

**Pharmacological modulation of processes
contributing to spinal hyperexcitability:
electrophysiological studies in the rat.**

By

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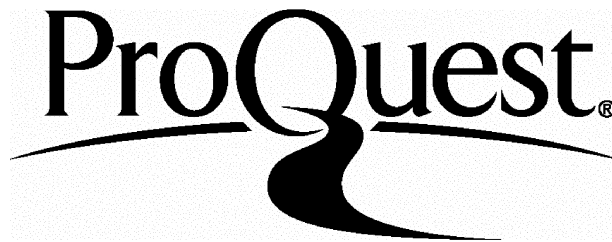
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Abstract

Two of the most effective analgesic strategies in man are (i) blockade of the NMDA receptor for glutamate, which plays a major role in nociceptive transmission and (ii) augmentation of inhibitory systems, exemplified by the use of ketamine and the opioids respectively. Both are, however, associated with side effects. Potential novel analgesic targets are investigated here using *in vivo* electrophysiology in the anaesthetised rat with pharmacological manipulation of spinal neuronal transmission.

Three different approaches were used to target NMDA receptors: (i) glycine site antagonists (Mrz 2/571 and Mrz 2/579), (ii) antagonists selective for receptors containing the NR2B subunit (ifenprodil and ACEA-1244), (iii) elevating the levels of N-acetyl-aspartyl-glutamate (NAAG), an endogenous peptide, by inhibition of its degradative enzyme. All agents produced selective inhibitions of noxious-evoked activity of dorsal horn neurones, with differing profiles. Effects of NAAG elevation were enhanced after inflammation and neuropathy, probably a result of agonist activity at the mGlu3 metabotropic glutamate receptor rather than reported NMDA receptor partial agonist properties.

The mu opioid receptor agonist methadone has affinity for the NMDA receptor. Spinal application of racemic methadone and the weak opioid optical isomer *d*-methadone, revealed that the inhibitory effects were predominantly opioid receptor mediated. Functional NMDA receptor blockade was not apparent.

Nociceptin/orphanin FQ (NC/OFQ) differs from the classical opioids and can, like NMDA receptor antagonists, attenuate spinal hyperexcitability. Pre-synaptic effects were enhanced after peripheral inflammation. Peripheral administration caused excitations of primary afferent fibres. Three putative antagonists were studied. [Phe¹ψ(CH₂-NH)Gly²]nociceptin-(1-13)-NH₂ was as potent as nociceptin in inhibition of neuronal responses and is now recognised as a partial agonist. [N-Phe¹]nociceptin(1-13)NH₂ was similarly unreliable, but J-113397, proved more fruitful.

Protons are implicated in inflammatory pain and peripheral hyperalgesia. I demonstrated activation of primary afferent fibres by peripheral administration of low pH solutions, the first electrophysiological study to do so *in vivo*.

The overall results provide a number of pharmacological strategies that may lead to a better control of pain. The thesis also sheds some light on a fundamental question in neurobiology, in describing both peripheral and central mechanisms that underlie normal transmission and aspects of plasticity in pain pathways.

Dedication

To Dorothy Carpenter, a wonderful grandmother, who would have been so proud to see this,
and to Jo Pearson, who will sadly never read my "Book about Drugs".

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Thanks must go first to Tony, for football chat Monday mornings, for learning about cerise pink and for teaching about lime green. If you had never given that course of lectures in Cambridge, I may never have found out that some scientists can be enthusiastic and passionate not only about their work, but about getting that message across. After all, what use is knowledge if you keep it to yourself? And what use is a lab with no one to work in it....I am indebted to everyone in the lab, old and new. Who would have guessed that I'd outlast Wahida, Vicky AND Louise? (Well, briefly Louise!) Thanks girls for everything...and for saying that you liked every haircut, thanks to Mark for a different perspective and for not noticing any haircuts, Lars for brief encounters, Vesa for longer, Katie for all things clinical, Rie for all things Latino, Sarah for running the London Marathon so that we didn't have to (surely 3 miles each would have been easier?), Ana Paula and Idil for fresh ideas and generosity, Lucinda for topping up the blonde quotient....and biggest love to the original blonde, Lizzy, we've got some stories eh? Thanks also to my project students, for their good times and hard work, Mansi, Sayan, Mash and Mei Mei.

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Chapter 1. Introduction

Perception of pain begins with detection of a stimulus — the process of transduction. This information is conveyed through primary afferent somatosensory nerve fibres into the dorsal horn of the spinal cord. Here the primary afferents synapse onto neurones within the dorsal horn where the information arriving from the periphery is subject to extensive positive and negative modulation. The 'output' of this spinal processing is then conveyed via several ascending tracts to integrative areas including the thalamus, midbrain nuclei and the hypothalamus, and then to higher centres where **sensation** becomes **perception**.

Many factors can alter the sensitivity of peripheral terminals of nociceptors and alter the transduction properties of these neurones. This signal arriving in the spinal cord is again subject to modulation. Better understanding the processes involved in this activity-dependent plasticity of nociceptive processing will improve treatment of persistent painful conditions. As a preface to this thesis, I will discuss the pathway of nociceptive processing — with emphasis on the changes that occur to increase activity in the system in persistent pain states.

1.1 Classification of primary afferent fibres

Functional, physiological, anatomical and histochemical properties have all been used to classify primary afferent neurones.

1.1.1 Functional classification denotes response properties

In most of the special senses relatively few, but highly specialised, 'receptor cells' exist (for example, rods and three types of cones in vision, the inner and outer hair cells in hearing). These specialised cells synapse onto sensory fibres, and the nature of the stimulus (colour hue, or pitch of a note) is coded by patterns of activity across fibres. In contrast, pain sensation is signalled by a 'line code', each axon carries only one sensory modality, and transduction takes place at the afferent fibre terminals. The modality conveyed by each axon in the pain pathway therefore depends on the complement of receptors and membrane channels expressed by the nerve endings. Thus, different types of sensory 'receptor' can be classified on a functional basis; that is, on the stimuli required for their activation.

Low threshold mechanoreceptors are activated by light touch. Specialised (but non-neuronal) structures surrounding the nerve ending dictate the precise modality of the stimulus that the fibre will respond to. The onion-like layers of the Pacinian corpuscles surround the nerve endings and confer sensitivity to high frequency vibration, Meissner's corpuscles respond to low frequency vibration and Merkel's disks respond to indentation of the skin. In hairy skin, Ruffini corpuscles are closely associated with the hair shaft and detect hair movement and low-frequency vibration. Thermoreceptors respond to warm and cool stimuli in the innocuous range.

Nociceptors are by definition activated by noxious (damaging, or potentially damaging) stimuli. Nociceptive nerve endings are therefore high threshold, and are often subclassified as AMH (A-fibre mechano-heat sensitive) or CMH (C-fibre mechano-heat sensitive) nociceptors (Raja *et al.*, 1997). Mechano-insensitive afferents (MIAs, or 'sleeping' nociceptors) have also been described. These are unresponsive to mechanical stimuli under normal circumstances, but may be sensitive to intense heat, to cold, to chemical activation, or become sensitised under pathological conditions and contribute to inflammatory hyperalgesia and receptive field expansion (Raja *et al.*, 1997). The chemosensitivity of nociceptors is discussed in detail in section 1.2.

1.1.2 Physiological properties of the axon can be classified

The extent of myelination and fibre diameter will determine the conduction velocity of an axon. This can be considered as the 'importance' the organism assigns to stimulus detection by that fibre type. For example fine, discriminative touch information requires rapid conduction as these sorts of stimuli change rapidly as the environment is explored. Conversely, the broad, burning pain of ill-defined locus conveyed by visceral nociceptive afferents does not require such rapid conduction, the stimulus is slowly changing and persistent. The diameter of the afferent cell bodies in the dorsal root ganglia (DRG) reflects afferent fibre diameter. The physiological boundaries used to classify A β -, A δ - and C-fibres are shown below in Table 1.1.

Table 1.1 Physiological properties of primary afferent fibre types

	Diameter (μm)	Myelination	Conduction velocity (ms^{-1})	Modality conveyed
A β -fibres	6–12	Heavy	25–70	Innocuous
A δ -fibres	1–5	Light	10–30	Innocuous/ noxious
C-fibres	0.2–1.5	None	<2.5	Noxious

Fine, unmyelinated, slowly conducting C-fibres convey noxious information, thinly-myelinated A δ -fibres convey both noxious and innocuous information, and the large diameter heavily-myelinated and rapidly conducting A β -fibres convey innocuous touch information. Describing the primary afferent type on this basis does not distinguish completely between nociceptive information and innocuous information (the 'grey' area being covered by the A δ -fibre classification). Furthermore, under pathological conditions A β fibres can signal pain (allodynia). As electrical stimulation of primary afferents and subsequent separation of fibre types on the basis of latency is used throughout this thesis, this is the criteria on which fibres will be identified for the remainder of this thesis.

1.1.3 Anatomical characterisation illuminates the pathways each fibre enters

Central processes of the sensory neurone types, as described in Table 1.1, have different termination patterns in the dorsal horn of the spinal cord (Sorkin & Carlton, 1997; and see this Chapter, section 1.3).

1.1.4 Histochemical classification reveals heterogeneity among nociceptors

Small diameter, presumably nociceptive afferent neurones can be divided broadly into two populations, those that stain for the plant-derived isolectin IB4, and those that express the NGF (nerve growth factor) receptor TrkA. The IB4-positive population was first identified after injection of IB4 conjugates into peripheral nerves. Non-peptidergic-small diameter primary afferents were selectively labelled (Kitchener *et al.*, 1993; Plenderleith & Snow, 1993; Silverman & Kruger, 1990). These small cells constitute 30% of dorsal root ganglion cells and virtually all IB4-labelled cells express Ret, a receptor tyrosine kinase required for glial cell line-derived neurotrophic factor (GDNF) signalling (Bennett *et al.*, 1998). Ret-immunoreactivity parallels staining for IB4 and is restricted to inner lamina II (Ili) (Molliver *et al.*, 1995; Molliver *et al.*, 1997).

A second population, which makes up 40–45% of adult rat DRG neurones, was identified by expression of TrkA and thus dependence on NGF in the adult nervous system (Molliver *et al.*, 1995; Wright & Snider, 1995). TrkA immunoreactivity in the dorsal horn is heaviest in laminae I and II outer (Ilo), and corresponds almost exactly with the peptide-producing population that stains positive for calcitonin gene-related peptide (CGRP) and substance P (SP) (Molliver *et al.*, 1995). The TrkA receptor is present not only on cell bodies but also on central terminals (Averill *et al.*, 1995), and expression is particularly high in visceral afferents (McMahon *et al.*, 1994).

Correlating expression of receptors and channels with these populations is beginning to give some indication of functional heterogeneity among nociceptors. For example, expression of the purine receptor P2X3 is restricted mainly to the GDNF-responsive/IB4-positive population in rat and human sensory nerves (Chen *et al.*, 1995; Vulchanova *et al.*, 1998; Yiangou *et al.*, 2000). Recent studies on null mutant mice reveal a probable role for this channel in initiating activity in the sub-noxious range in a population of afferent fibres (Cockayne *et al.*, 2000; Souslova *et al.*, 2000; and see this Chapter, section 1.2.8). The sensory neurone specific (SNS) sodium channel, which gives rise to the majority of TTX-resistant currents in DRG, is found in both TrkA- and IB4/ret-positive populations (Bennett *et al.*, 1998). The distribution of vanilloid receptor 1 (VR1) between these classes of nociceptor is still unclear, with contradictory results emerging from different studies (Guo *et al.*, 1999; Michael & Priestley, 1999; Tominaga *et al.*, 1998). It is likely that VR1 expression is not different between TrkA and GDNF-responsive/IB4-positive populations, although a small population of small-diameter cells that express Ret but do not label for IB4 show the highest relative levels of VR1 mRNA (Michael & Priestley, 1999).

Direct evidence of functional differences is also beginning to emerge. IB₄-positive neurones have longer duration action potentials, higher densities of TTX-resistant sodium currents, and smaller noxious heat activated currents than IB₄-negative neurones (Stucky & Lewin, 1999).

Work in this field is ongoing and it is an intriguing prospect that the histochemical dichotomy of small-diameter nociceptive sensory neurones could be paralleled by functional differences, and that different subclasses of nociceptors may relay distinct aspects of noxious stimuli both acutely and after injury in vivo.

