min sampling period.

All nociceptive dorsal horn neurons in these studies responded in this biphasic manner and over a similar time course. Cells varied widely in their firing rates per second at any one point in time, contributing to the large standard errors seen in summary figures and tables. Formalin resulted in some whitening of skin around the injection site, with no visible formation of oedema in the paw or toes over the hour-long sampling period. Therefore the neuronal responses described here occur prior to the formation of oedema, which is not noticeable until 2 to 3 hours after injection and is confined to the plantar region of the paw.

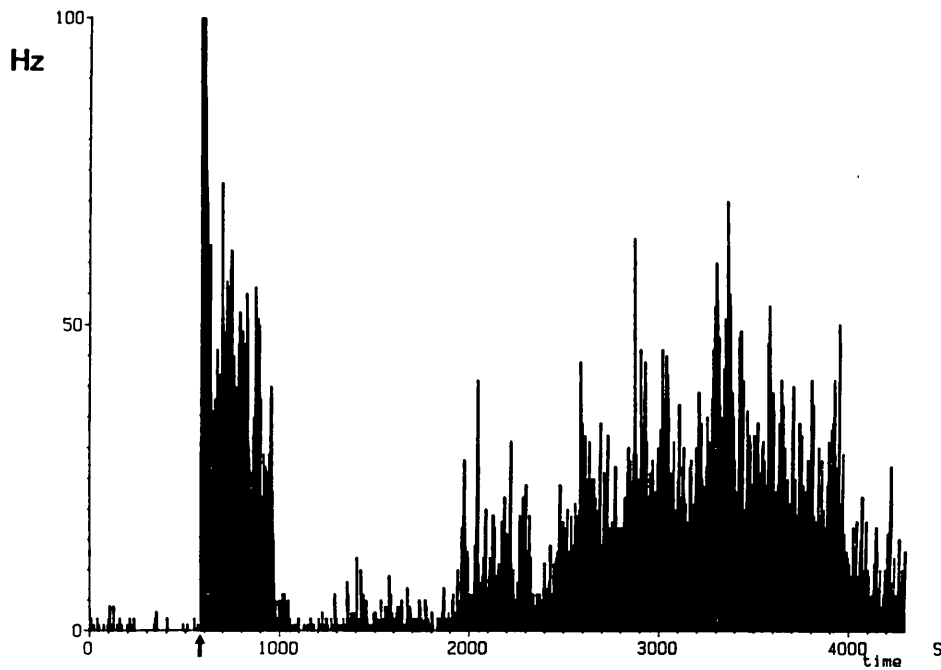


Figure 4.1. An extracellular recording of the activity (expressed as firing rate of the cell per second, in Hertz [Hz]) of a single rat dorsal horn neuron, showing the characteristic biphasic neuronal firing response obtained when using subcutaneous (s.c.) formalin as a long-lasting nociceptive stimulus. The arrow indicates the timepoint of s.c. injection of formalin (50 μ l of 5% solution in saline) into the rat hindpaw. Saline alone produces a similar injection artefact (reaching 100 Hz on the vertical axis) but produces little neuronal activity after the injection needle exits the tissue.

The trace in Figure 4.2 shows the result of a single experiment in which the kappa agonist U50488H (100 μg s.c. in a 50 μl volume of distilled water [dH_2O], administered 10 min before s.c. formalin) was tested for its peripheral effects on this prolonged noxious stimulus. As seen in the figure, U50488H reduces both peaks of formalin-induced activity as compared to the control trace (Figure 4.1).

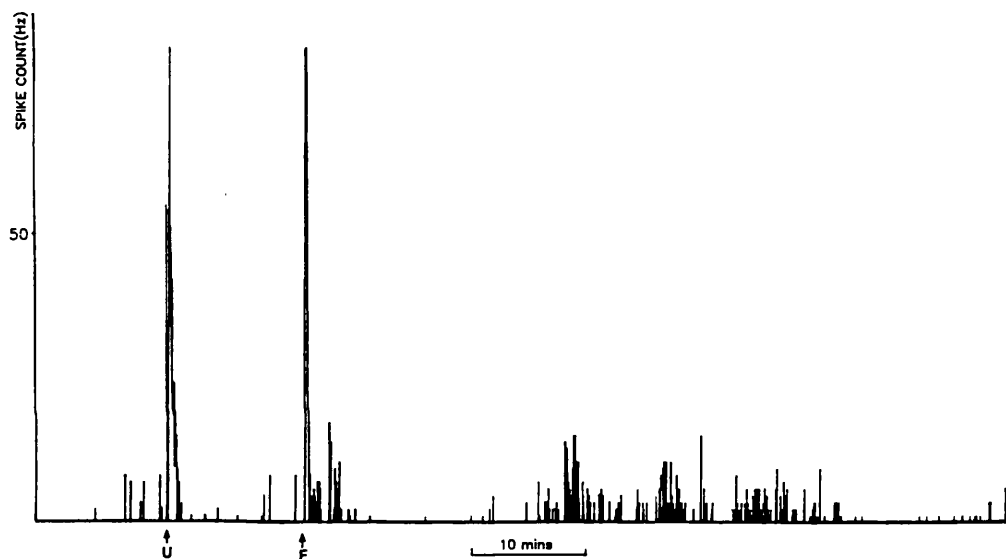


Figure 4.2. *An extracellular single-unit recording showing the inhibitory effect of U50488H (100 μg s.c. in 50 μl dH_2O) on both peaks of the formalin-induced firing response (expressed as firing rate of the cell per second, in Hz) of a single rat dorsal horn convergent neuron. The arrows indicate the points of injection of U50488H (U) and formalin (F), and are followed immediately by high levels of firing activity (approx. 100 Hz) representing injection artefacts which return to baseline after the needle exits the hindpaw.*

Figure 4.3 summarizes our work on the effects of peripherally administered U50488H. The left and right halves of this bargraph represent, respectively, the first and second peaks of the formalin response. In these experiments the first peak is defined as net neuronal activity over the first ten minutes after formalin

injection, and the second peak as neuronal activity over the remaining 50 minutes of the experiment. It can be seen that U50488H inhibits both peaks of the biphasic formalin response and the effect is dependent on dose. With 25 μg U50488H the slight inhibition is not statistically significant (P values in Table 4.1). But with the higher, 100 μg , dose both phases are inhibited significantly ($P=0.01$ for the first peak, and $P=0.006$ for the second peak). In order to show that U50488H was not exerting its inhibitory actions by acting systemically, we injected the kappa ligand into the contralateral paw and neither peak of formalin-induced activity was inhibited.

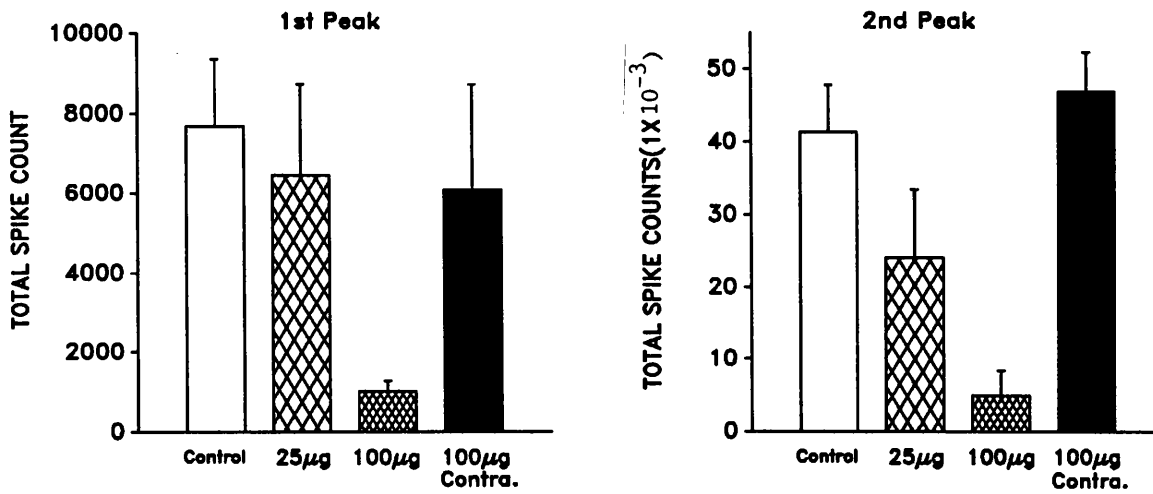


Figure 4.3. Peripherally administered U50488H (injected *s.c.*, in 50 µl dH₂O, into the same site as formalin) inhibits both the first (left) and second (right) peaks of formalin-induced activity in dorsal horn convergent neurons. Each bar on the x-axis represents the mean ± s.e.m. of a series of studies (see Table 4.1 for experimental numbers) in which the effects of 25 µg or 100 µg U50488H were compared with neuronal firing responses in formalin controls (Control) and tests for leakage of *s.c* U50488H into the systemic circulation (Contra.= contralateral paw). Note that the scale of the second peak y-axis is five times that of the first peak because, as described in the text, the second phase of the formalin response is more prolonged.

Table 4.1. Data used to calculate results for Figure 4.3. The first peak is defined as total neuronal firing over the first 10 min following formalin injection, and the second peak as neuronal activity for the remaining 50 min of recording. P values (n.s.=not significant) were calculated by the Mann-Whitney U-test. See section 2-2.1 for discussion of nonparametric analysis using this test. Median values for experimental results presented in this chapter were always found to fall within the limits of the mean.

U50488H TREATMENT	n	1 st Peak		2 nd Peak	
		Total Spike Counts (mean ± s.e.m.)	P	P	Total Spike Counts (mean ± s.e.m.)
Formalin control w/ dH ₂ O vehicle	7	7675 ± 1676	-----	-----	41361 ± 6401
25 µg <i>s.c.</i>	7	6439 ± 2285	n.s.	n.s.	24072 ± 9257
100 µg <i>s.c.</i>	5	1007 ± 258	0.01	0.006	4869 ± 3545
100 µg Contra.	3	6070 ± 2643	n.s.	n.s.	46934 ± 5424

4-2.3 Kappa depression of formalin-evoked activity is naloxone-reversible

Kappa (U50488H) inhibition of the biphasic formalin response was reversed by intraplantar administration of the opioid antagonist naloxone (100 µg in 100 µl saline; Figure 4.4). This dose of intraplantar naloxone had no effect of its own on formalin-induced activity.

Naloxone was also administered intraperitoneally (i.p.; 10 mg/kg, e.g. approx. 250 µg per animal) in order to try and reverse the effects of U50488H via a more conventional route of administration e.g systemically. Some reversal of the kappa inhibition was seen with i.p. naloxone (Figure 4.5), but due to variability in responses this effect is not statistically significant.

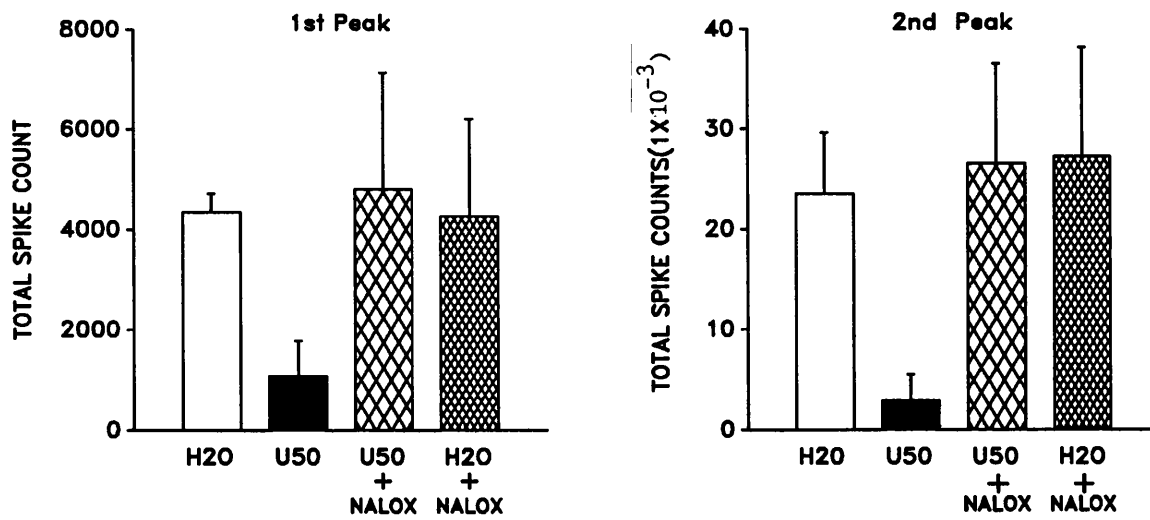


Figure 4.4. *Intraplantar administration of 100 µg naloxone (in 100 µl saline) along with 100 µg s.c. U50488H (in 50 µl dH₂O; U50 + Nalox., n=6) significantly reverses the inhibitory actions of U50488H (U50, n=4) on both the first (left) and second (right) peaks of the formalin response. Controls for the opioid vehicle (H₂O, n=9) show that this dose of naloxone has no effect on its own (H₂O + Nalox., n=9).*

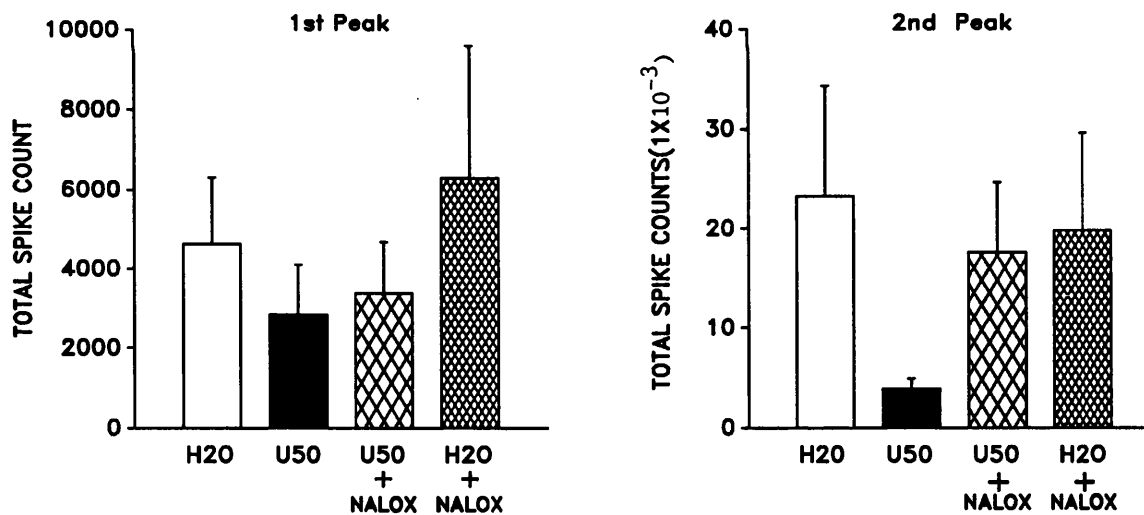


Figure 4.5. *Intraperitoneal (i.p.) injection of 10 mg/kg naloxone (in approx. 0.4 ml saline) along with 100 µg s.c. U50488H (in 50 µl dH₂O; U50 + Nalox., n=7) has no significant effect on reversing inhibition of the first peak by U50488H (U50, n=11), and shows only a slight (but insignificant, p=0.12) ability to reverse kappa inhibition of the second peak of the formalin response. Controls for the opioid vehicle (H₂O, n=11) show that this dose of naloxone has no effect on its own (H₂O + Nalox., n=7).*

4-2.4 Lack of kappa effect on electrically-evoked neuronal responses

Whereas the 100 μg dose of U50488H produced pronounced inhibitions of both phases of formalin-induced neuronal activity, this same dose of the kappa agonist did not affect responses evoked by electrical stimulation of C-fibers or A-fibers (Figure 4.6). This result shows that since transcutaneous electrical stimulation bypasses any potential opioid control of neuronal firing at the peripheral terminals of sensory neurons, U50488H is not acting by a local anesthetic-like action (see Frank, 1975). This would then suggest an action on the terminal or an interference with inflammatory mediators.

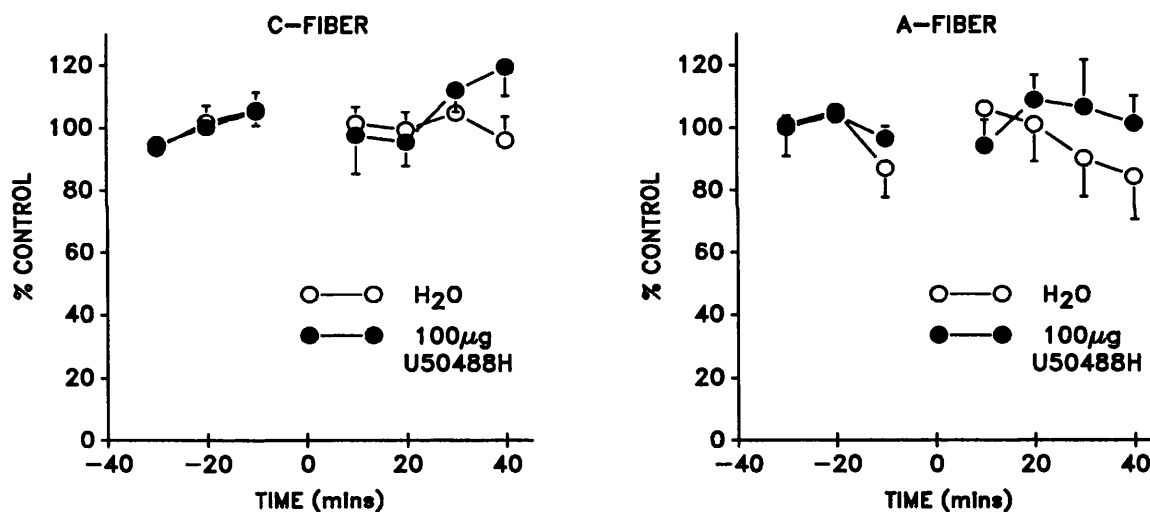


Figure 4.6. Peripheral (s.c.) administration of 100 μg U50488H does not significantly affect dorsal horn neuronal responses evoked by electrical stimulation at either C-fiber (left) or A-fiber (right) intensities when compared with opioid vehicle (H_2O) controls. Responses to electrically-evoked activation were determined by stimulating the hindpaw receptive field at three times the threshold for both A and C fibers (2msec wide pulses at 0.5 Hz) and counting the activity thus evoked over a trial of 16 stimuli. Trials were performed every 10 min and monitored for 40 min following administration of either distilled H_2O or U50488H directly into the receptive field (e.g. the site of electrical stimulation). All points represent means \pm SEM of six experiments.

This peripheral effect of U50488H is interesting when compared with the outcome of experiments done by Sullivan & Dickenson (personal communications), which show that intrathecal application of U50488H, at doses of

50-250 μ g, does not affect either phase of the formalin response. This work could be extended by doing parallel tests with U69593 and PD117302, non-peptide agonists which are reported to have increased selectivity for the kappa receptor. But, as shown in the next section (4-2.2) of this chapter, neither morphine, exogenous ligand for the mu receptor, nor a delta-selective ligand inhibit formalin-induced activity when applied peripherally into the receptive field, thus indicating that this effect is mediated via kappa-type opioid receptors. Other possible experiments to confirm the involvement of κ receptors include the use of selective kappa opioid antagonists such as norbinaltorphimine. These studies might also have benefitted from quaternary opioid antagonists (e.g. naloxone methylbromide) unable to penetrate the blood-brain barrier, especially given the well-documented central effects of naloxone (Le Bars et al., 1981b). Yet naloxone alone did not affect the formalin response. This observation suggests that the reversal of U50488H inhibition by the opioid antagonist can be attributed to an action at peripheral opioid receptors. Furthermore, there is some discussion in the literature concerning the possible central effects of quaternary antagonists *in vivo* (see review by Brown & Goldberg, 1985). These reported inconsistencies in delimiting the action of these compounds solely to the periphery suggest that this may not have been a conclusive experiment.

4-2.5 Lack of peripheral mu and delta opioid effects on formalin-induced activity

These studies have been extended to include morphine and Tyr-D-Ser-(tbu)-Gly-Phe-Leu-Thr (DSTBULET), agonists selective at mu and delta opioid receptors, respectively. DSTBULET, made by the insertion of a bulky tertiary butyl ether group into the sequence of the delta ligand DSLET, was chosen for its good selectivity for the δ -opioid receptor (Delay-Goyet et al., 1987). Many δ ligands, including the endogenous agonists [Leu]enkephalin and [Met]enkephalin (Kosterlitz & Paterson, 1985) and Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET; Zajac et al., 1983), show considerable cross-reactivity at mu receptors in spinal cord. [D-Pen²,D-Pen⁵]enkephalin (DPDPE) and [D-Pen²,L-Pen⁵]enkephalin (DPLPE), conformationally restricted cyclic enkephalin analogues, have been shown to have better delta opioid receptor selectivity in binding studies (Mosberg et al., 1983), but are reported to be less potent than DSTBULET (Delay-Goyet et al., 1987).

Intrathecal application of morphine and DSTBULET results in inhibition of both phases of the formalin response (Dickenson & Sullivan, 1987b; Sullivan et al., 1989). Peripheral administration of morphine into the hindpaw receptive field (100 μ g s.c. in 50 μ l saline), at a dose just below that which would produce systemic actions (Le Bars et al., 1983), affects neither phase significantly (Figure 4.7). The same dose of DSTBULET (100 μ g in 50 μ l dH₂O) also has no significant effect on either phase of the formalin response (Figure 4.8). This lack of an inhibition of formalin-induced activity by peripheral mu and delta ligands may emphasize further the involvement of the kappa receptor subtype in the peripheral action of U50488H on the formalin response. It must however be qualified that we have not investigated directly the possibility that the insignificant effects of morphine and DSTBULET on formalin-induced activity (when administered s.c.) may reflect that these mu and delta ligands are removed from the site of action faster than the kappa agonist U50488H. In future studies it would also be desirable to investigate the effects of higher doses of DSTBULET (e.g. 400-500 μ g s.c., or four to five times the dose used in our experiments) to confirm that our observations apply for several concentrations of this delta ligand. Although, as stated above, there are drawbacks associated with using higher concentrations of morphine, future studies would benefit from extending the range of ligands tested in the formalin model to incorporate another mu ligand e.g. [D-Ala², MePhe⁴, Gly(ol)⁵]enkephalin (DAGO), again at several doses to maintain consistency with the concentration regime followed for the kappa agonist experiments.

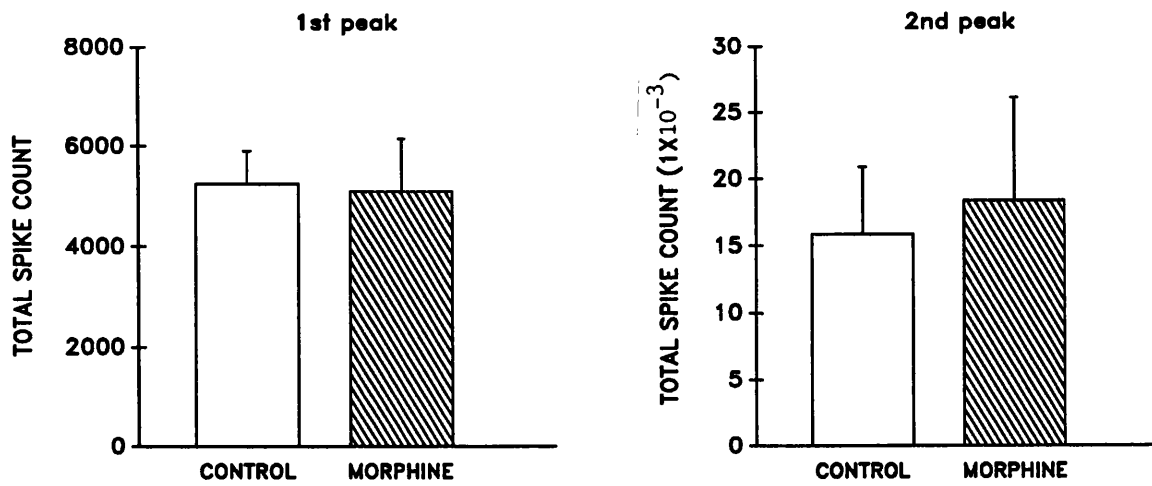


Figure 4.7. Morphine (100 μg in 50 μl of saline), an agonist at the μ opioid receptor, has no effect on the first (left) or second (right) peaks of formalin-induced neuronal activity when administered s.c. into the hindpaw receptive field ($n=11$ for controls with saline vehicle, and $n=10$ for morphine experiments).

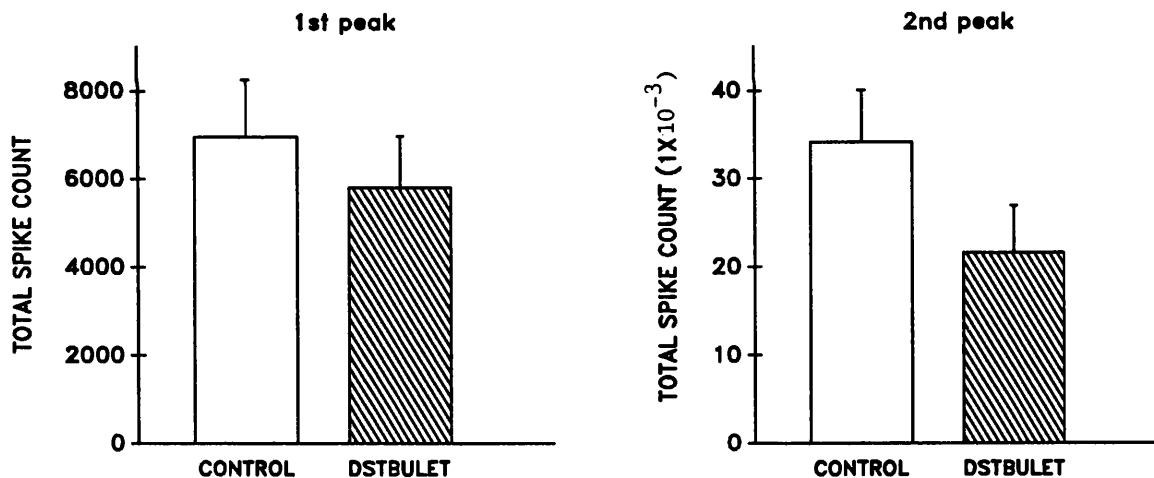


Figure 4.8. Summary bargraphs showing that s.c. administration of the delta opioid agonist DSTBULET (100 μg in 50 μl dH₂O) has no significant effect on either the first (left) or second (right) peaks of the formalin response ($n=14$ for controls with dH₂O vehicle; $n=13$ for DSTBULET experiments).

4-2.6 Superfusion studies of exposed spinal cord *in vivo*:
insignificant outflow of SP-LI centrally in response
to peripheral formalin stimulation

Unlike previous investigators who have measured spinal SP-LI outflow via push-pull cannulae (Kuraishi et al., 1983) or catheters inserted into the dorsal horn (Go & Yaksh, 1987), we opted for a non-invasive and potentially less damaging perfusion technique (see Duggan & Hendry, 1986). The full methodology of these experiments is presented in section 2-2.2 of the Methods and Materials. In short, the L₁-L₃ segments of the lumbar spinal cord were exposed by laminectomy and an agar well built around the exposed area. Urethane was used as the anesthetic in most of these experiments, though halothane was also employed in comparative studies. Urethane has been shown not to affect the responses of convergent neurons to peripheral stimulation (Smallman, 1988). Otherwise, the surgical procedure was kept as similar to that employed for the electrophysiological characterization of the formalin response in order to make more or less direct comparisons of SP-LI measurements to peaks of formalin-induced activity. Following a 30 min superfusion with artificial CSF to allow the tissue to accommodate after surgery, stimuli of a variety of sensory modalities were used to activate the central terminals. This activation was assessed by measuring SP-LI outflow from the exposed spinal region over a 90 min period, collecting 10 min fractions of the superfusate onto acetic acid (0.1 M final concn), lyophilizing, and then assaying resuspended lyophilizates for SP-LI (see section 2-3).

Formalin (5% in a 50 μ l volume) was injected s.c. into the hindpaw and SP-LI samples collected every 10 min. In addition to s.c. formalin, a variety of cutaneous stimuli were used to activate central terminals including noxious mechanical (continuous pinching of a single hindtoe for 5 min with blunt forceps; n=2), noxious thermal (70°C soldering iron held adjacent to, but not touching, hindtoe for 2 min; n=2), and transcutaneous electrical stimulation (as described in section 2-2.1, with 2 msec pulses at 15 volts/Hz applied by a Digitimer Ltd. isolated stimulator; n=2). Direct activation of the central terminals was also

attempted by superfusing the spinal cord with CSF buffers containing capsaicin (1 μ M for 3 min; n=2) and depolarizing concentrations of potassium (60 mM KCl, 3 min; n=5). In total, 17 experiments were carried out, using two different types of anesthesia (halothane and urethane), with only minor, inconsistent increases in the outflow of SP-LI. This neuropeptide release was not improved significantly by including peptidase inhibitors (10 μ M final concns of bestatin and bacitracin) in the synthetic CSF superfusing the spinal cord. | A preliminary experiment in which substance P (50 fmol) was added exogenously to the CSF superfusing the spinal cord and collected onto acetic acid (over a 10 min period according to the protocol described in section 2-2.2 of the Methods and Materials) showed that, while enzymatic degradation of released peptide may have occurred (e.g. approximately 35 fmol SP-LI was recovered in this experiment), SP-LI could be detected using this superfusion protocol. Studies were not carried out to determine the minimum amount of exogenously applied SP detectible by our protocol. As described in section 2-2.1, the pia mater was not removed (for reasons of maintaining the integrity of the spinal tissue) and we have not explored the possibility that SP-LI released from the spinal dorsal horn was unable to traverse the pial membrane.

We have seen that peripheral (s.c.) administration of the kappa agonist U50488H inhibits both peaks of formalin-induced activity in dorsal horn convergent neurons. The lack of effect of contralateral U50488H injection, and the reversibility of U50488H inhibition by local (intraplantar) naloxone administration suggest that this kappa event is mediated through a peripheral opioid receptor. Intraperitoneal (i.p.) injection of naloxone, at a high dose (10 mg/kg) reported to reverse morphine actions on formalin-induced pain (see North, 1978), did not reverse significantly this kappa effect on the formalin response. However, large doses of systemic naloxone are required to reverse kappa events (Kosterlitz et al., 1981,1986), and this may at least explain in part the better reversal seen with intraplantar naloxone. Naloxone alone, given either intraplantar or systemically, had no effect of its own on the formalin response. North (1978) and Abbott (1988) have confirmed these observations in reporting that the pretreatment of rats with naloxone via the systemic or other routes does not alter behavioral pain ratings following formalin injection. Intravenous (i.v.) administration of naloxone was not tested because the timeframe of the formalin response necessitates a longer duration of action than is provided by the i.v. route.

Abbott (1988) has reported recently a peripheral opioid effect with high doses of ethylketocyclazocine (EKC), but not morphine, in a behavioral test of formalin-induced nociception. Low doses of EKC, however, produced centrally-mediated antinociception in these studies so that Abbott implicated the involvement of both central and peripheral mechanisms in EKC modulation of behavioral responses to formalin injection. The opioid receptor type involved in this naloxone-sensitive action was not identified since, as discussed in section 1-3.4 and chapter 3, EKC has high affinity for both mu and kappa opioid binding sites.

Neither the mu agonist morphine, nor the delta-selective DSTBULET significantly affected the formalin response when administered s.c. into the hindpaw receptive field. Stein et al. (1989), in contrast, present evidence from

another model of prolonged nociceptive stimulation (Millan et al., 1988) that mu-, delta-, and kappa-selective opioid agonists can modulate antinociceptive responses via a peripheral receptor-specific mechanism. They, however, urge caution in drawing parallels to other *in vivo* studies due to the postacute nature of their unilateral Freund's adjuvant-induced inflammatory model in the rat hindpaw. Their suggestion that *de novo* synthesis and/or axonal transport of opioid receptors may be increased during inflammatory pain states (Stein et al., 1988,1989) may partially explain other reports of differential involvement of opioid receptor types in inflamed as compared to non-inflamed states. Thus, whereas Russell et al. (1987) find that U50488H displays powerful inhibition of spontaneous afferent activity in inflamed knee joint of the cat, Yaksh (1988) reports inhibition of SP-LI release from non-inflamed cat knee joint afferent terminals by mu and delta agonists but not by U50488H.

Attempts to correlate our observed peripheral kappa opioid inhibition of formalin-induced activity with altered outflow of SP-LI centrally proved negative. It seems unlikely that the depth of anesthesia produced with urethane was too deep and affected the release mechanism, especially since urethane is known to yield reproducible responses in dorsal horn neurons (Smallman, 1988), and since experiments using halothane as the anesthetic produced similar results. Studies by Hamon and colleagues, in which an excess of potassium has been used to directly stimulate rat spinal cord slices, show that the rate of basal outflow of SP-LI (Mauborgne et al., 1987) and calcitonin gene-related peptide-LI (Pohl et al., 1989) decreases progressively after the start of superfusion. This group showed that the repetitive presentation of potassium-enriched medium to this tissue from the dorsal half of the lumbar enlargement yields corresponding peaks of SP-LI release which are significantly above the respective background levels. However, these observations are clouded by the fact that the baseline deteriorates as a function of time to the extent that the second peak of SP-LI is not markedly different from the spontaneous outflow at the start of the experiment. Given these results in an *in vitro* preparation with greater access to SP stores, perhaps it is not surprising that

in the intact animal we do not detect appreciable increases in basal release of SP-LI using a non-invasive technique of perfusing the surface of the lumbar spinal cord.

A complementary approach is to turn to look at the peripheral terminals, which are increasingly thought to be analagous to central terminals (see Yaksh, 1988). As at the central terminals of primary afferent fibers, opioid modulation of SP-LI release from peripheral sensory nerve endings has been demonstrated *in vivo* (Brodin et al., 1983; Yaksh, 1988). Classifying opioid receptor type *in vivo*, however, presents several limitations. Firstly, even with supposedly very specific agonists cross-reactivity with other receptor types cannot be ruled out (Dray et al., 1989a; James et al., 1990). Diffusional problems also preclude definitive determination of drug concentrations at relevant sites. For these reasons we decided that greater progress could be made *in vitro* to further our understanding of the mechanism by which opioids modulate formalin-induced nociception. These efforts culminated with results presented in Chapter 7, in which we describe a model we have used to look at opioid effects on the stimulus-evoked (including formalin-induced) release of substance P-like immunoreactivity from peripheral sensory nerve terminals.

CHAPTER 5 -
OPIOID EFFECTS ON PRIMARY CULTURES OF RAT
DORSAL ROOT GANGLION (DRG) SENSORY NEURONS

Technical limitations to the *in vivo* study of sensory nerve endings can, to some extent, be surmounted by studying sensory neurons in cell culture. Dissociated cell preparations offer the advantage of being free of diffusional limitations, inherent to *in vivo* and isolated organ preparations, which make it difficult to gauge drug concentrations accurately at sensory endings. Other factors contributing to the extensive use of sensory neurons include their accessibility and the relative ease with which they can be cultured.

Nociceptive sensory information is transmitted from the periphery to the spinal cord via primary afferent sensory neurons. The cell bodies of these neurons are located in the dorsal root ganglia (DRG) and their central terminals synapse within the dorsal horn of the spinal cord. At the light microscopic level, DRG sensory neurons have been classified into two subpopulations based on a variety of morphological criteria including cell body diameter (Andres, 1961). Cells with large light somata are A-type neurons, while the B class of DRG neurons has small dark somata. Subclasses of sensory neurons can also be distinguished on the basis of functional criteria such as action potential duration and conduction velocity, and on the site of termination of the the three main groups of primary afferent sensory fibers (large myelinated, small myelinated, and small unmyelinated) within the spinal dorsal horn (see Lieberman, 1976). The large light A cells give rise to fast-conducting myelinated fibers activated by mechanical and thermal stimuli. Whereas the small dark B neurons include the polymodal nociceptors and give rise to slower conducting unmyelinated C fibers with long action potential durations.

Primary cultures of DRG neurons, prepared by dissociating intact dorsal root ganglia into individual cells by enzymatic digestion and mechanical disruption (see sections 2-4.1 and 2-4.2 of Methods and Materials), retain properties displayed by nociceptive sensory neurons *in vivo*. These properties include activation by bradykinin and capsaicin (Baccaglini & Hogan, 1983). Evidence suggests that opioid receptors exist on the presynaptic terminals of small diameter primary afferent fibers which subserve nociception (Gamse et al., 1979; Fields et

al., 1980). Substance P (SP) is also localized to these fine nociceptive afferents and has been implicated to perform a neuromodulatory role in nociceptive pathways (see review by Salt & Hill, 1983). Opioids may thus modulate nociceptive activity by interacting at presynaptic binding sites to inhibit the release of SP from the central endings of primary afferent sensory nerves. Mudge et al. (1979) have demonstrated that SP, contained mainly if not exclusively in the small dark B class of DRG cells (Hokfelt et al., 1976), is released from dissociated DRG cultures by depolarizing concentrations of potassium and that this outflow is inhibited by the opioid agonist [D-Ala²,Met⁵]enkephalinamide. Primary cultures of mouse, rat, and embryonic chick DRG neurons have therefore been used extensively for the biochemical and electrophysiological characterization of opioid receptor properties on sensory neurons.

Werz and Macdonald have reported two main effects of opioids on co-cultures of mouse DRG-spinal cord neurons. Dynorphin, an endogenous ligand for the kappa opioid receptor, causes a reduction in the duration and amplitude of calcium-dependent action potentials in cultured DRG neurons by decreasing voltage-dependent calcium conductance (Werz & Macdonald, 1984,1985; Macdonald & Werz, 1986). Mu and delta ligands also shorten action potentials (Werz & Macdonald, 1982) but by increasing calcium-dependent potassium conductance (Werz & Macdonald, 1983). Classical neurotransmitter release is stimulated by the influx of calcium ions into presynaptic nerve terminals (Llinas et al., 1976,1981). Therefore the activation of opioid receptors on primary afferent terminals to inhibit calcium entry, resulting from either a reduction in calcium conductance or an increase in potassium conductance, may translate into a reduction in the presynaptic release of neurotransmitter.