It must be remembered that these trophic factors are not simply markers for sub-populations of afferent fibres. During development, changing expression of receptors for different trophic factors is required for normal peripheral nerve development. In the adult trophic factors are required for maintenance of afferent phenotype and are implicated in changes that occur in persistent pain states (Fundin *et al.*, 1997 Molliver *et al.*, 1997; Silos Santiago *et al.*, 1995). Trophic factors, specifically brain derived neurotrophic factor (BDNF), may also have a role in neurotransmission and synaptic plasticity in the spinal cord (see this Chapter section 1.5.3).

1.2 Pharmacology of peripheral nociceptor terminals

In the presence of an ongoing stimulus, most of the special senses demonstrate adaptation to the stimulus. For example, in constant bright light conditions the sensitivity of rod and cone cells is reduced, olfactory receptors adapt to persistent odours. The mechanism of adaptation can result from reducing the generator potential evoked by the stimulus, or adaptation of the nerve fibre to the generator potential evoked by the stimulus-receptor interaction. Pronounced adaptation is displayed by low threshold mechano- and thermo-receptors. Pacinian corpuscles show complete adaptation to constant pressure — the onion-like lamellae surrounding the nerve terminal absorb displacement — so that action potentials are only recorded from A β -fibres innervating Pacinian corpuscles during onset and offset of stimulus (dynamic and not static phase). 'Warm' receptors also show pronounced adaptation by reducing the frequency of action potential generation. In contrast, no real adaptation is shown by nociceptors. Receptor-desensitisation — for example to prolonged application of bradykinin — can be demonstrated, but the physiological effect will be a *sensitisation* to a noxious stimulus. This is of obvious evolutionary importance to the organism. Thus in the presence of a repeated, noxious stimulus, the perceived intensity of the stimulus is much enhanced, and this hyperalgesia spreads to surrounding undamaged areas. Two processes contribute to this. First, peripheral sensitisation of nociceptors such that a lower intensity stimulus will generate activity in the fibre. Second, an enhanced central response that is induced by the increased activity in primary afferents, and which leads to an increased area of hyperalgesia. These are often termed primary and secondary hyperalgesia respectively. Secondary hyperalgesia is still considered by some to originate purely from peripheral mechanisms. I will discuss these two components, peripheral and central hyperalgesia, separately.

Although the thermal and mechanical sensitivity of nociceptors have been best characterised, (discussed in this chapter, section 1.1.1), the chemical sensitivity of these fibres is probably of much greater importance to the organism. The inflammatory mediators released by tissue injury can both activate directly and sensitise nociceptor terminal to subsequent thermal and mechanical stimuli.

1.2.1 The kinins are potent algogenic and proinflammatory peptides.

Released by enzymatic cleavage from high molecular weight kininogen and low molecular weight kininogen respectively, bradykinin and kallidin are produced during inflammation and tissue damage (Dray, 1997 for review).

Administration of bradykinin in human volunteers (intra-arterial, intra-peritoneal and intradermal) evokes sensations of pain and causes hyperalgesia to heat but not to mechanical stimuli (Coffman, 1966; Lim *et al.*, 1967; Manning *et al.*, 1991). Animal behavioural studies are in agreement with this and also reveal that the lowering of thermal

nociceptive thresholds induced by intraplantar bradykinin injection is largely due to B₂-receptor activation, generation of prostanoids and subsequent sensitisation of capsaicin-sensitive nociceptors (Schuligoi *et al.*, 1994).

Bradykinin can directly activate capsaicin-sensitive nociceptors *in vitro* (Belcher, 1979), evoke activity from nociceptive fibres in humans and monkeys (Khan *et al.*, 1992), and evoke activity in rat dorsal horn neurones after injection into their peripheral receptive field *in vivo* (Haley *et al.*, 1989). Effects of bradykinin first seemed to be mediated mostly through B₂ receptors (Borkowski *et al.*, 1995; Perkins *et al.*, 1993; Steranka *et al.*, 1989) and coupled to PKC activation (Dray *et al.*, 1992; Dray & Perkins, 1988; Kress *et al.*, 1996). Of the five PKC isoforms present in sensory nerves, the isoform PKC- ϵ may have a specific role in modulating the sensitivity of nociceptive neurones to noxious heat (Cesare *et al.*, 1999). The role of the B₁ inducible receptor is beginning to emerge and this may be of increased importance in persistent inflammatory states (Khasar *et al.*, 1995; Perkins *et al.*, 1993; Rupniak *et al.*, 1997; Seabrook *et al.*, 1997 and see Marceau *et al.*, 1998 for review). Recent evidence suggests that the B₁ receptor may be required for the development of inflammatory hyperalgesia, possibly via a spinal site (Pesquero *et al.*, 2000).

Importantly, bradykinin not only directly activates nociceptors, but also sensitises nociceptors to thermal stimuli and to other chemical mediators including IL-2, the prostanoids, 5-HT and histamine (Martin, 1996; Martin & Murphy, 1995; Rueff & Dray, 1993), and can stimulate release of such mediators from post-ganglionic sympathetic neurones.

1.2.2 Inflammatory mediators interact to sensitise nociceptors

In turn, inflammatory mediators including the prostanoids, 5-HT and histamine sensitise nociceptors to bradykinin (Belcher, 1979; Grubb *et al.*, 1991; Kirchhoff *et al.*, 1990; Mense, 1981; Rueff & Dray, 1992; Rueff & Dray, 1993). In certain situations, each of these mediators can also individually activate nociceptors or cause sensations of pain in humans (Belcher, 1979; Richardson *et al.*, 1985; Rueff & Dray, 1992; Schaible & Schmidt, 1988). Inflammation-induced sensitisation to bradykinin appears to require the presence of NGF (Koltzenburg *et al.*, 1999) and may involve a reduction in the nitric oxide (NO)/cGMP-mediated desensitisation (tachyphylaxis) that follows bradykinin application (Rueff *et al.*, 1994). However, NO is also implicated in generation of peripheral hyperalgesia (Aley *et al.*, 1998; Wang *et al.*, 1999).

The prostanoid PGE₂ plays an important role in the development of peripheral hyperalgesia. This is exemplified by the analgesic effectiveness of NSAIDs. PGE₂ has recently been shown to potentiate conductance through the TTX-resistant sodium channel which, as discussed later, plays an important role in nociception (Akopian *et al.*, 1999). PGE₂ also enhances Na⁺ currents through the capsaicin receptor VR1 via cAMP and PKA. The extent to which the effects of bradykinin are mediated by the prostanoids (PGE₂ in

particular) is debatable, as the effectiveness of NSAIDs in attenuating the effects of bradykinin varies, mostly depending on the concentration of bradykinin applied (Dray *et al.*, 1992; Rueff *et al.*, 1994).

1.2.3 The sympathetic nervous system

Sensitising effects of bradykinin and other inflammatory mediators have been suggested to occur, in part, via the sympathetic nervous system. Thus, bradykinin-induced hyperalgesia and prostaglandin release requires intact sympathetic post-ganglionic neurones (Green *et al.*, 1993; Khasar *et al.*, 1995; Levine *et al.*, 1986; Malik & Nasjletti, 1979). A similar requirement is apparent for NGF (Andreev *et al.*, 1995) and 5-HT (Pierce *et al.*, 1995). In addition, post-ganglionic activity may be enhanced by glutamate receptor activation during inflammation (Coggeshall & Carlton, 1999). However the sensitising effects of bradykinin were not seen to be altered in sympathectomised animals *in vitro* (intact rat skin) (Koltzenburg *et al.*, 1992) or in humans (Meyer *et al.*, 1992), nor was sympathectomy effective in reducing joint inflammatory responses in an arthritis model (Sluka *et al.*, 1994). Confusingly, after prolonged PGE₂-induced hyperalgesia, delayed sympathectomy was seen to enhance mechanical hyperalgesia (Aley *et al.*, 1996). The situation is likely to be complicated by the recently suggested process of negative feedback; activated nociceptive afferents inhibit post-ganglionic sympathetic neurone dependent plasma extravasation and vagotomy enhances the effects mechanical hyperalgesia evoked by bradykinin (Green *et al.*, 1998; Khasar *et al.*, 1998b; Khasar *et al.*, 1998c). Sympathetic- versus afferent-mediated neurogenic inflammation is discussed in a review by (Janig *et al.*, 1996).

1.2.4 Peptide transmitters are released from peripheral terminals

Further chemical, mechanical or thermal activation of nociceptors will also elicit antidromic release of CGRP and SP (Andrews & Helme, 1987; Averbeck *et al.*, 2000) and maybe PGE₂ (Ebersberger *et al.*, 1999). Such neurogenic inflammation widens the area of hyperalgesia (flare) and can go on to exacerbate the inflammatory process and cause nociceptor activation and sensitisation (Kessler *et al.*, 1992). However, neurogenic inflammation alone may not be sufficient to either activate nociceptors, or reduce thresholds for heat or mechanical stimuli (Handwerker *et al.*, 1987; Meyer *et al.*, 1988; Reeh *et al.*, 1986) and SP is not always seen to activate or sensitise C-fibres significantly (Cohen & Perl, 1990; Fitzgerald & Lynn, 1979 but see Carlton *et al.*, 1998b). In addition, SP release is not always evoked by antidromic electrical stimulation (Kress *et al.*, 1999). Although NK₁ receptor mRNA has been found in rat DRG and primary afferent fibre terminals (Andoh *et al.*, 1996; Carlton *et al.*, 1996; Li & Zhao, 1998 but see Brown *et al.*, 1995), treatment with an NK₁ receptor antagonist (SR140333) does not affect mechanical or thermal hyperalgesia after capsaicin insult (Amann *et al.*, 1995). Alternatively, release of inflammatory mediators (prostaglandins, histamine) from non-neuronal structures in the skin (blood vessels, mast

cells) by SP injection could be sufficient to cause activation of primary afferents, a process which appears to be receptor independent (Maggi, 1997).

This incomplete ability of local spread of peptide release to generate hyperalgesia is the main argument against a peripheral mechanism of secondary hyperalgesia.

These reciprocal interactions are illustrated in Figure 1.1, but this can in no way be a full account of what happens. A complicating factor in all studies of peripheral inflammatory processes arises when two or more agents are applied to the same target. When each has the ability to affect a response, the extent to which either sensitises or activates the substrate becomes indistinguishable, and in some ways irrelevant.

An important observation is that mechanical hyperalgesia is absent in human, behavioural and *in vitro* studies after application of any of the mediators discussed above. Even the combination of SP with 'inflammatory soup' (bradykinin, 5-HT, histamine and PGE₂) could not generate observable mechanical hyperalgesia in the *in vitro* dissected skin-nerve preparation (Kessler *et al.*, 1992). A small degree of mechanical hyperalgesia can be reproduced in human volunteers by co-administration of SP and bradykinin (Jensen *et al.*, 1991), but there are undoubtedly other important mechanisms. For example, interaction between peripherally released glutamate and SP may sensitise nociceptors (Carlton *et al.*, 1998b).

Small molecules and ion channels are also important in peripheral hyperalgesia. Molecular approaches have identified many ion channels specific to sensory neurones as important substrates in nociceptor activation and the generation of peripheral hyperalgesia. Although the activation and sensitisation mediated by ion channels and small molecule mediators undoubtedly occurs in parallel and in synergy with the mediators discussed above, I have considered them separately for clarity.

1.2.5 Protons can activate and sensitise nociceptors

Low pH has long been reported in inflamed tissue and in arthritic joints, skeletal and heart muscle during ischaemia, and in malignant tumours (Jacobus *et al.*, 1977; Lewis *et al.*, 1931; O'Donnell *et al.*, 1979; Pan *et al.*, 1988; Revici *et al.*, 1949). The build up of protons can occur as a result of both inadequate perfusion and altered local metabolism and is not just an innocent by-product of inflammatory processes. In human volunteers, generation of muscle ischaemia to cause a drop in muscle pH to below pH 7.0, is associated with sensations of pain (Issberner *et al.*, 1996; Pan *et al.*, 1988). Infusion of acidic solutions (at pH 5.2) into the skin of human volunteers gives rise to graded sustained pain and hyperalgesia to heat and to mechanical punctate stimuli (Guenther *et al.*, 1999; Steen & Reeh, 1993). Here therefore, the simplest of chemical substances can generate pain and hyperalgesia to thermal and mechanical stimuli.

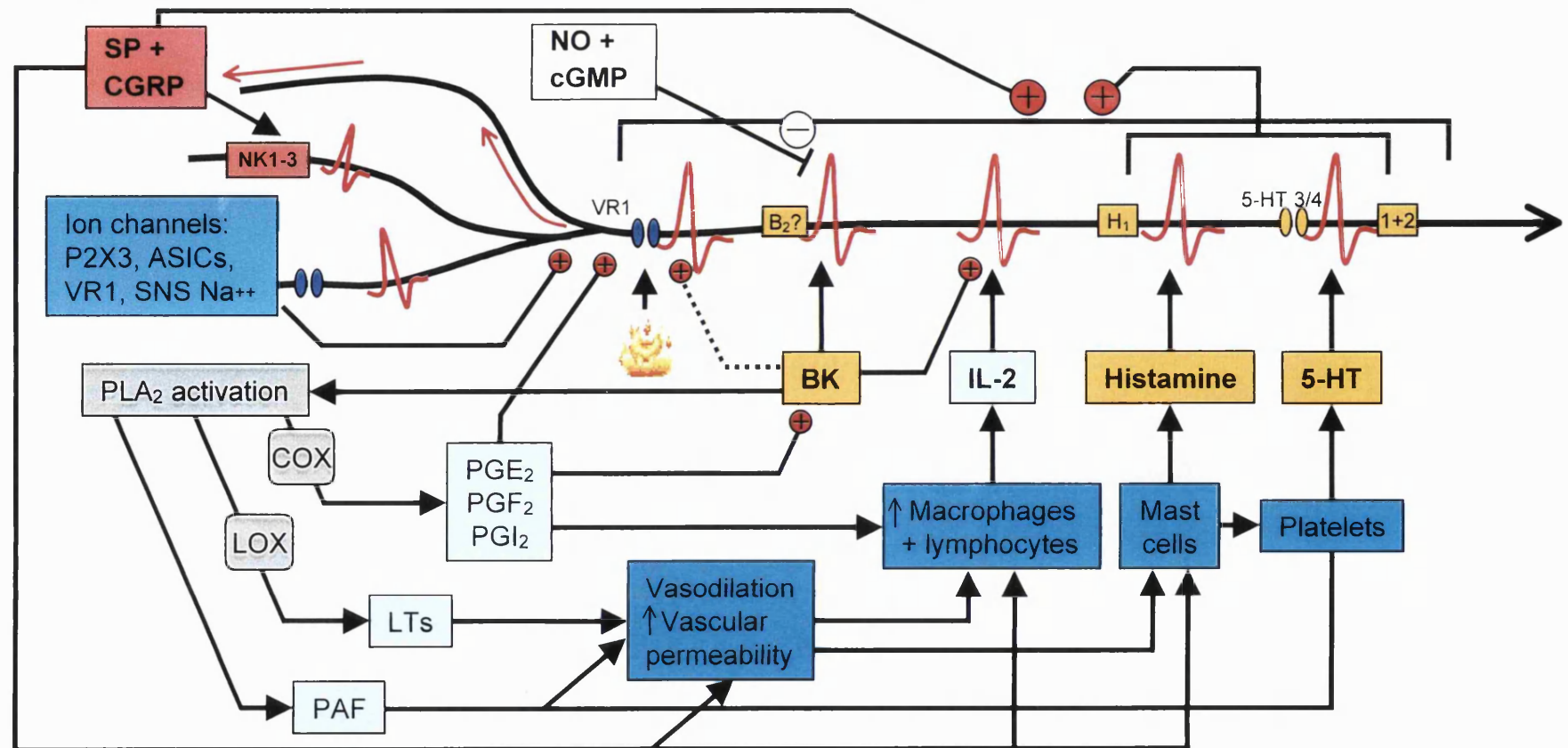


Figure 1.1 A variety of ion channels and small molecules are involved in peripheral activation (I_p) and sensitisation ($\bullet$) of nociceptors. Tissue damage releases bradykinin (BK, orange box), which acts via the B_2 receptor to activate nociceptors and sensitise neurones to heat and other chemical mediators such as IL-2, 5-HT and histamine. BK also activates phospholipase A_2 (PLA_2), generating cyclooxygenase (COX) and lipoxygenase (LOX) products — lilac boxes, the prostanooids, leukotrienes and platelet-activating factor (PAF) — which act to enhance inflammatory processes at the site of tissue injury (blue boxes). These processes in turn release more chemical mediators capable of activating and sensitising nociceptors (yellow boxes). Antidromic release of peptides (pink box) from activated nociceptors sensitises neurones to all these chemical mediators. Small molecules and ion channels can also activate and sensitise nociceptors, these are shown in more detail in Figure 1.2. More interactions than are indicated may occur. The divergence of nociceptor populations suggests that not all processes necessarily occur on the same individual fibre. Rectangular boxes on the neurone denote G-protein-coupled receptors and $\bullet$ denotes ion channels.

A sensor responsible for reception of pH changes in sensory nerves was first reported 20 years ago (Krishtal & Pidoplichko, 1980). Spinal and trigeminal ganglia neurones responded to pH changes with increased membrane permeability to sodium and potassium. This sensor was later noted to be present mainly on small diameter sensory nerve fibres, suggesting a role in nociception (Krishtal & Pidoplichko, 1981). Single unit recordings from sensory nerve endings has established that protons can activate subsets of primary afferent nerve fibres innervating skin (Steen *et al.*, 1992), cornea (Belmonte *et al.*, 1991; Chen *et al.*, 1995; Gallar *et al.*, 1993), cardiac muscle (Benson *et al.*, 1999; Pal *et al.*, 1989; Pan *et al.*, 1999; Uchida & Murao, 1975) and the viscera (Klement & Arndt, 1991) and cause release of SP and CGRP from these peripheral terminals (Geppetti *et al.*, 1991).

Voltage clamp recordings have further characterised the proton-evoked current in sensory neurones (frog DRG, Akaike *et al.*, 1990; rat DRG, Bevan & Yeats, 1991; and chick DRG, Davies *et al.*, 1988) as well as in CNS neurones (rat hypothalamus, Ueno *et al.*, 1992). The membrane current underlying activation of rat DRG neurones by low extracellular pH was described in detail by Bevan & Yeats (1991). Whole-cell recordings revealed a transient Na^+ current with a threshold for activation of pH 6.8 that could be recorded from most DRG neurones. A persistent H^+ -gated current was also recorded in a sub-population of DRG neurones, (diameter < 30 μm and capsaicin-sensitive). This sustained current requires a lower pH for activation (threshold pH 6.2) is poorly selective between monovalent cations (permeability $\text{K}^+ > \text{Cs}^+ > \text{Na}^+$) and is regarded as the current carried by the native H^+ -gated cation channel. Despite early reports to the contrary, proton-evoked currents in sensory neurones can be carried by Ca^{2+} (Kovalchuk *et al.*, 1990; Zeilhofer *et al.*, 1996). In fact, low pH-activated currents were initially thought to flow through proton-transformed voltage-gated calcium channels (Konnerth *et al.*, 1987) but with more careful investigation (Wegner *et al.*, 1996) and cloning of the channels, this idea became obsolete. The characteristics of proton-activated currents in sensory nerves are summarised in Chapter 5, Table 5.1.

(acid-sensing ion channel)

Following the cloning of the first ASIC by Waldmann *et al.* (1997), it became evident that these proton sensors belong to the amiloride-sensitive Na^+ /degenerin family of ion channels, and share the same structure. Two transmembrane domains bracket a long extracellular loop and both the C and N termini are located intracellularly (Canessa *et al.*, 1994; Renard *et al.*, 1994; Snyder *et al.*, 1994). Residues in the region preceding the first transmembrane domain are thought to contribute to pore formation and determine the ion selectivity of the channels (Coscoy *et al.*, 1999). Despite the cloning of a large number of ASICs there are fundamental differences between the characteristics of ASICs, either expressed alone or as heteromultimers, and the proton-evoked currents recorded from DRG sensory neurones. The current characteristics of homomeric and heteromeric assemblies of cloned ASICs are summarised in Chapter 5, Table 5.2 and 5.3. As-yet unidentified channels may alone or in combination underlie native proton-evoked nociception. The existing

channels may have hitherto unidentified roles in sensation, and not necessarily nociception. For example, BNC1 is required for normal touch sensation (Price *et al.*, 2000).

1.2.6 The capsaicin receptor, a putative endogenous sensor for noxious heat

Both the rat and human orthologue of the capsaicin receptor, a heat-activated ion channel, have now been cloned in rats and humans (Caterina *et al.*, 1997; Hayes *et al.*, 2000) and extensively characterised (see Szallasi & Blumberg, 1999 for review). Heat, protons and the endogenous cannabinoid anandamide may all function as the endogenous ligands for the VR1.

Capsaicin sensitivity, *in situ* hybridisation and immunohistochemistry all agree that VR1 is localised in small-diameter sensory (C-fibre) afferents (Caterina *et al.*, 1997). The immunohistochemistry work of Tominaga *et al.* (1998) reveals discrete immunoreactivity in unmyelinated afferent fibre terminals in the superficial dorsal horn, and in peripheral terminals of sensory nerves, including in the bladder wall. Staining was also seen in nodose ganglia and in medullary structures receiving projections from visceral organs. Co-localisation studies by Tominaga *et al.*, (1998) and more recently Michael & Priestley, (1999) revealed further heterogeneity in primary afferents. Expression of VR1 was found to be higher in TrkA-positive neurones (contain substance P and CGRP) than in IB4-positive neurones. However, it is found in both populations and is also absent from some cells of both types. High levels of VR1 staining (around 10% of VR1-positive cells) have been found in very small diameter and previously little characterised cells that express the receptor tyrosine kinase (Ret) and that do not label for either trkA or IB4. Conversely, it has also been reported that VR1-immunoreactivity co-localized with the P2X3 purinoceptor and IB4, whereas no substantial co-existence with SP- and CGRP-positive terminals was seen (Guo *et al.*, 1999).

VR1 null mice have now been generated. Their small-diameter DRG neurones lack the normal well-characterised capsaicin-, acid- and noxious heat-gated currents (Caterina *et al.*, 2000; Davis *et al.*, 2000). Behavioural responses to capsaicin injection were eliminated, but normal responses to acute noxious mechanical and thermal stimuli were observed. Development of thermal hyperalgesia after peripheral inflammation was severely limited. This indicates that other, not yet identified, heat-gated channels must exist and that the VR1 is necessary for development of normal inflammatory thermal hyperalgesia.

The VR1 receptor integrates many noxious inputs. VR1 expression confers sensitivity not only to mildly noxious heat (threshold 43–44°C), but also to low pH (pH 5.5) in expression systems (Caterina *et al.*, 1997). Low pH can also enhance the response of the channel to heat and capsaicin (Baumann & Martenson, 2000; Caterina *et al.*, 1997; Tominaga *et al.*, 1998). Small changes in pH (e.g. to 6.4) enhance VR1 responses to capsaicin and to heat, to such an extent that under even moderately acidic conditions, the temperature threshold for VR1 activation is reduced so much that the channel may be

activated at ambient temperatures (Tominaga *et al.*, 1998). This modulation appears to occur via a key extracellular site (Jordt *et al.*, 2000).

It has been suggested that the VR1 is *the* acid-sensing ion channel on nociceptor terminals. Currents evoked by capsaicin, heat and low pH do share many biophysical properties, but there are apparent discrepancies. Proton-activated channels in sensory neurones are less permeable to Ca^{2+} than capsaicin-activated channels (Zeilhofer *et al.*, 1996; Zeilhofer *et al.*, 1997) and show much slower (or no) inactivation (Bevan & Yeats, 1991; Caterina *et al.*, 1997). However, these properties may be altered by the presence of protons.

1.2.7 A sensory neurone specific, TTX-resistant sodium channel

Voltage-gated sodium channels are ubiquitous and subserve vital functions in excitable tissues throughout the body. Most are blocked by the application of tetrodotoxin (TTX). TTX-resistant (TTX-R) currents can be recorded from DRG cells (Rush *et al.*, 1998; Scholz *et al.*, 1998) and bradykinin-stimulated release of CGRP is TTX-insensitive (Jeftinija, 1994). At least three TTX-R sodium channels expressed in sensory neurones have been cloned and characterised (Akopian *et al.*, 1996; Dib-Hajj *et al.*, 1999; Sangameswaran *et al.*, 1996; Tate *et al.*, 1998). The sodium channels underlying the TTX-R currents in nociceptors may be an important site for the integration of sensitising stimuli. PGE_2 modulates TTX-R sodium currents in primary afferent neurones (England *et al.*, 1996) and antisense neutralisation of SNS reversibly blocks PGE_2 -induced inflammatory hyperalgesia (Khasar *et al.*, 1998a). PKA and PKC appear to interact to mediate modulation of TTX-R currents in nociceptors (Gold *et al.*, 1998). Null mutant mice with targeted disruption of the SNS (PN3) channel were expected to reveal much about the role of these channels in nociception. Null mice DRG neurones give rise to only TTX-sensitive sodium currents, and animals display reduced sensitivity to noxious mechanical stimuli (Akopian *et al.*, 1999). Surprisingly, only mild effects were seen on thermoreception and inflammatory hyperalgesia. Lowered thresholds for C-fibre activation of dorsal horn neurones and increased TTX-sensitive current density were observed, indicating the existence of compensatory mechanisms which may have masked further changes.