We are interested in opioid modulation of nociceptive signals in primary afferents, and have therefore used primary cultures of DRG neurons to investigate opioid effects on several indicators of neurotransmitter activity. Firstly we studied kappa effects on potassium depolarization-induced uptake of calcium-45 into neonatal rat DRG sensory neurons. We have also used potassium-rich solutions to

evoked release of substance P-like immunoreactivity (SP-LI) from neonatal and adult DRG cells, and have attempted to modulate this release with opioids.

5-2.1 Potassium (K⁺)-induced uptake of Calcium-45 (⁴⁵Ca²⁺) into populations of rat DRG neurons

Depolarization of cultured DRG cells by increasing the potassium (K⁺) concentration of the control buffer (Ca²⁺- and Mg²⁺-free Hanks balanced salt solution, pH 7.4) above 5.4 mM during a 10 min incubation period (see section 2-4.3 of Methods and Materials for uptake protocol) led to a significant increase in the influx of ⁴⁵Ca²⁺ into the cells (Figure 5.1). Enhancement of the 30 mM K⁺-induced influx of ⁴⁵Ca²⁺ was achieved by incubating the DRG cells with Bay K 8644, a dihydropyridine Ca²⁺ channel agonist (Schramm et al., 1983) known to prolong the opening of only the L-type class of voltage-sensitive Ca²⁺ channels (Fox et al., 1987). Although the response of cells was somewhat variable, 0.3 μM Bay K 8644 (Bay K) enhanced uptake by approximately doubling stimulation above levels attained by 30 mM K⁺ alone (Figure 5.2). The apparent decrease in the signal with micromolar Bay K concentrations (Figure 5.2) may reflect the reported partial agonist nature of Bay K outside of the nanomolar range (see White & Bradford, 1986). These levels of stimulation are comparable to those reported for K⁺/Bay K-induced ⁴⁵Ca²⁺ influx into DRG neurons (Wood et al., 1988) and co-cultures of spinal cord-DRG neurons (Attali et al., 1989). Basal uptake of ⁴⁵Ca²⁺, in low potassium (5.4 mM) medium was not affected by inclusion of Bay K 8644 in the incubation buffer (Figure 5.2).

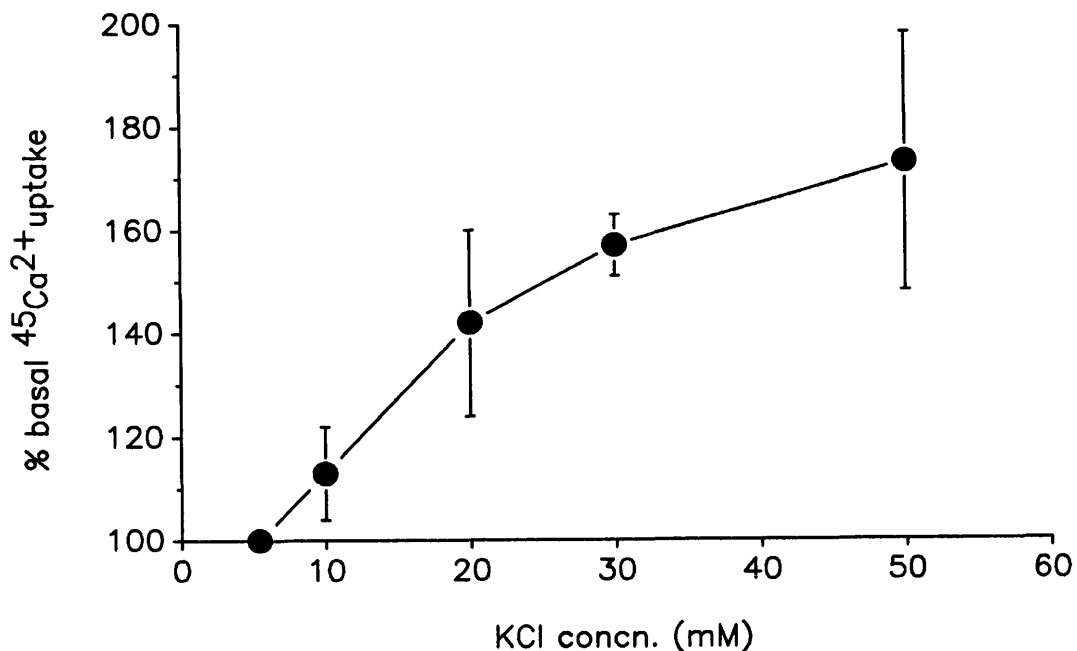


Figure 5.1. Concentration-response curve for potassium-induced $^{45}\text{Ca}^{2+}$ uptake into primary cultures of neonatal rat DRG neurons. Means \pm SEM are for replicates of six, in at least 2 separate experiments (with $n=2$ for 5 mM K^+ and $n=19$ for 30 mM K^+). For all figures in this chapter, "replicates" refers to the number of wells containing DRG cells from the same tissue preparation which were exposed to experimental stimuli. Results with "replicates" were averaged together (e.g. $n=1$), with the experimental "n" value referring to experiments conducted on different days and on different DRG cell preparations.

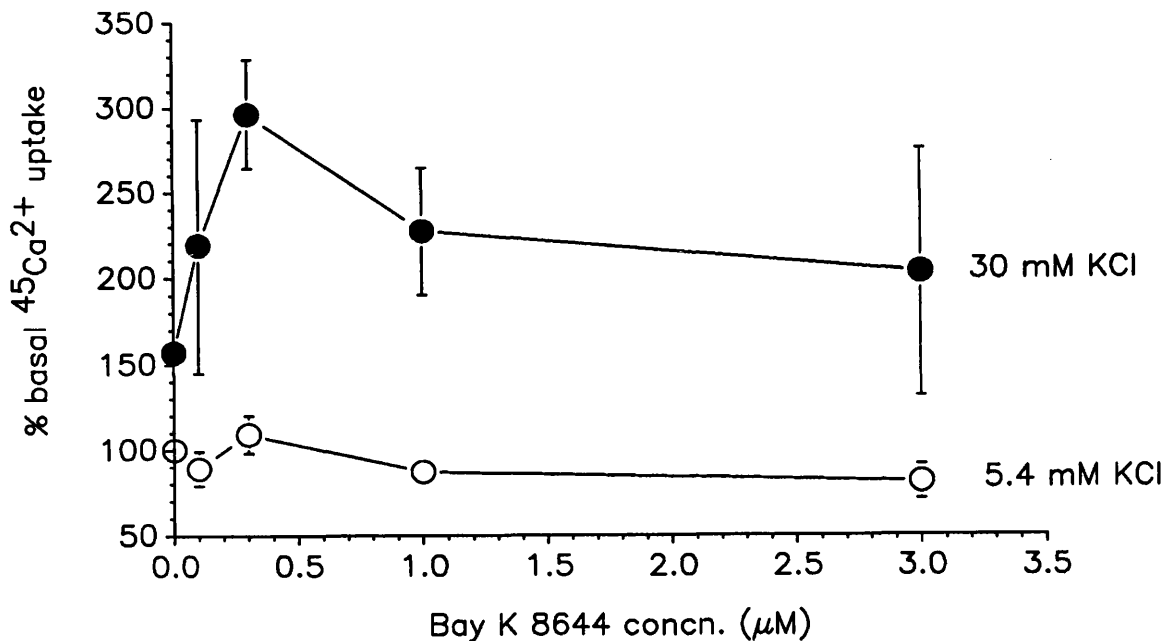


Figure 5.2. Concentration-response curves for the effect of Bay K 8644 on basal (5.4 mM KCl) and 30 mM K^+ -stimulated uptake of $^{45}\text{Ca}^{2+}$ into neonatal rat DRG cells. Means \pm SEM are for replicates of six, in at least 3 separate experiments, with $n=16$ for 30 mM K^+ /0.3 μM Bay K). The error bars for several (e.g. three) data points presented in this figure are smaller than the resolving capability of the SigmaPlot graphics package used to construct this figure.

than 5-fold increase (547%, 544% of basal $^{45}\text{Ca}^{2+}$ uptake; n=2) in the rate of $^{45}\text{Ca}^{2+}$ uptake. Wood et al. (1988) have also noted much higher levels of calcium accumulation with capsaicin than K^+ depolarization, and have suggested that this fact (combined with an insensitivity to Ca^{2+} channel antagonists) indicates that capsaicin-induced $^{45}\text{Ca}^{2+}$ influx involves mechanisms other than the opening of voltage-sensitive calcium channels.

The cultures of DRG neurons contained about 10% of non-neuronal cells. Purified primary cultures of these non-neuronal cells (i.e.: fibroblasts/Schwann cells) derived from DRG did not take up $^{45}\text{Ca}^{2+}$ in response to the 30 mM K^+ /0.3 μM Bay K 8644 stimulus (n=6). Hence the calcium uptake is a measure of neuronal activity.

Membrane depolarization has been shown to increase the uptake of calcium into peripheral neurons via the opening of voltage-sensitive calcium channels (VSCCs; see review by Miller, 1987b). Three different types of VSCCs (designated T, N, and L) have been identified in DRG neurons in culture on the basis of such characteristics as carrying conductances and the voltage ranges over which these channels activate and inactivate. A K^+ depolarizing stimulus first gives rise to a small transient calcium current via T channels, with stronger depolarization resulting in the activation of intermediate sized N channels and also the L-type of VSCCs. We tested that $^{45}\text{Ca}^{2+}$ uptake into primary cultures of neonatal rat DRG cells occurred by this mechanism by investigating the effect of several divalent cations and an organic calcium channel blocker on K^+ /Bay K-stimulated calcium uptake. Nifedipine, a dihydropyridine antagonist at L-type calcium channels (see Rane et al., 1987), blocked the K^+ /Bay K-induced uptake of $^{45}\text{Ca}^{2+}$ into DRG neurons (Figure 5.3), reaching a maximum of 50% inhibition at 1 μM (52%, 46% inhibition; n=2). This may result from the fact that though T-, N-, and L-type VSCCs would be activated by our strong depolarizing stimulus, the majority of the $^{45}\text{Ca}^{2+}$ influx would be mediated by L and N channels (as described above) of which only the L-type are sensitive to the dihydropyridine antagonist nifedipine. N-type VSCCs, but not L channels, are sensitive to the marine snail venom omega-conotoxin and this difference should be exploited in future experiments to test the hypothesis that our inability to block completely $^{45}\text{Ca}^{2+}$ uptake with nifedipine may be due to calcium influx through N channels. Attali et al. (1989) were also unable to inhibit $^{45}\text{Ca}^{2+}$ uptake completely with nifedipine. They reported that nifedipine gives a maximum inhibition of 80% in co-cultures of rat DRG and spinal cord neurons. The divalent cations cadmium and cobalt reduced stimulated $^{45}\text{Ca}^{2+}$ uptake to near background levels at 1 mM, with IC_{50} values in the range of 1-10 μM (Figure 5.4).

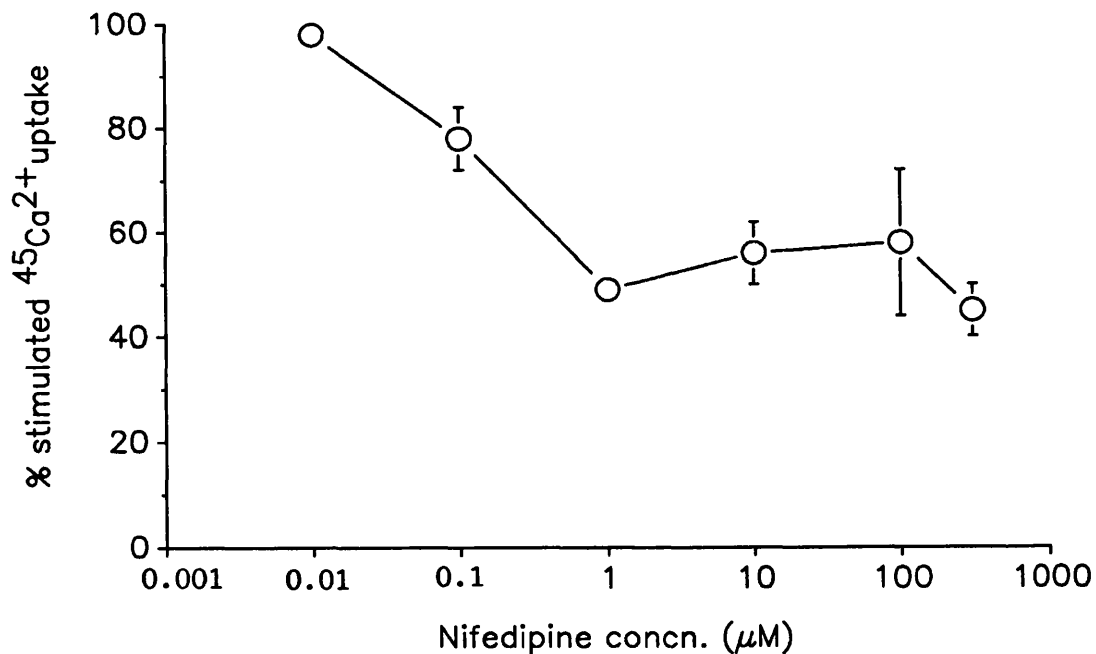


Figure 5.3. The effect of nifedipine, an organic calcium channel blocker, on the stimulation of $^{45}\text{Ca}^{2+}$ uptake by 30 mM K^+ /0.3 μM Bay K 8644 (K^+ /Bay K) into primary cultures of neonatal rat DRG neurons. Means \pm SEM are for replicates of six, in at least 2 separate experiments (with n=2 for 0.01 μM and 1 μM nifedipine).

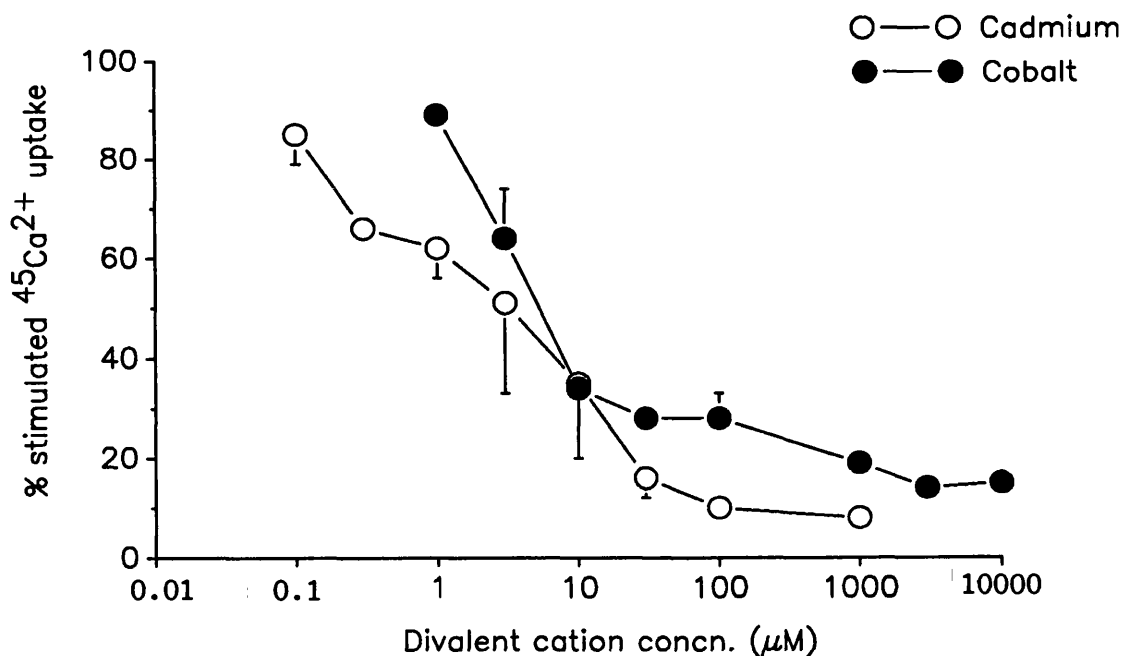


Figure 5.4. The divalent cations cadmium (Cd^{2+}) and cobalt (Co^{2+}) dose-dependently block K^+ /Bay K-stimulated uptake of $^{45}\text{Ca}^{2+}$ into neonatal rat DRG neurons. Means \pm SEM are for replicates of six, in at least 2 separate experiments (with n=2 for all data points without error bars).

Cells have other mechanisms of regulating Ca^{2+} entry besides voltage-sensitive calcium channels. One of these is the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Replacement of extracellular Na^+ by isosmotic sucrose, however, had little effect on 30 mM K^+ /1.0 μM Bay K-induced $^{45}\text{Ca}^{2+}$ uptake. Stimulated levels of $^{45}\text{Ca}^{2+}$ influx dropped slightly, but not significantly, to $181 \pm 26\%$ basal in Na^+ -free medium (n=3) as compared to $227 \pm 37\%$ in control buffer (n=3). Basal $^{45}\text{Ca}^{2+}$ uptake was increased by $18 \pm 7\%$ (n=3), perhaps indicating that low levels of Ca^{2+} may normally be extruded out of the DRG cells in exchange for Na^+ .

Having provided evidence that the accumulation of ⁴⁵Ca²⁺ into cultured DRG cells was occurring at least in part through voltage-sensitive Ca²⁺ channels, we moved on to investigate the effects of dynorphin on ⁴⁵Ca²⁺ uptake. Prodynorphin contains α- and β-neoendorphin, dynorphin A, and dynorphin B (Kakidani et al., 1982), all of which can be released at nerve terminals (Chavkin et al., 1983). We began our investigations with dynorphin B, which has been shown to be equieffective with dynorphin A(1-17) in decreasing calcium-dependent action potential duration in mouse neurons grown in culture (Werz and Macdonald, 1985). We were unable to detect significant inhibition of 30 mM KCl/0.3 μM Bay K (K⁺/Bay K) stimulated ⁴⁵Ca²⁺ uptake into primary cultures of neonatal rat DRG neurons when dynorphin B (0.1 nM-1 μM) was applied at the same time as the depolarizing stimulus (n=2). Pretreatment of the DRG neurons for 10 min with 100 nM dynorphin B, and continuation of the opioid administration throughout the 10 min K⁺/Bay K stimulus, produced a slight inhibition (33%, 45%; n=2) of stimulated ⁴⁵Ca²⁺ uptake compared to the non-pretreatment protocol (4 ± 14%, n=4).

We then expanded our studies of kappa opioid agonists, generating dose-response curves for dynorphin A and U50488H. As shown in Figure 5.5, pretreatment with dynorphin A inhibited calcium uptake, but the IC₅₀ value (1-10 μM) was very high compared to its affinity for the kappa receptor (see Goldstein & Naidu, 1989). U50488H at 10 μM did not substantially affect ⁴⁵Ca²⁺ uptake. Variability between experiments, already considerable in the absence of opioid agonists (Figures 5.1 & 5.2), increased in the presence of opioids to the extent that meaningful interpretation of experimental results was very difficult. Furthermore, the activity of the des-tyrosine dynorphin analogue (dynorphin A(2-17); Figure 5.5) in inhibiting the uptake of ⁴⁵Ca²⁺ into neonatal rat DRG cells did not differ significantly from that of dynorphin A even at the highest concentrations tested. If dynorphin A was acting through opioid receptors, one would have predicted noticeable differences in their relative activities, considering that dynorphin

A(2-17) is missing the terminal tyrosine residue necessary for opioid activity (Chavkin & Goldstein, 1981b). These results taken along with the inactivity of U50488H suggest that activation of kappa receptors is not affecting calcium uptake into neonatal DRG neurons.

In view of these problems, we compared the consistency of results using cultures of adult DRG cells to that of cultures from neonates. Levels of 30 mM K^+ /0.3 μ M Bay K-stimulated $^{45}Ca^{2+}$ uptake into adult DRG ($210 \pm 17\%$ basal, $n=10$) were comparable to those in neonates ($296 \pm 32\%$ basal, $n=16$; see Figure 5.2). However dynorphin A (1 nM-10 μ M) did not inhibit $^{45}Ca^{2+}$ influx into adult DRG by more than 30% ($n=$ at least 3, except $n=1$ for 10 μ M). Hence similar results were obtained with this kappa ligand in primary cultures of adult and neonatal DRG cells. Interassay variability in the $^{45}Ca^{2+}$ measurements did not improve with the adult tissue.

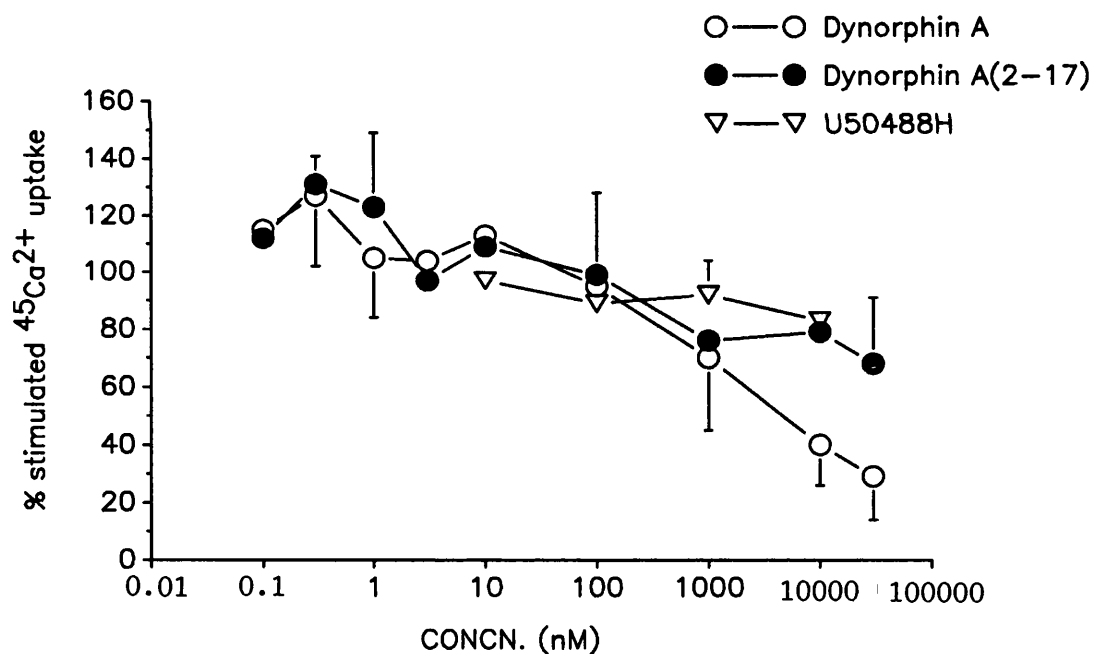


Figure 5.5. Dose-response curves for the inhibition of K^+ /Bay K-stimulated $^{45}Ca^{2+}$ uptake into neonatal rat DRG cells by the kappa opioid agonists U50488H, dynorphin A, and an inactive dynorphin fragment. Means \pm SEM are for replicates of six, in at least 2 separate experiments. Several error bars have been suppressed on this plot for reasons of clarity, though those error bars remaining reflect the general trend in variability.

5-2.3 Inconsistent opioid modulation of K⁺-evoked SP-LI release from DRG neurons

We also measured release of substance P from DRG cultures. In preliminary experiments, appreciable levels of substance P-like immunoreactivity (SP-LI) were measurable in primary cultures of neonatal DRG neurons beginning at 1 day postplating and continuing up to two weeks in culture (n=2), the longest timepoint tested. Subsequent release experiments were performed using neonatal DRG cells at 6-10 days postplating, when the average SP-LI content was 3-4 fmol/ μ g cell protein. In these experiments, control K⁺ (5.4 mM) or high K⁺ (10-50 mM) medium (Ca²⁺/Mg²⁺ free HEPES-buffered Hanks balanced salt solution) was added to sensory neurons (plated onto 4 well plastic dishes) for 5 min, removed, and assayed directly for SP-LI (see release protocol in section 2-4.4).

As shown in Figure 5.6, raising the extracellular potassium concentration above 5.4 mM increased the outflow of substance P-like immunoreactivity (SP-LI) from neonatal rat DRG cells. Levels of K⁺-stimulated SP-LI outflow reached an apparent plateau at 50 mM KCl, with experimental variability increasing to undesirable levels with the higher 100 mM KCl stimulus. Attempts to enhance the 50 mM K⁺-induced outflow of SP-LI with Bay K 8644 (0.1-1 μ M) indicated no effect at the lower concentrations, with a slight but insignificant facilitation of release (n=2) at 1 μ M Bay K (Figure 5.6). This contrasts with the findings of Perney et al. (1986) who reported that K⁺-evoked SP-LI release is enhanced by this dihydropyridine Ca²⁺ channel agonist at all K⁺ concentrations. However, in agreement with their findings, we found that 1 μ M Bay K increased 10, 20, and 30 mM K⁺-stimulated outflow of SP-LI (n=2), with the general trend of shifting the K⁺ concentration-response curve to the left. Basal release (5.4 mM KCl) of SP-LI was unaffected by 0.1-1 μ M Bay K 8644 (n=2).

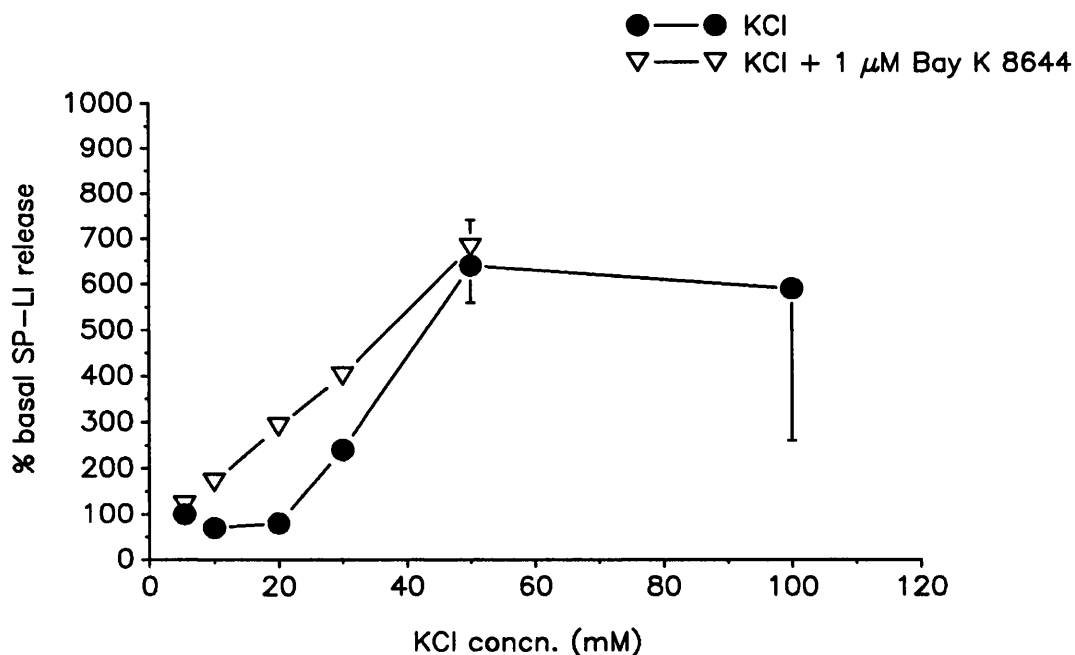


Figure 5.6. Dose-response curves for K^+ -induced release of substance P-like immunoreactivity (SP-LI) from primary cultures of neonatal rat DRG neurons in the absence or presence of $1 \mu\text{M}$ Bay K 8644. Means \pm SEM are for replicates of four, in at least 2 separate experiments (with $n=2$ for all data points without error bars, and $n=10$ for 50 mM KCl).

The amount of SP-LI released by the 50 mM K^+ stimulus was comparable to the level of SP-LI released by 100 nM capsaicin. A two minute exposure to a depolarizing concentration of KCl (50 mM) accounted for release of $8.5 \pm 0.7\%$ of total SP-LI content ($n=6$), with 8.1% (8.4%, 7.8%; $n=2$) released by 100 nM capsaicin. In our hands, bradykinin (10 nM-10 μM ; $n=3$), another activator of nociceptive sensory neurons, did not increase outflow of SP-LI above basal levels.

As observed in the $^{45}\text{Ca}^{2+}$ uptake assay, variability in SP-LI experiments increased in the presence of opioids. Cells were pre-incubated with opioids in control K^+ (5.4 mM) buffer for 5 min prior to a further 5 min exposure to the high K^+ (50 mM) plus opioid. We began by investigating the effects of the kappa agonist dynorphin A on 50 mM K^+ -induced outflow, and found that even at a high (1 μM) concentration this compound did not inhibit release of SP-LI by greater than $27 \pm 4\%$ ($n=3$). Another kappa agonist U50488H (100 nM-100 μM) did not inhibit release at all ($n=2$). Because mu and delta opioids may affect neurotransmitter release by increasing potassium conductance to reduce calcium

entry into DRG neurons (Werz & Macdonald, 1983), we extended the studies to include the mu ligands DAGO and morphine, and the delta opioids DPDPE and DADLE. DAGO produced highly variable results in the concentration range 3 nM-3 μ M (n=2), with standard errors obscuring the significance of up to 20% inhibition of 50 mM K⁺-induced release of SP-LI. Morphine, DPDPE, and DADLE (10 μ M) did not inhibit the K⁺-stimulated outflow of SP-LI from neonatal rat DRG neurons (n=2).

We also explored opioid inhibition of SP-LI release from adult rat DRG neurons. Raising the extracellular potassium concentration from 5.4 mM to 30 mM elicited a $500 \pm 120\%$ increase over basal levels of SP-LI outflow, amounting to a release of $3.6 \pm 0.3\%$ of total SP-LI content over the 5 min exposure (n=3). As with the neonates, potassium-induced SP-LI release from adult DRG neurons occurred in a concentration-dependent manner. A 5 min incubation of adult DRG cells in 50 mM K⁺ buffer increased levels of SP-LI release by $980 \pm 170\%$ above baseline, or $6.7 \pm 0.8\%$ of total SP-LI content (n=3). Subsequent attempts to modulate 30 mM K⁺-induced release of SP-LI with 1 μ M concentration of the mu agonists DAGO and morphine, the delta ligands DADLE and DPDPE, and the kappa ligands U50488H and dynorphin A failed to produce significant inhibition of the K⁺ stimulus (n=2).

Uptake of $^{45}\text{Ca}^{2+}$ into primary cultures of neonatal and adult rat dorsal root ganglion (DRG) sensory neurons was stimulated by increasing the extracellular potassium concentration from 5.4 mM to 30 mM. The $^{45}\text{Ca}^{2+}$ uptake signal was further optimized in the presence of the dihydropyridine (DHP) calcium channel agonist Bay K 8644 (0.3 μM), and was inhibited by the DHP antagonist nifedipine ($\text{IC}_{50} \approx 1 \mu\text{M}$). Our experiments suggest that $^{45}\text{Ca}^{2+}$ is, at least in part, entering the DRG cells through voltage-sensitive calcium channels.

One mechanism for regulating Ca^{2+} entry which we have not ruled out, however, is that of $^{45}\text{Ca}^{2+}$ influx occurring through voltage-dependent Na^+ channels. Attali et al. (1989) have shown that blockade of these channels by tetrodotoxin has no effect on either basal or K^+ /Bay K-stimulated $^{45}\text{Ca}^{2+}$ uptake into DRG/spinal cord co-cultures. Because of the equivocal results obtained with opioids in our experiments, further characterization of the specificity of the $^{45}\text{Ca}^{2+}$ uptake in our system was not deemed essential.

Attali et al. (1989) have recently demonstrated opioid regulation of $^{45}\text{Ca}^{2+}$ influx into co-cultures of rat spinal cord and dorsal root ganglion sensory neurons. In that study, uptake of $^{45}\text{Ca}^{2+}$ stimulated by 50 mM KCl in the presence of Bay K 8644 was inhibited significantly by kappa, but not mu or delta, opiates. Attali and co-workers (1989) have not, however, determined the relative contributions of the spinal cord and DRG neuronal cell types to kappa-opioid inhibition. They suggest however that, given the prevalence of spinal kappa sites, spinal cord neurons may be a substantial locus of opioid action. Although spinal cord-DRG co-cultures have been shown to be valuable in investigating spinal mechanisms of opioid analgesia, our target of opioid effects on sensory neurons dictates the use of cultures of DRG neurons alone.

We have used the calcium uptake assay in parallel with SP-LI release studies in an attempt to investigate the mechanism of action of opioids. However, none of a range of opioid ligands consistently inhibited $^{45}\text{Ca}^{2+}$ uptake. Similarly, opioid effects on release of SP-LI were inconsistent. Werz & Macdonald (1985)

report that only a small proportion of single DRG neurons in culture respond to opioids. In particular, this group reports that the responses of DRG neurons to dynorphin A were highly variable, producing a 30-40% mean reduction in calcium-dependent action potential duration. This suggests that only a small percentage of calcium channels, in those few DRG which express opioid receptors, are under control by opioid receptors. Perhaps this has led to our difficulty in detecting opioid effects on these few cells against a background of many neurons activated by the depolarizing potassium stimulus. Mudge et al. (1979) have reported enkephalin inhibition of SP-LI release from primary cultures of sensory neurons. This group has used 9-10 day embryonic chick DRG neurons tested at 12-18 days postplating. We however have used a dissociated DRG cell preparation from newborn rats, and have attempted to modulate evoked SP-LI release with opioids at 6-10 days postplating.

Chang et al. (1989) have recently reported mu (sufentanil, morphine), delta (met-enkephalin), and kappa opioid (U50488H) inhibition of electrically-evoked SP release from primary cultures of embryonic chick DRG neurons. Their efforts follow previous demonstrations from the same group that the increased outflow of SP from DRG cells using pulsed electrical stimulation is dihydropyridine-sensitive (Rane et al., 1987; Holz et al., 1988) and subject to presynaptic modulation by agonists at α -adrenergic and GABA_b receptors (Holz et al., 1989). Chang and colleagues (1989) argue that the brief electrical field stimulation of DRG cell cultures to generate action potentials is a more physiologic depolarizing stimulus than the elevation of extracellular potassium levels. The coupling of opioid receptors to secretory processes in DRG cells may therefore be investigated more appropriately using an electrical stimulus which generates action potentials of normal configuration, and which thereby facilitates the extrapolation of opioid effects *in vitro* to mechanisms of analgesia *in vivo*.

In view of our inconsistent results with primary cultures of sensory neurons, and since it is difficult to obtain enough cells from such cultures to do extensive biochemical experiments, part of our effort has been directed towards

finding DRG-derived cell lines that have some properties of sensory neurons and which express opioid receptors.

CHAPTER 6 -
OPIOID EFFECTS ON NEUROBLASTOMA x DRG (ND)
CLONAL CELL LINES

As discussed in the previous chapter, primary cultures of rat dorsal root ganglion (DRG) neurons have been used as an *in vitro* model of opioid receptors on sensory nerve terminals. The antinociceptive effects of opioids are thought to be partially due to actions on the smaller, unmyelinated afferent C fiber-type DRG neurons which contain the peptide neurotransmitter substance P (Fields et al., 1980). This B class of sensory neurons (Andres, 1961; Lieberman, 1976), however, comprises less than 20% of DRG cells (Hokfelt et al., 1976). Elucidation of opioid receptor function can, therefore, be complicated by the heterogeneity of neurons which makes it difficult to correlate opioid effects with a particular subpopulation of DRG cells. Biochemical studies on primary cultures are further frustrated by difficulties in obtaining sufficient numbers of cells.

In cases such as this, biochemical investigation of neuronal properties may be done more effectively with clonal cell lines than with authentic neuronal tissue. Continuous cell lines provide a homogeneous tissue source which can be grown in large quantities, factors contributing to the successful use of clonal neuroblastoma lines in studying the regulation of opioid receptor populations and their coupling to second messenger systems (see review by Chang, 1984). Clearly it would be advantageous to immortalize the features of selected subtypes of DRG sensory neurons, yielding clonal lines which retain properties relevant to the mechanism of activation of peripheral nociceptive sensory neurons. Such a clonal model may also prove useful in furthering our understanding of opioid modulation of nociceptive stimuli. Part of our effort has therefore been directed towards screening novel DRG-derived clonal cell lines for the expression of characteristics that distinguish nociceptive DRG neurons. Such properties include sensitivity to capsaicin and bradykinin (Baccaglini & Hogan, 1983), and opioid agonists (Mudge et al., 1979), and the presence of substance P-like immunoreactivity (SP-LI; Hokfelt et al., 1976; DiFiglia et al., 1982).

A series of mouse neuroblastoma N18TG2 x neonatal rat DRG neuron hybrid cell lines were constructed and kindly supplied by John Wood and Pat

Hogan. Wood and Hogan (unpublished results, Table 6.1) found that expression of the rat Thy1.1 neuronal surface marker, absent from the parental mouse neuroblastoma cells, was detectable on several of these neuroblastoma x DRG (ND) lines (e.g. NDE, ND8, and ND11). This provided evidence that these cells are true hybrids derived from rat DRG neurons rather than non-neuronal cells. Indirect evidence for the sensory neuronal character of these hybrid clones came from further studies showing that some of the ND lines expressed globo- and/or lacto-series glycolipids (Table 6.1), surface markers that are found on large- and small-/intermediate-diameter DRG neurons, respectively (Dodd & Jessell, 1985; Jessell & Dodd, 1985).

Sensory neurons of the small unmyelinated C fiber class are, furthermore, subject to selective destruction by capsaicin (see review by Fitzgerald, 1983). Opioid receptors have been demonstrated on capsaicin-sensitive primary afferent nerve fibers (Gamse et al., 1979). We have therefore screened a number of the ND cell lines for opioid binding sites.