1.2.8 P2X3, an ATP-gated ion channel

ATP is released from damaged tissue and causes sensations of pain in human volunteers (Bleehen & Keele, 1977). Seven ATP-gated cation-selective ion channels have been cloned and characterised, along with a number of G-protein coupled receptors. Of these, the P2X3 is selectively expressed by nociceptors. Two groups have recently generated P2X3 knock-out mice. Both found that the P2X3 channel was not involved in responses to acute thermal or mechanical stimuli, although dorsal horn neuronal responses to sub-noxious 'warm' stimuli were reduced, inflammatory pain processing was altered and bladder hyporeflexia were observed (Cockayne *et al.*, 2000; Souslova *et al.*, 2000).

1.2.9 Adenosine is generated by hydrolysis of ATP

Adenosine can cause pain and hyperalgesia, probably via activation of the A₂ receptor and cAMP production (Taiwo & Levine, 1990). The A₁ receptor is inhibitory and its activation can lead to antinociception (Taiwo & Levine, 1990), and thus presents an attractive target for drug development.

The interactions of these small molecules and ion channels are illustrated over the page in Figure 1.2.

1.2.10 Peripheral opioids

In the decade since the discovery of peripherals and the analgesic effects of opioid agonists at these peripheral sites, the understanding of peripheral opioid analgesia has much advanced. Importantly, a recent review of the increasing clinical trial data (mainly with intra-articular opioid agonists after knee surgery) shows returns for the research efforts invested (Shafer, 1999; Stein *et al.*, 2001).

Peripheral analgesic actions of opioids have been suggested to occur either mainly at peripheral afferent fibre terminals or sympathetic post-ganglionic terminals. The former now seems more likely (Zhou *et al.*, 1998) than the latter (Taiwo & Levine, 1991). This is supported in part by improved immunohistochemistry and receptor localisation (Wenk & Honda, 1999). As opioid receptors are synthesised in the DRG, their presence at peripheral terminals would not be unexpected. First shown in 1984 (Smith & Buchan, 1984), μ , δ and κ receptors have all now been localised to nociceptor terminals (Zhang *et al.*, 1998) and agonists at all receptor types can have antinociceptive effects in rat (Haley *et al.*, 1990a; Stein *et al.*, 1989) and monkey (Negus *et al.*, 1993). Mechanisms of action described include increased potassium conductance and decreased calcium conductance, with consequent inhibition of calcium-dependent release of neurogenic peptides from peripheral afferent fibre terminals, paralleling the central effector mechanisms for opioids (Machelska *et al.*, 1999).

Peripheral actions of opioids are enhanced after inflammation (Stein *et al.*, 1988). This occurs in the absence of increased receptor synthesis in the DRG and before increased peripheral receptor density is apparent (Schafer *et al.*, 1995), although increased peripherally-directed axonal transport of receptors does occur later (see Gebhart *et al.*, 2000; Machelska *et al.*, 1999; Stein *et al.*, 2001 for reviews). Increased cAMP levels and the numerous intracellular cascades initiated by inflammatory mediators may improve opioid receptor–G-protein coupling and underlie their increased effectiveness in inflammatory states, although physical effects to disrupt the parenchyma and improve access of opioids to the terminals in the inflammatory situation may also be important.

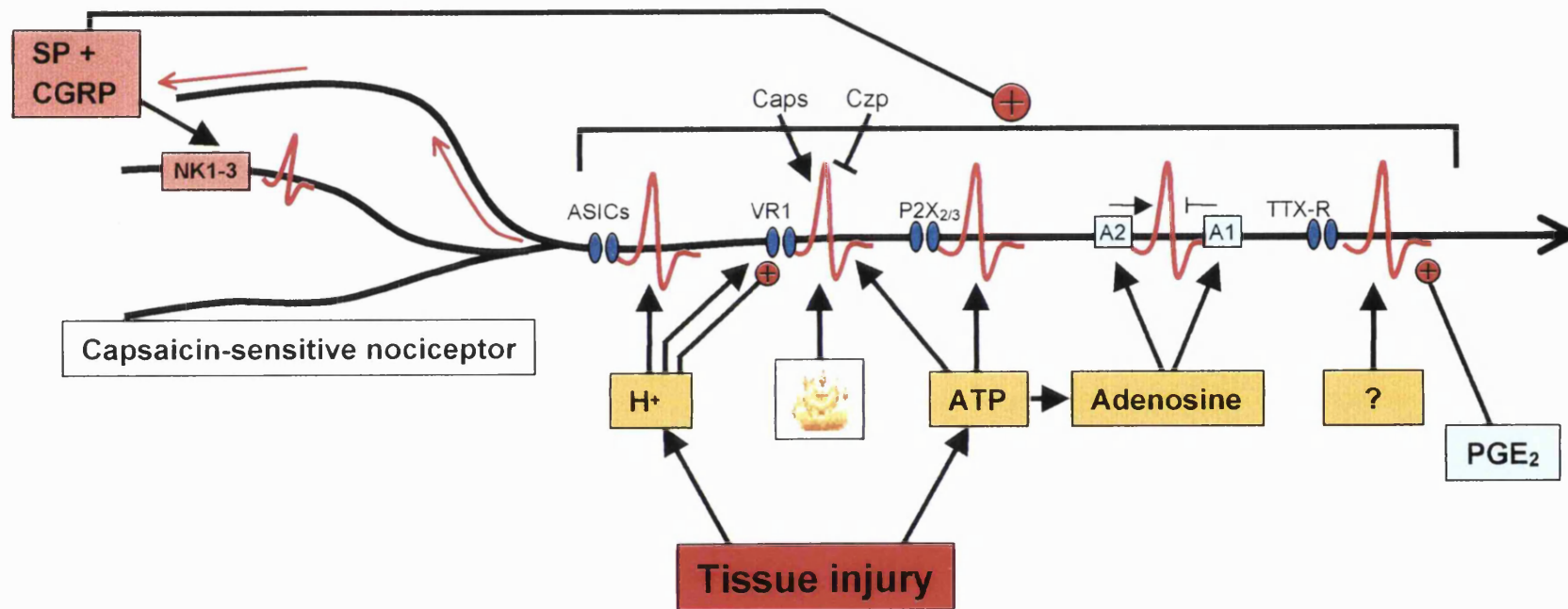


Figure 1.2 A variety of ion channels and small molecules are involved in peripheral activation (J_p) and sensitisation ($\bullet$) of nociceptors. Protons and ATP released by tissue injury act via acid-sensing ion channels (ASICs) and P2X_{2/3} ion channels to activate nociceptors. They also activate and/or sensitise neurones to heat via the VR1 receptor. This receptor can be studied by the application of the agonist capsaicin (Caps) and the antagonist capsazepine (Czp). Adenosine, generated from ATP, can activate nociceptive neurones via the G-protein-coupled A₂ receptor, or inhibit activity via the A₁ receptor. Several classes of TTX-resistant sodium channels (TTX-R) are expressed solely by nociceptive afferents and can be modulated by inflammatory mediators including prostaglandin E₂ (PGE₂). Antidromic release of peptides (pink box) from activated nociceptors sensitises neurones to all these chemical mediators. More interactions than are indicated may occur. The divergence of nociceptor populations suggests that not all processes necessarily occur on the same individual fibre. Rectangular boxes on the neurone denote G-protein-coupled receptors and $\bullet$ denotes ion channels.

The source of endogenous opioids in the periphery is probably immune cells, from which release is stimulated by corticotropin-releasing hormone and IL-1 β (Schafer *et al.*, 1996; Schafer *et al.*, 1997). Upregulation of binding sites on immune cells for these opioid-releasing agents is likely to represent part of the body's local response to combat inflammatory pain (Mousa *et al.*, 1996). Conversely, there is evidence that release of opioids, and application of morphine *in vitro* can attenuate immune function (for review see Gordon & Serafiem, 2001). The physiological benefit of this is less clear.

The role of the novel opioid-like peptide nociceptin/orphanin FQ (NC/OFQ) in peripheral nociception is less studied. Similarly synthesised in the DRG (Wick *et al.*, 1994), presence of the receptor at peripheral terminals of small diameter afferent fibres is expected, but has not yet been demonstrated. Unlike the opioid receptors, synthesis of the NC/OFQ receptor ORL1 (opioid receptor like receptor 1) appears to be rapidly upregulated after inflammation (Jia *et al.*, 1998). Interestingly, the synthesis of the precursor peptide is also increased in the DRG after inflammation (Andoh *et al.*, 1997), suggesting a possible peripheral release of the peptide itself. Northern blots have indicated that the NC/OFQ mRNA is localised in leukocytes (Nothacker *et al.*, 1996) and the receptor in mouse and human lymphocytes (Halford *et al.*, 1995; Wick *et al.*, 1995). After i.v. injection of the peptide, dose-dependent decreases in blood pressure and local vascular resistance, and suppressed antidromic (dorsal root stimulated) vasodilatation have been observed (Habler *et al.*, 1999).

1.2.11 Peripheral glutamate

The presence of the AMPA, kainate and NMDA receptors for glutamate in the dorsal root ganglia, the peripheral-directed transport of these receptors and their presence on peripheral sensory nerve terminals are now well established (Keast & Stephensen, 2000; Carlton, 2001). Behavioural studies show that peripheral administration of glutamate, AMPA, kainate or NMDA (hindpaw, knee joint and tail) evokes pain behaviours, thermal hyperalgesia and mechanical allodynia that is reversible by local administration of appropriate antagonists (Carlton *et al.*, 1995; Carlton *et al.*, 1998b; Lawland *et al.*, 1997; Zhou *et al.*, 1996). Although expression of receptors for glutamate does not appear to be limited to nociceptive afferents, selective activation of C-fibres has been demonstrated (Ault & Hildebrand, 1993; Du *et al.*, 2001). Interestingly, an increased involvement of peripheral receptors for glutamate has been suggested during inflammation (Carlton & Coggeshall, 1999; Davidson *et al.*, 1997; Jackson *et al.*, 1995; Lawland *et al.*, 1997; Omote *et al.*, 2000). Add to this the finding that peripheral excitatory actions of glutamate can be potentiated by co-administration of SP (Carlton *et al.*, 1998b) and that glutamate itself is released from peripheral afferent terminals (deGroot *et al.*, 2000) and a situation similar to that already discussed for other mediators of peripheral hyperalgesia begins to emerge. Interplay between sensory afferents and sympathetic efferents is again likely to occur (Coggeshall & Carlton, 1999).

1.2.12 Nociceptors are equipped to integrate many signals

Cellular cAMP, PKA and PKC are probable secondary mediators of nociceptor sensitisation and such intracellular signalling cascades seem to converge on 'effector' channels such as the VR1 and SNS (Fitzgerald *et al.*, 1999). Thus, cellular cAMP and PKA activity (specifically R1 β -PKA; Malmberg *et al.*, 1997a) is essential for maintenance of peripheral hyperalgesia (Aley & Levine, 1999; Cunha *et al.*, 1999). Of the five PKC isoforms present in sensory nerves, PKC- ϵ specifically mediates nociceptor sensitisation (Aley *et al.*, 2000; Cesare *et al.*, 1999; Khasar *et al.*, 1999). Guanylyl cyclase activation and cGMP formation appears to play an opposite role to cAMP in nociceptor sensitivity. The opposite effects of these second messenger systems is elegantly demonstrated in (Cunha *et al.*, 1999).

The VR1 is able to integrate the pain producing stimuli of noxious heat and low pH, and many inflammatory mediators through the interplay of PKC, PKA and intracellular calcium concentration ($[Ca^{2+}]_i$). In fact, the results obtained from the study of VR1-null mutant mice indicate that this channel is pivotal to the development of inflammatory hyperalgesia (Caterina *et al.*, 2000; Davis *et al.*, 2000). Heat-activated currents through VR1 are enhanced by PKC, rises in $[Ca^{2+}]_i$ are required for both capsaicin- and proton-induced heat sensitisation in rat nociceptors, (Guenther *et al.*, 1999), and Ca^{2+} - and PGE₂-stimulated cAMP formation and PKA activation can again increase capsaicin-evoked currents (Lopshire & Nicol, 1998). Elevated $[Ca^{2+}]_i$ can in addition evoke SP and CPRG release, but can also lead to desensitisation of the VR1 to capsaicin, heat and protons. A direct interaction has also been suggested between intracellular ATP and the VR1 (Kwak *et al.*, 2000).

The VR1 is a good candidate for integration of multiple pain-producing stimuli. Such integration is also likely to occur at many other nociceptor-specific receptors and channels. These complicated feedback augmentation and inhibitory controls, once elucidated, will provide potential for pharmacological manipulation.

1.3. Primary afferent fibre termination and transmitter profiles

Primary afferent fibres enter the spinal cord through the dorsal root. The intra-axonal injection of tracers (e.g. horse radish peroxidase, HRP) into functionally or physiologically identified fibre types reveals that different populations of fibres have distinct termination patterns within the dorsal horn. The spinal cord can be grossly separated into the white matter (axons) and grey matter (cell bodies and their processes). Further subdivision can then be made of the grey matter into ten layers (laminae) after Rexed (Rexed, 1952). Each lamina constitutes a functionally related group of cells, terminations and processes and extends the length of the spinal cord, rostro-caudally. Laminae I–V make up the dorsal horn, receiving sensory input, and laminae VI–X make up the motoneurone-containing ventral horn. These laminae are illustrated in transverse section in Figure 1.3, with the termination patterns of subtypes of primary afferent fibres indicated. The secondary neurones these inputs converge onto then project through different ascending tracts to higher centres of the brain. These are summarised in Figure 1.4.

Afferent fibres terminals contain a range of transmitters (for example Battaglia & Rustioni, 1988; Sorkin & Carlton, 1997; Wilcox & Seybold, 1997 for review). The particular neurotransmitters contained in a primary afferent fibre terminal and the receptor complement of the neurones it makes synaptic contact with will determine the nature of neurotransmission between the primary and secondary neurone in the dorsal horn, and also the susceptibility of this signal to modulation. This also has implications for understanding the coding mechanisms used by the system. It is unlikely, therefore, that specific transmitters are utilised by distinct classes of fibre. Information transfer probably depending more on the precise connectivity of each 'pathway'.

1.3.1 A β -fibres

A β -fibre afferents have large diameter cell bodies in the DRG (40–75 μ m) and appear light under the electron microscope. Such 'large light' cell bodies account for 35–45% of DRG cell bodies (Jessel & Dodd, 1989). The neurotransmitters utilised by these A β -fibre primary afferents is unclear. Although at least 50% of DRG neurones stain positive for glutamate in primates (rather more in rats), most glutamate-positive cells are of small diameter. Co-localisation of glutamate and aspartate has been reported in the DRG, but these amino acids appear to distribute to different populations of afferent fibre terminals in the dorsal horn (Merighi *et al.*, 1991). Aspartate is a possible candidate transmitter in A β -fibres (Sorkin & Carlton, 1997), although lack of evidence for mechanisms to terminate the action of this amino acid makes its role as a transmitter debatable. Peptides tend not to be localised in large diameter DRG cell bodies, although a significant level of CGRP-immunoreactivity has been detected in large, light DRG cell bodies (Merighi *et al.*, 1988). Although it would appear that there are many 'empty' A β -fibre terminals, it is probable that

glutamate or aspartate are in fact present in these neurones. Most immunohistochemistry studies are likely to underestimate the number of positive terminals or DRG cell bodies as human error can be introduced in the judging and counting of 'positive' cells. Additionally, levels of detection may be low, as demonstrated by the apparent increase in the proportion of DRG cell bodies that stain positive for glutamate after colchicine treatment to block axonal transport.

A β -fibre afferents terminate mainly in laminae III and IV but termination patterns are different depending on the type of receptor the afferents arise from. For instance, slowly adapting mechanoreceptors (Merckel disks) tend to terminate in the deeper laminae (IV or V) and send ascending collaterals to lamina III and IV.

Second order dorsal horn neurones exist that receive input almost exclusively from A β -fibres (Sorkin & Carlton, 1997). These low threshold (LT) cells are found mostly in laminae III and IV and project mainly into the spinocervical tract (SCT), postsynaptic dorsal column (PSDC) pathway, and are also found in the spinothalamic tract (STT). A β -fibre inputs can also converge with input from high-threshold primary afferents onto wide dynamic range (WDR) dorsal horn cells.

1.3.2 A δ -fibres

Intermediate diameter DRG cell bodies contain glutamate, aspartate and a wide range of peptides. It is difficult to say with precision which transmitters are utilised by A δ -fibre neurones for the same reasons as described above and due to problems identifying these fibre types on the basis of their diameter in DRG slices. Much of the discussion to follow regarding nociceptors and C-fibre terminals may apply equally well to A δ -fibre terminals originating from high threshold cutaneous mechanoreceptors.

Fine, myelinated afferent fibres with conduction velocities in the A δ -fibre range have different spinal termination patterns depending on their peripheral terminal specialisation. A δ -fibre afferents innervating hair cells terminate predominantly in lamina III and ventral lamina II, whereas high threshold A δ -fibre afferents, many of which can be classified as nociceptors, distribute to lamina I, or to laminae IV and V with projections to lamina I. Although some A δ -fibre afferents may contribute to inputs onto LT cells, it is predominantly WDR and high threshold (HT, nociceptor-specific) dorsal horn cells that receive input from A δ -fibre afferents. Subdividing response properties of HT cells reveals a population of noxious mechanosensitive cells that receive input predominantly from A δ -fibres (3A), and a class that receive input from both A δ - and C-fibre nociceptive afferents (3B) (Cervero *et al.*, 1976). HT cells are located mainly in lamina I (but also laminae V and X) and project into the STT, the spinomesencephalic tract (SMT) with a major component projecting to the parabrachial region and the spinohypothalamic tract (SHT).

1.3.3 C-fibres

Fine, unmyelinated C-fibres afferents have small diameter cell bodies in the DRG (<30µm). Around half of these cells contain a range of transmitters (for example Battaglia & Rustioni, 1988; see Sorkin & Carlton, 1997 and Wilcox & Seybold, 1997 for review), although as discussed earlier (this Chapter, section 1.1.4), approximately 50% of small diameter DRG neurones do not contain peptides. These two different populations of nociceptors have distinct termination patterns in the dorsal horn (Hunt & Rossi, 1985; Silverman & Kruger, 1990). The peptidergic, TrkA-expressing nociceptors terminate in lamina I and II, whereas IB4-staining and Ret-immunoreactivity is restricted to lamina III (Molliver *et al.*, 1995; Molliver *et al.*, 1997). Whether this separation of nociceptive afferents is maintained through functional connectivity onto distinct subsets of second order neurones and projection pathways is unclear.

As discussed earlier, inputs from C-, Aδ- and Aβ-fibres converge on WDR neurones in the deep dorsal laminae. Although it may appear that the peripheral separation of noxious- from innocuous-evoked activity is compromised by this convergence, WDR neurones are still able to code for the intensity of the stimulus. Firing frequency increases as the intensity of the stimulus increases from innocuous through to noxious (see Dickenson *et al.*, 1997b; Sorkin & Carlton, 1997). Most WDR neurones respond to a range of modalities, not only to mechanical stimuli but also to noxious cold and heat, and receive input from muscle and visceral afferents as well as from the skin. Their receptive fields are therefore larger than less 'dynamic' or 'convergent' dorsal horn cells. WDR cells are found predominantly around lamina V, but also in laminae I and X and project into the STT, SMT, spinoreticular tract (SRT), SCT and SHT. Therefore, although lamina V WDR neurones may receive direct input from Aβ-fibre afferents, multi-synaptic pathways are probably required to convey C- and Aδ-fibre input onto these dorsal horn cells. Such intrinsic spinal cord neurones are likely to utilise similar transmitters to the primary afferents and express receptors for a range of excitatory and inhibitory transmitters to allow modulation (Battaglia & Rustioni, 1992).

C- and Aδ-fibre nociceptors also converge on class 3B nociceptor-specific, HT cells. These cells are responsive to noxious mechanical stimulation and to noxious heat and cold but not to innocuous warming. HT cells are located mainly in lamina I (but also laminae V and X) and project into the STT, the SMT and the SHT.

C- and Aδ-fibres also synapse onto substantia gelatinosa (SG) neurones. The majority of cells in lamina II are interneurones projecting only to the surrounding segments and not supraspinally. The response properties of these neurones are complex and contradictory reports exist. A simple summary would be to say that there are two morphologically distinct populations, the islet and the stalked cells, both of which receive convergent afferent input, and are likely to represent inhibitory and excitatory interneurones, respectively (Dickenson *et al.*, 1997b).

Other classes of dorsal horn neurones, not discussed here, have complex response properties with both excitatory and inhibitory receptive fields, respond only to muscle or joint stimulation, receive input from both somatic and visceral afferents or respond exclusively to innocuous temperature or only to noxious thermal stimuli. These types of dorsal horn cells, as well as the better characterised and understood neurones, are described in more detail in Sorkin & Carlton (1997).

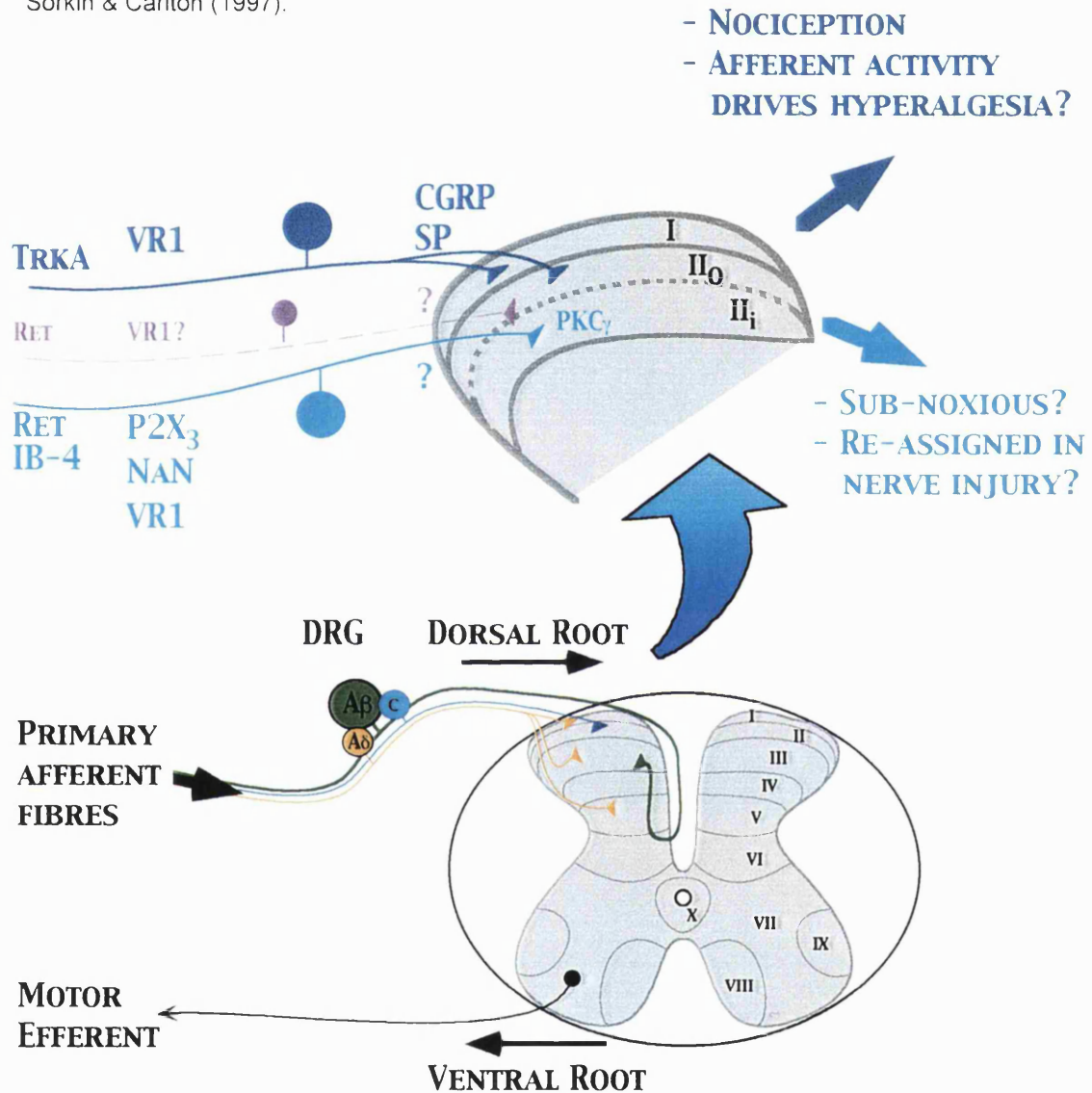


Figure 1.3 Termination patterns of primary afferent sensory fibres in the lumbar cord.