6-2.1 Naloxone-displaceable binding of [³H]etorphine to ND hybrid cells

In preliminary studies, we used 1 nM [³H]etorphine to label mu, delta, and kappa opioid receptors and challenged this binding with a saturating concentration (10 μM) of the unlabelled form of the opioid antagonist naloxone. NG108-15 cells are known to express opioid binding sites (Chang & Cuatrecasas, 1979; Akiyama et al., 1985) and were thus used as a positive control in these binding studies. Three undifferentiated ND hybrid lines (NDE, ND8, and ND27), unlike the parental N18TG2 mouse neuroblastoma cells, expressed naloxone-displaceable opioid binding sites (Table 6.1).

One of these cell lines, ND8, has also been shown to depolarize in response to capsaicin (Wood et al., 1989), a characteristic feature of nociceptive sensory neurons (Platika et al., 1985a). We therefore chose to concentrate our efforts on the ND8 hybrid, first seeking to characterize the nature of the opioid binding sites.

6-2.2 Opioid sites on ND cells: Saturation binding studies

More detailed [³H]etorphine saturation studies, covering a wide range of radioligand concentrations (0.1-5 nM), were carried out and revealed that opioid binding to undifferentiated ND8 cells displays saturability (Figure 6.1), a criterion for specific binding. Scatchard plots were linear and gave estimates of $K_d = 0.42 \pm 0.1$ nM and $B_{max} = 480 \pm 120$ fmol/mg protein (mean \pm SEM, n=4). A linear Scatchard plot, as was obtained in Figure 6.2, is consistent with the presence of a single class of binding site, though there are pitfalls in this interpretation (Klotz, 1982). The Hill slope was 1.06 ± 0.05 (n=4), a unity Hill slope (see Figure 6.3) providing further evidence for a single type of binding site.

Table 6.1. Summary table comparing the characteristics of five ND clonal cell lines to the parental line N18TG2 and the well-characterized NG108-15 neuroblastoma x glioma hybrid. Plus (+) and minus (-) signs indicate that cell lines tested positive or negative, respectively, for the characteristic in question. Blank spaces indicate that the experiment has not been done. The anti-Thy1.1 and glycolipid surface marker studies are unpublished results of Wood & Hogan. *Cell sensitivity to bradykinin (1 μ M), with the exception of the ND7 hybrid line (for which the bradykinin response was characterized electrophysiologically by Wood et al., 1989), was tested by monitoring changes in intracellular calcium levels with the calcium-chelating fluorescent indicator Fura 2 (see section 2-5.5 of the Methods and Materials for details of the protocol). Evidence for the sensitivity of NG108-15 cells to bradykinin has been reviewed by Miller (1987a).

Neuroblastoma x DRG neuron (ND) hybrid cell line	[³ H]etorphine binding	rat surface marker Thy1.1	glycolipid surface markers		bradykinin sensitivity*
			lacto-series	globo-series	
E	+	+	-	+	+
7	-		+	+	+ see above
8	+	+	-	+	+
11	-	+	+	+	+
27	+		-	-	+
N18TG2 (parental line)	-	-	-	-	-
NG108-15	+				+ see above

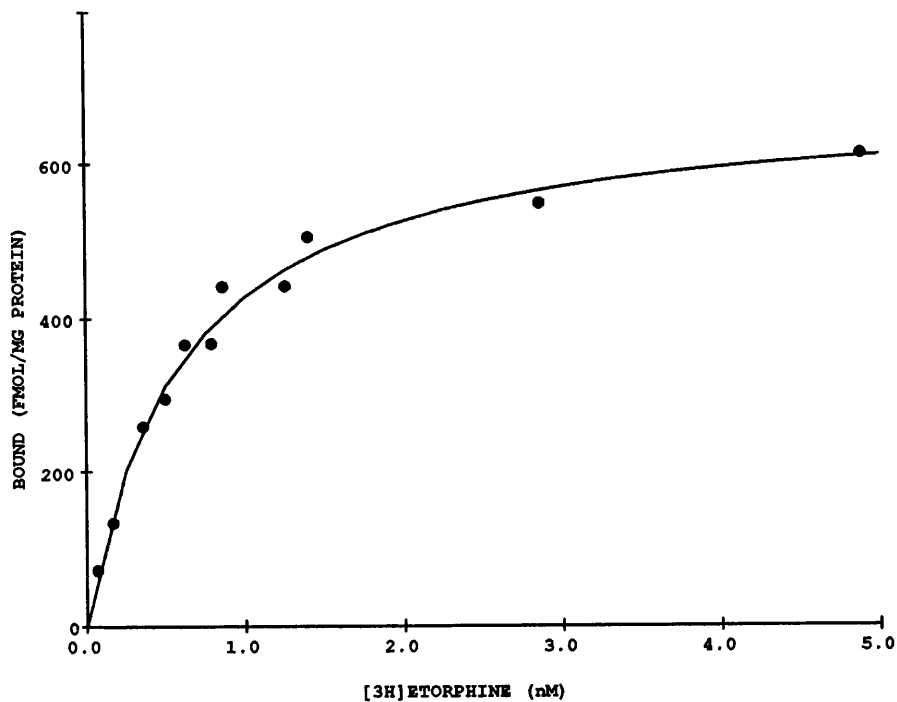


Figure 6.1. Results of a single saturation binding study using varying concentrations of [³H]etorphine to label opioid receptors on ND8 hybrid cells. In this saturation binding curve (and all other such curves presented in this thesis), nonspecific binding has been subtracted from total binding to yield the levels of specific binding (expressed in fmol/mg protein on the vertical axis). This curve was generated by a non-linear curve-fitting program (assuming a single type of non-interacting binding sites) in the RS/1 statistical computing package.

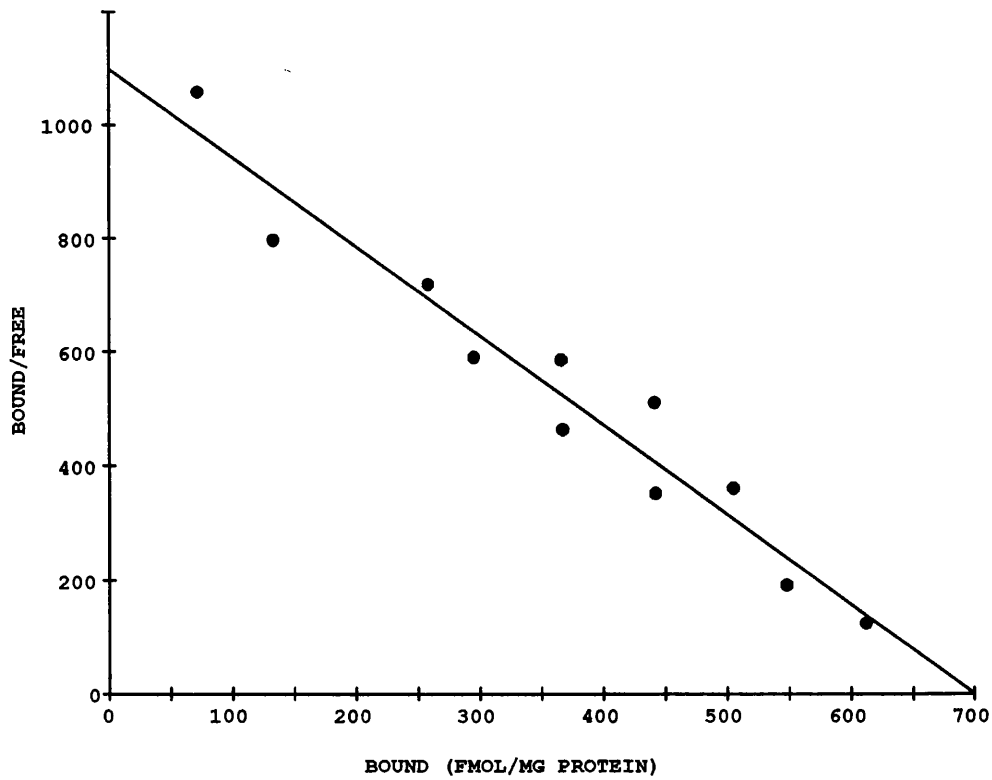


Figure 6.2. The Scatchard plot transformation of the data for saturable binding of [³H]etorphine to ND8 cells (from Figure 6.1) is linear, yielding estimates of $K_d = 0.64$ nM and $B_{max} = 700$ fmol per mg of cell protein for this experiment.

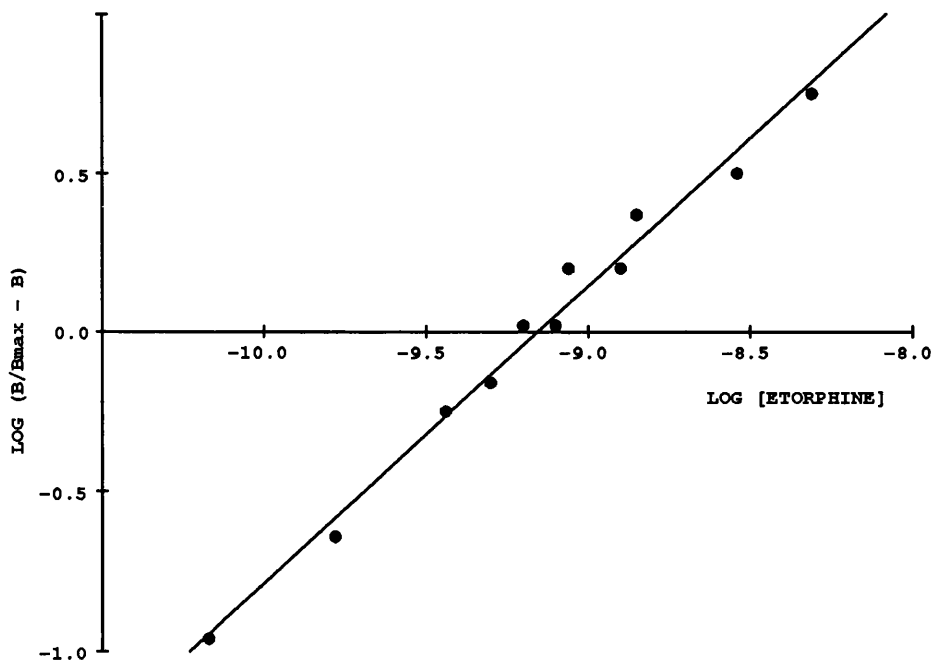


Figure 6.3. The Hill plot transformation of the Scatchard plot results (from Figure 6.2) gives a line of slope = 0.92 for this study on the saturable binding of [³H]etorphine to ND8 hybrids.

6-2.3 Opioid sites on ND cells: Competitive binding studies

Opioid receptor type on the ND8 clonal cell line was determined in competition experiments between 1 nM [³H]etorphine and unlabelled [D-Ala²,MePhe⁴ Gly(ol)⁵]enkephalin (DAGO, 10 nM to 10 μM), or [D-penicillamine²,L-penicillamine⁵]enkephalin (DPLPE, 1 nM to 10 μM), or U50488H (0.03 to 100 nM), ligands highly selective for μ , δ , and κ opioid receptors, respectively. As can be seen in Figure 6.4, the non-peptide U50488H did not displace the radioligand even at the highest concentration tested ($IC_{50} > 100$ nM). The μ opioid agonist DAGO gave an IC_{50} value of 4.1 ± 0.66 μM (n=3), with the structurally restricted enkephalin analogue DPLPE having an IC_{50} of 14 ± 1.3 nM (n=3). These results are consistent with expression of only δ -type opioid receptors on the ND8 hybrid cell line.

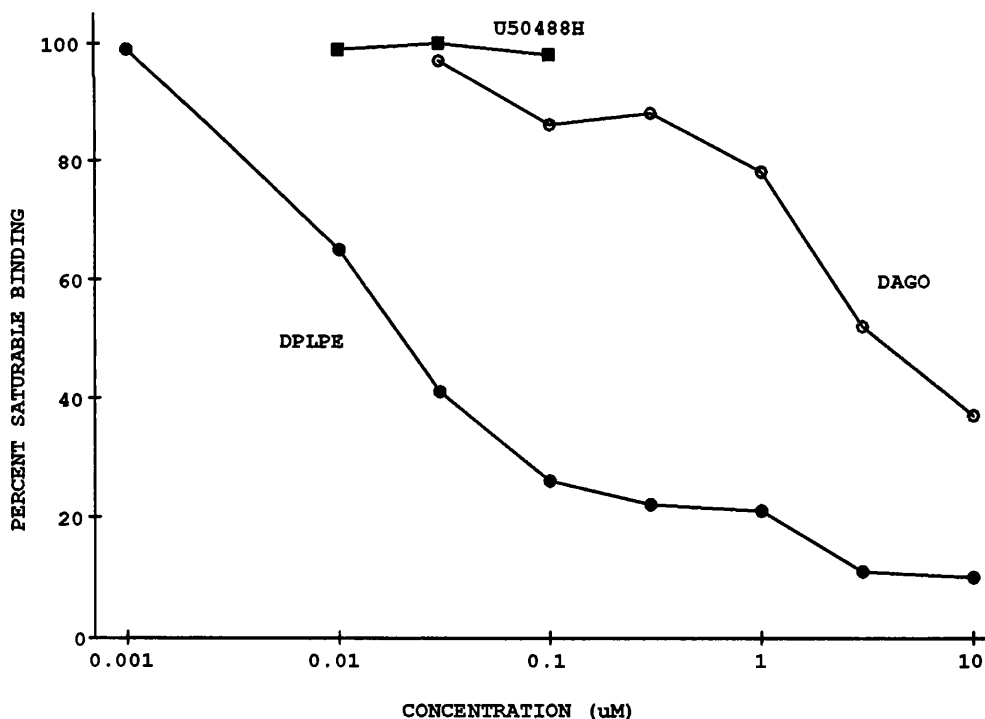


Figure 6.4. Results of a series of competition binding experiments (n=3) between 1 nM [³H]etorphine and unlabelled DAGO, DPLPE, or U50488H (ligands highly selective for μ , δ , and κ opioid receptors, respectively,) for opioid binding sites on ND8 cells.

6-2.4 Lack of delta opioid modulation of Rubidium-86 ($^{86}\text{Rb}^+$) efflux from populations of ND cells

As discussed in chapter 5, much is known about the electrical properties of DRG cells and the sensitivity of these ionic processes to opioid agonists. For example, Werz and Macdonald (1983) have reported that the delta opioid ligand leucine-enkephalin shortens action potentials by increasing calcium-dependent potassium conductance. Since several novel DRG x neuroblastoma cell lines are reported to possess action potential mechanisms similar to authentic DRG neurons (Platika et al., 1985b), we have looked at delta opioid effects on one biochemical correlate of ionic activity in ND8 hybrids.

Rubidium (Rb^+) is known to permeate a variety of potassium (K^+) channels and, though it will pass through other cation channels, is accepted as an indicator of K^+ fluxes (Arner & Stallcup, 1981; Tomozawa et al., 1985; Wood et al., 1988). We have therefore used $^{86}\text{Rb}^+$ efflux as a crude measure of K^+ currents to look at the effects of delta opioid agonists in ND8 cells. The efflux of $^{86}\text{Rb}^+$ from undifferentiated ND8 hybrids, however, was not stimulated by either 1 μM capsaicin or 50 mM KCl (n=3 for each stimulus). Similar results were obtained when ND8 cells were differentiated with nerve growth factor (200 ng/ml), retinoic acid (1 μM), dibutyl cyclic AMP (1 mM), low serum (1% foetal calf serum), or a cocktail of these (n=2 for each stimulus under each differentiating condition; see Figure 6.5.A) to induce visible morphological changes which are reported to be accompanied by alterations in the expression of cell surface structures (Jessell et al., 1984) and functions characteristic of sensory neurons (Wood et al., 1990).

The NDE clonal cell line, another hybrid which was found to express only delta-type opioid receptors, was also unresponsive to capsaicin in the $^{86}\text{Rb}^+$ efflux assay under a variety of differentiating regimes (n=2). Depolarizing concentrations of potassium, however, did increase the efflux of $^{86}\text{Rb}^+$ from populations of both non-differentiated and differentiated NDE cells (n=2; see Figure 6.5.B).

Subsequent investigation of the relative ability (compared to 50 mM KCl) of the delta-selective opioid ligand [D-Pen², D-Pen⁵]enkephalin (DPDPE, 1 μM) to stimulate efflux from NDE hybrids yielded no significant effect on the ⁸⁶Rb⁺ signal (n=2; see Figure 6.5.C).

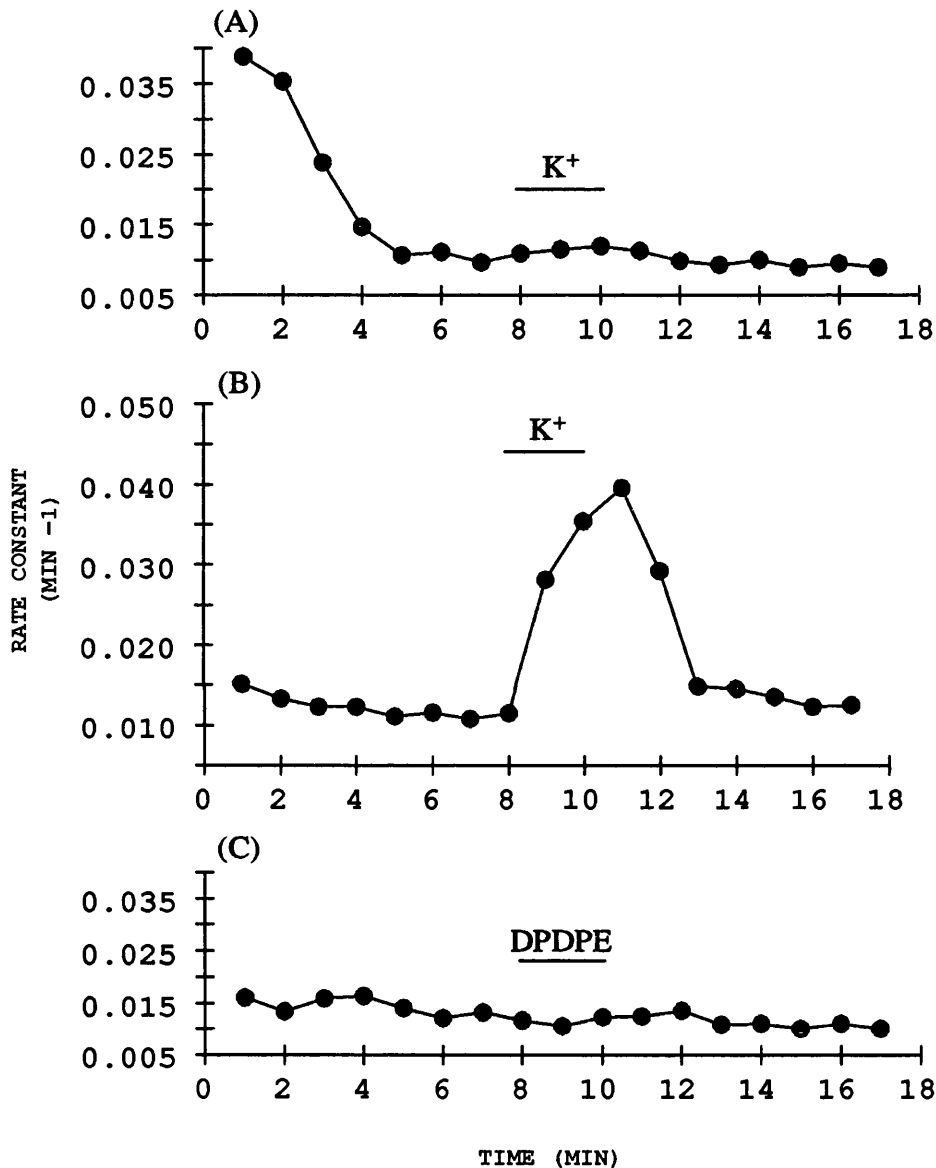


Figure 6.5. Results of single experiments, showing that (A) the efflux of rubidium-86 (⁸⁶Rb⁺) from populations of ND8 cells was not stimulated significantly by a 2 min application (indicated by the solid line) of 50 mM KCl. Potassium-stimulated ⁸⁶Rb⁺ efflux was, however, seen in the clonal line NDE (B), another cell line expressing only delta-type opioid binding sites. Further investigation of this cell line revealed that the delta opioid ligand [D-Pen²,D-Pen⁵]enkephalin (DPDPE, 1μM), was unable to increase the efflux of ⁸⁶Rb⁺ from NDE hybrids (C). See methods section for a description of how the rate constant was calculated. In these experiments, cells were differentiated with dibutyl cyclic AMP (1 mM).

6-2.5 SP-LI content of ND clonal cell lines

The smaller dark B class of DRG neurons contain a variety of neurotransmitters, including numerous neuropeptides. One of these neuropeptides, substance P, is believed to be involved in conveying nociceptive information (see review by Salt & Hill, 1983). Enkephalins, acting presumably through delta opioid receptors, have been shown to inhibit the release of substance P from primary cultures of dorsal root ganglion neurons (Mudge et al., 1979). ND cell lines testing positive for opioid binding sites were therefore analyzed for the presence of substance P-like immunoreactivity (SP-LI) by specific radioimmunoassay (see section 2-5.4 of the Methods and Materials chapter for protocol). Subsequent experiments would then have focused on ascertaining whether SP-LI stores were releasible and subject to modulation by opioids. Non-differentiated ND8, NDE, and ND27 cells, however, did not contain measureable amounts of SP-LI. Neither the parental cell line N18TG2 nor NG108-15 cells were found to contain appreciable levels of SP-LI (n=2). Differentiating the above hybrid lines with nerve growth factor, retinoic acid, dibutyl cyclic AMP, low serum, or a cocktail of these (see section 6-2.4) had no significant effect on the SP-LI content of these cells (n=2).

We have demonstrated that several ND clonal cell lines (NDE, ND8, and ND27) express opioid binding sites. Further characterization of these sites on the NDE and ND8 hybrids has revealed that they are delta-type opioid receptors. The clonal cell line NG108-15, a hybrid of rat glioma C6BU1 and mouse neuroblastoma N18TG2 cells, is known to possess delta opioid receptors (Akiyama et al., 1985) and tested positive in our binding assays. The parent C6BU1 cells are reported not to express opioid receptors (Klee & Nirenberg, 1974; Blosser et al., 1976; Law et al., 1979a; Hammonds & Li, 1981; Cabon et al., 1987), and in agreement with several groups we were unable to detect substantial levels of opioid binding sites on the parental N18TG2 cells (Klee & Nirenberg, 1974; Blosser et al., 1976). This prompts the question as to the identity of the parental origin of the opioid receptors in NG108-15 cells, which are known to contain chromosomes from both parents (Amano et al., 1974). One possibility is that a previously silent gene from the neuroblastoma or glioma parent cells became activated in the NG108-15 cell fusion product. In the course of addressing this issue, Law et al. (1979a) reported low levels of high affinity [³H]naloxone and [³H]dihydromorphine binding sites on N18TG2 cells. Sharma et al. (1975) had demonstrated previously a small amount of opioid binding activity in the N18TG2 parents with [³H]naloxone but not with [³H]dihydromorphine, while Blosser et al. (1976) failed to detect significant numbers of [³H]naloxone binding sites.

The opioid sites described by Law et al. (1979a), accounting for only 20% of the reported density of opioid receptors on the neuroblastoma x glioma NG108-15 hybrid (Klee & Nirenberg, 1974), have since been detected by several groups (Devane et al., 1986; Cabon et al., 1987; Icard-Liepkałns & Bochet, 1988). Cabon et al. (1987) reported low levels of sites for [³H]bremazocine, a narcotic analgesic with mixed agonist and antagonist properties that binds with roughly equal affinity to μ -, δ -, and κ -opioid receptors. Devane et al. (1986), using radioligands selective for μ , δ , and κ sites, have characterized the N18TG2 opioid receptors as exclusively of the delta type. Delta opioid sites on N18TG2 cells have

also been labelled with the tritiated form of the selective delta agonist Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET), though again at densities significantly lower than on NG108-15 cells (Icard-Liepkalns & Bochet, 1988). To further complicate the picture, Hammonds and Li (1981) have detected specific, saturable binding sites for β -endorphin but not [Leu]enkephalin on N18TG2 cells. The inconsistency of opioid binding studies on N18TG2 cells means that we are unable to discern whether the delta opioid receptors expressed on the NDE and ND8 clonal cell lines originate from the parental neonatal rat DRG neurons or from the parental mouse neuroblastoma N18TG2 cells.

Delta opioid modulation of adenylate cyclase has been demonstrated in the N18TG2 (Law et al., 1982), NG108-15 (Brandt et al., 1976; Wahlstrom et al., 1977), and several other cell lines. Although such second messenger systems as cyclic AMP, cyclic GMP, inositol trisphosphate (IP₃) and intracellular calcium play important roles in the control of sensory neuronal excitability (see review by Miller, 1987a), we have chosen instead to focus on the potential coupling of ND delta opioid receptors to ion fluxes and neurotransmitter release. The ND8 and NDE cell lines did not, however, contain SP-LI, nor were the observed delta opioid binding sites on these hybrids coupled to K⁺ channel activity as measured by ⁸⁶Rb⁺ efflux. North et al. have reported that opioid effects on potassium conductances in slices of rat locus coeruleus neurons can be blocked in solutions containing high (2 mM) concentrations of rubidium chloride (North & Williams, 1985; Williams et al., 1988). The amount of rubidium used in our assays was significantly lower, at an approximate concentration of 7 μ M (see section 2-5.3 of Methods and Materials for experimental details), thus reducing the likelihood that our observations can be explained by the observations of North and colleagues. Platika et al. (1985b) have suggested that differences in the electrical properties of different DRG x neuroblastoma cell lines may result from selective expression of ion channels or putative transmitters. We have not characterized the ND lines electrophysiologically, and so are unable to comment on the presence or absence of the various types of K⁺ channels on different ND lines. More complete characterization of peptide and non-peptide transmitters expressed in these hybrid cells also remains to be performed.

Primary cultures of DRG sensory neurons are used widely in biochemical and electrophysiological studies relating to peripheral pain mechanisms, and therefore several other groups have also sought to generate dorsal root ganglion-derived clonal cell lines which reflect accurately the characteristics of these neurons. Francel et al. (1987) have recently presented reports of a dorsal root ganglion x neuroblastoma hybrid cell line expressing delta opioid receptors coupled functionally to adenylate cyclase. These F-11 cells contain substance P-like immunoreactivity (SP-LI), though at much lower levels than in primary cultures of neonatal rat DRG neurons. Release of this SP-LI can be increased by depolarizing concentrations of potassium in a dihydropyridine-sensitive manner. The F-11 cell line has also proved useful in elucidating intracellular events subsequent to bradykinin receptor activation (Francel et al., 1989).

Our general strategy for screening ND hybrids represents a valid methodological approach, though unfortunately the clonal cell lines supplied by John Wood and Pat Hogan did not express properties consistent with our interests. The final experimental chapter of this thesis describes our use of a guinea pig heart slice preparation with which we have had more positive results in characterizing the properties of peripheral terminals of sensory nerves.

CHAPTER 7 -
OPIOID MODULATION OF SP-LI RELEASE
FROM THE PERIPHERAL TERMINALS OF SENSORY NEURONS
IN A GUINEA PIG RIGHT VENTRICULAR SLICE
PREPARATION

In this chapter I will discuss the use of guinea pig heart slices as a general model for studying opioid modulation of neuropeptide release from the peripheral terminals of sensory neurons. First I will survey the literature which implicates roles for both opioids and neuropeptides in cardiovascular control. Enkephalin-containing neurons (Hughes et al., 1977; Simantov et al., 1977) and opioid receptors (Atweh & Kuhar, 1977; Goodman et al., 1980) have been shown to be distributed densely in brain regions critical to cardiovascular control (e.g. nucleus tractus solitarius, hypothalamus, nucleus ambiguus, and dorsal vagal nucleus). This is consistent with evidence that endogenous opioid systems can exert a complex variety of effects on cardiac function (see review by Holaday, 1983). There is also evidence that, in addition to their influence within the cardiovascular centers of the brain, opioid peptides can influence hemodynamic processes by acting at peripheral opioid receptors in cardiac neural (Mantelli et al., 1989), muscular (Laurent et al., 1985), or vascular elements (see Holaday, 1983).

Immunoreactive nerve fibers for dynorphin (Spampinato & Goldstein, 1983), [Met]enkephalin (Lang et al., 1983), and [Leu]enkephalin (Lang et al., 1983) have been detected in the heart. Weihe et al. (1983,1985) have used high performance liquid chromatography (HPLC) fractionation and the mouse vas deferens assay to characterize opioid peptides in guinea pig heart extracts. They have found guinea pig atria and ventricles to contain a variety of different biologically active opioid peptides derived from proenkephalin and prodynorphin.

Localization of opioid receptors at peripheral sites in the rodent heart is less resolved than for cardiovascular centers in the CNS. *In vitro* autoradiographic studies reported the absence of [³H]naloxone binding sites in rat cardiac tissue (Dashwood & Spyer, 1986). Simantov et al. (1978) have demonstrated opioid binding in whole guinea pig heart homogenates, though these binding sites lacked stereospecificity. Burnie (1981) reported stereospecific opioid binding sites for [³H]diprenorphine ([³H]DPN), which binds with equal affinity to all three of the major types of opioid receptor (Chang et al., 1981), on homogenates of rat right

ventricular cardiac muscle though he did not characterize these sites in detail. Opioid binding sites for [³H]DPN in membranes prepared from individual chambers of the rat heart have been examined more recently (Krumins et al., 1985; Krumins, 1987) though resolution between saturable and total binding was poor, a difficulty arising from the apparent low densities of such sites in the heart. We have been unable to detect appreciable levels of specific high affinity opioid binding to homogenates of individual chambers of the guinea pig heart (see section 7-2.1).

Several reports suggest that cardiac opioid peptides may nonetheless interact with functional opioid receptors in the heart and play an important role in the peripheral control of myocardial and coronary performance. Activation of kappa opioid receptors, for example, is reported to cause relaxation of cerebral blood vessels (Altura et al., 1984), lending the possibility that a similar situation may occur with coronary vasculature. Saxon et al. (1982) have shown that morphine and [Met]enkephalin reduce contractile strength in a rabbit papillary muscle bioassay, postulating that this negative inotropic effect may be mediated via presynaptic myocardial opioid receptors with mu-type characteristics. Investigation of neurotransmitter systems involved follows logically from these observed opioid effects. Mantelli et al. (1987,1989), for example, have investigated the sympathetic and peptidergic components of the cardiac response to nerve stimulation. They conclude that circulating opioid peptides may modulate the efferent functions of guinea pig cardiac sensory nerves via activation of atrial and ventricular opioid receptors to influence the prejunctional release of noradrenaline or peptide transmitters. We, however, are interested in opioid effects at the peripheral terminals of afferent, rather than efferent, sensory nerves.

Many studies have shown efferent innervation of the heart to be composed of vagal (cholinergic) and sympathetic (catecholaminergic) components. Progress on establishing the origins of afferent innervation has lagged behind perhaps because of the lack, until relatively recently, of a biochemical marker for identifying sensory neurons. Substance P (SP) is now considered to be a marker

for a subpopulation of primary sensory neurons (see review by Nicoll et al., 1980). Immunohistochemical and ultrastructural studies of mammalian cardiac tissues have mapped the localization of SP to neural elements distributed throughout the hearts of various species, including the guinea pig (Wharton et al., 1981; Hua et al., 1985; Dalsgaard et al., 1986; Hougland et al., 1986; Papka & Urban, 1987). SP-immunoreactive (IR) nerve fibers are associated with coronary vessels and cardiac muscle, and are particularly dense in the atria. The neurotoxin capsaicin is known to deplete SP-like immunoreactivity (LI) from small diameter primary afferents in several peripheral organs (e.g. skin, eye, ureter, tooth pulp, and airways) including those fibers supplying the cardiovascular system. Capsaicin has therefore become a useful tool, sometimes used in combination with nerve sectioning experiments, to determine the possible pathways for cardiac afferent fibers. Some reports suggest that SP-IR fibers in the guinea pig heart originate predominantly from dorsal root ganglia (Urban & Papka, 1985; Papka & Urban, 1987), though others suggest that cardiac sensory innervation arises from vagal (nodose) ganglia as well as thoracic spinal ganglia (Lundberg et al., 1983; Dalsgaard et al., 1986). Most reports consider these SP-IR nerve fibers to be exclusively of extrinsic origin, with several studies demonstrating the absence of SP-immunoreactivity in autonomic ganglion nerve cell bodies (Wharton et al., 1981). One recent report (Baluk & Gabella, 1989), however, provides evidence for a small subpopulation of intrinsic cardiac neurons which show immunoreactivity for SP. The physiological significance of such SP-positive autonomic ganglion cell bodies in guinea pig heart is unclear, though Baluk and Gabella (1989) suggest that such neurons may contribute to the control of cardiac reflexes.

Studies on guinea pig heart have also revealed SP-IR fibers in the sinoatrial and atrioventricular nodes, proximal parts of the conduction system (Wharton et al., 1981; Franco-Cereceda et al., 1987). There is limited information on the localization of SP-positive fibers to other parts of the conduction system in the guinea pig heart, however a recent study on bovine heart has revealed dense SP-IR innervation in the conduction cells of the atrioventricular bundle (Forsgren, 1989).

Thus it is possible that SP may be important functionally in the regulation of the heart beat.

Despite such suggestions the pharmacological role of SP in the heart is not completely understood. Exogenous SP can produce powerful vasodilation of the coronary vasculature and increase vascular permeability in the walls of the large vessels serving the heart (Lundberg et al., 1984a). SP has not, however, been shown to exert a significant influence on myocardial cells, having only slight direct chronotropic or inotropic effects on the heart (see review by Franco-Cereceda, 1988).

Afferent fibers innervating atrial and ventricular tissue are known to convey nociceptive information and mediate powerful cardiovascular reflexes (see review by Malliani et al., 1986). Given that SP may transmit nociceptive afferent information (see review by Salt & Hill, 1983), Wharton et al. (1981) have postulated the involvement of SP-containing neurons in the transmission of cardiac pain. This interpretation that SP has an afferent function in the heart is strengthened by recent demonstrations that SP-IR nerve fibers in the guinea pig heart are sensitive to degeneration by capsaicin (Urban & Papka, 1985; Dalsgaard et al., 1986; Franco-Cereceda et al., 1987; Papka & Urban, 1987). Unlike other tissues such as respiratory tract, skin, eye, and stomach which come into contact with irritants from the external environment (i.e. particulate smoke, pollen, and spicy foods), it is not fully understood what natural stimuli cause activation of SP-containing primary afferent C-fibers in the heart. Franco-Cereceda (1988), however, has reported that ischaemia increases the outflow of calcitonin gene-related peptide (CGRP), another neuropeptide found in capsaicin-sensitive sensory neurons, from the isolated perfused guinea pig heart.

Activation of cardiac nociceptors, therefore, may not only increase activity in cardiac afferents and centrally-mediated reflexes, but also result in the local release of neuropeptides which then affect cardiovascular function (e.g. by altering neural excitability, smooth muscle contraction, or coronary blood flow). Recent work shows that bradykinin-, capsaicin-, and potassium-depolarization can

stimulate the release of SP-LI (Hoover, 1987; Geppetti et al., 1988) and CGRP-LI (Geppetti et al., 1988) from capsaicin-depletible neuropeptide pools in the isolated perfused guinea pig heart. Data from these groups suggest that this evoked release of neuropeptides from cardiac sensory nerves could mediate some of the functional responses of the isolated perfused heart to bradykinin (Manzini et al., 1989) and capsaicin (Hoover, 1987; Manzini et al., 1989).

Opioid modulation of neuropeptide release has been demonstrated in several peripheral tissue preparations. For example, persuasive evidence exists to suggest that the electrical stimulation-evoked release of SP-LI from rat tooth pulp (Brodin et al., 1983) and cat knee joint (Yaksh, 1988) primary afferents is subject to modulation by opioid receptors on these peripheral terminals. Technical limitations have so far prevented investigators from correlating opioid effects on bronchoconstriction and neurogenic plasma extravasation with reduced release of SP-LI from sensory nerve endings in guinea pig airways (Belvisi et al., 1989). However, presynaptic modulation of SP release by opioids is indicated in these studies by the fact that morphine did not inhibit intravenous SP-induced increases in microvascular permeability. Others have suggested, but not demonstrated directly, that opioids modulate neuropeptide release from cardiac primary afferents. To examine this hypothesis, we have quantified the evoked release of SP-LI from the peripheral terminals of guinea pig heart sensory neurons in the presence and absence of opioids.

For these experiments the guinea pig heart was chosen since this tissue contains high concentrations of SP localized almost exclusively to capsaicin-sensitive primary afferent nerve fibers. The extent of SP innervation of the heart varies markedly between different species, with few SP-IR nerve fibers in rat heart compared to guinea pig (Hougland & Hoover, 1983; Papka & Urban, 1987). This species variability has been further substantiated by radioimmunoassay results which indicate that the levels of detectable SP-LI material in tissue extracts of rat heart (Holzer et al., 1982) is approximately 40 times less than that in guinea pig heart (Papka et al., 1984).

First, in preliminary studies, we measured the SP-LI content of individual chambers of the guinea pig heart, and assayed atrial and ventricular tissue for opioid binding sites. We then used potassium depolarization (100 mM KCl) and capsaicin (100 nM-3 μ M) to activate nociceptive sensory neurons and increase the outflow of SP-LI from guinea pig right ventricular slices. Formalin (0.2%) was also used to stimulate heart tissue because of our *in vivo* interest in the sensitivity of this prolonged chemogenic nociceptive stimulus to peripherally administered opioids, as demonstrated in Chapter 4. Finally, mu (DAGO), delta (DADLE), and kappa (U50488H, U69593) opioid ligands were tested for their relative abilities to influence SP-LI release induced by each of these activating stimuli.

In these SP-LI release studies, right ventricles from two or three guinea pigs were chopped into 700 micron slices, pooled, and placed in a perfusion chamber. Depolarizing agents and opioids, dissolved in an oxygenated Krebs solution, were administered by peristaltic pump and fractions collected each minute onto acetic acid. Fractions were then lyophilized and assayed for substance P-like immunoreactivity (SP-LI). After each experiment, the heart slices were boiled in acetic acid and assayed for total SP-LI content (see section 2-6.3 of Methods and Materials for full details), with the SP-LI released during each 1 min fraction expressed as a percentage of the total SP-LI content at the beginning of the respective collection period.