1.3.4 Transmitter profile of C-fibre afferents

Terminals arising from small diameter DRG cell bodies contain both small clear and large dense granular vesicles, indicating the co-transmission of glutamate and peptides. Glutamate-containing terminals are more numerous than those containing aspartate, and

these amino acids do not co-localise (Merighi *et al.*, 1991). DRG neurones immunopositive for peptides tend to be small diameter and the co-localisation of CGRP and tachykinins, CGRP and somatostatin, and tachykinins and somatostatin to single secretory vesicles has been demonstrated (Battaglia & Rustioni, 1988; Merighi *et al.*, 1988; Merighi *et al.*, 1989; Merighi *et al.*, 1991; Plenderleith *et al.*, 1990) and see (Wilcox & Seybold, 1997; Sorkin & Carlton, 1997 for review). Other putative transmitters localised to nociceptor terminals include the peptides N-acetyl-aspartylglutamate (NAAG), thyrotropin-releasing hormone (TRH), corticotrophin-releasing hormone (CRF), oxytocin, vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), cholecystokinin (CCK), nociceptin/orphanin FQ (NC/OFQ), galanin and possibly a number of other mediators such as adenosine and the potential for the production of nitric oxide (NO).

The finding that more than one peptide is localized to the same secretory granule supports the idea that peptides are co-released upon nerve stimulation. This correlates with physiological and pharmacological data demonstrating an interaction between different peptides in the modulation of synaptic activity. The transmitters listed above are also present in the superficial dorsal horn in intrinsic spinal neurones or descending pathways, along with the opioid peptides enkephalin, dynorphin, the monoamines dopamine, noradrenaline, adrenaline and serotonin, the inhibitory amino acids γ -aminobutyric acid (GABA) and glycine, acetylcholine (ACh), and neurotensin. Although most of these studies have been conducted in the rat, distribution of peptides in DRG, dorsal root and spinal cord laminae appears to be similar in most mammals studied, including primates (Bonfanti *et al.*, 1991; Merighi *et al.*, 1990).

1.4 Primary afferent fibre projection characteristics

The ascending pathways from dorsal horn cells are summarised in Figure 1.4, but for a more comprehensive review, see Sorkin & Carlton (1997). Briefly, evidence from lesion studies indicated that the major ascending pathway for conveying information about noxious stimuli was the spinothalamic tract. STT cells are mainly high threshold or WDR, and nuclei in the thalamus receive, integrate and project this information further. The spinomesencephalic tract is emerging as an important pathway, and the large area covered by the target area assignation 'mesencephalon' has received recent attention. A particular midbrain destination for many superficial high threshold nociceptive dorsal horn neurones is the parabrachial nucleus (PBN) (Bester *et al.*, 1995; Bester *et al.*, 1997b; Bester *et al.*, 2000; Iwata *et al.*, 1998; Menendez *et al.*, 1996). As the source of projections to limbic forebrain structures (hypothalamus, amygdala and preoptic area) (Bester *et al.*, 1997a; Bester *et al.*, 1999; Chamberlin *et al.*, 1999) the PBN could be an important determinant of the emotive and motivational responses to 'pain'. The PBN is also subject to modulation from periaqueductal grey (PAG) efferents (Krout *et al.*, 1998). Further discussion of the anatomy of supraspinal processing of nociceptive information is beyond the scope of this thesis. The reader is directed to several recent and comprehensive reviews (Sorkin & Carlton, 1997)

The mechanisms and circuits that are involved in generating the perception of pain are still unclear, but important areas are emerging. Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) studies in animals and humans of brain activity in response to noxious stimuli have mapped important areas. These include the thalamus, somatosensory cortices I and II (SI and SII), anterior cingulate gyrus, insula, prefrontal cortex, lentiform nucleus and the cerebellum (Bushnell *et al.*, 1999; Casey, 1999; Davis, 2000; Hudson, 2000; Ingvar, 1999; Jones, 1999; Laurent *et al.*, 2000; Treede *et al.*, 2000). SI and SII are most likely to be responsible for the sensori-discriminatory aspects and the anterior cingulate cortex the most likely candidate for generating the affective and motivational aspects of pain perception.

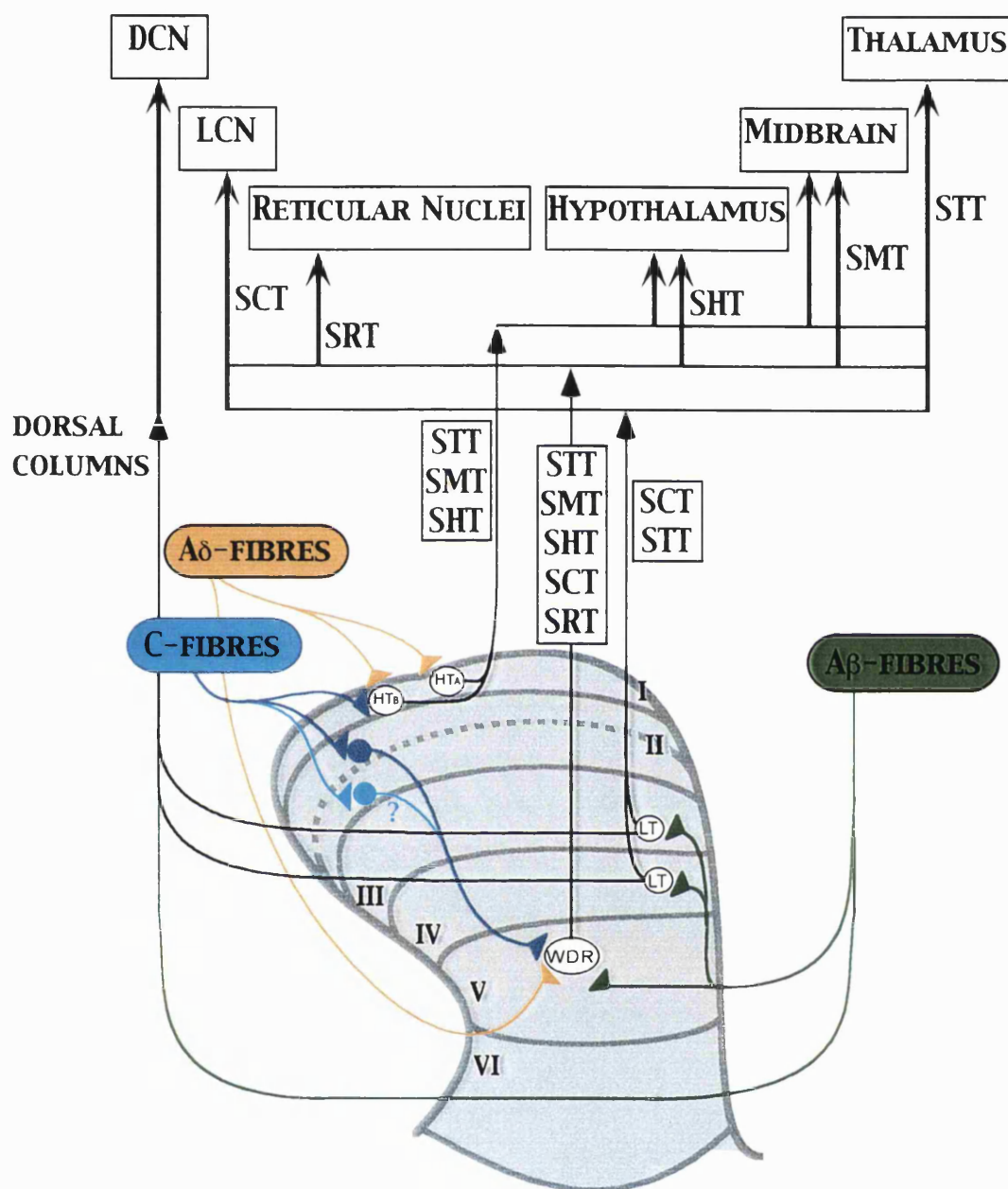


Figure 1.4 Summary of projection pathways taken by three different types of dorsal horn neurone. LT, low threshold cells; HT, high threshold cells (HT_A and HT_B); WDR, wide dynamic range cells. STT, spinothalamic tract; SMT, spinomesencephalic tract; SHT, spinohypothalamic tract; SCT, spinocervical tract; SRT, spinoreticular tract. These tracts project to the thalamus, midbrain, hypothalamus, lateral cervical nucleus (LCN) and reticular nuclei respectively. The dorsal columns project to the dorsal column nuclei (DCN). For more detailed summary see (Sorkin & Carlton, 1997).

1.5 Excitatory pharmacology of spinal processing

The pharmacology of the dorsal horn of the spinal cord is rich and complex. This provides a huge scope for both positive and negative modulation, and also provides the pharmacologist with many potential targets for manipulation. It is the balance between activity in the excitatory and inhibitory systems of the dorsal horn that determines the 'amount' of information entering ascending tracts to the higher centres.

1.5.1 Peptides

Present in terminals and released by a distinct population of nociceptive afferents, substance P and CGRP are the predominant excitatory peptide transmitters in the dorsal horn. Due to the lack of pharmacological tools, SP has been studied in more detail than CGRP.

Innocuous stimuli are insufficient to elicit release of SP. Noxious and persistent stimuli that activate C-fibre afferents are required to evoke release of SP (Duggan *et al.*, 1987; Duggan *et al.*, 1988; Go & Yaksh, 1987; Schaible *et al.*, 1990; Klein, 1992). Once released, SP can diffuse large distances and persist for up to an hour (Hope *et al.*, 1990). Evoked release of SP in the superficial dorsal horn has been seen both to be reduced by opioids (Go & Yaksh, 1987; Yaksh *et al.*, 1980) and to be unaffected by opioids (Morton *et al.*, 1990). More recently, modern techniques have again provided evidence for noxious-evoked release of SP from C-fibre afferent terminals (Allen *et al.*, 1997; Trafton *et al.*, 1999). The requirement for a noxious stimulus for release is not purely because peptides are located in terminals of high threshold C-fibre afferents, but also that neuropeptide vesicles are stored at some distance from release sites, ensuring that peptides may not be released from the afferent fibre terminal except in conditions of high frequency stimulation and significant Ca^{2+} entry (Nusbaum *et al.*, 2001).

Three tachykinins are derived from the preprotachykinin peptide, SP, neurokinin A (NKA) and NKB, which prefer the tachykinin receptors NK_1 , NK_2 and NK_3 respectively, although a large degree of cross-affinity exists (Radhakrishnan & Henry, 1997). Important neuronal targets for released substance P include the NK_1 -expressing HT lamina I neurones that project via the SMT to the PBN and via the SRT to the reticular formation (Todd *et al.*, 2000). NK_1 -expressing neurones in deeper laminae may also send dendrites to the superficial laminae and receive nociceptive input in this way. Thus both spinal and supraspinal circuits utilise the tachykinins. Neurones responsive to released SP from primary afferents are not absolutely required for normal responses to acute noxious stimuli, but are necessary for the generation of spinal hyperexcitability and manifestation of behavioural inflammatory hyperalgesia. Ablation of lamina I SP receptor-expressing neurones by saporin leaves acute nociception intact but eliminates hyperalgesia (Mantyh *et al.*, 1997). Both behavioural and electrophysiological pharmacological studies using NK_1

receptor antagonists have revealed a role for SP in generating spinal hyperexcitability. To cite several recent examples, NK₁ antagonists attenuate thermal hyperalgesia induced by carrageenan injection (Okano *et al.*, 1998), reduce pain behaviours in the formalin test (Henry *et al.*, 1999) and impair the induction of LTP of C-fibre field potentials, a putative experimental model of spinal hyperalgesia (Liu & Sandkuhler, 1997). Results from NK₁-knockout mice support the pharmacology, and these animals show maintenance of normal acute nociception, but reduced behavioural hyperalgesia (De Felipe *et al.*, 1998; Mansikka *et al.*, 1999; Mansikka *et al.*, 2000). Additionally, over-expression of SP leads to increased pain behaviours and hyperalgesia (McLeod *et al.*, 1999). However, both transgenic studies and classical pharmacological manipulation of the tachykinin system must be treated with caution as there could be a significant cross-over between the other tachykinins (NKA and NKB) and the receptors NK₁–NK₃.