The first stimulus in these experiments was always 100 mM KCl and was used as an internal control. Opioid inhibition and/or stimulation of SP-LI release from guinea pig right ventricular slices was then quantified against a subsequent KCl, capsaicin, or formalin stimulus. Areas under the two stimulus peaks were calculated, and the second peak expressed as a percentage of the first. All peak ratios were then normalized to control experiments in which heart tissue was exposed to two KCl stimuli. Throughout this chapter, unless otherwise stated, results are expressed as a percentage of these KCl controls.

7-2.1 Opioid binding studies on homogenates of individual guinea pig heart chambers

We carried out a series of opioid binding studies on crude membranes prepared from individual chambers of the guinea pig heart to determine whether opioid sites in this tissue were concentrated in any particular chamber. Using a modified version of the protocol employed by Kruminis et al. (1985) to detect opioid binding sites in rat heart, we could not detect opioid binding sites in membranes prepared from single guinea pig atria or ventricles (see section 2-6.2 of Methods and Materials for full protocol). No saturable binding was observed in any of the chambers when using 1-10 nM [³H]DAGO, [³H]DPDPE, [³H]U69593, or [³H]naloxone and challenging these radioligands with either unlabelled naloxone (10 μM) or the unlabelled forms of these opioid ligands (1 μM). As discussed in the introduction to this chapter, previous binding studies on rodent heart have yielded similar results.

7-2.2 Content of SP-LI in extracts of individual guinea pig heart chambers

Substance P-like immunoreactivity (SP-LI) was detected in extracts from all of the chambers of the guinea pig heart (Table 7.1). In these studies individual guinea pig heart chambers were assayed directly for SP-LI content after homogenization and boiling in acetic acid (see section 2-6.1 of Methods and Materials for full protocol). Although atria appear to have 3 to 4 times more SP-LI than ventricles, interchamber variation in SP-LI content is not as evident when absolute levels of SP-LI are approximated by taking into account the differing chamber weights (e.g. a guinea pig ventricle weighs on average 5-6 times more than an atrium).

Table 7.1. *Levels of substance P-like immunoreactivity (SP-LI), as determined by specific radioimmunoassay, in extracts of individual chambers of the guinea pig heart.*

chamber	n	pmol SP-LI / g protein	pmol SP-LI / g wet wt.
left atrium	10	58.7 ± 10.6	4.9 ± 0.9
right atrium	9	44.3 ± 4.9	3.4 ± 0.4
left ventricle	13	15.7 ± 1.8	1.4 ± 0.1
right ventricle	16	10.7 ± 1.2	1.1 ± 0.1

**Releasability of SP-LI from pooled slices of guinea pig
right ventricle by potassium (100 mM K⁺) depolarization**

Given our previous demonstration that all chambers of the guinea pig heart contain appreciable levels of SP-LI (Table 7.1), our efforts now focused on correlating content with releasability. In preliminary experiments 60 mM KCl was used to depolarize heart tissue, with results showing that this stimulus did not stimulate consistently the release of measureable amounts of SP-LI from guinea pig right ventricular slices. The time investment required for the radioimmunological detection of SP-LI in superfusate samples dictated that we use a higher (>60 mM) KCl concentration to detect reliably the stimulated release of SP-LI. Extensive KCl concentration-response studies were not carried out, but 100 mM KCl always increased the outflow of SP-LI from heart tissue. This stimulus enabled us to progress with later studies looking at opioid modulation of KCl-evoked SP-LI release. For all experiments reported here the first stimulus was 100 mM KCl. Responses to subsequent stimuli in the same preparation are reported as a percentage of this first response. 'In this way we can normalize the data and reduce variation between experiments.

It was possible to release SP-LI with two consecutive potassium stimuli in preliminary experiments carried out on single chambers, but pooling two or three chambers improved significantly the release signal and allowed us to select a chamber to investigate further. We chose the right ventricle because comparative studies indicated that SP-LI release from right ventricular slices was reproducible between different heart preparations (Table 7.2) and showed the most consistently stable baseline.

Table 7.2. Substance P-like immunoreactivity (SP-LI) could be released from three pooled chambers of the guinea pig heart in response to two consecutive 100 mM KCl stimuli (n=3).

pooled chamber	peak ratio	1 st KCl stimulus	
		times basal release	% total SP-LI
left atrium	79 ± 4 %	3.8 ± 1.4	0.3 ± 0.0
right atrium	56 ± 6	4.9 ± 1.1	0.7 ± 0.1
left ventricle	68 ± 8	7.1 ± 2.8	0.5 ± 0.3
right ventricle	82 ± 11	8.2 ± 1.1	0.5 ± 0.2

Release experiments on two pooled right ventricles yielded an even steadier basal release and greater reproducibility between experiments. Superfusion of slices from two right ventricles with Krebs buffer containing a depolarizing concentration of KCl (100 mM) increased the outflow of SP-LI from heart tissue

(Figure 7.1). This release returned to basal levels during a washout period, and a subsequent 3 min application of KCl was also able to stimulate SP-LI release, though to a slightly lesser extent. The ratio of the second KCl-stimulated peak of SP-LI release to the first was $66 \pm 4\%$ (mean \pm sem, $n=3$), with the first KCl stimulus releasing $0.9 \pm 0.2\%$ of total SP-LI content, or 4.8 ± 0.3 times above background release. These values are slightly different from those reported in Table 7.2 since, as mentioned previously, the reduction of the pool size to two right ventricles improved reproducibility. Recovery of SP-LI released from heart slices was not improved significantly by the inclusion of 0.1% bovine serum albumin (BSA) or a cocktail of peptidase inhibitors (10 μ M [final concn] leupeptin, 1 μ M pepstatin A, 10 μ M bestatin, 10 μ M bacitracin, and 10 μ M phosphoramidon) in the Krebs superfusion buffer.

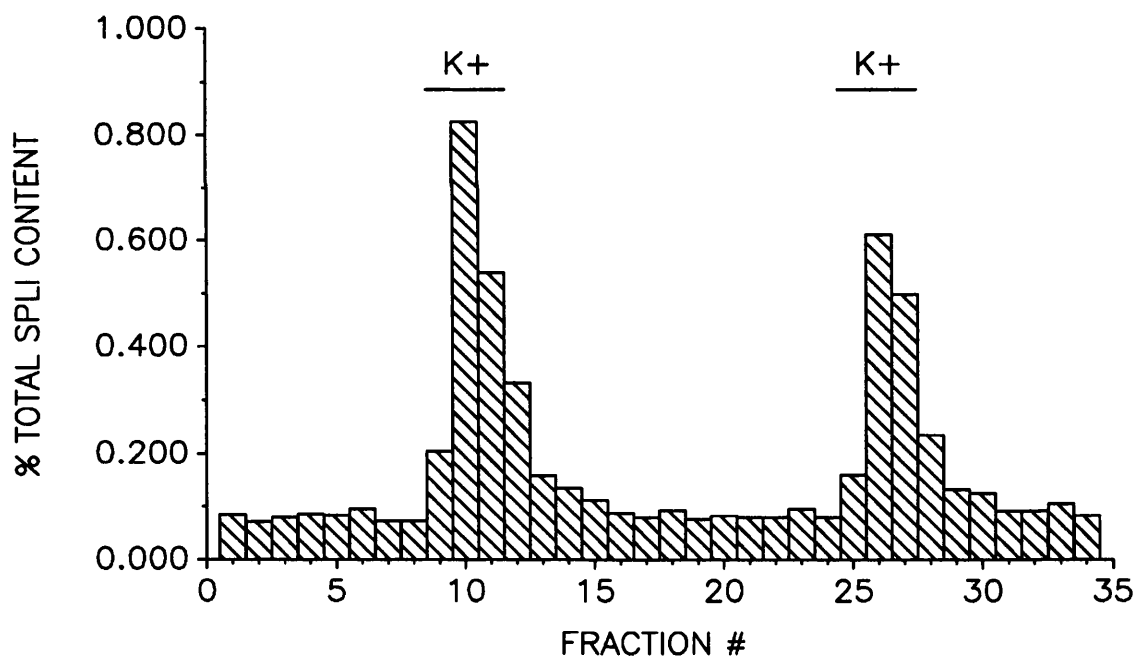


Figure 7.1. *SP-LI release from pooled slices of two guinea pig right ventricles can be stimulated by repetitive 3 min applications of 100 mM KCl (K^+) separated by a washout period.*

Since our research efforts are directed towards opioid events at the peripheral terminals of sensory neurons, we also investigated stimulation of SP-LI release with more selective activators of nociceptive neurons. Manzini et al. (1989) have recently investigated the potential involvement of neuropeptide release in the cardiac actions of bradykinin in an isolated perfused guinea-pig heart preparation. In that study, high concentrations of bradykinin (10 μ M) increased the outflow of SP-LI and were found to produce rapid increases in heart rate and coronary flow, coupled with a significant reduction in contractile strength. Bradykinin, furthermore, was observed not to produce measurable release of SP-LI after destruction of cardiac sensory neurons by *in vitro* or *in vivo* pretreatment of guinea pig hearts with the neurotoxin capsaicin. Preliminary results from our laboratory suggested that bradykinin (100 nM-10 μ M) also gave a robust and reproducible response in the guinea pig right ventricular slice preparation (n=2 for each concentration tested). Appropriate controls, however, have shown that the apparent response can be explained completely by cross-reaction of the SP 3 antibody with bradykinin (Figure 7.2).

Though the antibody cross-reacts only about 1:30,000 with bradykinin, this is sufficient to detect the rather high concentrations that we have used. In subsequent experiments with 10 nM bradykinin, which does not interfere with the radioimmunoassay, SP-LI release was not stimulated from guinea pig right ventricular slices (n=2). Obvious extensions of these studies are to attempt to remove bradykinin from superfusate samples with e.g. an affinity resin.

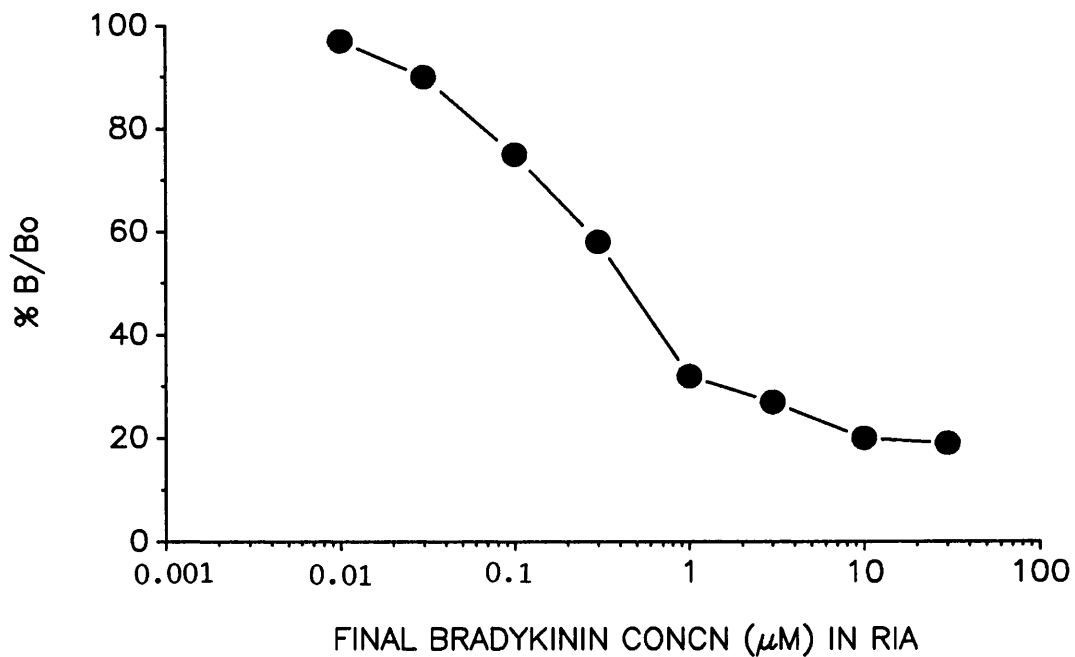


Figure 7.2. Apparent release of SP-LI by bradykinin can be explained by cross-reactivity of the SP 3 antibody with bradykinin (see section 2-3 of Methods and Materials for full details of this antibody). This cross-reactivity is expressed as a percentage ratio of the amount of ^{125}I -SP bound to antibody in the presence of bradykinin (B) compared to the amount of iodinated SP bound in its absence (B_0). Points represent the means of triplicate measurements from two experiments.

Another characteristic feature of nociceptive sensory neurons is activation by capsaicin (see reviews by Fitzgerald, 1983; Buck & Burks, 1986). Capsaicin stimulation (100 nM-3 μ M) released significantly larger amounts of SP-LI from pooled right ventricular slices than did 100 mM KCl (see Figures 7.3 and 7.4). A concentration-response curve for capsaicin is shown in Figure 7.3. Capsaicin stocks were diluted in Krebs superfusion buffer from a 10 mM stock in dimethylsulphoxide (DMSO) to a final concentration of <0.1% DMSO. DMSO vehicle was added to Krebs in lieu of capsaicin in control experiments. The capsaicin vehicle had no effect on SP-LI release.

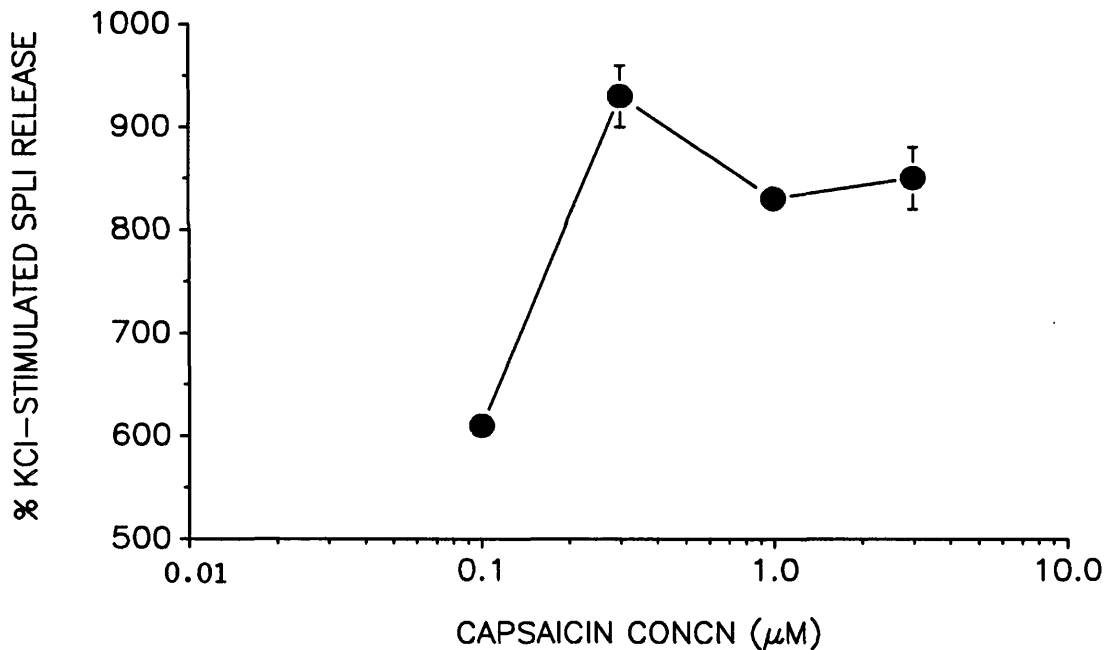


Figure 7.3. Capsaicin stimulates the release of SP-LI from slices of guinea pig right ventricle. Note that the scale of the vertical axis ranges from 500-1000% of the KCl controls. Points indicate the mean \pm sem of at least 3 experiments (with $n=9$ for 1 μ M capsaicin).

In vitro capsaicin pretreatment (1 μ M for 3 min) abolishes the response of guinea pig right ventricular slices to a subsequent KCl stimulus (Figure 7.4). Levels of SP-LI released by the second potassium stimulus were thus reduced to $14 \pm 5\%$ (n=3) of the first KCl, compared to 72% (68%, 76%; n=2) in experiments controlling for the capsaicin pretreatment stimulus (DMSO, 3 min). Capsaicin pretreatment *in vitro* also abolishes the response to a subsequent application of capsaicin (Figure 7.5), suggesting that the tissue desensitizes. Whereas the first capsaicin application induced the outflow of SP-LI to $670 \pm 65\%$ (n=3) of the first KCl, a second capsaicin stimulus, applied 20 min later after a washout in drug-free buffer, stimulates release to $11 \pm 1.4\%$ (n=3) of the first KCl. This contrasts with the results of vehicle controls for capsaicin pretreatment in which a subsequent dose of capsaicin increased SP-LI outflow to 800% (675%, 925%; n=2) of the first KCl. Obvious extensions of these studies would be to treat guinea pigs neonatally with capsaicin, though Hoover (1987) and Geppetti et al. (1988) found that capsaicin treatment *in vivo* yielded similar results to pretreatment of tissue *in vitro*. And a wealth of data has already been accumulated to show that SP exists in capsaicin-depletable sensory fibers in the guinea pig heart (see Papka & Urban, 1987).

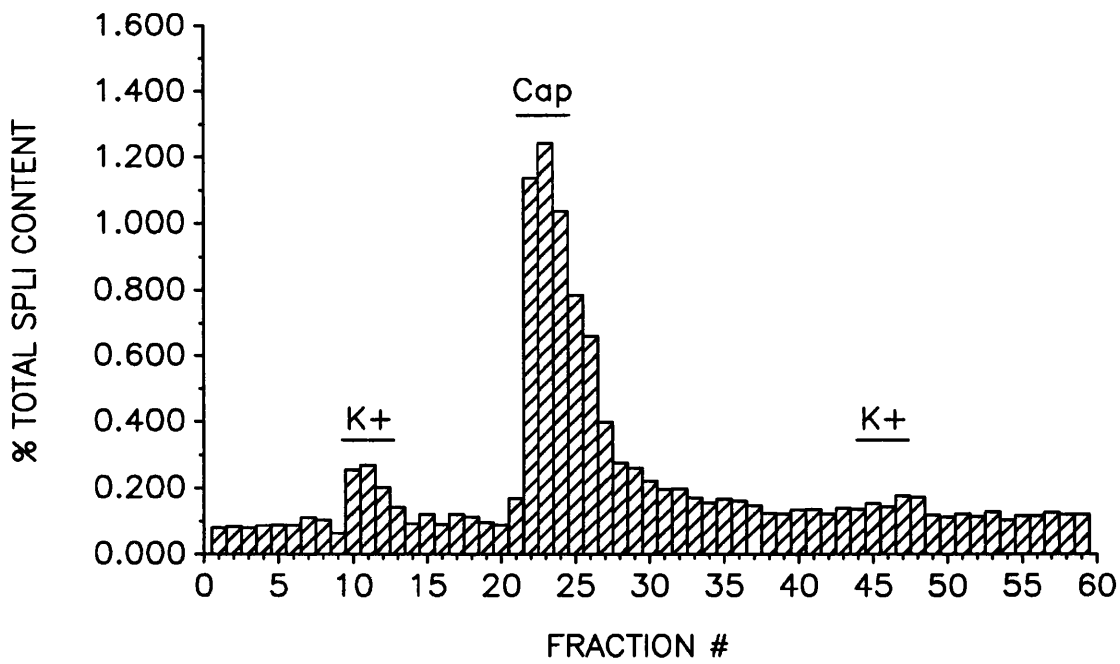


Figure 7.4. Capsaicin ($1 \mu\text{M}$ for 3 min), administered 10 min after the end of a KCl (100 mM, 3 min) stimulus, causes a significantly greater outflow of SP-LI from guinea pig right ventricular slices than KCl. Exposure of the heart tissue to capsaicin causes desensitization to a subsequent KCl stimulus even after a 20 min recovery period.

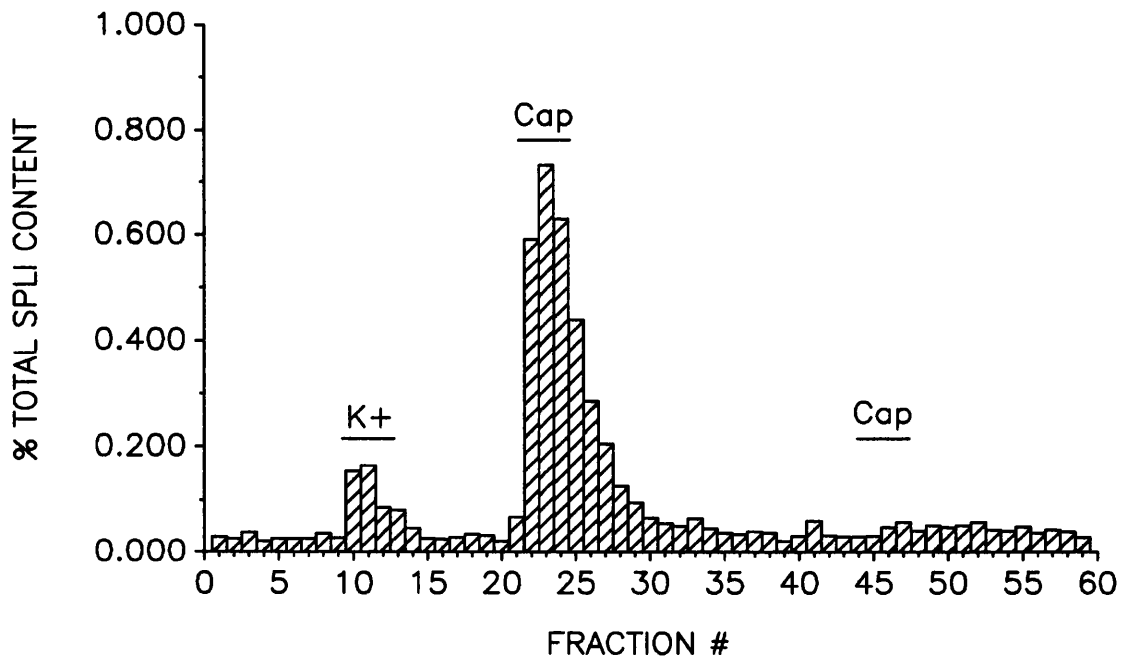


Figure 7.5. Capsaicin administration ($1 \mu\text{M}$, 3 min) also causes tachyphylaxis to a subsequent dose of capsaicin.

7-2.6 Formalin (0.2%)-evoked outflow of SP-LI

Subcutaneous injection of a dilute (0.5%) formaldehyde solution (formalin) was used as a long-lasting nociceptive stimulus in earlier experiments *in vivo*. As discussed in Chapter 4, little is known about the mechanism of formalin-induced nociception apart from indications that both direct activation of nociceptors and inflammatory processes are involved. We have investigated the ability of formalin to stimulate the *in vitro* release of SP-LI from pooled slices of guinea pig right ventricles.

Prior to the release experiments we tested for the interference of formalin with the SP radioimmunoassay (RIA). In these control studies we added known amounts of SP (1-15 fmol) to tubes containing Krebs buffer with varying concentrations of added formalin (0-5%), lyophilized the solutions, and assayed for SP-LI after resuspending the lyophilizates.

These results indicate that raising the final concentrations of formalin in the superfusate above 0.2% will interfere significantly with the RIA. This was reflected not only by a change in the gross shape of the SP standard curve with higher formalin concentrations (Figure 7.6), but also by the formation of an insoluble white precipitate upon lyophilization. The exact cause of this is unknown, though formaldehyde is commonly used as a tissue fixative and one hypothesis is that, at concentrations greater than 0.2%, formalin may be crosslinking substance P molecules and denaturing the protein so that the SP 3 antibody is no longer able to recognize the function and/or conformation of the antigenic C-terminus. The effects of fixative agents on antigenicity are reported to vary greatly with different antisera (see Kosaka et al., 1986; Morris et al., 1989).

Subsequent release experiments show that 0.2% formalin, like capsaicin and depolarizing concentrations of potassium, can increase SP-LI outflow from guinea pig right ventricular slices (Figure 7.7). Normalizing the results to KCl control peak ratios, 0.2% formalin stimulates release of SP-LI to $80 \pm 10\%$ (mean \pm sem, n=3) of KCl control values.

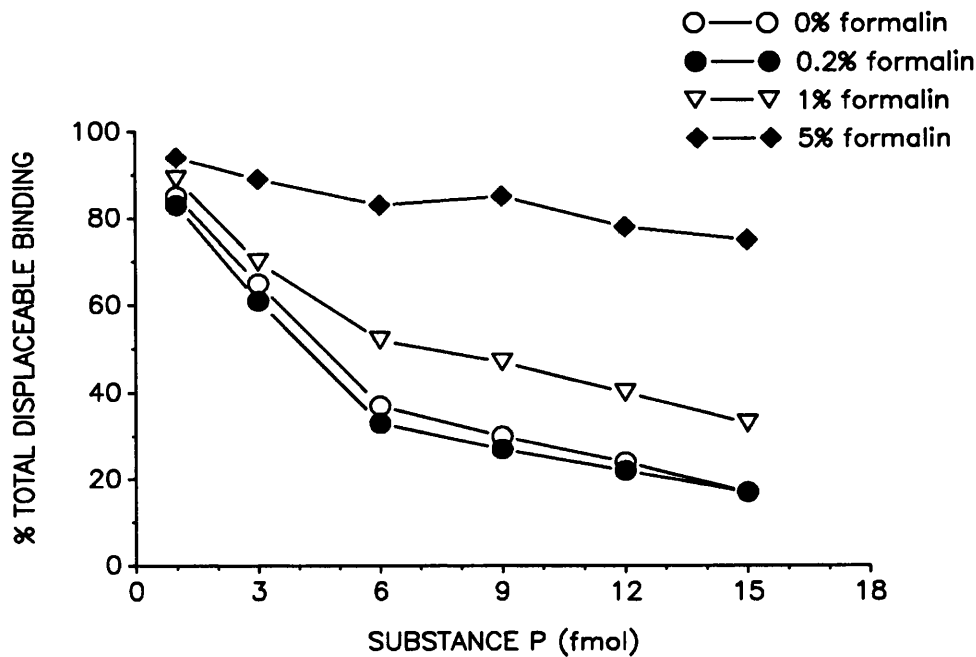


Figure 7.6. Curves demonstrating the effects of dilute formaldehyde (formalin) solutions on radioimmunoassay measurements of substance P standards. Points represent the means of triplicate measurements from two experiments.

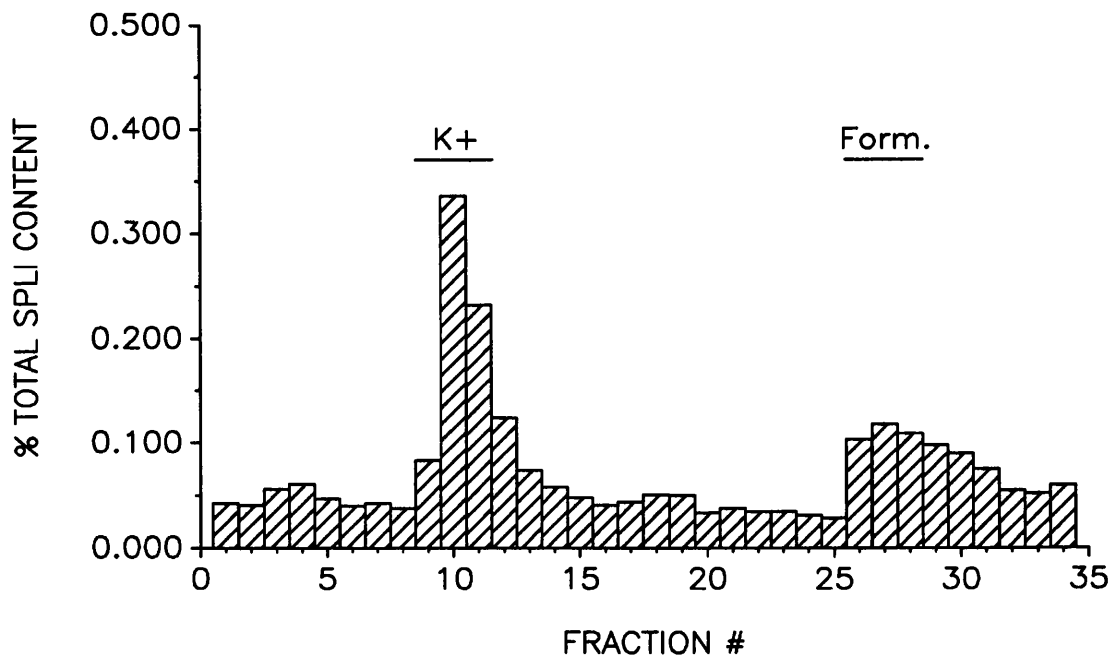


Figure 7.7. Formalin, at a concentration (0.2%) which does not interfere with the RIA, also stimulates SP-LI release.

Potassium (100 mM, 3 min)-evoked release of SP-LI was undetectable when calcium (CaCl_2) was omitted from the Krebs perfusion buffer and 1 mM EGTA (ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetracetic acid) added (n=2). Under these Ca^{2+} -free conditions, formalin (0.2%, 3 min)-evoked release was largely unaffected, with SP-LI outflow reaching 70% of KCl control values (67%, 73%; n=2) as compared to $80 \pm 10\%$ of KCl controls in the presence of calcium (n=3).

7-2.8 Noradrenaline modulates K⁺-evoked SP-LI outflow from guinea pig heart slices

After characterizing stimuli which increased outflow of SP-LI, we then proceeded to assess the ability of a variety of compounds to modulate this release. Noradrenaline, like opioid peptides, is reported to modulate presynaptically neuropeptide release from primary afferent nerve endings (Kuraishi et al., 1985b; Pang & Vasko, 1986; Holz et al., 1989). Prior to embarking on extensive studies with opioids, we therefore challenged guinea pig right ventricular slices with a saturating concentration (see Holz et al., 1989) of noradrenaline (NA). In these experiments, superfusion of heart tissue with 50 μ M NA for 3 min prior to the onset of, and throughout, the 3 min KCl stimulus inhibited the evoked release of SP-LI to 11% of KCl controls (16%, 5%; n=2, see Figure 7.8).

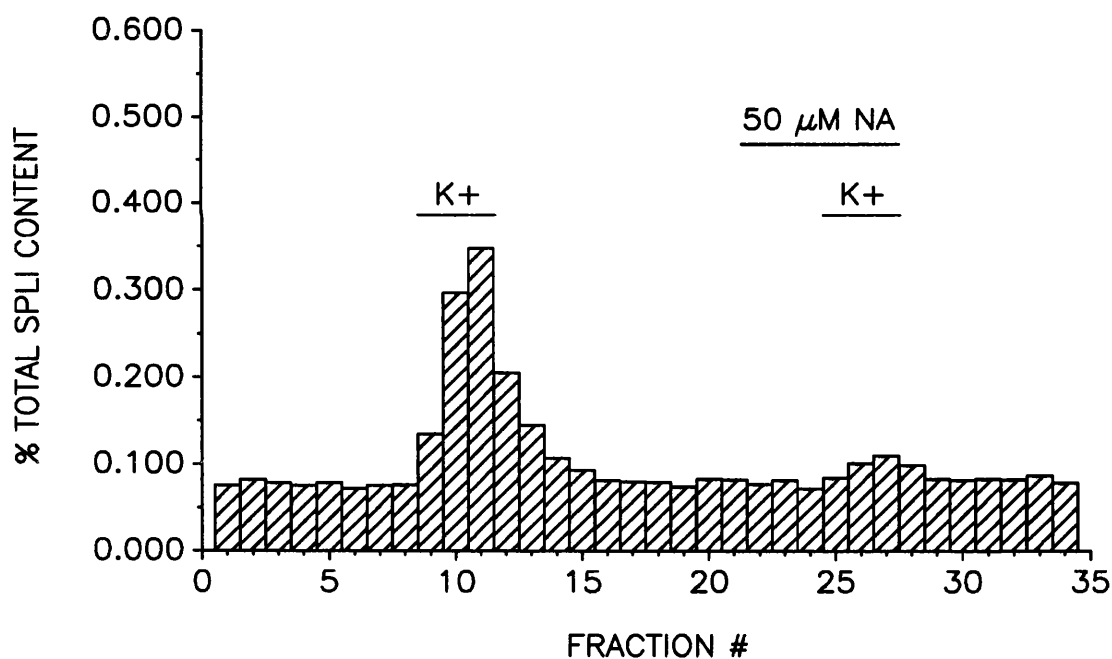


Figure 7.8. This trace shows that KCl-stimulated SP-LI release from this preparation is sensitive to blockade by 50 μ M noradrenaline (NA).

Studies on opioid modulation of K⁺-evoked SP-LI release from guinea pig right ventricular slices also employed this pretreatment protocol. At the lower concentrations tested (10 nM-1 μ M), the mu opioid ligand DAGO was found to inhibit K⁺-stimulated SP-LI release in a concentration-dependent manner (Figure 7.9). Potassium-stimulated SP-LI release was never inhibited completely by DAGO within the concentration range 10 nM-10 μ M, reaching a maximum of 80% inhibition at 1 μ M DAGO.

The agonist IC₅₀ for DAGO was 60 nM. Inhibition of K⁺-evoked SP-LI release by an approximate IC₅₀ concentration (100 nM) of DAGO is partially reversed by 1 μ M naloxone (39% of KCl control [43%, 36%; n=2] raised to 58% of KCl control [51%, 64%; n=2] in the presence of naloxone).

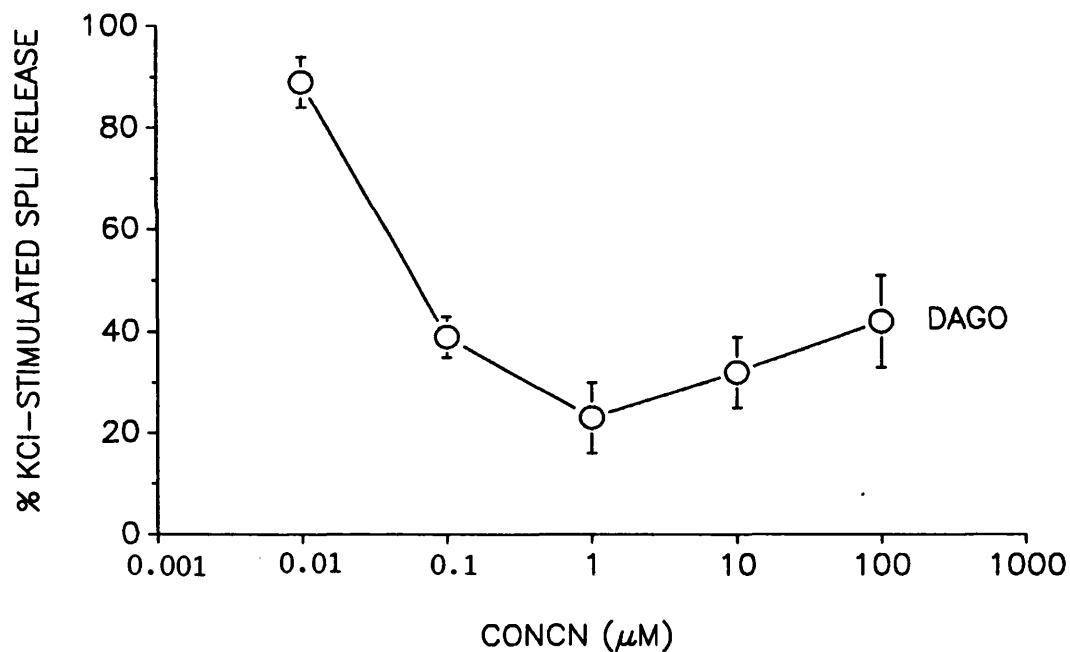


Figure 7.9. The mu agonist [*D*-Ala²,MePhe⁴ Gly(ol)⁵]enkephalin (DAGO) dose-dependently inhibits KCl-stimulated outflow of SP-LI from guinea pig right ventricular slices (*n* equal to at least 2) with an IC₅₀ value of 60 nM.

Superfusing the delta ligand [D-Ala²,D-Leu⁵] enkephalin (DADLE) onto heart slices also produced concentration-dependent inhibitions of KCl-evoked SP-LI release (Figure 7.10). DADLE, with an IC₅₀ value equal to 140 nM, was slightly less effective than DAGO (IC₅₀= 60 nM) in modulating SP-LI outflow. As seen with DAGO, approximately 20% of the tissue response to KCl controls was insensitive to DADLE at those concentrations tested (10 nM-100 μM). The inhibitory effects produced by an approximate IC₅₀ concentration of DADLE (100 nM) were less sensitive to reversal by 1 μM naloxone than was DAGO (n=3).

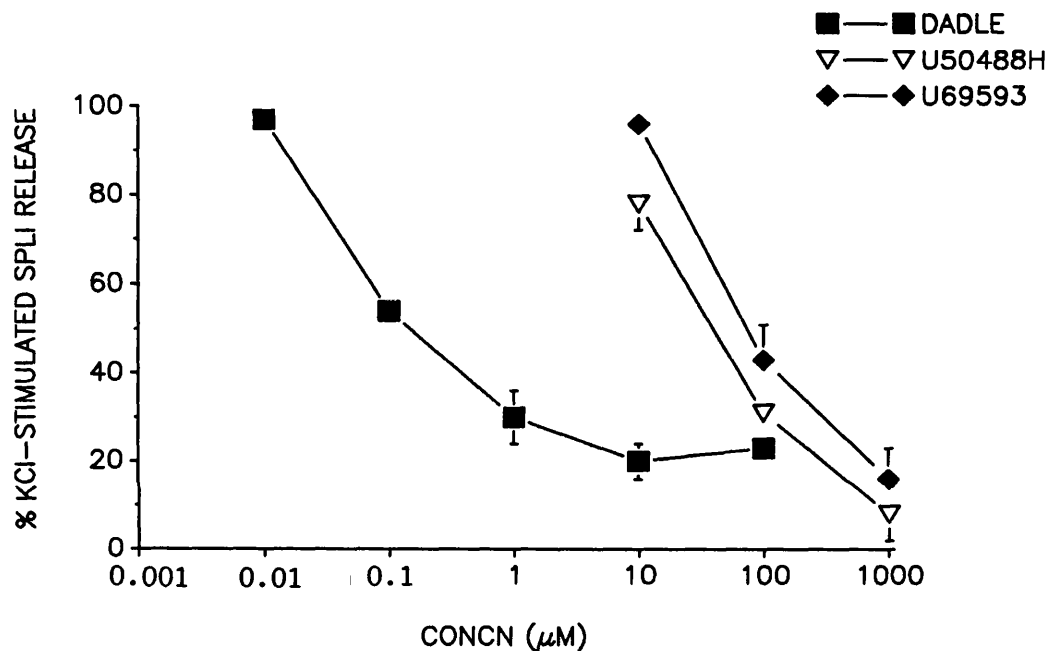


Figure 7.10. The delta ligand [D-Ala²,D-Leu⁵]enkephalin (DADLE), and the kappa-selective agonists U50488H and U69593 dose-dependently inhibit KCl-evoked release of SP-LI from pooled slices of guinea pig right ventricle. IC₅₀ values for DADLE, U50488H, and U69593 were 140 nM, 40 μM, and 75 μM, respectively. Points represent the means ± SEM from 2 to 4 experiments.

Kappa ligands were less potent than mu and delta agonists in inhibiting the potassium-induced outflow of SP-LI from guinea pig right ventricular slices (Figure 7.10). The IC₅₀ values for U50488H and U69593 were 40 μM and 75 μM, respectively. Although the IC₅₀ values for these kappa agonists are similar,

U69593 is much more sensitive to reversal by naloxone than is U50488H. Challenging 100 μ M U69593 with an equimolar concentration of naloxone completely blocks the ability of this kappa ligand to reduce SP-LI outflow (see Figures 7.11 and 7.12). In this way, 100 μ M U69593 reduces the outflow of SP-LI to 43% of KCl control (36%, 51%; n=2) in the absence of 100 μ M naloxone, compared to 86% of KCl control (81%, 91%; n=2) in the presence of naloxone. A 100 μ M concentration of U50488H, however, which reduces K^+ -stimulated SP-LI outflow to $31 \pm 3\%$ of KCl controls (n=4), is not affected by inclusion of 100 μ M naloxone in the superfusion buffer (with SP-LI release at $21 \pm 2\%$ of KCl control levels, n=3).

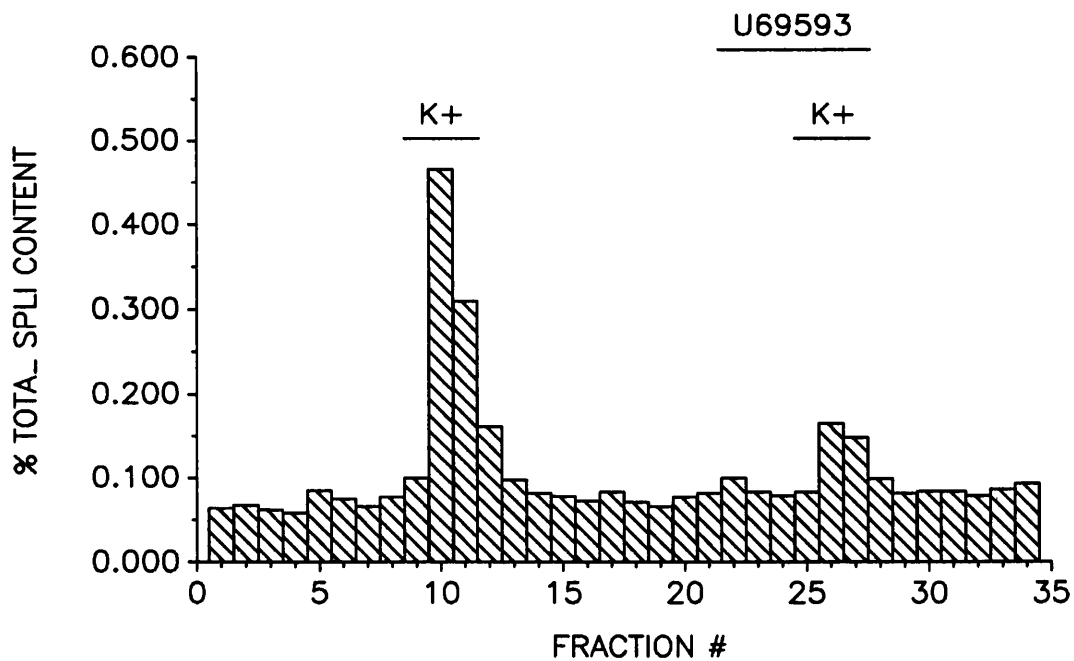


Figure 7.11. *100 μ M U69593 inhibits KCl-evoked SP-LI release from guinea pig right ventricular slices.*

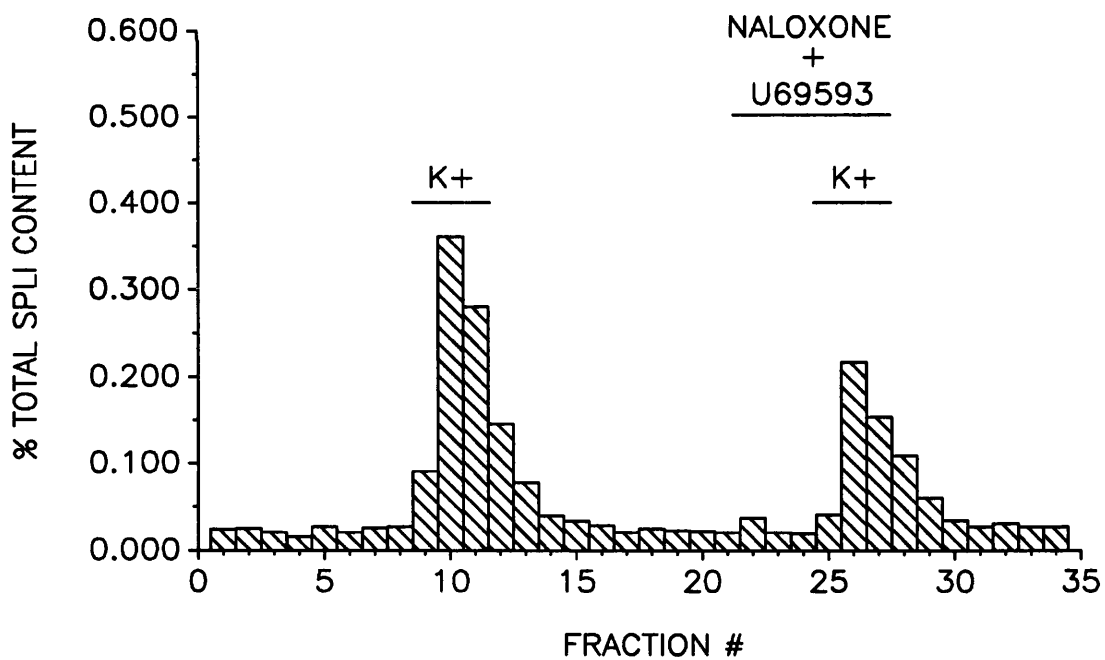


Figure 7.12. *100 μ M naloxone reverses the inhibitory effect of 100 μ M U69593 on K⁺-stimulated SP-LI outflow from heart slices.*

7-2.10 Stimulation of SP-LI outflow by a mu opioid
and high concentrations of kappa ligands

In addition to its inhibitory influence, DAGO stimulates the release of SP-LI from heart slices at the higher concentrations tested (10 μM and 100 μM ; Figure 7.13). Blockade of opioid receptors by 1 μM naloxone increases the ability of 10 μM DAGO to stimulate release from 61% of KCl control (84%, 37%; $n=2$) in the absence of 1 μM naloxone compared to 740% of KCl control (810%, 670%; $n=2$) in the presence of naloxone. Naloxone alone did not stimulate the release of SP-LI from guinea pig right ventricular slices ($n=2$).

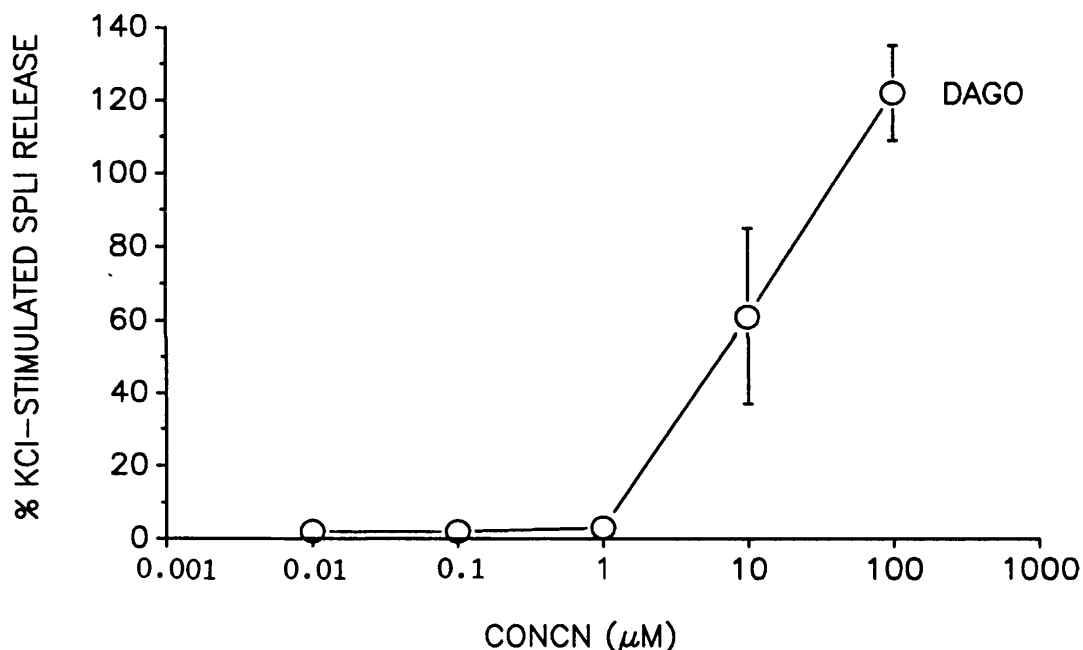


Figure 7.13. DAGO, at concentrations from 10-100 μM , stimulates SP-LI release from right ventricular slices. Points represent the means \pm SEM from at least two experiments.

Unlike DAGO, neither DADLE nor the kappa ligands U50488H and U69593 stimulated SP-LI release within the concentration range 1-100 μ M (n equal to between 2 and 4). However, 1 mM concentrations of U50488H and U69593 stimulated the outflow of SP-LI to 50% (67%, 33%; n=2) and 22% (29%, 15%; n=2) of KCl control values, respectively. Stimulation of SP-LI release by 1 mM U50488H was not reversed significantly by 100 μ M naloxone (n=2).

Our use of formalin as a chronic pain stimulus *in vivo*, coupled with our observations that peripheral administration of kappa (but not mu and delta) opioids can modulate this nociceptive activity, influenced us to test the ability of opioids to modulate formalin-stimulated SP-LI release. As for the studies on K⁺-evoked release, mu (morphine), delta (DADLE), and kappa (U50488H, U69593) opioids were superfused onto heart slices for 3 min prior to and throughout the 3 min formalin (0.2%) stimulus.

Morphine, instead of DAGO, was chosen as the mu ligand for these experiments *in vitro* in order to parallel the *in vivo* formalin work presented in Chapter 4. Supplies of DSTBULET (kindly provided by Dr. B.P. Roques for our *in vivo* studies) were not sufficient to carry out superfusion studies. Therefore DADLE, shown by Yaksh (1988) to inhibit the electrically-evoked release of SP-LI from knee joint primary afferents (at a concentration of 100 μ M), was used as the delta ligand. Neither morphine (100 μ M) nor DADLE (100 μ M) significantly inhibited formalin-evoked SP-LI release (n=2 for each drug). Levels of formalin-induced release in the presence of the mu and delta ligands were 90% (91%, 89%; n=2) and 82% (82%, 81%; n=2) of KCl control, respectively, compared to 80 \pm 10% of KCl for formalin alone (n=3).

Similar results were obtained with equally high concentrations of the selective kappa agonist U69593. Neither 100 μ M (82% of KCl control [90%, 74%; n=2]) nor 1 mM U69593 (83 \pm 13% of KCl control; n=4) attenuated the stimulation of guinea pig right ventricular slices by formalin. The kappa agonist U50488H, however, partially inhibited formalin-evoked SP-LI release at the two concentrations tested. The lower, 10 μ M, concentration of U50488H reduced formalin-stimulated release to 64% of KCl controls (67%, 61%; n=2) with 1 mM U50488H diminishing the formalin-evoked outflow of SP-LI to 38 \pm 11% of KCl control levels (n=4).

We have developed a guinea pig heart slice preparation *in vitro* for investigating opioid effects at the peripheral terminals of sensory neurons. Holaday (1983) has suggested that one factor which may hinder binding studies on heart is the proportionally high level of muscle tissue compared to neural tissue. This may explain why we were unable to detect opioid binding sites in homogenates of guinea pig heart. Our results, which indicate that SP-LI is 3 to 4 times more plentiful in atrial than ventricular tissue, are in agreement with other radioimmunoassay data (Dalsgaard et al., 1986; Manzini et al., 1989) and with immunohistochemical studies describing denser SP-IR innervation of atria than ventricles (Urban & Papka, 1985; Papka & Urban, 1987).

The results of subsequent experiments show that a range of stimuli including elevation of extracellular potassium ion concentration (100 mM KCl), capsaicin (100 nM-3 μ M), and formalin (0.2%) can increase the outflow of SP-LI from slices of guinea pig right ventricle. Potassium-evoked release of SP-LI was calcium-dependent, but formalin was found to stimulate neuropeptide release in a largely calcium-independent manner. Although the calcium-dependency of capsaicin-stimulated SP-LI release was not investigated in these studies, there is some suggestion from work in other tissues that capsaicin-induced release is largely Ca^{2+} -dependent (Dray et al., 1989b; Maggi et al., 1989).

Capsaicin has been shown to stimulate the release of SP-LI from the peripheral terminals of sensory nerves in a variety of tissues including the heart (see reviews by Buck & Burks, 1986; Holzer, 1988). Expressed as a percentage of total tissue content, the levels of SP-LI released by capsaicin (1 μ M) in our experiments on right ventricular slices are comparable to those reported by Hoover (1987) in the isolated perfused guinea pig heart.

A second exposure to capsaicin failed to evoke any further SP-LI release from heart slices. Desensitization to the effects of capsaicin on SP-LI release has been shown previously in studies on the isolated heart (Hoover, 1987; Manzini et al., 1989). Approximately 90% of the SP-LI content of the right ventricular slices

remained after the first exposure to capsaicin, so that the failure of a repeated capsaicin challenge to stimulate release is probably not explained by depletion of tissue SP-LI.

Both opioids (Mudge et al., 1979; Dunlap & Fischbach, 1981; Werz & Macdonald, 1982) and noradrenaline (Dunlap & Fischbach, 1981; Canfield & Dunlap, 1984) inhibit voltage-dependent calcium currents and decrease the duration of calcium-dependent action potentials in sensory neurons grown in culture. In our preliminary characterization of the heart slice system we have shown that noradrenaline can inhibit release of SP-LI from peripheral terminals of sensory neurons. This work could be extended to include the use of selective α_1 and α_2 adrenergic agonists and antagonists to establish, firstly, that this was an alpha adrenoceptor-mediated effect and, secondly, to characterize the subtype(s) of receptor involved. Holz et al. (1989) demonstrated that the inhibitory action of noradrenaline on SP-LI release from DRG cell cultures was mediated by α_2 -receptors. There is some recent evidence in the literature to suggest that α_2 receptors (Adamson et al., 1989; Lipscombe & Tsien, 1989) and kappa opioid receptors (Gross & Macdonald, 1987; Adamson et al., 1989) are coupled to N-type voltage-sensitive Ca^{2+} channels.

As discussed in Chapter 5, opioids generally exert an inhibitory influence on sensory neuronal cultures by shortening the duration of calcium dependent action potentials. This opioid receptor-mediated effect on DRG somata, occurring either via opioid-induced opening of K^+ channels (Werz & Macdonald, 1983) or closing of Ca^{2+} channels (Werz & Macdonald, 1985), is postulated to be functionally analogous to opioid receptors on primary afferent terminals. Reduction in the entry of calcium through these opioid receptors on presynaptic DRG terminals may then translate into a reduction in transmitter release. SP release from dissociated cell cultures of DRG neurons, for example, is inhibited by opioids at concentrations (1-10 μM) similar to those reported to shorten the duration of somatic calcium-dependent action potentials in these cells (Mudge et al., 1979; Chang et al., 1989).

Crain and Shen, however, report that opioids have excitatory modulatory effects in addition to these well-characterized inhibitory influences (Shen & Crain, 1989; Crain & Shen, 1990). In work on cultures of mouse spinal cord-DRG explants, they have presented evidence that the mu ligand DAGO prolongs action potential duration (APD) at nanomolar concentrations while shortening APD at micromolar concentrations. This group has proposed a mechanistic scheme for this dual modulatory influence (Crain & Shen, 1990), which they suggest may involve differential coupling of 'excitatory' and 'inhibitory' opioid receptors to ionic conductances via distinct G protein-linked pathways.

Our observations on heart slices contrast to those of Crain and Shen in that DAGO was inhibitory at lower and stimulatory at higher concentrations. In agreement with our observations, however, Mauborgne et al. (1987) have shown that micromolar concentrations of the mu ligands DAGO and morphine enhance the potassium-evoked outflow of SP-LI from rat dorsal spinal cord slices. These results give a complicated picture of mu opioid modulation of the SP release mechanism in guinea pig right ventricular tissue. The picture is complicated further by our results with naloxone, which blocked the inhibitory effects of DAGO, but enhanced the stimulatory effects. It may be that at the high concentration of DAGO the net stimulation we see is a combination of a strong stimulatory effect, which is not mediated by opioid receptors, and an inhibitory effect which is opioid receptor-mediated. Thus the presence of naloxone may block DAGO inhibition and allow full expression of the stimulatory effect.

The delta ligand DADLE inhibited K⁺-evoked SP-LI release nearly as potently as DAGO. This DADLE effect was not as naloxone-sensitive as the mu inhibitory event. Inhibition of K⁺-stimulated SP-LI release by U69593, but not by U50488H, was reversed by the opioid receptor antagonist naloxone. This suggests that the inhibitory effects of U69593 in the guinea pig right ventricular slice preparation are mediated by specific opioid receptors. DADLE and low concentrations of kappa agonists did not induce SP-LI release on their own.

High (1 mM) concentrations of the kappa ligands U69593 and U50488H

were able to inhibit K^+ -stimulated SP-LI release from heart slices and to stimulate neuropeptide outflow in the absence of a depolarizing stimulus. This dual modulatory kappa effect is, as described above for the mu agonist DAGO, contrary to the results of Shen & Crain (1990). In a recent report they present evidence that, in addition to the well-documented inhibitory effects of high concentrations of kappa ligands (e.g. Werz & Macdonald, 1985), low concentrations of the kappa agonists U50488H and dynorphin prolong action potential duration in a naloxone-sensitive manner. Shen & Crain (1990) suggest that the effects of these ligands are mediated through different subtypes of kappa receptor, with U50488H exerting its effect by increasing a Ca^{2+} conductance. Dynorphin, they report, prolongs the action potential by decreasing a voltage-sensitive K^+ conductance. The increase in SP-LI outflow from heart slices observed in the presence of 1 mM U50488H was not sensitive to naloxone (100 μ M), indicating that this stimulatory effect is not opioid receptor-mediated.

There is evidence from the literature that effects of capsaicin are not modulated by opioids. Capsaicin-induced release of CGRP-LI from the isolated perfused guinea-pig heart was not influenced by morphine (Franco-Cereceda, 1988). The capsaicin-evoked stimulation of guinea pig atrial contractility was similarly unaffected by morphine (Lundberg et al., 1984b). Bartho et al. (1987) reported an inability of [D-Met²,Pro⁵]-enkephalinamide to modulate capsaicin-induced bronchoconstriction, whereas it has been shown that opioids can modulate electrically-evoked bronchoconstriction and plasma leakage in guinea pig airways. Collectively, these results suggest that the mechanism of capsaicin-induced neuropeptide release is not subject to presynaptic regulation by activation of opioid receptors to the same degree as release induced by K^+ depolarization or nerve stimulation. Capsaicin opens a non-specific cation channel in sensory neurons (Bevan & Forbes, 1988; Wood et al., 1988), so that the Ca^{2+} influx which leads to SP release is probably via a capsaicin-gated channel, and not through voltage-sensitive calcium channels as for K^+ -stimulated SP-LI release. For example capsaicin-evoked calcium uptake into DRG neurons is not inhibited

by dihydropyridines or omega conotoxin (Wood et al., 1988).

Our results with formalin-evoked release agreed well with our *in vivo* experiments (see Chapter 4). Formalin-evoked release of SP-LI, unlike that stimulated by KCl, was largely calcium-independent. One possible explanation for these results is that low quantities of Ca^{2+} remain even after prolonged superfusion of heart slices with Ca^{2+} -free medium, and these concentrations of extracellular Ca^{2+} are sufficient for formalin, but not potassium, activation of sensory fibers. In which case, the SP-LI released by formalin must be from another pool than that released by KCl. The only source of extracellular Ca^{2+} would be from impurities present in the salts used to prepare the Krebs solution, and the relevance of impurities inducing neurotransmitter release is unlikely given the chelating capability of the 1 mM EGTA included in the superfusate. Formalin could, alternatively, be stimulating the release of Ca^{2+} from intracellular stores e.g. by an inositol trisphosphate-mediated mechanism. It cannot be ruled out that formalin is destroying the structure of sensory neurons, though most of the tissue SP-LI content (>95%) remains at the end of the experiment.

Effects of formalin were not sensitive to mu or delta opioids, but were blocked by U50488H. In the heart slice preparation there is some indication that U50488H is not working at opioid receptors. Firstly, when KCl was used as the stimulus the effects of U50488H were not sensitive to naloxone, but the effects of U69593 were antagonized. Secondly, with formalin as the stimulus, U50488H inhibited SP-LI release from right ventricular slices but U69593 had no effect. In this latter case, if U50488H were acting at kappa receptors U69593 should also have been active in modulating SP-LI outflow. Non-opioid effects of U50488H have been reported previously (Hayes et al., 1988).

The mechanism of action of formalin may differ between the heart slice and *in vivo* preparations. One possibility is that the observed effect of U50488H *in vivo* may not be directly on neurons that respond to formalin. This would explain why naloxone-sensitive U50488H effects were not seen in the heart slice setup where all tissue is exposed to the stimulus. *In vivo* the signal measured may be due

in part to mediators that diffuse to sensory endings somewhat removed from the site of formalin injection. Alternatively, SP-LI measurements *in vitro* may not be an appropriate model for the activation of sensory neurons *in vivo*. For example, other neuropeptides (e.g. CGRP) or inflammatory mediators such as bradykinin, prostaglandins, and interleukins may play a more important role than substance P in the generation of the formalin response *in vivo*. Cardiac afferents may, furthermore, be different from skin afferents in that kappa receptors may not be expressed to the same extent in the two tissues. This might explain the high concentrations of U50488H and U69593 necessary to inhibit the potassium stimulated release of SP-LI.

CHAPTER 8 -
CONCLUSIONS

Evidence has accumulated from behavioral, functional, and biochemical studies pointing to possible roles for peripheral opioid receptors in modulating nociception (see e.g. Ferreira & Nakamura, 1979; Joris et al., 1987; Stein et al., 1988,1989). This thesis presents evidence from preparations *in vivo* and *in vitro* that opioids can modulate nociceptive activity at the peripheral terminals of sensory neurons.

The formalin test, in which a small volume of dilute formaldehyde solution is injected s.c. into the animal hindpaw, was introduced to reduce problems of assessing behavioral analgesia using transient nociceptive stimuli (Dubuisson & Dennis, 1977). We have used an electrophysiological correlate of the formalin model (Dickenson & Sullivan, 1987a,b) to investigate the actions of peripherally administered receptor-selective opioid ligands on this index of sensory neuronal activation by a prolonged noxious chemogenic stimulus. In this setup, the activity of a single dorsal horn neuron is recorded extracellularly. The firing activity of the cell in response to formalin injection into the hindpaw is characteristically biphasic. The biphasic nature and the relative durations of the peaks of dorsal horn neuronal activity correlate well with the profile of activity seen in behavioral studies with formalin. The first peak may represent a direct effect of this chemical stimulus on C fibers while the second phase is an inflammatory-type response. The administration of 100 μ g of either the mu ligand morphine or the delta-selective DSTBULET directly (s.c.) into the hindpaw receptive field 10 min before formalin injection had no effect on the subsequent response of the neurons to the stimulus. However, injection of the kappa agonist U50488H into the hindpaw produced a concentration-related inhibition of both the first and second peaks of formalin-induced activity. Administration of the same dose of U50488H into the contralateral paw, to control for the possibility that this kappa ligand was exerting its inhibitory actions by acting systemically, did not alter either peak of the formalin response. Kappa inhibition of formalin-induced activity was reversed significantly by intraplantar naloxone, indicating that U50488H is producing its effects via opioid receptors located in the periphery.

This peripheral profile of opioid effects on formalin-induced activity differs from that seen centrally. Intrathecal application of mu (Dickenson & Sullivan, 1987b) and delta (Sullivan et al., 1989) but not kappa opioids (except at high concentrations; personal communications) depress formalin-induced activity. We have addressed this relative lack of a central kappa effect in binding studies on adult rat spinal cord homogenates. Results indicate that, although kappa sites are present, levels of [³H]U69593 binding to spinal cord are 6-7 times lower in the adult as compared to the neonate.

In saturation and competition studies, we have demonstrated the presence of specific high affinity binding sites for the kappa ligands [³H]U69593 and blocked [³H]EKC, and the mu ligand [³H]DAGO on neonatal rat spinal cord membranes. Few or no delta sites labelled by [³H]DPDPE were detected. This data agrees with results from studies showing functional mu and kappa receptors in the neonatal rat spinal cord (Dray et al., 1989a; James et al., 1990). A similar pattern of receptor types has been found in brains from neonatal rats (Spain et al., 1985; McDowell & Kitchen, 1987; Milligan et al., 1987). The functional significance of the presence and developmental changes in opioid receptors is, however, unclear.

Increasingly, the peripheral terminals of primary afferent sensory neurons are thought to be analogous to central endings terminating in the superficial laminae of the spinal dorsal horn (see Yaksh, 1988). For example, opioids modulate release of the neuropeptide substance P (SP) from the peripheral as well as central endings of sensory nerves (see review by Holzer, 1988). SP is reported to exert local effects following its release from the peripheral terminals and is implicated to play a role in neurogenic inflammation (see Holzer, 1988). In addition to this, SP may convey afferent information via release at central terminals (see reviews by Nicoll et al., 1980; Salt & Hill, 1983).

There is some suggestion in the literature that SP-LI levels increase in the dorsal horn after formalin injection into the hindpaw and that this buildup of the neuropeptide is naloxone-reversible (Kantner et al., 1985,1986; McCarson &

Goldstein, 1989). Several groups have shown that spinal preprodynorphin levels also increase in response to peripheral noxious stimuli (see Iadorola et al., 1988). Thus McCarson & Goldstein (1989) suggest that endogenous opioids may modulate the formalin-evoked release of SP-LI in the dorsal horn. Prior to moving *in vitro* to further examine formalin stimulation of peripheral sensory nerve endings, we therefore investigated whether the observed modulation of the formalin response *in vivo* (measured centrally as decreased dorsal horn neuronal activity) by peripherally administered U50488H resulted in a change in SP-LI release at the central terminals.

Noxious thermal, chemical, mechanical, and electrical cutaneous stimuli are known to activate primary afferents and have been shown recently to cause release of SP centrally (Kuraishi et al., 1983; Duggan et al., 1987,1988; Go & Yaksh, 1987). These groups have either used push-pull cannulae, antibody-coated microprobes, or catheters inserted directly into the dorsal horn. We however have used a noninvasive technique of superfusing the surface of the exposed (L₁-L₃) segments of the adult rat spinal cord. Attempts to correlate peripheral opioid modulation of formalin-induced dorsal horn neuronal activity with changes in levels of SP-LI release at central terminals were unsuccessful. A possible explanation is that, as Kantner et al. (1985) hypothesized, SP-LI built up in the spinal dorsal horn may reflect increased synthesis of the neuropeptide, and the release mechanism may be under endogenous opioid control. Alternatively, SP-LI released in response to the peripheral formalin stimulus is being degraded more quickly than it is collected, despite the presence of peptidase inhibitors in the superfusion buffer.

Given the fact that classification of receptor-type *in vivo* can involve certain complexities (e.g. diffusional limitations and drug redistribution) we have searched for alternative, more simplistic models to investigate opioid events in the periphery. We have therefore turned to *in vitro* assays to study opioid effects on the peripheral terminals of primary afferents in preparations devoid of central inputs.

Problems of reproducibility with opioid responses on $^{45}\text{Ca}^{2+}$ uptake and SP-LI release in primary cultures of dorsal root ganglion (DRG) neurons may reflect low densities and/or differential expression of functionally-coupled opioid receptors (Werz & Macdonald, 1985; Werz et al., 1987).

Hybrid cell lines have certain advantages over primary cultures. Although the properties of such tumor-derived cells may not be entirely analagous to those of intact DRG (e.g. as primary cultures are), clonal cell lines do provide a useful means of analyzing receptor properties. Cell lines have therefore been used extensively to elucidate the coupling of opioid receptors to ion channels and neurotransmitter release (see review by McFadzean, 1988). We screened a series of neuroblastoma x DRG (ND) cell line fusion products for opioid binding sites and found that two ND hybrid lines expressed delta, but not mu or kappa, opioid receptors. Our attempts to correlate delta opioid ligand-receptor interaction with a tissue effect e.g. $^{86}\text{Rubidium}$ efflux, as a crude indicator of potassium currents, or SP-LI release proved negative.

Our *in vitro* efforts culminated in the development of a guinea pig right ventricular slice preparation to investigate opioid effects on SP-LI release from the peripheral endings of sensory nerves. Localization studies have pinpointed SP to exist almost entirely within the peripheral terminals of capsaicin-sensitive sensory neurons in the heart (see Papka & Urban, 1987). Several groups have provided evidence that SP may directly affect cardiac function subsequent to release from the peripheral endings of sensory neurons in the guinea pig heart (Hoover, 1987; Manzini et al., 1989).

The work presented in this thesis shows that the outflow of SP-LI from pooled slices of guinea pig right ventricle is increased in response to depolarization by elevated extracellular potassium levels (100 mM K^+), capsaicin (100 nM-3 μM), and formalin (0.2%). Superfusion of heart slices in calcium-free medium buffered with EGTA prevented stimulation of SP-LI release by K^+ , but formalin-evoked release was largely calcium-independent.

Potassium-evoked release from right ventricular slices was modulated to

differing extents by mu (DAGO), delta (DPDPE), and kappa (U50488H, U69593) opioid ligands, and the naloxone-sensitivity of these effects were, again, variable. Micromolar concentrations of DAGO and millimolar concentrations of the kappa ligands also stimulated SP-LI release on their own. Such excitatory opioid effects have been reported previously (Mauborgne et al., 1987), though others suggest that this occurs at low ligand concentrations (Crain & Shen, 1990; Shen & Crain, 1990). Although opioid binding sites were not detected in crude membranes of ventricular tissue, this work has demonstrated that opioid receptors are present in the guinea pig heart and are coupled to processes capable of modulating SP-LI release. Previous binding studies on the heart have encountered similar difficulties, which may be explained partially by the high ratio of muscular to neural tissue in this organ.

In contrast to K^+ -evoked release, formalin-stimulated SP-LI release was not modulated by mu (morphine) or delta (DADLE) ligands. The kappa agonist U50488H, at high (1 mM) concentrations, inhibited formalin-evoked release, but equimolar amounts of the more selective kappa ligand U69593 did not attenuate significantly stimulated SP-LI release. The observed differences in U50488H effects on formalin-induced responses *in vivo* and *in vitro* suggest that there might be differential expression of opioid receptors on sensory neurons in the rat hindpaw as compared to guinea pig heart.

The inhibitory actions of endogenous opioid peptides on neuronal activity are suggested to result, at least in part, from their inhibition of the release of SP-LI and other neurotransmitters from the peripheral endings of sensory nerves (see Brodin et al., 1983). This point of view is supported by the fact that opioid peptides have been shown not to inhibit the effects of exogenous SP in a peripheral tissue preparation e.g. rat saphenous nerve (Smith & Buchan, 1984). However it is unclear whether the opioid peptides which modulate neuropeptide release are themselves released from sensory nerve terminals or exert their actions as circulating hormones.

Given the abuse potential of morphine, the exogenous ligand for the mu

opioid receptor, there has been much interest in developing ligands selective for non-mu opioid receptors. Studies such as those presented in this thesis, by characterizing the effects of activating different opioid receptors at different sites, could provide information on likely therapeutic targets for selective opioids. The potential benefits of targeting ligands for specific receptor types are emphasized by the fact that, recently, several kappa agonists have begun clinical trials (see Millan, 1990).

REFERENCES

- Adamson, P., Xiang, J.-Z., Mantzourides, T., Brammer, M.J., & Campbell, I.C. (1989) Presynaptic α_2 -adrenoceptor and κ -opiate receptor occupancy promotes closure of neuronal (N-type) calcium channels. *Eur. J. Pharmacol.* 174: 63-70.
- Ahlquist, R.P. (1948) A study of the adrenergic receptors. *Am. J. Physiol.* 153: 586-600.
- Akil, H., Hewlett, W.A., Barchas, J.D., & Li, C.H. (1980) Binding of [3 H] β -endorphin to rat brain membranes: Characterization of opiate properties and interaction with ACTH. *Eur. J. Pharmacol.* 64: 1-8.
- Akil, H., Richardson, D.E., Barchas, J.D., & Li, C.H. (1978) Appearance of β -endorphin-like immunoreactivity in human ventricular cerebrospinal fluid upon analgesic electrical stimulation. *Proc. Natl. Acad. Sci. U.S.A.* 75: 5170-5172.
- Akil, H., Watson, S.J., Young, E., Lewis, M.E., Khachaturian, H., & Walker, J.M. (1984) Endogenous opioids: Biology and function. *Annu. Rev. Neurosci.* 7: 223-255.
- Akiyama, K., Gee, K.W., Mosberg, H.I., Hruby, V.J., & Yamamura, H.I. (1985) Characterization of [3 H][2-D-penicillamine, 5-D-penicillamine] -enkephalin binding to δ opiate receptors in the rat brain and neuroblastoma-glioma hybrid cell line (NG108-15). *Proc. Natl. Acad. Sci. U.S.A.* 82: 2543-2547.
- Allerton, C.A., Boden, P.R., Hill, R.G., Hughes, J., Hunter, J.C., & Smith, J.A.M. (1989a) Correlation of [3 H]U69593 binding sites with antinociceptive activity in the 9- to 16-day-old rat spinal cord. *J. Physiol.(Lond.)* 409: 38P.
- Allerton, C.A., Smith, J.A.M., Hunter, J.C., Hill, R.G., & Hughes, J. (1989b) Correlation of ontogeny with function of [3 H]U69593 labelled κ opiate binding sites in the rat spinal cord. *Brain Res.* 502: 149-157.
- Alreja, M., Mutalik, P., Nayar, U., & Manchanda, S.K. (1984) The formalin test: A tonic pain model in the primate. *Pain* 20: 97-105.
- Altura, B.T., Altura, B.M., & Quirion, R. (1984) Identification of benzomorphan- κ opiate receptors in cerebral arteries which subserve relaxation. *Br. J. Pharmacol.* 82: 459-466.
- Amano, T., Hamprecht, B., & Kemper, W. (1974) High activity of choline acetyltransferase induced in neuroblastoma x glia hybrid cells. *Exp. Cell. Res.* 85: 399-408.
- Andres, K.H. (1961) Untersuchungen über den Feinbau von Spinalganglien. *Z. Zellforsch. Mikrosk. Anat.* 55: 1-48.
- Arner, L.S. & Stallcup, W.B. (1981) Rubidium efflux from neural cell lines through voltage-dependent potassium channels. *Dev. Biol.* 83: 138-145.
- Arunlakshana, O. & Schild, H.O. (1959) Some quantitative uses of drug antagonists. *Br. J. Pharmacol.* 14: 48-58.

- Attali, B., Gouarderes, C., Mazarguil, H., Audigier, Y., & Cros, J. (1982) Evidence for multiple "kappa" binding sites by use of opioid peptides in the guinea-pig lumbo-sacral spinal cord. *Neuropeptides* 3: 53-64.
- Attali, B., Saya, D., Nah, S.-Y., & Vogel, Z. (1989) κ Opiate agonists inhibit Ca^{2+} influx in rat spinal cord-dorsal root ganglion cocultures: Involvement of a GTP-binding protein. *J. Biol. Chem.* 264 (1): 347-353.
- Atweh, S.F. & Kuhar, M.J. (1977) Autoradiographic localization of opiate receptors in rat brain. I. Spinal cord and lower medulla. *Brain Res.* 124: 53-67.
- Azami, J., Llewelyn, M.B., & Roberts, M.H.T. (1982) The contribution of nucleus reticularis paragigantocellularis and nucleus raphe magnus to the analgesia produced by systemically administered morphine, investigated with the microinjection technique. *Pain* 12: 229-246.
- Baccaglioni, P.I. & Hogan, P.G. (1983) Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells. *Proc. Natl. Acad. Sci. U.S.A.* 80: 594-598.
- Bach, S., Noreng, M.F., & Tjellden, N.U. (1988) Phantom limb pain in amputees during the first 12 months following limb amputation, after preoperative lumbar epidural blockade. *Pain* 33: 297-301.
- Baluk, P. & Gabella, G. (1989) Some intrinsic neurons of the guinea-pig heart contain substance P. *Neurosci. Lett.* 104: 269-273.
- Barde, Y.A., Lindsay, R.M., Monard, D., & Thoenen, H. (1978) New factor released by cultured glioma cells supporting survival and growth of sensory neurones. *Nature* 274: 818.
- Barrett, R.W. & Vaught, J.L. (1982) The effects of receptor selective opioid peptides on morphine-induced analgesia. *Eur. J. Pharmacol.* 80: 427-430.
- Barrett, R.W. & Vaught, J.L. (1983) Evaluation of the interactions of mu and delta selective ligands with [3H]D-Ala²-D-Leu⁵-enkephalin binding to mouse brain membranes. *Life Sci.* 33: 2439-2448.
- Bartho, L., Amann, R., Saria, A., Szolcsanyi, J., & Lembeck, F. (1987) Peripheral effects of opioid drugs on capsaicin-sensitive neurones of the guinea-pig bronchus and rabbit ear. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 336: 316-320.
- Bartho, L. & Szolcsanyi, J. (1981) Opiate agonists inhibit neurogenic plasma extravasation in the rat. *Eur. J. Pharmacol.* 73: 101-104.
- Basbaum, A.I. & Fields, H.L. (1984) Endogenous pain control systems: Brainstem spinal pathways and endorphin circuitry. *Annu. Rev. Neurosci.* 7: 309-338.
- Bayliss, W.M. (1901) On the origin from the spinal cord of the vaso-dilator fibres of the hind-limb, and on the nature of these fibres. *J. Physiol. (Lond.)* 26: 173-209.