The role of these other tachykinin receptors is beginning to emerge. For instance, NK₃ receptors are present on primary afferent terminals and enhance release of SP (Schmid *et al.*, 1998). Blockade of NK₃ receptors reduces inflammatory hyperalgesia (Zaratin *et al.*, 2000). It is worth noting that presynaptic NK₁ receptors also exist (Li & Zhao, 1998) where they may have an autoregulatory role.

Despite convincing pre-clinical evidence that substance P and its receptors form an important part of the nociceptive pathway, NK₁ receptor antagonists have been disappointing in clinical trials for analgesia in a variety of clinical pain states. Substantial heterogeneity among mammalian tachykinin receptors is evident (Beresford *et al.*, 1991; Maggi, 1995) and has been blamed for the lack of efficacy as analgesics. Fortunately for the pharmaceutical companies that invested in these drugs, they are proving to be effective in other systems. Clinical trials are now underway with NK₁ antagonists in anti-emesis and depression. The complex issues are discussed in an excellent review by Hill (2000).

The tachykinin receptors couple to G proteins, and several intracellular pathways have been linked to tachykinin receptor activation (Quartara & Maggi, 1997; Wilcox & Seybold, 1997). For example, SP-induced calcium entry and potentiation of capacitative calcium entry might be mediated by IP₃/diacylglycerol (Zhou *et al.*, 2000). Downstream from Ca²⁺ entry, processes that may lead to spinal hyperexcitability include the release of PGE₂, activation of calcium-dependent kinases (perhaps PKC γ in particular, as it is restricted to lamina I and II), activation of nitric oxide synthase (NOS) and subsequent release of NO and activation of the arachidonic acid pathway. These processes will be discussed in more detail later, but one of the most important effects resulting from the release of excitatory peptides and subsequent receptor activation, is the recruitment of the NMDA receptor for glutamate.

A peptide with an emerging role in nociception is N-acetyl-aspartylglutamate (NAAG). However, as this peptide is intimately involved in glutamatergic signalling, it will be discussed with the glutamatergic pharmacology below.

1.5.2 Glutamate

Glutamate is arguably the most important excitatory transmitter in the vertebrate central nervous system and the cell surface receptors that mediate the effects of released glutamate are located at most excitatory synapses in the CNS. These receptors are divided into the slow, G-protein-coupled (metabotropic) receptors, and the fast, ligand-gated ion channels (ionotropic receptors). The latter class is again divided — according to the synthetic agonists initially used to characterise them — into AMPA receptors, mediating the majority of fast excitatory neurotransmission, kainate-preferring receptors and NMDA receptors. (See Hollmann & Heinemann, 1994; Ozawa *et al.*, 1998 for reviews).

The NMDA receptor has been studied most extensively over the last decade as a full set of pharmacological tools has become available, and it is perhaps the most interesting glutamate receptor in terms of physiology and pathology. This ligand-gated ion channel has a Na^+ and a high Ca^{2+} permeability and slow gating kinetics, such that its activation, and the ensuing Ca^{2+} influx is powerful enough to trigger long-term changes within and around that cell. The NMDA receptor is implicated in plasticity in many systems such as memory, epileptic activity, motor function, vision and spinal sensory transmission. Excessive NMDA receptor activation is thought to be responsible for some of the excitotoxic neuronal death seen after cerebral ischaemia, and is also implicated as a final mechanism of neuronal death in many neurodegenerative diseases (Coyle & Puttfarcken, 1993; Meldrum & Garthwaite, 1990).

The NMDA receptor is also important in modulation of nociceptive transmission in the spinal cord, its activation being crucial to the development of central hypersensitivity. There is evidence for involvement of the NMDA receptor in inflammatory pain (Haley *et al.*, 1990b), neuropathic pain (Mao *et al.*, 1993), allodynia (Yaksh, 1989) and ischaemic pain (Newell *et al.*, 1995). In these persistent pain states, where the relation between stimulus and magnitude of response breaks down, the NMDA receptor is vital both in establishing the state of heightened responsiveness, and in maintaining this state. As such, parallels can be made to the phenomenon of long-term potentiation (Rygh *et al.*, 2000). 'Wind-up' is an experimental technique used to study the development of spinal hypersensitivity, and although the term wind-up is often used interchangeably with hypersensitivity, they are not the same thing. Repeated application of a noxious stimulus increases the pain ratings reported in human psychophysical experiments, the electrophysiological correlate is the enhanced response of dorsal horn neurones to repeated electrical C-fibre stimulation (Davies & Lodge, 1987; Dickenson & Sullivan, 1987; Mendell, 1966).

Several determinants of NMDA receptor function allow it to play this pivotal role. A number of events must combine for its activation, hence it is often described as a 'coincidence detector'. Apart from binding of glutamate, glycine is also needed as a co-agonist for channel opening to occur (Johnson & Ascher, 1987). In addition, at resting membrane potentials, the channel is blocked by physiological concentrations of Mg^{2+} ,

preventing Ca^{2+} influx even when glutamate binds (Nowak *et al.*, 1984). To relieve the Mg^{2+} block, a sustained membrane depolarisation is required, such as occurs after prolonged intense C-fibre stimulation, for example resulting from peripheral hyperalgesia. Released glutamate can now open the NMDA receptor channel. The ensuing Ca^{2+} influx triggers a number of intracellular events (discussed in this Chapter, section 1.5.3), the end-point of which is the generation of a hyperexcited state in the neurone. Its response is then enhanced to any subsequent input, regardless of its nature. Thus, central sensitisation is thought to be responsible for allodynia associated with tissue injury, a phenomenon that cannot be explained by peripheral events alone.

The pivotal role of the NMDA receptor as a co-incidence detector in the plasticity of nociceptive transmission makes it an attractive target for development of new analgesics. However, the NMDA receptor is not restricted to spinal pain pathways and it is not surprising that NMDA receptor antagonists such as the channel blocker ketamine and competitive antagonists such as APV (AP-5) are associated with a range of adverse effects. Possible approaches to avoiding or lessening the adverse effects associated with global block of NMDA receptors are to target a particular receptor type by its subunit makeup, and to antagonise binding of glycine at the receptor complex. Both of these approaches to avoiding ubiquitous blockade of NMDA receptors are discussed in more detail in Chapter 3. Ifenprodil is a non-competitive NMDA receptor antagonist, selective for receptors containing the NR2B subunit (Williams, 1993). It and other similar compounds with this selectivity have reduced side effect profiles *in vivo* (Duval *et al.*, 1992). Therefore, if the functional significance of these various receptor types is determined, subunit selective drugs could target the relevant systems more accurately, reducing the side effects of ubiquitous NMDA receptor block.

Brain-derived neurotrophic factor (BDNF) appears to be an important modulator of NMDA receptor function, although its role is complex. BDNF fulfils the criteria used to define a neurotransmitter, and its expression in sensory ganglia is enhanced after inflammation and nerve injury (Lee *et al.*, 1999; Michael *et al.*, 1999; Wheeler *et al.*, 1998). BDNF is reported to enhance NMDA receptor function and to be required for the inflammation-induced development of hyperexcitability *in vitro* (Kerr *et al.*, 1999) and hypersensitivity *in vivo* (Mannion *et al.*, 1999). However, long-term spinal administration of BDNF is reported to markedly attenuate the development of allodynia and hyperalgesia after sciatic nerve constriction (Cejas *et al.*, 2000). Interestingly, in the primate, a discrete upregulation of BDNF in the inferior temporal cortex has been observed that is specific to declarative learning, suggesting involvement of BDNF in NMDA receptor-mediated learning and memory (Tokuyama *et al.*, 2000).

AMPA-preferring receptors, comprised of subunits GluR1-4, and kainate-preferring receptors, comprised of subunits GluR5-7 and KA1-2, are arranged as either homo- or heteromeric pentamers (Hollmann & Heinemann, 1994). mRNA coding for GluR1-4 subunits is found throughout the dorsal horn of the spinal cord, with strong expression of GluR2 and

to a lesser extent GluR1 in the superficial laminae of the dorsal horn (Furuyama *et al.*, 1993; Tölle *et al.*, 1993). Lower levels of mRNA coding for kainate-preferring subunits are found in the dorsal horn, with no GluR6 mRNA detected (Furuyama *et al.*, 1993). Both AMPA and kainate preferring subunits are found in dorsal root ganglia (Partin *et al.*, 1993; Sato *et al.*, 1993) with evidence for presynaptic AMPA and particularly kainate preferring receptors containing GluR5 subunits (Agrawal & Evans, 1986; Carlton *et al.*, 1998a; Huettner, 1990; Partin *et al.*, 1993).

Evidence points to a functional role of kainate-preferring receptors at both pre- and postsynaptic sites on dorsal horn neurones (Procter *et al.*, 1998; Stanfa & Dickenson, 1999). The role of presynaptic kainate-preferring receptors is increased under conditions of inflammation (Simmons *et al.*, 1998; Stanfa & Dickenson, 1999). This may result from the increased release of glutamate within the spinal cord (Sluka & Westlund, 1992; Sorkin *et al.*, 1992), leading to increased activation of putative presynaptic kainate receptors.

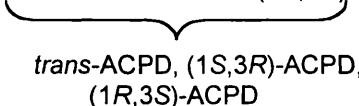
In contrast, a significant proportion of AMPA receptors are thought to be located postsynaptically in the spinal cord, on both intrinsic and projection neurones (Furuyama *et al.*, 1993; Tölle *et al.*, 1993; Ye & Westlund, 1996). Under normal conditions, AMPA receptors contribute to only fast, 'faithful' transmission of both innocuous and noxious stimuli. Following inflammation, AMPA receptors make a greater contribution to previously NMDA receptor-mediated spinal mechanisms of central amplification and wind-up (Simmons *et al.*, 1998; Stanfa & Dickenson, 1999; but see Hunter & Singh, 1994).

The majority of AMPA receptors are impermeable to Ca^{2+} (Bleakman & Lodge, 1998) but the absence of the GluR2 subunit confers Ca^{2+} permeability on AMPA receptors (GluR1-4) and significant levels of the Ca^{2+} permeable non-NMDA receptors are present in the adult CNS (Egebjerg & Heinemann, 1993; Sommer *et al.*, 1991). Functional Ca^{2+} -permeable AMPA receptors are found in the superficial laminae of the spinal cord, suggesting a role in nociceptive transmission (Engelman *et al.*, 1999; Spike *et al.*, 1998). Although Ca^{2+} entry through Ca^{2+} -permeable AMPA receptors in the spinal cord may enhance spinal nociceptive transmission (Gu *et al.*, 1996), recent studies have suggested a link between these receptors and inhibitory systems in the dorsal horn of the spinal cord (Spike *et al.*, 1998; Stanfa *et al.*, 2000a). This is consistent with anatomical data suggesting that GABAergic interneurons in the superficial dorsal horn express Ca^{2+} -permeable AMPA receptors (Albuquerque *et al.*, 1999; Spike *et al.*, 1998). The localisation and functional role of these receptors clearly differs from the distinct roles of the other ionotropic glutamate receptors in the spinal cord. Ca^{2+} -impermeable AMPA receptors mediate the fast basal excitatory response to nociceptive and non-nociceptive stimuli, NMDA receptors are responsible for the spinal amplification of nociceptive responses, and kainate receptors play a facilitatory role, possibly at presynaptic receptors on primary afferent fibres (see references in Dickenson, 1997). This diversity in the spinal receptors for glutamate, each with a distinct and defined role, allows this transmitter to play a complex role in nociceptive

transmission and modulation, with much scope for plasticity in the balance between excitatory and inhibitory pathways in chronic pain states.

The G-protein coupled (metabotropic) receptors for glutamate generate even more diversity in excitatory amino acid transmission. These mGlu receptors mediate the slower modulatory events, and can be divided into 3 groups; group I (mGlu1 and 5), group II (mGlu2 and 3) and group III (mGlu4, 6, 7, 8), as shown below.

Table 1.2 Classification of metabotropic glutamate receptors

	Group I	Group II	Group III
Receptors	mGlu1 + 5	mGlu2 +3	mGlu4, 6-8
G-protein	G _{q/11}	G _i /G _o	G _i /G _o
Effector	↑ IP ₃ and DAG	↓ cAMP	↓ cAMP
Agonists	DHPG (RS)-3,5-DHPG	(1S,3S)-ACPD L-CCG-I LY354740 (2R,4R)-APDC	L-AP4 ACPT-I 4CI-3,5-DHPG L-SOP
			
Antagonists	L-AP3 L-AP3 MCPG, LY393053	EGLU MCCG LY341495	MAP4 MSOP CPPG
			
			

Information taken from the 2000 edition of the *Trends in Pharmacological Sciences Nomenclature Supplement* and TOCRIS information sheet 'Muddling through the mGlu Maze'.