- Beckett, A.H. & Casy, A.F. (1954) Synthetic analgesics: stereochemical considerations. *J. Pharm. Pharmacol.* 6: 986-1001.
- Belvisi, M.G., Rogers, D.F., & Barnes, P.J. (1989) Neurogenic plasma extravasation: inhibition by morphine in guinea pig airways *in vivo*. *J. Appl. Physiol.* 66 (1): 268-272.
- Berthelsen, S. & Pettinger, W.A. (1977) A functional basis for classification of α -adrenergic receptors. *Life Sci.* 21: 595-606.
- Besson, J.-M. & Chaouch, A. (1987) Peripheral and spinal mechanisms of nociception. *Physiol. Rev.* 67 (1): 67-186.
- Besson, J.-M., Conseiller, C., Hamann, K.-F., & Maillard, M.-C. (1972) Modifications of dorsal horn cell activities in the spinal cord after intra-arterial injection of bradykinin. *J. Physiol. (Lond.)* 221: 189-205.
- Bevan, S.J. & Forbes, C.A. (1988) Membrane effects of capsaicin on dorsal root ganglion neurons in cell culture. *J. Physiol. (Lond.)* 398: 28P.
- Birch, P.J., Hayes, A.G., Sheehan, M.J., & Tyers, M.B. (1987) Norbinaltorphimine: antagonist profile at κ opioid receptors. *Eur. J. Pharmacol.* 144: 405-408.
- Blosser, J., Abbott, J., & Shain, W. (1976) Sympathetic ganglion cell x neuroblastoma hybrids with opiate receptors. *Biochem. Pharmacol.* 25: 2395-2399.
- Bonner, T.I. (1989) New subtypes of muscarinic acetylcholine receptors. *Trends in Pharmacol. Sci.* 10 (Suppl. *Subtypes of Muscarinic Receptors IV*): 11-15.
- Bonnet, K.A., Groth, J., Gioannini, T., Cortes, M., & Simon, E.J. (1981) Opiate receptor heterogeneity in human brain regions. *Brain Res.* 221: 437-440.
- Botticelli, L.J., Cox, B.M., & Goldstein, A. (1981) Immunoreactive dynorphin in mammalian spinal cord and dorsal root ganglia. *Proc. Natl. Acad. Sci. U.S.A.* 78: 7783-7786.
- Bowen, W.D., Gentleman, S., Herkenham, M., & Pert, C.B. (1981) Interconverting μ and δ forms of the opiate receptor in rat striatal patches. *Proc. Natl. Acad. Sci. U.S.A.* 78: 4818-4822.
- Bowker, R.M., Abbott, L.C., & Ditts, R.P. (1988) Peptidergic neurons in the nucleus raphe magnus and the nucleus gigantocellularis: their distributions, interrelationships, and projections to the spinal cord. In *Progress in Brain Research, Vol. 77: Pain Modulation*, (eds. Fields, H.L. & Besson, J.-M.), pp. 95-127. Elsevier, Oxford.
- Bradbury, A.F., Smyth, D.G., & Snell, C.R. (1976) Lipotropin: precursor to two biologically active peptides. *Biochem. Biophys. Res. Commun.* 69 (4): 950-956.
- Brandt, M., Gullis, R.J., Fischer, K., Buchen, C., Hamprecht, B., Moroder, L., & Wunsch, E. (1976) Enkephalin regulates the levels of cyclic nucleotides in neuroblastoma x glioma hybrid cells. *Nature* 262: 311-313.

- Brodin, E., Gazelius, B., Olgart, L., & Nilsson, G. (1981) Tissue concentration and release of substance P-like immunoreactivity in the dental pulp. *Acta. Physiol. Scand.* 111: 141-149.
- Brodin, E., Gazelius, B., Panopoulos, P., & Olgart, L. (1983) Morphine inhibits substance P release from peripheral sensory nerve endings. *Acta. Physiol. Scand.* 117: 567-570.
- Bromage, P.R. (1989) Epidural anaesthetics and narcotics. In *Textbook of Pain*, 2nd edition, (eds. Wall, P.D. & Melzack, R.), pp. 744-753. Churchill Livingstone, Edinburgh.
- Brown, D.R. & Goldberg, L.I. (1985) The use of quaternary narcotic antagonists in opiate research. *Neuropharmacol.* 24: 181-192.
- Bruce, A.N. (1913) Vaso-dilator axon-reflexes. *Q. J. Exp. Physiol.* 6: 339-354.
- Buck, S.H. & Burks, T.F. (1986) The neuropharmacology of capsaicin: review of some recent observations. *Pharmacol. Rev.* 38: 179-226.
- Burnie, J. (1981) Naloxone in shock. *Lancet* 1 (8226): 942.
- Cabon, F., Rhyner, T., Blanot, F., Goujet-Zalc, C., Mallet, J., & Zalc, B. (1987) Cell-free synthesis of opiate binding sites. *Neurochem. Int.* 11 (2): 219-221.
- Canfield, D.R. & Dunlap, K. (1984) Pharmacological characterization of amine receptors on embryonic chick sensory neurones. *Br. J. Pharmacol.* 82: 557-563.
- Cervero, F., Iggo, A., & Ogawa, H. (1976) Nociceptor-driven dorsal horn neurones in the lumbar spinal cord of the cat. *Pain* 2: 5-24.
- Cesselin, F., Montastruc, J.L., Gros, C., Bourgoin, S., & Hamon, M. (1980) Met-enkephalin levels and opiate receptors in the spinal cord of chronic suffering rats. *Brain Res.* 191: 289-293.
- Cesselin, F., Oliveras, J.L., Bourgoin, S., Sierralta, F., Michelot, R., Besson, J.M., & Hamon, M. (1982) Increased levels of met-enkephalin-like material in the CSF of anaesthetized cats after tooth pulp stimulation. *Brain Res.* 237: 325-338.
- Chaillet, P., Coulaud, A., Zajac, J.-M., Fournie-Zaluski, M.-C., Costentin, J., & Roques, B.P. (1984) The μ rather than the δ subtype of opioid receptors appears to be involved in enkephalin-induced analgesia. *Eur. J. Pharmacol.* 101: 83-90.
- Chang, H.M., Berde, C.B., Holz, G.G., Steward, G.F., & Kream, R.M. (1989) Sufentanil, morphine, met-enkephalin, and κ -agonist (U-50,488H) inhibit substance P release from primary sensory neurons: a model for presynaptic spinal opioid actions. *Anesthesiology* 70 (4): 672-677.
- Chang, K.-J. (1984) Opioid receptors: Multiplicity and sequelae of ligand-receptor interactions. In *The Receptors, Vol. I*, (ed. Conn, P.M.), pp. 1-81. Academic Press, Inc., London.
- Chang, K.-J., Blanchard, S.G., & Cuatrecasas, P. (1984) Benzomorphan sites are ligand recognition sites of putative ϵ -receptors. *Mol. Pharmacol.* 26: 484-488.

- Chang, K.-J., Cooper, B.R., Hazum, E., & Cuatrecasas, P. (1979) Multiple opiate receptors: Different regional distribution in the brain and differential binding of opiates and opioid peptides. *Mol. Pharmacol.* 16: 91-104.
- Chang, K.-J. & Cuatrecasas, P. (1979) Multiple opiate receptors: enkephalins and morphine bind to receptors of different specificity. *J. Biol. Chem.* 254: 2610-2618.
- Chang, K.-J., Hazum, E., & Cuatrecasas, P. (1980) Possible role of distinct morphine and enkephalin receptors in mediating actions of benzomorphan drugs (putative κ and σ agonists). *Proc. Natl. Acad. Sci. U.S.A.* 77: 4469-4473.
- Chang, K.-J., Hazum, E., & Cuatrecasas, P. (1981) Novel opiate binding sites selective for benzomorphan drugs. *Proc. Natl. Acad. Sci. U.S.A.* 78: 4141-4145.
- Chavkin, C., Bakhit, C., Weber, E., & Bloom, F.E. (1983) Relative contents and concomitant release of prodynorphin/neoendorphin-derived peptides in rat hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* 80: 7669-7673.
- Chavkin, C. & Goldstein, A. (1981a) Demonstration of a specific dynorphin receptor in guinea pig ileum myenteric plexus. *Nature* 291: 591-593.
- Chavkin, C. & Goldstein, A. (1981b) Specific receptor for the opioid peptide dynorphin: Structure-activity relationships. *Proc. Natl. Acad. Sci. U.S.A.* 78 (10): 6543-6547.
- Chavkin, C., James, I.F., & Goldstein, A. (1982) Dynorphin is a specific endogenous ligand of the κ opioid receptor. *Science* 215: 413-415.
- Childers, S.R., Creese, I., Snowman, A.M., & Snyder, S.H. (1979) Opiate receptor binding affected differentially by opiates and opioid peptides. *Eur. J. Pharmacol.* 55: 11-18.
- Clark, C.R., Birchmore, B., Sharif, N.A., Hunter, J.C., Hill, R.G., & Hughes, J. (1988) PD117302: a selective agonist for the κ -opioid receptor. *Br. J. Pharmacol.* 93: 618-626.
- Contreras, P.C., DiMaggio, D.A., & O'Donohue, T.L. (1987) An endogenous ligand for the sigma opioid binding site. *Synapse* 1: 57-61.
- Cook, A.J., Woolf, C.J., & Wall, P.D. (1986) Prolonged C-fibre mediated facilitation of the flexion reflex in the rat is not due to changes in afferent terminal or motoneurone excitability. *Neurosci. Lett.* 70: 91-96.
- Couture, R. & Cuello, A.C. (1984) Studies on the trigeminal antidromic vasodilatation and plasma extravasation in the rat. *J. Physiol. (Lond.)* 346: 273-285.
- Cox, B.M. & Chavkin, C. (1983) Comparison of dynorphin-selective kappa receptors in mouse vas deferens and guinea pig ileum: spare receptor fraction as a determinant of potency. *Mol. Pharmacol.* 23: 36-43.

- Cox, B.M., Goldstein, A., & Li, C.H. (1976) Opioid activity of a peptide, β -lipotropin-(61-91), derived from β -lipotropin. *Proc. Natl. Acad. Sci. U.S.A.* 73: 1821-1823.
- Crain, S.M. & Shen, K.-F. (1990) Opioids can evoke direct receptor-mediated excitatory effects on sensory neurons. *Trends in Pharmacol. Sci.* 11: 77-81.
- Crain, B.J., Valdes, F., Chang, K.-J., & McNamara, J.O. (1985) Autoradiographic localization of benzomorphan binding sites in rat brain. *Eur. J. Pharmacol.* 113: 179-186.
- Cruz, L. & Basbaum, A.I. (1985) Multiple opioid peptides and the modulation of pain: Immunohistochemical analysis of dynorphin and enkephalin in the trigeminal nucleus caudalis and spinal cord of the cat. *J. Comp. Neurol.* 240: 331-348.
- Czlonkowski, A., Costa, T., Przewlocki, R., Pasi, A., & Herz, A. (1983) Opiate receptor binding sites in human spinal cord. *Brain Res.* 267: 392-396.
- Dale, H.H. (1906) On some physiological actions of ergot. *J. Physiol. (Lond.)* 34: 163-206.
- Dale, H.H. (1935) Pharmacology and nerve endings. *Proc. R. Soc. Med.* 28: 319-332.
- Dalsgaard, C.-J., Franco-Cereceda, A., Saria, A., Lundberg, J.M., Theodorsson-Norheim, E., & Hokfelt, T. (1986) Distribution and origin of substance P- and neuropeptide Y-immunoreactive nerves in the guinea-pig heart. *Cell Tissue Res.* 243: 477-485.
- Dashwood, M.R. & Spyer, K.M. (1986) Autoradiographic localization of α -adrenoceptors, muscarinic acetylcholine receptors and opiate receptors in the heart. *Eur. J. Pharmacol.* 127: 279-282.
- Daval, G., Verge, D., Basbaum, A.I., Bourgoin, S., & Hamon, M. (1987) Autoradiographic evidence of serotonin₁ binding sites on primary afferent fibres in the dorsal horn of the rat spinal cord. *Neurosci. Lett.* 83: 71-76.
- Delay-Goyet, P., Seguin, C., Dauge, V., Calenco, G., Morgat, J.-L., Gacel, G., & Roques, B.P. (1987) [³H]DSTBULET, a new linear hexapeptide with both an improved selectivity and a high affinity for δ -opioid receptors. In *Progress in Opioid Research: Proceedings of the 1986 International Narcotics Research Conference*, pp. 197-200. NIDA Research Monograph 75, Rockville, MD.
- Dennis, S.G. & Melzack, R. (1979) Comparison of phasic and tonic pain in animals. In *Advances in Pain Research and Therapy, Vol.3*, (eds. Bonica, J.J., Liebeskind, J.C., & Albe-Fessard, D.G.), pp. 747-760. Raven Press, New York.
- Devane, W.A., Spain, J.W., Coscia, C.J., & Howlett, A.C. (1986) An assessment of the role of opioid receptors in the response to cannabimimetic drugs. *J. Neurochem.* 46 (6): 1929-1935.
- Dickenson, A.H. & Le Bars, D. (1987) Supraspinal morphine and descending inhibitions acting on the dorsal horn of the rat. *J. Physiol. (Lond.)* 384: 81-107.

- Dickenson, A.H., Oliveras, J.-L., & Besson, J.-M. (1979) Role of the nucleus raphe magnus in opiate analgesia as studied by the microinjection technique in the rat. *Brain Res.* 170: 95-111.
- Dickenson, A.H. & Sullivan, A.F. (1986) Electrophysiological studies on the effects of intrathecal morphine on nociceptive neurones in the rat dorsal horn. *Pain* 24: 211-222.
- Dickenson, A.H. & Sullivan, A.F. (1987a) Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurones. *Neurosci. Lett.* 83: 207-211.
- Dickenson, A.H. & Sullivan, A.F. (1987b) Subcutaneous formalin-induced activity of dorsal horn neurones in the rat: differential response to an intrathecal opiate administered pre or post formalin. *Pain* 30: 349-360.
- Dickenson, A.H., Sullivan, A.F., Knox, R., Zajac, J.M., & Roques, B.P. (1987) Opioid receptor subtypes in the rat spinal cord: Electrophysiological studies with μ - and δ -opioid receptor agonists in the control of nociception. *Brain Res.* 413: 36-44.
- DiFiglia, M., Aronin, N., & Leeman, S.E. (1982) Light microscopic and ultrastructural localization of immunoreactive substance P in the dorsal horn of monkey spinal cord. *Neurosci.* 7: 1127-1139.
- Dodd, J. & Jessell, T.M. (1985) Lactoseries carbohydrates specify subsets of dorsal root ganglion neurons projecting to the superficial dorsal horn of rat spinal cord. *J. Neurosci.* 5 (12): 3278-3294.
- Donnerer, J., Oka, K., Brossi, A., Rice, K.C., & Spector, S. (1986) Presence and formation of codeine and morphine in the rat. *Proc. Natl. Acad. Sci. U.S.A.* 83: 4566-4567.
- Dray, A., Hankins, M.W., & Yeats, J.C. (1989b) Desensitization and capsaicin-induced release of substance P-like immunoreactivity from guinea-pig ureter *in vitro*. *Neurosci.* 31 (2): 479-483.
- Dray, A., James, I.F., Ketchum, S.B., & Perkins, M.N. (1989a) Opioid receptor types in neonatal rat spinal cord: ligand binding and inhibition of nociception *in vitro*. *Advances in the Biosciences* 75: 65-68.
- Dray, A. & Perkins, M.N. (1987) Blockade of nociceptive responses in the neonatal rat spinal cord *in vitro* by excitatory amino acid antagonists. *J. Physiol. (Lond.)* 382: 177P.
- Dubuisson, D. & Dennis, S.G. (1977) The formalin test: A quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* 4: 161-174.
- Duggan, A.W., Hall, J.G., & Headley, P.M. (1976) Morphine, enkephalin and the substantia gelatinosa. *Nature* 264: 456-458.
- Duggan, A.W., Hall, J.G., & Headley, P.M. (1977a) Enkephalins and dorsal horn neurones of the cat: Effects on responses to noxious and innocuous skin stimuli. *Br. J. Pharmacol.* 61: 399-408.

- Duggan, A.W., Hall, J.G., & Headley, P.M. (1977b) Suppression of transmission of nociceptive impulses by morphine: selective effects of morphine administered in the region of the substantia gelatinosa. *Br. J. Pharmacol.* 61: 65-76.
- Duggan, A.W. & Hendry, I.A. (1986) Laminar localization of the sites of release of immunoreactive substance P in the dorsal horn with antibody-coated microelectrodes. *Neurosci. Lett.* 68: 134-140.
- Duggan, A.W., Hendry, I.A., Morton, C.R., Hutchison, W.D., & Zhao, Z.Q. (1988) Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. *Brain Res.* 451: 261-273.
- Duggan, A.W., Morton, C.R., Zhao, Z.Q., & Hendry, I.A. (1987) Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat: a study with antibody microprobes. *Brain Res.* 403: 345-349.
- Duggan, A.W. & North, R.A. (1984) Electrophysiology of opioids. *Pharmacol. Rev.* 35: 219-281.
- Duka, T., Schubert, P., Wuster, M., Stoiber, R., & Herz, A. (1981) A selective distribution pattern of different opiate receptors in certain areas of rat brain as revealed by *in vitro* autoradiography. *Neurosci. Lett.* 21: 119-124.
- Dunlap, K. & Fischbach, G.D. (1981) Neurotransmitters decrease the calcium conductance activated by depolarization of embryonic chick sensory neurones. *J. Physiol. (Lond.)* 317: 519-535.
- Erspermer, V. (1981) The tachykinin peptide family. *Trends in Neurosci.* 4: 267-269.
- Faccini, E., Uzumaki, H., Govoni, S., Missale, C., Spano, P.F., Covelli, V., & Trabucchi, M. (1984) Afferent fibers mediate the increase of met-enkephalin elicited in rat spinal cord by localized pain. *Pain* 18: 25-31.
- Ferrara, P. & Li, C.H. (1980) β -Endorphin: Characteristics of binding sites in rabbit spinal cord. *Proc. Natl. Acad. Sci. U.S.A.* 77: 5746-5748.
- Ferreira, S.H. (1983) Prostaglandins: Peripheral and central analgesia. In *Advances in Pain Research Therapy, Vol.5*, (eds. Bonica, J.J., Lindblom, U., & Iggo, A.), pp. 627-634. Raven Press, New York.
- Ferreira, S.H., Molina, N., & Vettore, O. (1982) Prostaglandin hyperalgesia V.: A peripheral analgesic receptor for opiates. *Prostaglandins* 23 (1): 53-60.
- Ferreira, S.H. & Nakamura, M. (1979) Prostaglandin hyperalgesia: the peripheral analgesic activity of morphine, enkephalins and opioid antagonists. *Prostaglandins* 18: 191-200.
- Fields, H.L., Emson, P.C., Leigh, B.K., Gilbert, R.F.T., & Iversen, L.L. (1980) Multiple opiate receptor sites on primary afferent fibres. *Nature* 284: 351-353.
- Fitzgerald, M. (1983) Capsaicin and sensory neurones--A review. *Pain* 15: 109-130.

- Fitzgerald, M. (1989) Course and termination of primary afferent fibres. In *Textbook of Pain*, 2nd edition, (eds. Wall, P.D. & Melzack, R.), pp. 46-62. Churchill Livingstone, Edinburgh.
- Foreman, J. & Jordan, C. (1983) Histamine release and vascular changes induced by neuropeptides. *Agents-Actions* 13 (2-3): 105-116.
- Forsgren, S. (1989) The distribution of nerve fibers showing substance P-like immunoreactivity in the conduction system of the bovine heart: dense innervation in the atrioventricular bundle. *Anat. Embryol.* 179: 485-490.
- Fox, A.P., Nowycky, M.C., & Tsien, R.W. (1987) Single-channel recordings of three types of calcium channels in chick sensory neurones. *J. Physiol. (Lond.)* 394: 173-200.
- Francel, P.C., Harris, K., Smith, M., Fishman, M.C., Dawson, G., & Miller, R.J. (1987) Neurochemical characteristics of a novel dorsal root ganglion x neuroblastoma hybrid cell line, F-11. *J. Neurochem.* 48 (5): 1624-1631.
- Francel, P.C., Keefer, J.F., & Dawson, G. (1989) Bradykinin analogs antagonize bradykinin-induced second messenger production in a sensory neuron cell line. *Mol. Pharmacol.* 35: 34-38.
- Franco-Cereceda, A. (1988) Calcitonin gene-related peptide and tachykinins in relation to local sensory control of cardiac contractility and coronary vascular tone. *Acta Physiol. Scand.* 133 (Suppl. 569): 1-63.
- Franco-Cereceda, A., Henke, H., Lundberg, J.M., Petermann, J.B., Hokfelt, T., & Fischer, J.A. (1987) Calcitonin gene-related peptide (CGRP) in capsaicin-sensitive substance P-immunoreactive sensory neurons in animals and man: Distribution and release by capsaicin. *Peptides* 8: 399-410.
- Franco-Cereceda, A., Lundberg, J.M., Saria, A., Schreiber, W., & Tritthart, H.A. (1988) Calcitonin gene-related peptide: Release by capsaicin and prolongation of the action potential in the guinea-pig heart. *Acta Physiol. Scand.* 132: 181-190.
- Frank, G.B. (1975) Two mechanisms for the meperidine block of action potential production in frog's skeletal muscle; non-specific and opiate drug receptor mediated blockade. *J. Physiol. (Lond.)* 252: 585-601.
- Furchgott, R.F. (1972) The classification of adrenoceptors (adrenergic receptors): An evaluation from the standpoint of receptor theory. In *Handbook of Experimental Pharmacology: Catecholamines, Vol.33*, (eds. Blaschko, H. & Muscholl, E.), pp.283-335. Springer-Verlag, Berlin.
- Gamse, R., Holzer, P., & Lembeck, F. (1979) Indirect evidence for presynaptic location of opiate receptors on chemosensitive primary sensory neurones. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 308: 281-285.
- Gamse, R., Holzer, P., & Lembeck, F. (1980) Decrease of substance P in primary afferent neurones and impairment of neurogenic plasma extravasation by capsaicin. *Br. J. Pharmacol.* 68: 207-213.

- Garzon, J., Schulz, R., & Herz, A. (1985) Evidence for the ϵ -type of opioid receptor in the rat vas deferens. *Mol. Pharmacol.* 28: 1-9.
- Geppetti, P., Maggi, C.A., Perretti, F., Frilli, S., & Manzini, S. (1988) Simultaneous release by bradykinin of substance P- and calcitonin gene-related peptide immunoreactivities from capsaicin-sensitive structures in guinea-pig heart. *Br. J. Pharmacol.* 94: 288-290.
- Gilbert, P.E. & Martin, W.R. (1976) The effects of morphine- and nalorphine-like drugs in the nondependent, morphine-dependent and cyclazocine-dependent chronic spinal dog. *J. Pharmacol. Exp. Ther.* 198: 66-82.
- Gillan, M.G.C. & Kosterlitz, H.W. (1982) Spectrum of the μ -, δ -, and κ -binding sites in homogenates of rat brain. *Br. J. Pharmacol.* 77: 461-469.
- Gillan, M.G.C., Kosterlitz, H.W., & Paterson, S.J. (1980) Comparison of the binding characteristics of tritiated opiates and opioid peptides. *Br. J. Pharmacol.* 70: 481-490.
- Glazer, E.J. & Basbaum, A.I. (1983) Opioid neurons and pain modulation: an ultrastructural analysis of enkephalin in cat superficial dorsal horn. *Neurosci.* 10: 357-376.
- Go, V.L.W. & Yaksh, T.L. (1987) Release of substance P from the cat spinal cord. *J. Physiol. (Lond.)* 391: 141-167.
- Goldstein, A., Barrett, R.W., James, I.F., Lowney, L.I., Weitz, C.J., Knipmeyer, L.L., & Rapoport, H. (1985) Morphine and other opiates from beef brain and adrenal. *Proc. Natl. Acad. Sci. U.S.A.* 82: 5203-5207.
- Goldstein, A., Fischli, W., Lowney, L.I., Hunkapiller, M., & Hood, L. (1981) Porcine pituitary dynorphin: Complete amino acid sequence of the biologically active heptadecapeptide. *Proc. Natl. Acad. Sci. U.S.A.* 78: 7219-7223.
- Goldstein, A. & James, I.F. (1983) The dynorphin opioid peptides and the kappa opioid receptor. In *Mechanism of Drug Action*, (eds. Singer, T.P., Mansour, T.E., & Ondarza, R.N.), pp. 49-60. Academic Press, Inc., San Francisco.
- Goldstein, A. & James, I.F. (1984a) Multiple opioid receptors: criteria for identification and classification. *Trends in Pharmacol. Sci.* 5 (12): 503-505.
- Goldstein, A. & James, I.F. (1984b) Site-directed alkylation of multiple opioid receptors: II. Pharmacological selectivity. *Mol. Pharmacol.* 25: 343-348.
- Goldstein, A., Lowney, L.I., & Pal, B.K. (1971) Stereospecific and nonspecific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* 68: 1742-1747.
- Goldstein, A. & Naidu, A. (1989) Multiple opioid receptors: Ligand selectivity profiles and binding site signatures. *Mol. Pharmacol.* 36: 265-272.

- Goodman, R.R., Adler, B.A., & Pasternak, G.W. (1985) Regional differences in μ_1 -binding of [^3H][D-Ala²,D-Leu⁵]-enkephalin: Comparisons of thalamus and cortex in the rat. *Neurosci. Lett.* 59: 155-158.
- Goodman, R.R., Houghten, R.A., & Pasternak, G.W. (1983) Autoradiography of [^3H] β -endorphin binding in brain. *Brain Res.* 288: 334-337.
- Goodman, R.R. & Snyder, S.H. (1982a) Autoradiographic localization of kappa opiate receptors to deep layers of the cerebral cortex may explain unique sedative and analgesic effects. *Life Sci.* 31: 1291-1294.
- Goodman, R.R. & Snyder, S.H. (1982b) κ opiate receptors localized by autoradiography to deep layers of cerebral cortex: Relation to sedative effects. *Proc. Natl. Acad. Sci. U.S.A.* 79: 5703-5707.
- Goodman, R.R., Snyder, S.H., Kuhar, M.J., & Young, W.S. (1980) Differentiation of delta and mu opiate receptor localizations by light microscopic autoradiography. *Proc. Natl. Acad. Sci. U.S.A.* 77: 6239-6243.
- Gregor, M. & Zimmermann, M. (1972) Characteristics of spinal neurones responding to cutaneous myelinated and unmyelinated fibres. *J. Physiol. (Lond.)* 221: 555-576.
- Grevel, J. & Sadee, W. (1983) An opiate binding site in the rat brain is highly selective for 4,5-epoxymorphinans. *Science* 221: 1198-1201.
- Grevel, J., Yu, V., & Sadee, W. (1985) Characterization of a labile naloxone binding site (λ site) in rat brain. *J. Neurochem.* 44: 1647-1656.
- Gross, R.A. & Macdonald, R.L. (1987) Dynorphin A selectively reduces a large transient (N-type) calcium current of mouse dorsal root ganglion neurons in cell culture. *Proc. Natl. Acad. Sci. U.S.A.* 84: 5469-5473.
- Grynkiewicz, G., Poenie, M., & Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260 (6): 3440-3450.
- Haley, J.E., Dickenson, A.H., & Schachter, M. (1989) Electrophysiological evidence for a role of bradykinin in chemical nociception in the rat. *Neurosci. Lett.* 97: 198-202.
- Haley, J.E., Sullivan, A.F., & Dickenson, A.H. (1990) Evidence for spinal N-methyl-D- aspartate receptor involvement in prolonged chemical nociception in the rat. *Brain Res.* (in press).
- Hammonds, R.G., Ferrara, P., & Li, C.H. (1981a) β -Endorphin: Characteristics of binding sites in a neuroblastoma-glioma hybrid cell. *Proc. Natl. Acad. Sci. U.S.A.* 78: 2218-2220.
- Hammonds, R.G., Ferrara, P., & Li, C.H. (1984) β -Endorphin: Binding activity of synthetic analogs with various chain lengths in neuroblastoma x glioma NG108-15 cell membranes. *Int. J. Pept. Prot. Res.* 24: 597-599.

- Hammonds, R.G., Hammonds, A.S., Ling, N., & Puett, D. (1982) β -endorphin and deletion peptides. A correlation of opiate receptor affinity with helix potential. *J. Biol. Chem.* 257: 2990-2995.
- Hammonds, R.G. & Li, C.H. (1981) Human β -endorphin: Specific binding in neuroblastoma N18TG2 cells. *Proc. Natl. Acad. Sci. U.S.A.* 78 (11): 6764-6765.
- Hammonds, R.G., Ling, N., & Puett, D. (1981b) Interaction of tritiated β -endorphin with rat brain membranes. *Anal. Biochem.* 114: 75-84.
- Hamon, M., Gallissot, M.C., Menard, F., Gozlan, H., Bourgoin, S., & Verge, D. (1989) 5-HT₃ receptor binding sites are on capsaicin-sensitive fibres in the rat spinal cord. *Eur. J. Pharmacol.* 164: 315-322.
- Hamprecht, B. (1977) Structural, electrophysiological, biochemical, and pharmacological properties of neuroblastoma-glioma cell hybrids in cell culture. In *International Review of Cytology*, Vol. 49, pp. 99-170. Academic Press, Inc., London.
- Handa, B.K., Lane, A.C., Lord, J.A.H., Morgan, B.A., Rance, M.J., & Smith, C.F.C. (1981) Analogs of β -LPH₆₁₋₆₄ possessing selective agonist activity at μ -opiate receptors. *Eur. J. Pharmacol.* 70: 531-540.
- Handwerker, H.O., Iggo, A., & Zimmermann, M. (1975) Segmental and supraspinal actions on dorsal horn neurons responding to noxious and non-noxious skin stimuli. *Pain* 1: 147-165.
- Harmar, A. & Keen, P. (1982) Synthesis, and central and peripheral axonal transport of substance P in a dorsal root ganglion-nerve preparation *in vitro*. *Brain Res.* 231: 379-385.
- Harris, D.W. & Sethy, V.H. (1980) High affinity binding of [³H]ethylketocyclazocine to rat brain homogenate. *Eur. J. Pharmacol.* 66: 121-123.
- Hayes, A.G., Birch, P.P., & Cavicchini, E. (1988) Evidence that the kappa agonist U50488H has non-opioid actions. *J. Pharm. Pharmacol.* 40: 718-720.
- Hayes, A. & Kelly, A. (1985) Profile of activity of κ receptor agonists in the rabbit vas deferens. *Eur. J. Pharmacol.* 110: 317-322.
- Hayes, A.G., Sheehan, M.J., & Tyers, M.B. (1987) Differential sensitivity of models of antinociception in the rat, mouse, and guinea-pig to μ - and κ -opioid receptor agonists. *Br. J. Pharmacol.* 91: 823-832.
- Hazum, E., Chang, K.-J., & Cuatrecasas, P. (1979a) Interaction of iodinated human [D-Ala²] β -endorphin with opiate receptors. *J. Biol. Chem.* 254 (6): 1765-1767.
- Hazum, E., Chang, K.-J., & Cuatrecasas, P. (1979b) Specific nonopiate receptors for β -endorphin. *Science* 205: 1033-1035.
- Hazum, E., Chang, K.-J., Cuatrecasas, P., & Pasternak, G.W. (1981) Naloxazone irreversibly inhibits the high affinity binding of [¹²⁵I]D-Ala²-D-Leu⁵-enkephalin. *Life Sci.* 28: 2973-2979.

- Heapy, C.G., Jamieson, A., & Russell, N.J.W. (1987) Afferent C-fibre and A-delta activity in models of inflammation. *Br. J. Pharmacol.* 90: 164P.
- Henderson, G., Hughes, J., & Kosterlitz, H.W. (1972) A new example of a morphine-sensitive neuro-effector junction: adrenergic transmission in the mouse vas deferens. *Br. J. Pharmacol.* 46 (4): 764-766.
- Herkenham, M. & Pert, C.B. (1980) *In vitro* autoradiography of opiate receptors in rat brain suggests loci of "opiate" pathways. *Proc. Natl. Acad. Sci. U.S.A.* 77 (9): 5532-5536.
- Herkenham, M. & Pert, C.B. (1982) Light microscopic localization of brain opiate receptors: A general autoradiographic method which preserves tissue quality. *J. Neurosci.* 2: 1129-1149.
- Herkenham, M., Rice, K.C., Jacobson, A.E., & Rothman, R.B. (1986) Opiate receptors in rat pituitary are confined to the neural lobe and are exclusively kappa. *Brain Res.* 382: 365-371.
- Hill, A.W. (1910) The possible effects of the aggregation of the molecules of hemoglobin on its dissociation curves. *J. Physiol. (Lond.)* 40: iv-vii.
- Hiller, J.M., Pearson, J., & Simon, E.J. (1973) Distribution of stereospecific binding of the potent narcotic analgesic etorphine in the human brain: Predominance in the limbic system. *Res. Commun. Chem. Pathol. Pharmacol.* 6: 1052-1062.
- Hiller, J.M. & Simon, E.J. (1980) Specific high affinity [³H]ethylketocyclazocine binding in rat central nervous system: Lack of evidence for κ receptors. *J. Pharmacol. Exp. Ther.* 214: 516-519.
- Hirota, N., Kuraishi, Y., Hino, Y., Sato, Y., Satoh, M., & Takagi, H. (1985) Met-enkephalin and morphine but not dynorphin inhibit noxious stimuli-induced release of substance P from rabbit dorsal horn *in situ*. *Neuropharmacol.* 24: 567-570.
- Hokfelt, T., Elde, R., Johansson, O., Luft, R., Nilsson, G., & Arimura, A. (1976) Immunohistochemical evidence for separate populations of somatostatin-containing and substance P-containing primary afferent neurons in the rat. *Neurosci.* 1: 131-136.
- Hokfelt, T., Johansson, O., Ljungdahl, A., Lundberg, J.M., & Schultzberg, M. (1980) Peptidergic neurones. *Nature* 284: 515-521.
- Hokfelt, T., Ljungdahl, A., Terenius, L., Elde, R., & Nilsson, G. (1977) Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: Enkephalin and substance P. *Proc. Natl. Acad. Sci. U.S.A.* 74: 3081-3085.
- Holaday, J.W. (1983) Cardiovascular effects of endogenous opiate systems. *Annu. Rev. Pharmacol. Toxicol.* 23: 541-594.
- Hollt, V., Tulunay, F.C., Woo, S.K., Loh, H.H., & Herz, A. (1982) Opioid peptides derived from pro-enkephalin A but not that from pro-enkephalin B are substantial analgesics after administration into brain of mice. *Eur. J. Pharmacol.* 85: 355-356.

- Holmdahl, G., Hakanson, R., Leander, S., Rosell, S., Folkers, K., & Sundler, F. (1981) A substance P antagonist [D-Pro²,D-Trp^{7,9}]SP, inhibits inflammatory responses in the rabbit eye. *Science* 214: 1029-1031.
- Holz, G.G., Dunlap, K., & Kream, R.M. (1988) Characterization of the electrically evoked release of substance P from dorsal root ganglion neurons: methods and dihydropyridine sensitivity. *J. Neurosci.* 8 (2): 463-471.
- Holz, G.G., Kream, R.M., Spiegel, A., & Dunlap, K. (1989) G proteins couple α -adrenergic and GABA_B receptors to inhibition of peptide secretion from peripheral sensory neurons. *J. Neurosci.* 9 (2): 657-666.
- Holzer, P. (1988) Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neurosci.* 24 (3): 739-768.
- Holzer, P., Bucsics, A., & Lembeck, F. (1982) Distribution of capsaicin-sensitive nerve fibres containing immunoreactive substance P in cutaneous and visceral tissues of the rat. *Neurosci. Lett.* 31: 253-257.
- Hoover, D.B. (1987) Effects of capsaicin on release of substance P-like immunoreactivity and physiological parameters in isolated perfused guinea-pig heart. *Eur. J. Pharmacol.* 141: 489-492.
- Hosobuchi, Y., Rossier, J., Bloom, F.E., & Guillemin, R. (1979) Stimulation of human periaqueductal gray for pain relief increases immunoreactive β -endorphin in ventricular fluid. *Science* 203: 279-281.
- Hough, L.B. & Green, J.P. (1984) Histamine and its receptors in the nervous system. In *Handbook of Neurochemistry, Vol. 6: Receptors in the Nervous System* (Second Edition), (ed. A. Lajtha), pp. 145-211. Plenum Press, New York.
- Hougland, M.W., Durkee, K.H., & Hougland, A.E. (1986) Innervation of guinea pig heart by neurons sensitive to capsaicin. *J. Auton. Nerv. Syst.* 15: 217-225.
- Hougland, M.W. & Hoover, D.B. (1983) Detection of substance P-like immunoreactivity in nerve fibers in the heart of guinea-pigs but not rats. *J. Auton. Nerv. Syst.* 8: 295-301.
- Hua, X.-Y., Theodorsson-Norheim, E., Brodin, E., Lundberg, J.M., & Hokfelt, T. (1985) Multiple tachykinins (neurokinin A, neuropeptide K and substance P) in capsaicin-sensitive sensory neurons in the guinea-pig. *Regulatory Peptides* 13: 1-19.
- Hughes, J. (1975) Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. *Brain Res.* 88: 295-308.
- Hughes, J., Kosterlitz, H.W., & Smith, T.W. (1977) The distribution of methionine-enkephalin and leucine-enkephalin in the brain and peripheral tissues. *Br. J. Pharmacol.* 61: 639-647.

- Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A., & Morris, H.R. (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 258: 577-579.
- Huidobro-Toro, J.P., Caturay, E.M., Ling, N., Lee, N.M., Loh, H.H., & Way, E.L. (1982) Studies on the structural prerequisites for the activation of the β -endorphin receptor on the rat vas deferens. *J. Pharmacol. Exp. Ther.* 222: 262-269.
- Hunnskaar, S., Fasmer, O.B., & Hole, K. (1985) Formalin test in mice, a useful technique for evaluating mild analgesics. *J. Neurosci. Methods* 14: 69-76.
- Hunnskaar, S. & Hole, K. (1987) The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* 30: 103-114.
- Hunt, S.P., Kelly, J.S., & Emson, P.C. (1980) The electron microscopic localization of methionine-enkephalin within the superficial layers (I and II) of the spinal cord. *Neurosci.* 5: 1871-1890.
- Hunter, W.M. & Greenwood, F.C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* 194: 495-496.
- Hutchinson, M., Kosterlitz, H.W., Leslie, F.M., & Waterfield, A.A. (1975) Assessment in the guinea-pig ileum and mouse vas deferens of benzomorphans which have strong antinociceptive activity but do not substitute for morphine in the dependent monkey. *Br. J. Pharmacol.* 55: 541-546.
- Iadarola, M.J., Brady, L.S., Draisci, G., & Dubner, R. (1988) Enhancement of dynorphin gene expression in spinal cord following experimental inflammation: stimulus specificity, behavioral parameters and opioid receptor binding. *Pain* 35: 313-326.
- Icard-Liepkalns, C. & Bochet, P. (1988) Photoaffinity labeling of a 33 kDa protein subunit of the δ -opioid receptor in neuroblastoma and hybrid cell lines. *FEBS Lett.* 233 (1): 167-172.
- Iggo, A. (1974) Activation of cutaneous nociceptors and their actions on dorsal horn neurons. *Adv. Neurol.* 4: 1-9.
- Itzhak, Y. (1989) Different modulation of the binding to two phencyclidine (PCP) receptor subtypes: effects of N-methyl-D-aspartate agonists and antagonists. *Neurosci. Lett.* 104: 314-319.
- Iversen, L.L. (1983) Neuropeptides--what next? *Trends in Neurosci.* 6: 293-294.
- James, I.F. (1986) Opioid receptors for the dynorphin peptides. In *Opioid peptides: Molecular pharmacology, biosynthesis and analysis*, (eds. Rapaka, R.S. & Hawks, R.L.), pp. 192-208. NIDA Research Monograph 70, Rockville, MD.
- James, I.F., Bettaney, J., Perkins, M.N., Ketchum, S.B., & Dray, A. (1990) Opioid receptor ligands in the neonatal rat spinal cord: binding and *in vitro* depression of the nociceptive responses. *Br. J. Pharmacol.* 99: 503-508.

- James, I.F., Chavkin, C., & Goldstein, A. (1982) Preparation of brain membranes containing a single type of opioid receptor highly selective for dynorphin. *Proc. Natl. Acad. Sci. U.S.A.* 79: 7570-7574.
- James, I.F. & Goldstein, A. (1984) Site-directed alkylation of multiple opioid receptors: I. Binding selectivity. *Mol. Pharmacol.* 25: 337-342.
- Jancso, N., Jancso-Gabor, A., & Szolcsanyi, J. (1967) Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *Br. J. Pharmacol.* 31: 138-151.
- Jancso, N., Jancso-Gabor, A., & Szolcsanyi, J. (1968) The role of sensory nerve endings in neurogenic inflammation induced in human skin and in the eye and paw of the rat. *Br. J. Pharmacol.* 33: 32-41.
- Jancso, G., Kiraly, E., & Jancso-Gabor, A. (1977) Pharmacologically induced selective degeneration of chemosensitive primary sensory neurons. *Nature* 270: 741-743.
- Jessell, T.M. & Dodd, J. (1985) Structure and expression of differentiation antigens on functional subclasses of primary sensory neurons. *Philos. Trans. R. Soc. Lond. (Biol.)* 308: 271-281.
- Jessell, T.M. & Iversen, L.L. (1977) Opiate analgesics inhibit substance P release from rat trigeminal nucleus. *Nature* 268: 549-551.
- Jessell, T.M., Voyvodic, J., Matthew, W.D., & Dodd, J. (1984) Dorsal root ganglion x neuroblastoma cell hybrids express sensory neuron surface antigens. *Soc. Neurosci. Abstr.* 10 (2): 763.
- Johansson, S., Kjellen, L., Hook, M., & Timpl, R. (1981) Substrate adhesion of rat hepatocytes: A comparison of laminin and fibronectin as attachment proteins. *J. Cell Biol.* 90 (1): 260-264.
- Johnson, S.M. & Duggan, A.W. (1981) Evidence that the opiate receptors of the substantia gelatinosa contribute to the depression, by intravenous morphine, of the spinal transmission of impulses in unmyelinated primary afferents. *Brain Res.* 207: 223-228.
- Jonsson, C.-E., Brodin, E., Dalsgaard, C.-J., & Haegerstrand, A. (1986) Release of substance-P like immunoreactivity in dog paw lymph after scalding injury. *Acta Physiol. Scand.* 126: 21-24.
- Joris, J.L., Dubner, R., & Hargreaves, K.M. (1987) Opioid analgesia at peripheral sites: A target for opioids released during stress and inflammation? *Anesth. Analg.* 66: 1277-1281.
- Juma, I. & Grossman, W. (1976) The effect of morphine on the activity evoked in ventrolateral tract axons of the cat spinal cord. *Exp. Brain Res.* 24: 473-484.
- Juma, I. & Heinz, G. (1979) Differential effects of morphine and opioid analgesics on A and C fibre-evoked activity in ascending axons of the rat spinal cord. *Brain Res.* 171: 573-576.

- Kakidani, H., Furutani, Y., Takahashi, H., Noda, M., Morimoto, Y., Hirose, T., Asai, M., Inayama, S., Nakanishi, S., & Numa, S. (1982) Cloning and sequence analysis of cDNA for porcine β -neo-endorphin/dynorphin precursor. *Nature* 298: 245-249.
- Kantner, R.M., Goldstein, B.D., & Kirby, M.L. (1986) Regulatory mechanisms for substance P in the dorsal horn during a nociceptive stimulus: axoplasmic transport vs electrical activity. *Brain Res.* 385: 282-290.
- Kantner, R.M., Kirby, M.L., & Goldstein, B.D. (1985) Increase in substance P in the dorsal horn during a chemogenic nociceptive stimulus. *Brain Res.* 338: 196-199.
- Kebabian, J.W. & Calne, D.B. (1979) Multiple receptors for dopamine. *Nature* 277: 93-96.
- Khachaturian, H., Watson, S.J., Lewis, M.E., Coy, D., Goldstein, A., & Akil, H. (1982) Dynorphin immunocytochemistry in the rat central nervous system. *Peptides* 3: 941-954.
- Kirby, M.L. (1981) Development of opiate receptor binding in rat spinal cord. *Brain Res.* 205: 400-404.
- Kirby, M.L. & Mattio, T.G. (1982) Developmental changes in serotonin and 5-hydroxyindoleacetic acid concentrations and opiate receptor binding in rat spinal cord following neonatal 5,7-dihydroxytryptamine treatment. *Dev. Neurosci.* 5: 394-402.
- Kitchen, I., Kelly, M., & Paz Viveros, M. (1990) Ontogenesis of κ -opioid receptors in rat brain using [³H]U-69593 as a binding ligand. *Eur. J. Pharmacol.* 175: 93-96.
- Klee, W.A. & Nirenberg, M. (1974) A neuroblastoma x glioma hybrid cell line with morphine receptors. *Proc. Natl. Acad. Sci. U.S.A.* 71 (9): 3474-3477.
- Klotz, I.M. (1982) Numbers of receptor sites from Scatchard graphs: Facts and fantasies. *Science* 217: 1247-1249.
- Knapp, R.J., Porreca, F., Burks, T.F., & Yamamura, H.I. (1989) Mediation of analgesia by multiple opioid receptors. In *Advances in Pain Research and Therapy, Vol. 11*, (eds. Hill, C.S. & Fields, W.S.), pp. 247-289. Raven Press, New York.
- Knox, R.J. & Dickenson, A.H. (1987) Effects of selective and non-selective κ -opioid receptor agonists on cutaneous C-fibre-evoked responses of rat dorsal horn neurones. *Brain Res.* 415: 21-29.
- Kosaka, T., Nagatsu, I., Wu, J.-Y., & Hama, K. (1986) Use of high concentrations of glutaraldehyde for immunocytochemistry of transmitter-synthesizing enzymes in the central nervous system. *Neurosci.* 18 (4): 975-990.
- Kosterlitz, H.W., Corbett, A.D., Gillan, M.G.C., McKnight, A.T., Paterson, S.J., & Robson, L.E. (1986) Recent developments in bioassay using selective ligands and selective *in vitro* preparations. In *Opioid peptides: Molecular pharmacology, biosynthesis and analysis*, (eds. Rapaka, R.S. & Hawks, R.L.), pp. 223-236. NIDA Research Monograph Series 70, Rockville, MD.

- Kosterlitz, H.W. & Paterson, S.J. (1985) Types of opioid receptors: relation to antinociception. *Phil. Trans. R. Soc. Lond. (Biol.)* 308: 291-297.
- Kosterlitz, H.W., Paterson, S.J., & Robson, L.E. (1981) Characterization of the κ -subtype of the opiate receptor in the guinea-pig brain. *Br. J. Pharmacol.* 73: 939-949.
- Kosterlitz, H.W. & Waterfield, A.A. (1975) *In vitro* models in the study of structure-activity relationship of narcotic analgesics. *Annu. Rev. Pharmacol. Toxicol.* 15: 29-47.
- Kosterlitz, H.W. & Watt, A.J. (1968) Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). *Br. J. Pharmacol.* 33: 266-276.
- Kream, R.M., Schoenfeld, T.A., Mancuso, R., Clancy, A.N., El-Bermani, W., & Macrides, F. (1985) Precursor forms of substance P (SP) in nervous tissue: Detection with antisera to SP, SP-Gly, and SP-Gly-Lys. *Proc. Natl. Acad. Sci. U.S.A.* 82: 4832-4836.
- Krumins, S.A. (1987) Characterization of dermorphin binding to membranes of rat brain and heart. *Neuropeptides* 9: 93-102.
- Krumins, S.A., Faden, A.I., & Feuerstein, G. (1985) Opiate binding in rat hearts: Modulation of binding after hemorrhagic shock. *Biochem. Biophys. Res. Commun.* 127 (1): 120-128.
- Kuraishi, Y., Hirota, N., Sato, Y., Hino, Y., Satoh, M., & Takagi, H. (1985a) Evidence that substance P and somatostatin transmit separate information related to pain in the spinal dorsal horn. *Brain Res.* 325: 294-298.
- Kuraishi, Y., Hirota, N., Sato, Y., Kaneko, S., Satoh, M., & Takagi, H. (1985b) Noradrenergic inhibition of the release of substance P from the primary afferents in the rabbit spinal dorsal horn. *Brain Res.* 359: 177-182.
- Kuraishi, Y., Hirota, N., Sugimoto, M., Satoh, M., & Takagi, H. (1983) Effects of morphine on noxious stimuli-induced release of substance P from rabbit dorsal horn *in vivo*. *Life Sci.* 33 (Suppl. I.): 693-696.
- Laduron, P.M. (1984) Axonal transport of opiate receptors in capsaicin-sensitive neurones. *Brain Res.* 294: 157-160.
- Lahti, R.A., Mickelson, M.M., McCall, J.M., & VonVoigtlander, P.F. (1985) [³H]U-69593 a highly selective ligand for the κ opioid receptor. *Eur. J. Pharmacol.* 109: 281-284.
- Lamotte, C. (1977) Distribution of the tract of Lissauer and the dorsal root fibers in the primate spinal cord. *J. Comp. Neurol.* 172: 529-562.
- Lamotte, C., Pert, C.B., & Snyder, S.H. (1976) Opiate receptor binding in primate spinal cord: distribution and changes after dorsal root section. *Brain Res.* 112: 407-412.
- Lands, A.M., Arnold, A., McAuliff, J.P., Luduena, F.P., & Brown, T.G. (1967) Differentiation of receptor systems activated by sympathomimetic amines. *Nature* 214: 597-598.

- Lang, R.E., Hermann, K., Dietz, R., Gaida, W., Ganten, D., Kraft, K., & Unger, T. (1983) Evidence for the presence of enkephalins in the heart. *Life Sci.* 32: 399-406.
- Largent, B.L., Gundlach, A.L., & Snyder, S.H. (1986) Pharmacological and autoradiographic discrimination of sigma and phencyclidine receptor binding sites in brain with (+)-[³H]SKF 10,047, (+)-[³H]-3-[3-hydroxyphenyl]-N-(1-propyl) piperidine and [³H]-1-[1-(2-thienyl) cyclohexyl]piperidine. *J. Pharmacol. Exp. Ther.* 238 (2): 739-748.
- Laurent, S., Marsh, J.D., & Smith, T.W. (1985) Enkephalins have a direct positive inotropic effect on cultured cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.* 82: 5930-5934.
- Law, P.Y., Herz, A., & Loh, H.H. (1979a) Demonstration and characterization of a stereospecific opiate receptor in the neuroblastoma N18TG2 cells. *J. Neurochem.* 33: 1177-1187.
- Law, P.Y., Koehler, J.E., & Loh, H.H. (1982) Comparison of opiate inhibition of adenylate cyclase activity in neuroblastoma N18TG2 and neuroblastoma x glioma NG108-15 hybrid cell lines. *Mol. Pharmacol.* 21: 483-491.
- Law, P.Y., Loh, H.H., & Li, C.H. (1979b) Properties and localization of β -endorphin receptor in rat brain. *Proc. Natl. Acad. Sci. U.S.A.* 76: 5455-5459.
- Lawson, S.N. (1981) Dorsal root ganglion neurones and dorsal roots: Effects of neonatal capsaicin. In *Spinal Cord Sensation: Sensory Processing in the Dorsal Horn*, (ed. Brown, A.G.), pp. 57-58. Scottish Press, Edinburgh.
- Lawson, S.N. (1987) The morphological consequences of neonatal treatment with capsaicin on primary afferent neurones in adult rats. *Acta Physiologica Hungarica* 69 (3-4): 315-321.
- Le Bars, D., Chitour, D., Kraus, E., Clot, A.M., Dickenson, A.H., & Besson, J.-M. (1981a) The effect of systemic morphine upon diffuse noxious inhibitory controls (DNIC) in the rat: evidence for a lifting of certain descending inhibitory controls of dorsal horn convergent neurones. *Brain Res.* 215: 257-274.
- Le Bars, D., Chitour, D., Kraus, E., Dickenson, A.H., & Besson, J.M. (1981b) Effect of naloxone upon diffuse noxious inhibitory controls (DNIC) in the rat. *Brain Res.* 204: 387-402.
- Le Bars, D., Dickenson, A.H., & Besson, J.-M. (1979a) Diffuse Noxious Inhibitory Controls (DNIC): I. Effects on dorsal horn convergent neurones in the rat. *Pain* 6: 283-304.
- Le Bars, D., Dickenson, A.H., & Besson, J.-M. (1979b) Diffuse Noxious Inhibitory Controls (DNIC): II. Lack of effect on non-convergent neurones, supraspinal involvement and theoretical implications. *Pain* 6: 305-327.
- Le Bars, D., Dickenson, A.H., & Besson, J.-M. (1983) Opiate analgesia and descending control systems. In *Advances in Pain Research and Therapy, Vol. 5*, (eds. Bonica, J.J. et al.), pp. 341-371. Raven Press, New York.

- Le Bars, D., Dickenson, A.H., Besson, J.-M., & Villanueva, L. (1986) Aspects of sensory processing through convergent neurons. In *Spinal Afferent Processing*, (ed. Yaksh, T.L.), pp.467-504. Plenum Press, New York.
- Le Bars, D., Menetrey, D., Conseiller, C., & Besson, J.M. (1975) Depressive effects of morphine upon lamina V cells activities in the dorsal horn of the spinal cat. *Brain Res.* 98: 261-277.
- Le Bars, D., Rivot, J.P., Guilbaud, G., Menetrey, D., & Besson, J.M. (1979c) The depressive effect of morphine on the C fibre response of dorsal horn neurones in the spinal rat pretreated or not by pCPA. *Brain Res.* 176: 337-353.
- Le Bars, D. & Villanueva, L. (1988) Electrophysiological evidence for the activation of descending inhibitory controls by nociceptive afferent pathways. In *Progress in Brain Research, Vol. 77: Pain Modulation*, (eds. Fields, H.L. & Besson, J.-M.), pp. 275-299. Elsevier, Oxford.
- Lee, N.M., Leybin, L., Chang, J.-K., & Loh, H.H. (1980b) Opiate and peptide interaction: Effect of enkephalins on morphine analgesia. *Eur. J. Pharmacol.* 68: 181-185.
- Leighton, G.E., Rodriguez, R.E., Hill, R.G., & Hughes, J. (1988) κ -Opioid agonists produce antinociception after i.v. and i.c.v. but not intrathecal administration in the rat. *Br. J. Pharmacol.* 93: 553-560.
- Leivo, I., Vaheri, A., Timpl, R., & Wartiovaara, J. (1980) Appearance and distribution of collagens and laminin in the early mouse embryo. *Dev. Biol.* 76: 100-114.
- Lemaire, S., Berube, A., Derome, G., Lemaire, I., Magnan, J., Regoli, D., & St.-Pierre, S. (1978a) Synthesis and biological activity of β -endorphin and analogues. Additional evidence for multiple opiate receptors. *J. Med. Chem.* 21: 1232-1235.
- Lemaire, S., Magnan, J., & Regoli, D. (1978b) Rat vas deferens: A specific bioassay for endogenous opioid peptides. *Br. J. Pharmacol.* 64: 327-329.
- Lembeck, F. & Donnerer, J. (1985) Opioid control of the function of primary afferent substance P fibres. *Eur. J. Pharmacol.* 114: 241-246.
- Lembeck, F., Donnerer, J., & Bartho, L. (1982) Inhibition of neurogenic vasodilatation and plasma extravasation by substance P antagonists, somatostatin and [D-Met²,Pro⁵]enkephalinamide. *Eur. J. Pharmacol.* 85: 171-176.
- Leslie, F.M. (1987) Methods used for the study of opioid receptors. *Pharmacol. Rev.* 39: 197-249.
- Leslie, F.M., Chavkin, C., & Cox, B.M. (1980) Opioid binding properties of brain and peripheral tissues: Evidence for heterogeneity in opioid ligand binding sites. *J. Pharmacol. Exp. Ther.* 214: 395-402.
- Lewis, V.A. & Gebhart, G.F. (1977) Evaluation of the periaqueductal central gray (PAG) as a morphine-specific locus of action and examination of morphine-induced and stimulation-produced analgesia at coincident PAG loci. *Brain Res.* 124: 283-303.

- Lewis, M.E., Mishkin, M., Bragin, E., Brown, R.M., Pert, C.B., & Pert, A. (1981) Opiate receptor gradients in monkey cerebral cortex: Correspondence with sensory processing hierarchies. *Science* 211: 1166-1169.
- Lewis, M.E., Pert, A., Pert, C.B., & Herkenham, M. (1983) Opiate receptor localization in rat cerebral cortex. *J. Comp. Neurol.* 216: 339-358.
- Leysen, J.E., Gommeren, W., & Niemegeers, C.J.E. (1983) [³H]Sufentanil, a superior ligand for μ -opiate receptors: binding properties and regional distribution in rat brain and spinal cord. *Eur. J. Pharmacol.* 87: 209-225.
- Lieberman, A.R. (1976) Sensory ganglia. In *The Peripheral Nerve*, (ed. D.N. Landon), pp. 178-188. Chapman and Hall, London.
- Lindsay, R.M. (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. *J. Neurosci.* 8 (7): 2394-2405.
- Lindsay, R.M., Lockett, C., Sternberg, J., & Winter, J. (1989) Neuropeptide expression in cultures of adult sensory neurons: modulation of substance P and calcitonin gene-related peptide levels by nerve growth factor. *Neurosci.* 33 (1): 53-65.
- Lin-Shiau, S.Y. & Li, C.H. (1984) Studies on characterization of human β -endorphin binding to membrane preparations of rat vas deferens. *J. Chin. Biochem. Soc.* 13: 10-18.
- Lipscombe, D., Kongsamut, S., & Tsien, R.W. (1989) α -adrenergic inhibition of sympathetic neurotransmitter release by modulation of N-type calcium-channel gating. *Nature* 340: 639-642.
- Llinas, R., Steinberg, I.Z., & Walton, K. (1976) Presynaptic calcium currents and their relation to synaptic transmission: Voltage clamp study in squid giant synapse and theoretical model for the calcium gate. *Proc. Natl. Acad. Sci. U.S.A.* 73: 2918-2922.
- Llinas, R., Steinberg, I.Z., & Walton, K. (1981) Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophys. J.* 33 (3): 323-351.
- Lord, J.A.H., Waterfield, A.A., Hughes, J., & Kosterlitz, H.W. (1976) Multiple opiate receptors. In *Opiates and Endogenous Opioid Peptides*, (ed. Kosterlitz, H.W.), pp. 275-280. Elsevier/North-Holland, Amsterdam.
- Lord, J.A.H., Waterfield, A.A., Hughes, J., & Kosterlitz, H.W. (1977) Endogenous opioid peptides: Multiple agonists and receptors. *Nature* 267: 495-499.
- Lowry, O.H., Rosebrough, N.J., Carr, A.L., & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Lundberg, J.M., Brodin, E., Hua, X., & Saria, A. (1984a) Vascular permeability changes and smooth muscle contraction in relation to capsaicin-sensitive substance P afferents in the guinea-pig. *Acta Physiol. Scand.* 120: 217-227.

- Lundberg, J.M., Brodin, E., & Saria, A. (1983) Effects and distribution of vagal capsaicin-sensitive substance P neurons with special reference to the trachea and lungs. *Acta Physiol. Scand.* 119: 243-252.
- Lundberg, J.M., Hua, X., & Fredholm, B.B. (1984b) Capsaicin-induced stimulation of the guinea pig atrium. Involvement of a novel sensory transmitter or a direct action on myocytes? *Naunyn-Schmiedeberg's Arch. Pharmacol.* 325: 176-182.
- Lutz, R.A., Cruciani, R.A., Costa, T., Munson, P.J., & Rodbard, D. (1984) A very high affinity opioid binding site in rat brain: Demonstration by computer modeling. *Biochem. Biophys. Res. Comm.* 122: 265-269.
- Lutz, R.A., Cruciani, R.A., Munson, P.J., & Rodbard, D. (1985) μ_1 : A very high affinity subtype of enkephalin binding sites in rat brain. *Life Sci.* 36: 2233-2238.
- Macdonald, R.L. & Werz, M.A. (1986) Dynorphin A decreases voltage-dependent calcium conductance of mouse dorsal root ganglion neurons. *J. Physiol. (Lond.)* 377: 237-249.
- Maggi, C.A., Santicioli, P., Geppetti, P., Parlani, M., Astolfi, M., Del Bianco, E., Patacchini, R., Giuliani, S., & Meli, A. (1989) The effect of calcium free medium and nifedipine on the release of substance P-like immunoreactivity and contractions induced by capsaicin in the isolated guinea-pig and rat bladder. *Gen. Pharmac.* 20 (4): 445-456.
- Malliani, A., Lombardi, F., & Pagani, M. (1986) Sensory innervation of the heart. In *Progress in Brain Research, Vol. 67*, (eds. Cervero, F. & Morrison, J.F.B.), pp. 39-48. Elsevier, Oxford.
- Mansour, A., Khachaturian, H., Lewis, M.E., Akil, H., & Watson, S.J. (1987) Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. *J. Neurosci.* 7 (8): 2445-2464.
- Mansour, A., Lewis, M.E., Khachaturian, H., Akil, H., & Watson, S.J. (1986) Pharmacological and anatomical evidence of selective μ , δ , and κ opioid receptor binding in rat brain. *Brain Res.* 399: 69-79.
- Mantelli, L., Amerini, S., & Ledda, F. (1989) Effects of opioid drugs on capsaicin-sensitive neurones in guinea-pig atria. *Eur. J. Pharmacol.* 170: 217-223.
- Mantelli, L., Corti, V., & Ledda, F. (1987) On the presence of opioid receptors in guinea-pig ventricular tissue. *Gen. Pharmac.* 18 (3): 309-313.
- Manzini, S., Perretti, F., De Benedetti, L., Pradelles, P., Maggi, C.A., & Geppetti, P. (1989) A comparison of bradykinin- and capsaicin-induced myocardial and coronary effects in isolated perfused heart of guinea-pig: involvement of substance P and calcitonin gene-related peptide release. *Br. J. Pharmacol.* 97: 303-312.

- Martin, W.R., Eades, C.G., Thompson, J.A., Huppler, R.E., & Gilbert, P.E. (1976) The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J. Pharmacol. Exp. Ther.* 197: 517-532.
- Mauborgne, A., Lutz, O., Legrand, J.-C., Hamon, M., & Cesselin, F. (1987) Opposite effects of δ and μ opioid receptor agonists on the *in vitro* release of substance P-like material from the rat spinal cord. *J. Neurochem.* 48 (2): 529-537.
- Maurer, R., Cortes, A., Probst, A., & Palacios, J.M. (1983) Multiple opiate receptors in human brain: An autoradiographic investigation. *Life Sci.* 33 (Suppl. I): 231-234.
- McCarson, K.E. & Goldstein, B.D. (1989) Naloxone blocks the formalin-induced increase of substance P in the dorsal horn. *Pain* 38: 339-345.
- McDowell, J. & Kitchen, I. (1987) Development of opioid systems: peptides, receptors and pharmacology. *Brain Res. Rev.* 12: 397-421.
- McFadzean, I. (1988) The ionic mechanisms underlying opioid actions. *Neuropeptides* 11: 173-180.
- McGregor, G.P. (1982) Substance P. In *Radioimmunoassay of Gut Regulatory Peptides*, (eds. Bloom, S.R. & Long, R.G.), pp. 154-163. W.B. Saunders Co., Ltd., London.
- McGregor, G.P. & Bloom, S.R. (1983) Radioimmunoassay of substance P and its stability in tissue. *Life Sci.* 32: 655-662.
- McLean, S. & Weber, E. (1988) Autoradiographic visualization of haloperidol-sensitive sigma receptors in guinea-pig brain. *Neurosci.* 25 (1): 259-269.
- McQuay, H.J., Carroll, D., & Moore, R.A. (1988) Postoperative orthopaedic pain-- the effect of opiate premedication and local anaesthetic blocks. *Pain* 33: 291-295.
- McQuay, H.J., Sullivan, A.F., Smallman, K., & Dickenson, A.H. (1989) Intrathecal opioids, potency and lipophilicity. *Pain* 36: 111-115.
- Mendell, L.M. (1966) Physiological properties of unmyelinated fiber projection to the spinal cord. *Exp. Neurol.* 16: 316-332.
- Mickley, G.A., Stevens, K.E., & Galbraith, J.A. (1985) Quaternary naltrexone reverses morphine-induced behaviors. *Physiol. Behav.* 35 (2): 249-254.
- Millan, M.J. (1989) Kappa-opioid receptor-mediated antinociception in the rat. I. Comparative actions of mu- and kappa-opioids against noxious thermal, pressure, and electrical stimuli. *J. Pharmacol. Exp. Ther.* 251 (1): 334-341.
- Millan, M.J. (1990) κ -Opioid receptors and analgesia. *Trends in Pharmacol. Sci.* 11: 70-76.
- Millan, M.J., Czlonkowski, A., Lipkowski, A., & Herz, A. (1989) Kappa-opioid receptor-mediated antinociception in the rat. II. Supraspinal in addition to spinal sites of action. *J. Pharmacol. Exp. Ther.* 251: 342-350.

- Millan, M.J., Czlonkowski, A., Morris, B., Stein, C., Arendt, R., Huber, A., Holtt, V., & Herz, A. (1988) Inflammation of the hind limb as a model of unilateral, localized pain: influence on multiple opioid systems in the spinal cord of the rat. *Pain* 35: 299-312.
- Millan, M.J., Czlonkowski, A., Pilcher, C.W.T., Almeida, O.F.X., Millan, M.H., Colpaert, F.C., & Herz, A. (1987) A model of chronic pain in the rat: functional correlates of alterations in the activity of opioid systems. *J. Neurosci.* 7 (1): 77-87.
- Millan, M.J., Millan, M.H., Czlonkowski, A., Holtt, V., Pilcher, C.W.T., Herz, A., & Colpaert, F.C. (1986) A model of chronic pain in the rat: response of multiple opioid systems to adjuvant-induced arthritis. *J. Neurosci.* 6 (4): 899-906.
- Miller, R.J. (1982) Multiple opiate receptors for multiple opioid peptides. *Medical Biology* 60: 1-6.
- Miller, R.J. (1987a) Bradykinin highlights the role of phospholipid metabolism in the control of nerve excitability. *Trends in Neurosci.* 10 (6): 226-228.
- Miller, R.J. (1987b) Multiple calcium channels and neuronal function. *Science* 235: 46-52.
- Milligan, G., Streaty, R.A., Gierschik, P., Spiegel, A.M., & Klee, W.A. (1987) Development of opiate receptors and GTP-binding regulatory proteins in neonatal rat brain. *J. Biol. Chem.* 262: 8626-8630.
- Monard, D., Rentsch, M., Schuerch-Rathgeb, Y., & Lindsay, R.M. (1977) Morphological differentiation of neuroblastoma cells in medium supplemented with delipidated serum. *Proc. Natl. Acad. Sci. U.S.A.* 74 (9): 3893-3897.
- Monard, D., Stockel, K., Goodman, R., & Thoenen, H. (1975) Distinction between nerve growth factor and glial factor. *Nature* 258: 444-445.
- Morris, J.F., Shaw, F.D., & Pow, D.V. (1989) Strategies for the ultrastructural study of peptide-containing neurons. In *IBRO Handbook Series: Methods in the Neurosciences, Vol. 11, Neuropeptides: A Methodology*, (eds. Fink, G. & Harmar, A.J.), pp. 83-124. John Wiley & Sons Ltd., Chichester.
- Mosberg, H.I., Hurst, R., Hruby, V.J., Gee, K., Yamamura, H.I., Galligan, J.J., & Burks, T.F. (1983) Bis-penicillamine enkephalins possess highly improved specificity towards δ opioid receptors. *Proc. Natl. Acad. Sci. U.S.A.* 80: 5871-5874.
- Moskowitz, A.S. & Goodman, R.R. (1985) Autoradiographic distribution of μ_1 and μ_2 opioid binding in the mouse central nervous system. *Brain Res.* 360: 117-129.
- Mudge, A.W., Leeman, S.E., & Fischbach, G.D. (1979) Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. *Proc. Natl. Acad. Sci. U.S.A.* 76 (1): 526-530.

- Nagy, J.I., Hunt, S.P., Iversen, L.L., & Emson, P.C. (1981) Biochemical and anatomical observations on the degeneration of peptide-containing primary afferent neurons after neonatal capsaicin. *Neurosci.* 6 (10): 1923-1934.
- Nagy, J.I., Iversen, L.L., Goedert, M., Chapman, D., & Hunt, S.P. (1983) Dose-dependent effects of capsaicin on primary sensory neurons in the neonatal rat. *J. Neurosci.* 3 (2): 399-406.
- Nagy, J.I., Vincent, S.R., Staines, W.A., Fibiger, H.C., Reisine, T.D., & Yamamura, H.I. (1980) Neurotoxic action of capsaicin on spinal substance P neurons. *Brain Res.* 186: 435-444.
- Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.N., & Numa, S. (1979) Nucleotide sequence of cloned cDNA for bovine corticotropin- β -lipotropin precursor. *Nature* 278: 423-427.
- Nicoll, R.A., Schenker, C., & Leeman, S.E. (1980) Substance P as a transmitter candidate. *Annu. Rev. Neurosci.* 3: 227-268.
- Ninkovic, M., Hunt, S.P., Emson, P.C., & Iversen, L.L. (1981a) The distribution of multiple opiate receptors in bovine brain. *Brain Res.* 214: 163-167.
- Ninkovic, M., Hunt, S.P., & Gleave, J.R.W. (1982) Localization of opiate and histamine H₁-receptors in the primate sensory ganglia and spinal cord. *Brain Res.* 241: 197-206.
- Ninkovic, M., Hunt, S.P., & Kelly, J.S. (1981b) Effect of dorsal rhizotomy on the autoradiographic distribution of opiate and neurotensin receptors and neurotensin-like immunoreactivity within the rat spinal cord. *Brain Res.* 230: 111-119.
- Nock, B., Rajpara, A., O'Connor, L.H., & Cicero, T.J. (1988) [³H]U-69593 labels a subtype of kappa opiate receptor with characteristics different from that labelled by [³H]ethylketocyclazocine. *Life Sci.* 42: 2403-2412.
- Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., Nakanishi, S., & Numa, S. (1982) Cloning and sequence analysis of cDNA for bovine adrenal preproenkephalin. *Nature* 295: 202-206.
- Noguchi, K., Morita, Y., Kiyama, H., Sato, M., Ono, K., & Tohyama, M. (1989) Preproenkephalin gene expression in the rat spinal cord after noxious stimuli. *Molecular Brain Res.* 5: 227-234.
- Oka, K., Kantrowitz, J.D., & Spector, S. (1985) Isolation of morphine from toad skin. *Proc. Natl. Acad. Sci. U.S.A.* 82: 1852-1854.
- Oka, T., Negishi, K., Suda, M., Matsumiya, T., Inazu, T., & Ueki, M. (1981) Rabbit vas deferens: A specific bioassay for opioid κ -receptor agonists. *Eur. J. Pharmacol.* 73: 235-236.
- Oka, T., Negishi, K., Suda, M., Sawa, A., Fujino, M., & Wakimasu, M. (1982) Evidence that dynorphin-(1-13) acts as an agonist on opioid κ -receptors. *Eur. J. Pharmacol.* 77: 137-141.
- Olgart, L., Gazelius, B., Brodin, E., & Nilsson, G. (1977) Release of substance P-like immunoreactivity from the dental pulp. *Acta Physiol. Scand.* 101: 510-512.

- Otsuka, M. & Konishi, S. (1976) Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat. *Nature* 264: 83-84.
- Otsuka, M. & Yanagisawa, M. (1988) Effect of a tachykinin antagonist on a nociceptive reflex in the isolated spinal cord-tail preparation of the newborn rat. *J. Physiol. (Lond.)* 395: 255-270.
- Pang, I.-H. & Vasko, M.R. (1986) Morphine and norepinephrine but not 5-hydroxytryptamine and γ -aminobutyric acid inhibit the potassium-stimulated release of substance P from rat spinal cord slices. *Brain Res.* 376: 268-279.
- Papka, R.E., Furness, J.B., Della, N.G., Murphy, R., & Costa, M. (1984) Time course of effect of capsaicin on ultrastructure and histochemistry of substance P-immunoreactive nerves associated with the cardiovascular system of the guinea-pig. *Neurosci.* 12 (4): 1277-1292.
- Papka, R.E. & Urban, L. (1987) Distribution, origin and sensitivity to capsaicin of primary afferent substance P-immunoreactive nerves in the heart. *Acta Physiologica Hungarica* 69 (3-4): 459-468.
- Pasternak, G.W. (1980) Multiple opiate receptors: [³H]ethylketocyclazocine receptor binding and ketocyclazocine analgesia. *Proc. Natl. Acad. Sci. U.S.A.* 77: 3691-3694.
- Pasternak, G.W., Carroll-Buatti, M., & Spiegel, K. (1981) The binding and analgesic properties of a sigma opiate, SKF 10,047. *J. Pharmacol. Exp. Ther.* 219: 192-198.
- Pasternak, G.W., Childers, S.R., & Snyder, S.H. (1980a) Naloxazone, a long-lasting opiate antagonist: Effects on analgesia in intact animals and on opiate receptor binding *in vitro*. *J. Pharmacol. Exp. Ther.* 214: 455-462.
- Pasternak, G.W., Childers, S.R., & Snyder, S.H. (1980b) Opiate analgesia: Evidence for mediation by a subpopulation of opiate receptors. *Science* 208: 514-516.
- Pasternak, G.W. & Wood, P.J. (1986) Minireview: Multiple mu opiate receptors. *Life Sci.* 38: 1889-1898.
- Paton, W.D.M. (1955) The response of the guinea-pig ileum to electrical stimulation by coaxial electrodes. *J. Physiol. (Lond.)* 127: 40-41P.
- Paton, W.D.M. (1957) The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br. J. Pharmacol.* 12: 119-127.
- Payan, D.G., Levine, J.D., & Goetzl, E.J. (1984) Modulation of immunity and hypersensitivity by sensory neuropeptides. *J. Immunol.* 132 (4): 1601-1604.
- Perney, T.M., Hirning, L.D., Leeman, S.E., & Miller, R.J. (1986) Multiple calcium channels mediate neurotransmitter release from peripheral neurons. *Proc. Natl. Acad. Sci. U.S.A.* 83: 6656-6659.
- Pernow, B. (1983) Substance P. *Pharmacol. Rev.* 35 (2): 85-141.

- Peroutka, S.J. & Snyder, S.H. (1982) Recognition of multiple serotonin receptor binding sites. In *Advances in Biochemical Psychopharmacology, Vol. 34: Serotonin in Biological Psychiatry*, (eds. Ho, B.T., Schoolar, J.C., & Usdin, E.), pp. 155-172. Raven Press, New York.
- Pert, C.B. & Snyder, S.H. (1973) Opiate receptor: Demonstration in nervous tissue. *Science* 179: 1011-1014.
- Pfeiffer, A., Pasi, A., Mehraein, P., & Herz, A. (1981) A subclassification of κ -sites in human brain by use of dynorphin 1-17. *Neuropeptides* 2: 89-97.
- Piercey, M.F., Lahti, R.A., Schroeder, L.A., Einspahr, F.J., & Barsuhn, C. (1982) U-50488H, a pure kappa receptor agonist with spinal analgesic loci in the mouse. *Life Sci.* 31: 1197-1200.
- Platika, D., Baizer, L., & Fishman, M.C. (1985a) Sensory neurons "immortalized" by fusion with neuroblastoma cells. *Trans. Assoc. Am. Phys.* 98: 301-304.
- Platika, D., Boulos, M.H., Baizer, L., & Fishman, M.C. (1985b) Neuronal traits of clonal cell lines derived by fusion of dorsal root ganglia neurons with neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.* 82: 3499-3503.
- Pohl, M., Lombard, M.C., Bourgoin, S., Carayon, A., Benoliel, J.J., Mauborgne, A., Besson, J.M., Hamon, M., & Cesselin, F. (1989) Opioid control of the *in vitro* release of calcitonin gene-related peptide from primary afferent fibres projecting in the rat cervical cord. *Neuropeptides* 14: 151-159.
- Porreca, F., Mosberg, H.I., Hurst, R., Hruby, V.J., & Burks, T.F. (1984) Roles of mu, delta and kappa opioid receptors in spinal and supraspinal mediation of gastrointestinal transit effects and hot-plate analgesia in the mouse. *J. Pharmacol. Exp. Ther.* 230: 341-348.
- Porreca, F., Mosberg, H.I., Omnaas, J.R., Burks, T.F., & Cowan, A. (1987) Supraspinal and spinal potency of selective opioid agonists in the mouse writhing test. *J. Pharmacol. Exp. Ther.* 240: 890-894.
- Portoghese, P.S. (1965) A new concept on the mode of interaction of narcotic analgesics with receptors. *J. Med. Chem.* 8: 609-616.
- Portoghese, P.S., Larson, D.L., Jiang, J.B., Caruso, T.P., & Takemori, A.E. (1979) Synthesis and pharmacologic characterization of an alkylating analogue (chlornaltrexamine) of naltrexone with ultralong-lasting narcotic antagonist properties. *J. Med. Chem.* 22: 168-173.
- Portoghese, P.S., Larson, D.L., Sayre, L.M., Fries, D.S., & Takemori, A.E. (1980) A novel opioid receptor site directed alkylating agent with irreversible narcotic antagonistic and reversible agonistic activities. *J. Med. Chem.* 23: 233-234.
- Portoghese, P.S., Lipkowski, A.W., & Takemori, A.E. (1987) Binaltorphimine and nor-binaltorphimine, potent and selective κ -opioid receptor antagonists. *Life Sci.* 40: 1287-1292.

- Price, D.D., Hayashi, H., Dubner, R., & Ruda, M.A. (1979) Functional relationships between neurons of marginal and substantia gelatinosa layers of primate dorsal horn. *J. Neurophysiol.* 42: 1590-1608.
- Price, D.D., Hull, C.D., & Buchwald, N.A. (1971) Intracellular responses of dorsal horn cells to cutaneous and sural nerve A and C fiber stimuli. *Exp. Neurol.* 33: 291-309.
- Price, D.D. & Wagman, I.H. (1970) Physiological roles of A and C fiber inputs to the spinal dorsal horn of *Macaca mulatta*. *Exp. Neurol.* 29: 383-399.
- Proudfit, H.K. (1988) Pharmacologic evidence for the modulation of nociception by noradrenergic neurons. In *Progress in Brain Research, Vol. 77: Pain Modulation*, (eds. Fields, H.L. & Besson, J.-M.), pp. 357-370.
- Quirion, R., Chicheportiche, R., Contreras, P.C., Johnson, K.M., Lodge, D., Tam, S.W., Woods, J.H., & Zukin, S.R. (1987) Classification and nomenclature of phencyclidine and sigma receptor sites. *Trends in Neurosci.* 10 (11): 444-446.
- Quirion, R., Zajac, J.M., Morgat, J.L., & Roques, B.P. (1983) Autoradiographic distribution of mu and delta opiate receptors in rat brain using highly selective ligands. *Life Sci.* 33 (Suppl. I): 227-230.
- Ralston, H.J. & Ralston, D.D. (1979) The distribution of dorsal root axons in laminae I, II, and III of the macaque spinal cord: a quantitative electron microscope study. *J. Comp. Neurol.* 184: 643-684.
- Rane, S.G., Holz, G.G., & Dunlap, K. (1987) Dihydropyridine inhibition of neuronal calcium current and substance P release. *Pflugers Arch.* 409: 361-366.
- Rexed, B. (1954) A cytoarchitectonic atlas of the spinal cord in the cat. *J. Comp. Neurol.* 100: 297-380.
- Rice, K.C., Jacobson, A.E., Burke, T.R., Bajwa, B.S., Streaty, R.A., & Klee, W.A. (1983) Irreversible ligands with high selectivity toward δ or μ opiate receptors. *Science* 220: 314-316.
- Robson, L.E., Foote, R.W., Maurer, R., & Kosterlitz, H.W. (1984) Opioid binding sites of the κ -type in guinea-pig cerebellum. *Neurosci.* 12: 621-627.
- Robson, L.E. & Kosterlitz, H.W. (1979) Specific protection of the binding sites of D-Ala²-D-Leu⁵-enkephalin (δ -receptors) and dihydromorphine (μ -receptors). *Proc. R. Soc. Lond. B.* 205: 425-432.
- Rodriguez, R.E., Leighton, G., Hill, R.G., & Hughes, J. (1986) *In vivo* evidence for spinal delta-opiate receptor-operated antinociception. *Neuropeptides* 8: 221-241.
- Romer, D., Buscher, H., Hill, R.C., Maurer, R., Petcher, T.J., Welle, H.B.A., Bakel, H.C.C.K., & Akkerman, A.M. (1980) Bremazocine: a potent, long-acting opiate kappa-agonist. *Life Sci.* 27: 971-978.

- Rosell, S., Olgart, L., Gazelius, B., Panapoulos, P., Folkers, K., & Horig, J. (1981) Inhibition of antidromic and substance P-induced vasodilatation by a substance P antagonist. *Acta Physiol. Scand.* 111: 381-382.
- Rosenbaum, J.S., Holford, N.H.G., Richards, M.L., Aman, R.A., & Sadee, W. (1984) Discrimination of three types of opioid binding sites in rat brain *in vivo*. *Mol. Pharmacol.* 25: 242-248.
- Rothman, R.B., Bowen, W.D., Herkenham, M., Jacobson, A.E., Rice, K.C., & Pert, C.B. (1985) A quantitative study of [³H][D-Ala²-D-Leu⁵]enkephalin binding to rat brain membranes: Evidence that oxymorphone is a noncompetitive inhibitor of the lower affinity δ -binding site. *Mol. Pharmacol.* 27: 399-408.
- Rothman, R.B., Bowen, W.D., Schumacher, U.K., & Pert, C.B. (1983) Effect of β -FNA on opiate receptor binding: Preliminary evidence for two types of μ receptors. *Eur. J. Pharmacol.* 95: 147-148.
- Rothman, R.B. & Westfall, T.C. (1982a) Allosteric coupling between morphine and enkephalin receptors *in vitro*. *Mol. Pharmacol.* 21: 548-557.
- Rothman, R.B. & Westfall, T.C. (1982b) Morphine allosterically modulates the binding of [³H]leucine enkephalin to a particulate fraction of rat brain. *Mol. Pharmacol.* 21: 538-547.
- Ruda, M.A. (1982) Opiates and pain pathways: Demonstration of enkephalin synapses on dorsal horn projection neurons. *Science* 215: 1523-1525.
- Russell, N.J.W., Jamieson, A., Callen, T.S., & Rance, M.J. (1985) Peripheral opioid effects upon neurogenic plasma extravasation and inflammation. *Br. J. Pharmacol.* 86: 788P.
- Russell, N.J.W., Schaible, H.-G., & Schmidt, R.F. (1987) Opiates inhibit the discharges of fine afferent units from inflamed knee joint of the cat. *Neurosci. Lett.* 76: 107-112.
- Sakurada, T., Le Greves, P., Stewart, J., & Terenius, L. (1985) Measurement of substance P metabolites in rat CNS. *J. Neurochem.* 44 (3): 718-722.
- Salt, T.E. & Hill, R.G. (1983) Neurotransmitter candidates of somatosensory primary afferent fibres. *Neurosci.* 10 (4): 1083-1103.
- Saria, A. (1984) Substance P in sensory nerve fibres contributes to the development of oedema in the rat hind paw after thermal injury. *Br. J. Pharmacol.* 82: 217-222.
- Saria, A., Lundberg, J.M., Skofitsch, G., & Lembeck, F. (1983) Vascular protein leakage in various tissues induced by substance P, capsaicin, bradykinin, serotonin, histamine and by antigen challenge. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 324: 212-218.
- Saria, A., Martling, C.-R., Yan, Z., Theodorsson-Norheim, E., Gamse, R., & Lundberg, J.M. (1988) Release of multiple tachykinins from capsaicin-sensitive sensory nerves in the lung by bradykinin, histamine, dimethylphenyl piperazinium, and vagal nerve stimulation. *Am. Rev. Respiratory Dis.* 137: 1330-1335.

- Sarson, D.L. (1982) Quality control and assay mathematics. In *Radioimmunoassay of Gut Regulatory Peptides*, (eds. Bloom, S.R. & Long, R.G.), pp. 154-163. W.B. Saunders Co., Ltd., London.
- Saxon, M.E., Ivanitsky, G.R., Beloyartsev, F.F., Safronova, V.G., Kokoz, Y.M., & Freydin, A.A. (1982) Myocardial opiate receptors. *General Physiology and Biophysics* 1: 447-452.
- Sayre, L.M., Larson, D.L., Takemori, A.E., & Portoghese, P.S. (1984) Design and synthesis of naltrexone-derived affinity labels with nonequilibrium opioid agonist and antagonist activities. Evidence for the existence of different μ receptor subtypes in different tissues. *J. Med. Chem.* 27: 1325-1335.
- Scatchard, G. (1949) The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51: 660-672.
- Schaumann, W. (1957) Inhibition by morphine of the release of acetylcholine from the intestine of the guinea-pig. *Br. J. Pharmacol.* 12: 115-118.
- Schmauss, C. & Yaksh, T.L. (1984) *In vivo* studies on spinal opiate receptor systems mediating antinociception. II. Pharmacological profiles suggesting a differential association of mu, delta, and kappa receptors with visceral, chemical, and cutaneous thermal stimuli in the rat. *J. Pharmacol. Exp. Ther.* 228: 1-12.
- Schramm, M., Thomas, G., Towart, R., & Franckowiak, G. (1983) Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. *Nature* 303: 535-537.
- Schulz, R., Faase, E., Wuster, M., & Herz, A. (1979) Selective receptors for β -endorphin on the rat vas deferens. *Life Sci.* 24: 843-850.
- Schulz, R. & Wuster, M. (1981) Are there subtypes (isoreceptors) of multiple opiate receptors in the mouse vas deferens? *Eur. J. Pharmacol.* 76: 61-66.
- Schulz, R., Wuster, M., & Herz, A. (1981a) Differentiation of opiate receptors in the brain by the selective development of tolerance. *Pharmacol. Biochem. Behav.* 14: 75-79.
- Schulz, R., Wuster, M., & Herz, A. (1981b) Pharmacological characterization of the ϵ -opiate receptor. *J. Pharmacol. Exp. Ther.* 216: 604-606.
- Schulz, R., Wuster, M., & Herz, A. (1982) Endogenous ligands for κ -opiate receptors. *Peptides* 3: 973-976.
- Schulz, R., Wuster, M., Krenss, H., & Herz, A. (1980) Lack of cross-tolerance on multiple opiate receptors in the mouse vas deferens. *Mol. Pharmacol.* 18: 395-401.
- Schulz, R., Wuster, M., Rubini, P., & Herz, A. (1981c) Functional opiate receptors in the guinea-pig ileum: Their differentiation by means of selective tolerance development. *J. Pharmacol. Exp. Ther.* 219: 547-550.
- Sharma, S.K., Nirenberg, M., & Klee, W.A. (1975) Morphine receptors as regulators of adenylate cyclase activity. *Proc. Natl. Acad. Sci. U.S.A.* 72: 590-594.

- Shaw, J.S., Rourke, J.D., & Burns, K.M. (1988) Differential sensitivity of antinociceptive tests to opioid agonists and partial agonists. *Br. J. Pharmacol.* 95: 578-584.
- Shen, K.-F. & Crain, S.M. (1989) Dual opioid modulation of the action potential duration of mouse dorsal root ganglion neurons in culture. *Brain Res.* 491: 227-242.
- Shen, K.-F. & Crain, S.M. (1990) Dynorphin prolongs the action potential of mouse sensory ganglion neurons by decreasing a potassium conductance whereas another specific kappa opioid does so by increasing a calcium conductance. *Neuropharmacol.* 29 (4): 343-349.
- Shibata, M., Ohkubo, T., Takahashi, H., & Inoki, R. (1989) Modified formalin test: Characteristic biphasic pain response. *Pain* 38: 347-352.
- Shibata, M., Ohkubo, T., Takahashi, H., Kudo, T., & Inoki, R. (1986a) Peripheral analgesic action of opioid peptides and morphine analogues. *Folia Pharmacol. Jpn.* 88 (2): 101-108.
- Shibata, M., Ohkubo, T., Takahashi, H., Kudo, T., & Inoki, R. (1986b) Studies of inflammatory pain response related pain producing substance and endogenous opioid system. *Folia Pharmacol. Jpn.* 87 (4): 405-416.
- Simantov, R., Childers, S.R., & Snyder, S.H. (1978) [³H]Opiate binding: anomalous properties in kidney and liver membranes. *Mol. Pharmacol.* 14: 69-76.
- Simantov, R., Kuhar, M.J., Uhl, G.R., & Snyder, S.H. (1977) Opioid peptide enkephalin: immunohistochemical mapping in rat central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 74: 2167-2172.
- Simantov, R., Snowman, A.M., & Snyder, S.H. (1976) Temperature and ionic influences on opiate receptor binding. *Mol. Pharmacol.* 12: 977-986.
- Simmonds, M.A. (1983) Multiple GABA receptors and associated regulatory sites. *Trends in Neurosci.* 6: 279-281.
- Simon, E.J. (1986) Progress in the characterization of the opioid receptor subtypes: Peptides as probes. Future directions. In *Opioid peptides: Molecular pharmacology, biosynthesis, and analysis*, (eds. Rapaka, R.S & Hawks, R.L.), pp. 155-174. NIDA Research Monograph 70, Rockville, MD.
- Simon, E.J., Bonnet, K.A., Crain, S.M., Groth, J., Hiller, J.M., & Smith, J.R. (1980) Recent studies on interaction between opioid peptides and their receptors. In *Neural Peptides and Neuronal Communication*, (eds. Costa, E. & Trabucchi, M.), pp. 335-346. Raven Press, New York.
- Simon, E.J., Hiller, J.M., & Edelman, I. (1973) Stereospecific binding of the potent narcotic analgesic [³H]etorphine to rat-brain homogenate. *Proc. Natl. Acad. Sci. U.S.A.* 70: 1947-1949.

- Sircar, R., Nichtenhauser, R., Ieni, J.R., & Zukin, S.R. (1986) Characterization and autoradiographic visualization of (+)-[³H]SKF 10,047 binding in rat and mouse brain: Further evidence for phencyclidine/"sigma opiate" receptor commonality. *J. Pharmacol. Exp. Ther.* 237 (2): 681-688.
- Smallman, K. (1988) The interaction of high pressure with analgesia produced by morphine and pethidine. Ph.D. thesis submitted to University of London.
- Smith, T.W. & Buchan, P. (1984) Peripheral opioid receptors located on the rat saphenous nerve. *Neuropeptides* 5: 217-220.
- Smith, J.R. & Simon, E.J. (1980) Selective protection of stereospecific enkephalin and opiate binding against inactivation by N-ethylmaleimide: Evidence for two classes of opiate receptors. *Proc. Natl. Acad. Sci. U.S.A.* 77: 281-284.
- Snyder, S.H. & Goodman, R.R. (1980) Multiple neurotransmitter receptors. *J. Neurochem.* 35: 5-15.
- Sonders, M.S., Keana, J.F.W., & Weber, E. (1988) Phencyclidine and psychotomimetic sigma opiates: recent insights into their biochemical and physiological sites of action. *Trends in Neurosci.* 11 (1): 37-40.
- Spain, J.W., Roth, B.L., & Coscia, C.J. (1985) Differential ontogeny of multiple opioid receptors (μ , δ , and κ). *J. Neurosci.* 5: 584-588.
- Spampinato, S. & Goldstein, A. (1983) Immunoreactive dynorphin in rat tissues and plasma. *Neuropeptides* 3: 193-212.
- Stautner, C., Wuster, M., Schulz, R., & Herz, A. (1982) A differential interaction of putative μ -selective agonists with opiate binding sites in rat brain. *Neurochem. Int.* 4: 557-561.
- Stein, C., Millan, M.J., Shippenberg, T.S., Peter, K., & Herz, A. (1989) Peripheral opioid receptors mediating antinociception in inflammation: Evidence for involvement of mu, delta, and kappa receptors. *J. Pharmacol. Exp. Ther.* 248 (3): 1269-1275.
- Stein, C., Millan, M.J., Yassouridis, A., & Herz, A. (1988) Antinociceptive effects of μ - and κ -agonists in inflammation are enhanced by a peripheral opioid receptor-specific mechanism. *Eur. J. Pharmacol.* 155: 255-264.
- Straughan, D.W. (1984) 5-HT; peripheral and central receptors, and function. *Trends in Pharmacol. Sci.* 5: 410-411.
- Su, T.-P., Weissman, A.D., & Yeh, S.-Y. (1986) Endogenous ligands for sigma opioid receptors in the brain ("sigmaphin"): evidence from binding assays. *Life Sci.* 38: 2199-2210.
- Sullivan, A.F., Dickenson, A.H., & Roques, B.P. (1989) δ -Opioid mediated inhibitions of acute and prolonged noxious-evoked responses in rat dorsal horn neurones. *Br. J. Pharmacol.* 98: 1039-1049.

- Szolcsanyi, J. (1984) Capsaicin-sensitive chemoreceptive neural system with dual sensory-efferent function. In *Antidromic Vasodilatation and Neurogenic Inflammation*, (eds. Chahl, L., Szolcsanyi, J., & Lembeck, F.), pp. 27-52. Akademiai Kiado, Budapest.
- Takemori, A.E., Ho, B.Y., Naeseth, J.S., & Portoghese, P.S. (1988) Nor-binaltorphimine, a highly selective kappa-opioid antagonist in analgesic and receptor binding assays. *J. Pharmacol. Exp. Ther.* 246: 255-258.
- Takemori, A.E., Larson, D.L., & Portoghese, P.S. (1981) The irreversible narcotic antagonistic and reversible agonistic properties of the fumaramate methyl ester derivative of naltrexone. *Eur. J. Pharmacol.* 70 (4): 445-451.
- Takemori, A.E. & Portoghese, P.S. (1985) Receptors for opioid peptides in the guinea-pig ileum. *J. Pharmacol. Exp. Ther.* 235: 389-392.
- Tam, S.W. & Liu-Chen, L.-Y. (1986) Reversible and irreversible binding of β -funaltrexamine to mu, delta and kappa opioid receptors in guinea pig brain membranes. *J. Pharmacol. Exp. Ther.* 239: 351-357.
- Terenius, L. (1973) Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta. Pharmacol. et Toxicol. (Copen.)* 32: 317-320.
- Terenius, L. & Wahlstrom, A. (1975) Search for an endogenous ligand for the opiate receptor. *Acta Physiol. Scand.* 94: 74-81.
- Tiseo, P.J., Geller, E.B., & Adler, M.W. (1988) Antinociceptive action of intracerebroventricularly administered dynorphin and other opioid peptides in the rat. *J. Pharmacol. Exp. Ther.* 246: 449-453.
- Toll, L., Keys, C., Polgar, W., & Loew, G. (1984) The use of computer analysis in describing multiple opiate receptors. *Neuropeptides* 5: 205-208.
- Tomozawa, Y., Sueoka, N., & Miyake, M. (1985) Clonal sublines of rat neurotumor RT4 and cell differentiation: V. Comparison of Na⁺ influx, Rb⁺ efflux, and action potential among stem-cell, neuronal, and glial cell types. *Dev. Biol.* 108: 503-512.
- Toogood, C.I.A., McFarthing, K.G., Hulme, E.C., & Smyth, D.G. (1986) Use of ¹²⁵I-Tyr²⁷ β -endorphin for the study of β -endorphin binding sites in rat cortex. *Neuroendocrinol.* 43: 629-634.
- Traynor, J. (1989) Subtypes of the κ -opioid receptor: fact or fiction? *Trends in Pharmacol. Sci.* 10: 52-53.
- Traynor, J.R., Kelly, P.D., & Rance, M.J. (1982) Multiple opiate binding sites in rat spinal cord. *Life Sci.* 31: 1377-1380.
- Traynor, J.R. & Rance, M.J. (1984) Opiate binding in the rat spinal cord: evidence for mu and delta sites. *Neuropeptides* 5: 81-84.
- Traynor, J.R. & Wood, M.S. (1987) Distribution of opioid binding sites in spinal cord. *Neuropeptides* 10: 313-320.

- Tulunay, F.C., Jen, M.-F., Chang, J.-K., Loh, H.H., & Lee, N.M. (1981) Possible regulatory role of dynorphin on morphine- and β -endorphin-induced analgesia. *J. Pharmacol. Exp. Ther.* 219: 296-298.
- Tyers, M.B. (1980) A classification of opiate receptors that mediate antinociception in animals. *Br. J. Pharmacol.* 69: 503-512.
- Upton, N., Sewell, R.D.E., & Spencer, P.S.J. (1982) Differentiation of potent μ - and κ -opiate agonists using heat and pressure antinociceptive profiles and combined potency analysis. *Eur. J. Pharmacol.* 78: 421-429.
- Urban, L. & Papka, R.E. (1985) Origin of small primary afferent substance P-immunoreactive nerve fibers in the guinea-pig heart. *J. Auton. Nerv. Syst.* 12: 321-331.
- Vignon, J., Chaudieu, I., Allaoua, H., Journod, L., Javoy-Agid, F., Agid, Y., & Chicheportiche, R. (1989) Comparison of [3 H]phencyclidine ([3 H]PCP) and [3 H]N-[1-(2-thienyl)cyclohexyl]piperidine ([3 H]TCP) binding properties to rat and human brain membranes. *Life Sci.* 45: 2547-2555.
- Vignon, J., Privat, A., Chaudieu, I., Thierry, A., Kamenka, J.-M., & Chicheportiche, R. (1986) [3 H]Thienyl-phencyclidine ([3 H]TCP) binds to two different sites in rat brain. Localization by autoradiographic and biochemical techniques. *Brain Res.* 378: 133-141.
- VonVoigtlander, P.F., Lahti, R.A., & Ludens, J.H. (1983) U-50,488: a selective and structurally novel non-mu (kappa) opioid agonist. *J. Pharmacol. Exp. Ther.* 224: 7-12.
- Wahlstrom, A., Brandt, M., Moroder, L., Wunsch, E., Lindeberg, G., Ragnarsson, U., Terenius, L., & Hamprecht, B. (1977) Peptides related to β -lipotropin with opioid activity: effects on levels of adenosine 3':5'-cyclic monophosphate in neuroblastoma x glioma hybrid cells. *FEBS Lett.* 77: 28-32.
- Wall, P.D. (1960) Cord cells responding to touch, damage, and temperature of skin. *J. Neurophysiol.* 23: 197-210.
- Wall, P.D. (1967) The laminar organization of dorsal horn and effects of descending impulses. *J. Physiol. (Lond.)* 188: 403-423.
- Wall, P.D. (1988) Editorial: The prevention of postoperative pain. *Pain* 33: 289-290.
- Ward, S.J., Fries, D.S., Larson, D.L., Portoghese, P.S., & Takemori, A.E. (1985) Opioid receptor binding characteristics of the non-equilibrium μ antagonist, β -funaltrexamine (β -FNA). *Eur. J. Pharmacol.* 107: 323-330.
- Ward, S.J., Portoghese, P.S., & Takemori, A.E. (1982) Pharmacological profiles of β -funaltrexamine (β -FNA) and β -chlornaltrexamine (β -CNA) on the mouse vas deferens preparation. *Eur. J. Pharmacol.* 80: 377-384.
- Watkins, J.C. (1984) Excitatory amino acids and central synaptic transmission. *Trends in Pharmacol. Sci.* 5: 373-376.

- Weber, E., Sonders, M., Quarum, M., McLean, S., Pou, S., & Keana, J.F.W. (1986) 1,3-Di(2-[5-³H]tolyl)guanidine: a selective ligand that labels σ -type receptors for psychotomimetic opiates and antipsychotic drugs. *Proc. Natl. Acad. Sci. U.S.A.* 83: 8784-8788.
- Weihe, E., McKnight, A.T., Corbett, A.D., Hartschuh, W., Reinecke, M., & Kosterlitz, H.W. (1983) Characterization of opioid peptides in guinea-pig heart and skin. *Life Sci.* 33 (Suppl.I): 711-714.
- Weihe, E., McKnight, A.T., Corbett, A.D., & Kosterlitz, H.W. (1985) Proenkephalin- and prodynorphin-derived opioid peptides in guinea-pig heart. *Neuropeptides* 5: 453-456.
- Weitz, C.J., Faull, K.F., & Goldstein, A. (1987) Synthesis of the skeleton of the morphine molecule by mammalian liver. *Nature* 330: 674-677.
- Werz, M.A., Grega, D.S., & Macdonald, R.L. (1987) Actions of mu, delta and kappa opioid agonists and antagonists on mouse primary afferent neurons in culture. *J. Pharmacol. Exp. Ther.* 243 (1): 258-263.
- Werz, M.A. & Macdonald, R.L. (1982) Heterogeneous sensitivity of cultured dorsal root ganglion neurones to opioid peptides selective for μ - and δ -opiate receptors. *Nature* 299: 730-733.
- Werz, M.A. & Macdonald, R.L. (1983) Opioid peptides selective for mu- and delta-opiate receptors reduce calcium-dependent action potential duration by increasing potassium conductance. *Neurosci. Lett.* 42: 173-178.
- Werz, M.A. & Macdonald, R.L. (1984) Dynorphin reduces calcium-dependent action potential duration by decreasing voltage-dependent calcium conductance. *Neurosci. Lett.* 46: 185-190.
- Werz, M.A. & Macdonald, R.L. (1985) Dynorphin and neoendorphin peptides decrease dorsal root ganglion neuron calcium-dependent action potential duration. *J. Pharmacol. Exp. Ther.* 234 (1): 49-56.
- Wharton, J., Polak, J.M., McGregor, G.P., Bishop, A.E., & Bloom, S.R. (1981) The distribution of substance P-like immunoreactive nerves in the guinea-pig heart. *Neurosci.* 6 (11): 2193-2204.
- White, E.J. & Bradford, H.F. (1986) Enhancement of depolarization-induced synaptosomal calcium uptake and neurotransmitter release by Bay K 8644. *Biochem. Pharmacol.* 35 (13): 2193-2197.
- White, D.M. & Helme, R.D. (1985) Release of substance P from peripheral nerve terminals following electrical stimulation of the sciatic nerve. *Brain Res.* 336: 27-31.
- Willis, W.D. (1982) Control of nociceptive transmission in the spinal cord. In *Progress in Sensory Physiology, Vol.3*, (ed. Ottoson, D.), pp. 1-155. Springer-Verlag, Berlin.
- Wolozin, B.L. & Pasternak, G.W. (1981) Classification of multiple morphine and enkephalin binding sites in the central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 78: 6181-6185.

- Woo, S.K., Tulunay, F.C., Loh, H.H., & Lee, N.M. (1983) Effect of dynorphin-(1-13) and related peptides on respiratory rate and morphine-induced respiratory rate depression. *Eur. J. Pharmacol.* 96: 117-122.
- Wood, J.N., Bevan, S.J., Coote, P.R., Dunn, P.M., Harmar, A., Hogan, P., Latchman, D.S., Morrison, C., Rougon, G., Theveniau, M., & Wheatley, S. (1990) Novel cell lines display properties of nociceptive sensory neurons. (submitted to *Proc. Natl. Acad. Sci. U.S.A.*)
- Wood, J.N., Dunn, P.M., Hogan, P., & Rang, H.P. (1989) Bradykinin evokes an inward current in a sensory neuron-derived cell line. *Soc. Neurosci. Abstr.* 15: 973.
- Wood, J.N., Winter, J., James, I.F., Rang, H.P., Yeats, J., & Bevan, S. (1988) Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J. Neurosci.* 8 (9): 3208-3220.
- Wood, P.L. (1982) Multiple opiate receptors: Support for unique mu, delta and kappa sites. *Neuropharmacol.* 21: 487-497.
- Wood, P.L., Charleson, S.E., Lane, D., & Hudgin, R.L. (1981) Multiple opiate receptors: Differential binding of μ , κ , and δ agonists. *Neuropharmacol.* 20: 1215-1220.
- Woolf, C.J. (1983) Evidence for a central component of post-injury pain hypersensitivity. *Nature* 306: 686-688.
- Woolf, C.J. & Wall, P.D. (1986) Morphine-sensitive and morphine-insensitive actions of C-fibre input on the rat spinal cord. *Neurosci. Lett.* 64: 221-225.
- Wuster, M., Schulz, R., & Herz, A. (1978) Specificity of opioids towards the μ -, δ -, and ϵ -opiate receptors. *Neurosci. Lett.* 15: 193-198.
- Wuster, M., Schulz, R., & Herz, A. (1980) The direction of opioid agonists towards μ -, δ -, and ϵ -receptors in the vas deferens of the mouse and the rat. *Life Sci.* 27: 163-170.
- Wuster, M., Schulz, R., & Herz, A. (1981) Multiple opiate receptors in peripheral tissue preparations. *Biochem. Pharmacol.* 30: 1883-1887.
- Wuster, M., Schulz, R., & Herz, A. (1983) A subclassification of multiple opiate receptors by means of selective tolerance development. *J. Receptor Res.* 3: 199-214.
- Yaksh, T.L. (1979) Direct evidence that spinal serotonin and noradrenaline terminals mediate the spinal antinociceptive effects of morphine in the periaqueductal gray. *Brain Res.* 160: 180-185.
- Yaksh, T.L. (1988) Substance P release from knee joint afferent terminals: modulation by opioids. *Brain Res.* 458: 319-324.
- Yaksh, T.L., Al-Rodhan, N.R.F., & Jensen, T.S. (1988) Sites of action of opiates in production of analgesia. In *Progress in Brain Research, Vol. 77: Pain Modulation*, (eds. Fields, H.L. & Besson, J.-M.), pp. 371-394. Elsevier, Oxford.

- Yaksh, T.L. & Elde, R.P. (1980) Release of methionine-enkephalin immunoreactivity from the rat spinal cord *in vivo*. *Eur. J. Pharmacol.* 63: 359-362.
- Yaksh, T.L., Jessell, T.M., Gamse, R., Mudge, A.W., & Leeman, S.E. (1980) Intrathecal morphine inhibits substance P release from mammalian spinal cord *in vivo*. *Nature* 286: 155-157.
- Yaksh, T.L. & Noueihed, R. (1985) The physiology and pharmacology of spinal opiates. *Annu. Rev. Pharmacol. Toxicol.* 25: 433-462.
- Yaksh, T.L., Yeung, J.C., & Rudy, T.A. (1976) Systematic examination in the rat of brain sites sensitive to the direct application of morphine: Observation of differential effects within the periaqueductal gray. *Brain Res.* 114: 83-103.
- Yanagisawa, M., Murakoshi, T., Tamai, S., & Otsuka, M. (1985) Tail-pinch method *in vitro* and the effects of some antinociceptive compounds. *Eur. J. Pharmacol.* 106: 231-239.
- Yanagisawa, M. & Otsuka, M. (1984) The effect of a substance P antagonist on chemically induced nociceptive reflex in the isolated spinal cord-tail preparation of the newborn rat. *Proc. Japan. Acad. B* 60: 427-430.
- Yonehara, N., Imai, Y., & Inoki, R. (1988) Effects of opioids on the heat stimulus-evoked substance P release and thermal edema in the rat hind paw. *Eur. J. Pharmacol.* 151: 381-387.
- Yonehara, N., Shibutani, T., & Inoki, R. (1987) Contribution of substance P to heat-induced edema in rat paw. *J. Pharmacol. Exp. Ther.* 242 (3): 1071-1076.
- Young, W.S. & Kuhar, M.J. (1979) A new method for receptor autoradiography: [³H]opioid receptors in rat brain. *Brain Res.* 179: 255-270.
- Young, W.S., Wamsley, J.K., Zarbin, M.A., & Kuhar, M.J. (1980) Opioid receptors undergo axonal flow. *Science* 210: 76-78.
- Zajac, J.-M., Gacel, G., Petit, F., Dodey, P., Rossignol, P., & Roques, B.P. (1983) Deltakephalin, Tyr-D-Thr-Gly-Phe-Leu-Thr: a new highly potent and fully specific agonist for opiate δ -receptors. *Biochem. Biophys. Res. Comm.* 111: 390-397.
- Zhang, A.Z. & Pasternak, G.W. (1981a) Ontogeny of opioid pharmacology and receptors: High and low affinity site differences. *Eur. J. Pharmacol.* 73: 29-40.
- Zhang, A.Z. & Pasternak, G.W. (1981b) Opiates and enkephalins: A common binding site mediates their analgesic actions in rats. *Life Sci.* 29: 843-851.
- Zieglansberger, W. & Bayerl, H. (1976) The mechanism of inhibition of neuronal activity by opiates in the spinal cord of cat. *Brain Res.* 115: 111-128.
- Zieglansberger, W. & Tulloch, I.F. (1979) The effects of methionine- and leucine-enkephalin on spinal neurones of the cat. *Brain Res.* 167: 53-64.

Zimmermann, M. (1977) Encoding in dorsal horn interneurons receiving noxious and nonnoxious afferents. *J. Physiol. (Paris)* 73: 221-232.

Zukin, R.S., Eghbali, M., Olive, D., Unterwald, E.M., & Tempel, A. (1988) Characterization and visualization of rat and guinea pig brain κ opioid receptors: Evidence for κ_1 and κ_2 opioid receptors. *Proc. Natl. Acad. Sci. U.S.A.* 85: 4061-4065.

ADDITIONAL REFERENCES

- Abbott, F.V. (1988) Peripheral and central antinociceptive actions of ethylketocyclazocine in the formalin test. *Eur.J.Pharmacol.* 152:93-100.
- Bruning, J.L. & Kintz, B.L. (1977) Nonparametric tests, miscellaneous tests of significance, and indexes of relationships. In Computational Handbook of Statistics, Second edition, pp.218-228. Scott, Foresman & Co.; Glenview, Illinois.
- Chalazonitis, A. & Crain, S.M. (1986) Maturation of opioid sensitivity of fetal mouse dorsal root ganglion neuron perikarya in organotypic cultures: regulation by spinal cord. *Neurosci.* 17(4):1181-1198.
- Cheng, Y.-C. & Prusoff, W.H. (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem.Pharmacol.* 22:3099-3102.
- Christie, M.J., Williams, J.T., & North, R.A. (1988) Cellular mechanisms of opioid tolerance: Studies in single brain neurons. *Mol.Pharmacol.* 32:633-638.
- North, M.A. (1978) Naloxone reversal of morphine analgesia but failure to alter reactivity to pain in the formalin test. *Life Sci.* 22(4):295-302.
- North, R.A. & Williams, J.T. (1985) On the potassium conductance increased by opioids in rat locus coeruleus neurones. *J.Physiol.(Lond.)* 364:265-280.
- Parsons, C.G. & Headley, P.M. (1989a) Spinal antinociceptive actions of mu- and kappa-opioids: the importance of stimulus intensity in determining 'selectivity' between reflexes to different modalities of noxious stimulus. *Br.J.Pharmacol.* 98:523-532.
- Parsons, C.G. & Headley, P.M. (1989b) On the selectivity of intravenous mu- and kappa-opioids between nociceptive and non-nociceptive reflexes in the spinalized rat. *Br.J.Pharmacol.* 98:544-551.
- Parsons, C.G., West, D.C., & Headley, P.M. (1989) Spinal antinociceptive actions and naloxone reversibility of intravenous mu- and kappa-opioids in spinalized rats: potency mismatch with values reported for spinal administration. *Br.J.Pharmacol.* 98:533-543.
- Sheehan, M.J., Hayes, A.G., & Tyers, M.B. (1988) Lack of evidence for epsilon-opioid receptors in the rat vas deferens. *Eur.J.Pharmacol.* 154:237-245.
- Smith, C.F.C. & Rance, M.J. (1983) Opiate receptors in the rat vas deferens. *Life Sci.* 33(Suppl.I):327-330.
- Williams, J.T., North, R.A., & Tokimasa, T. (1988) Inward rectification of resting and opiate-activated potassium currents in rat locus coeruleus neurons. *J.Neurosci.* 8(11):4299-4306.
- Yoshimura, M. & North, R.A. (1983) Substantia gelatinosa neurones hyperpolarized in vitro by enkephalin. *Nature* 305:529-530.