Pharmacological tools for probing the function of these receptors are far from perfect, but relatively selective compounds do exist, as detailed above. The group III mGlu receptors are least well characterised as pharmacological tools are still limited, although a role in nociception is likely as mGlu7 receptor-like immunoreactivity has been demonstrated on terminals of nociceptive primary afferents in superficial laminae of the spinal cord (Li *et al.*, 1997; Ohishi *et al.*, 1995). Much evidence points to a role for group I receptors in the transmission and positive modulation of nociceptive information in the spinal cord (Boxall *et al.*, 1996; Chen *et al.*, 2000; Dolan & Nolan, 2000; Neugebauer *et al.*, 1994; Neugebauer *et al.*, 1999; Stanfa & Dickenson, 1998; Ugolini *et al.*, 1999; Vidnyanszky *et al.*, 1994; Young *et al.*, 1997; Young *et al.*, 1998). However, both facilitation and inhibition of evoked neuronal

responses has been demonstrated on application of the mixed group I/II agonist *trans*-ACPD and its isomers (Cuesta *et al.*, 1999; Stanfa & Dickenson, 1998) and inhibition of peptide release from primary afferent terminals by the group I agonist DHPG (Cuesta *et al.*, 1999). A similar duality has been demonstrated for group I mGlu receptors in neurotoxicity and neuroprotection (Nicoletti *et al.*, 1999).

The role of group I mGlu receptors in nociception is therefore complex. It has been suggested that group I mGlu receptors are expressed on GABAergic interneurons, and that facilitations result from disinhibition (Jia *et al.*, 1999). It is also possible that much of these conflicting results stem from the complex pharmacology, whereby the use of non-specific agonists is justified by demonstrating reversal using more selective antagonists. One recent study used intrathecal administration of blocking-antibodies to group I mGlu receptors, an approach that had no effect on acute noxious tests or the formalin test, but attenuated the development of cold allodynia in a model of neuropathy (Fundytus *et al.*, 1998).

In contrast, the role of group II mGlu receptors appears to be predominantly antinociceptive (Dolan & Nolan, 2000), especially after inflammation (Stanfa & Dickenson, 1998). The mGlu2 and 3 receptors are inhibitory. Activation of the mGlu3 receptor reduces cAMP formation and inhibits presynaptic voltage-dependent Ca^{2+} channels (Glaum & Miller, 1995). The mGlu3 receptor may be of particular interest in nociception. Its discrete expression in the dorsal horn is enhanced after the development of peripheral inflammation (Boxall *et al.*, 1998) where it may function as an autoregulatory control on the excessive glutamate release seen in inflammation. Group II mGlu receptors are also expressed on spinal cord astrocytes, and may play a role in neuronal–glial cell signalling (Silva *et al.*, 1999).

While we wait for development of drugs that can manipulate the complex and many-faceted involvement of glutamate in nociceptive transmission and modulation, an endogenous neuropeptide may already possess the ideal pharmacological profile. N-acetyl-aspartylglutamate (NAAG) is a neuropeptide localised in subsets of glutamatergic, GABAergic, cholinergic, noradrenergic and serotonergic neurones in the mammalian brain and spinal cord. It fulfils the criteria used to define a neurotransmitter: it is stored in synaptic vesicles at the presynaptic terminal (Williamson & Neale, 1988), exhibits Ca^{2+} dependent release (Zollinger *et al.*, 1988; Zollinger *et al.*, 1994), is removed from the synapse both by direct uptake (Cassidy & Neale, 1993a) and by enzymatic degradation (Robinson *et al.*, 1987) and has a very interesting pharmacological profile.

NAAG is an agonist at the inhibitory mGlu3 receptor (Wroblewska *et al.*, 1997) and a partial agonist at the NMDA receptor (Puttfarcken *et al.*, 1993; Sekiguchi *et al.*, 1992), where the high affinity but low efficacy of NAAG can interfere with normal glutamatergic transmission. This profile therefore confers a predominantly inhibitory function. However, the breakdown of NAAG by NAALADase (N-acetylated- α -linked-acidic dipeptidase, also known as glutamate carboxypeptidase II) liberates NAA and glutamate, so NAAG may also function

as a source of extra-synaptic glutamate. (Fuhrman *et al.*, 1994; Slusher *et al.*, 1992). NAALADase is bound to the extracellular membrane of astrocytes (Cassidy & Neale, 1993b), and therefore regulates the balance between levels of synaptic NAAG and extra-synaptic glutamate. This activity of NAALADase is altered, and thereby implicated, in many disease states that involve dysregulation of glutamatergic transmission including epilepsy, Huntington's Disease and Alzheimer's Disease, amyotrophic lateral sclerosis (ALS) and schizophrenia (Coyle, 1997).

Protecting NAAG from breakdown by inhibition of NAALADase activity with the selective inhibitor 2-PMPA increases brain levels of NAAG and protects against ischaemic injury *in vitro* (Slusher *et al.*, 1999) and *in vivo* (Vornov *et al.*, 1999). Pharmacological strategies that are effective in neuroprotection paradigms can often translate into effective analgesic strategies, as an underlying factor in each condition is excessive glutamate release and excess excitability. Partial agonism at the NMDA receptor, as displayed by NAAG, may be an effective strategy to circumvent ubiquitous NMDA receptor blockade, and target only areas where NMDA receptor activation is excessive. There is little literature on the role of the mGlu3 receptor in nociception. However, emerging evidence as discussed above suggests that group II mGlu receptor agonists may be anti-nociceptive and the mGlu3 receptor is expressed strongly in laminae II–V of the dorsal horn and is enhanced after inflammation (Boxall *et al.*, 1998). Endogenous NAAG, the enzyme responsible for its breakdown and the receptor targets of released NAAG are all present in the dorsal horn of the spinal cord. Increasing NAAG levels using drugs like 2-PMPA to protect it from breakdown could be a valid strategy for analgesia.

The different functional roles of the spinal receptors for glutamate, roles that are subject to plasticity, allow this transmitter to play a complex role in nociceptive transmission and modulation. There is much scope for novel therapies and recent clinical studies have indicated that targeting the NMDA receptor may be an effective strategy in the control of pain (Sang, 2000). Similar advances could soon be made through targeting non-NMDA receptors, but the issue is still going to be side effects rather than efficacy. In this context, manipulation of an endogenous transmitter may be useful and thus investigation of NAAG in nociception will be illuminating, and may also elucidate the role of the mGlu3 receptor, which is still poorly understood.

1.5.3 Mechanisms of spinal hyperexcitability

The end result of spinal mechanisms of injury-induced hyperexcitability is generation of secondary hyperalgesia, lowering the threshold required for a 'painful' behavioural response. This can be observed, for instance, as thermal hyperalgesia after carrageenan inflammation in behavioural paradigms. Electrophysiological correlates include the observation that after inflammation, non-noxious stimulation can release substance P (Honore *et al.*, 1999), and facilitate the response of SG neurones to A β -fibre mediated inputs

(Baba *et al.*, 1999). Analogous to the situation in the periphery, interaction between different transmitters determines the state of excitability of the system. Elimination of any one single transmitter molecule or receptor may well have little effect on nociception unless that substance has a dominant role. So far, the only such substrate identified is the NMDA receptor for glutamate. Although this powerful receptor switch requires a specific combination of events for its activation, the parallel use of several transmitters ensures that elimination of any one is not sufficient to prevent NMDA receptor recruitment and subsequent hypersensitivity. It is possible that individual transmitter systems take on more dominant roles in particular pathological states. The release of glutamate (Sluka & Westlund, 1992; Sorkin *et al.*, 1992), SP (Henry *et al.*, 1999; Honore *et al.*, 1999; Schaible *et al.*, 1990) and CGRP (Calza *et al.*, 2000) is increased during inflammation, providing even more scope for interactions between these systems.

It is probably not an overstatement to say that the mechanisms discussed below all depend absolutely on NMDA receptor activation for their initiation and in turn, full development of NMDA receptor mediated hyperexcitability depends on each of these mechanisms. The key element is increased intracellular calcium ($[Ca^{2+}]_i$), which is not generated in sufficient amounts from any other source than influx through the NMDA receptor channel.

This hyperexcitability leads to the expansion of receptive fields that is thought to be crucial to the development of hyperalgesia, and possibly also allodynia. A more excitable dorsal horn neurone will respond to weak inputs — those that do not normally release enough transmitter to drive an action potential — expanding the peripheral receptive field and so increasing the peripheral area where stimulation will evoke a response. Thus, more dorsal horn cells will respond to a given stimulus, increasing the spinal nociceptive drive.

Ca^{2+} influx through NMDA receptors enhances receptor function (increased open probability). This feedback ' Ca^{2+} amplification' is thought to be mediated through PKC (protein kinase C) phosphorylation of sites in the C-terminus of the NR1 subunit (Dingledine *et al.*, 1999; Zheng *et al.*, 1997). Whereas PKC ϵ appears to be the major isoform responsible for sensitisation of nociceptors in the periphery (Aley *et al.*, 2000; Cesare *et al.*, 1999; Khasar *et al.*, 1999), a specific role for PKC γ has been suggested in spinal neurones. PKC γ is expressed in mostly non-GABAergic dorsal horn neurones, concentrated in lamina II, but also found from the ventral edge of lamina I to the dorsal edge of lamina III (Polgár *et al.*, 1999). Mice that lack PKC γ retain normal responses to acute noxious stimuli but exhibit reduced development of both behavioural and neurochemical changes characteristic of neuropathy after partial sciatic nerve ligation (Malmberg *et al.*, 1997b). PKC γ immunoreactivity increases in lamina II after chronic constrictive sciatic nerve injury in the rat. This can be blocked by chronic spinal administration of MK-801, lending support to the theory that PKC γ activity is injury- and NMDA receptor-dependent (Mao *et al.*, 1995). It

should be remembered that PKC may also mediate Ca^{2+} -dependent inactivation of the NMDA receptor (Lu *et al.*, 2000; Snell *et al.*, 1994).

Tyrosine kinases also enhance NMDA receptor function (Chen & Leonard, 1996; Lancaster & Rogers, 1998; Xiong *et al.*, 1999; Yu & Salter, 1999; Yu *et al.*, 1997), and may in fact mediate the effects of PKC on NMDA receptor activity (Lu *et al.*, 1999; Zheng *et al.*, 1999). Tyrosine kinases such as Src may mediate the NMDA receptor-potentiating effects of BDNF, and G-protein independent effects of mGlu receptor activation may include signalling through the Src family of tyrosine kinases (Heuss *et al.*, 1999).

Many of the transmitter systems previously discussed act to increase intracellular levels of cAMP; activation of PKA by cAMP may also enhance NMDA receptor function, but the mechanism is still not clear (see for example Urban *et al.*, 1994). A neuronal-specific modulatory subunit of PKA ($\text{R1}\beta$) is required for the development of inflammatory, but not neuropathic pain (Malmberg *et al.*, 1997a), in contrast to the situation described above for $\text{PKC}\gamma$. It is an intriguing possibility that in different injury states, distinct subsets of afferent neurones may drive the development of dorsal horn hyperexcitability by distinct intracellular pathways.

Prostaglandins are generated as increased $[\text{Ca}^{2+}]_i$ activates phospholipase A_2 and leads to formation of COX (cyclooxygenase) and LOX (lipoxygenase) products. Spinal application of NSAIDs is effective in attenuating spinal hyperexcitability and behavioural hyperalgesia (Malmberg & Yaksh, 1992). Of the inflammatory mediators generated, PGE_2 has again received much attention. Noxious thermal stimulation (Coderre *et al.*, 1990), peripheral formalin injection (Malmberg & Yaksh, 1995), induction of chronic inflammation in the knee joint (Yang *et al.*, 1996), and intrathecal application of SP (Hua *et al.*, 1999) can all increase spinal levels of PGE_2 . Application of NMDA receptor antagonists, NK_1 receptor antagonists and morphine can all attenuate this release, again demonstrating the importance of interactions between several systems rather than the absolute importance of any one system. Released PGE_2 may function, like NO, as a retrograde messenger in the spinal cord, effecting changes in the presynaptic terminal (Park *et al.*, 2000). Upregulation of PGE_2 receptors in the spinal cord has been demonstrated in a naturally occurring chronic inflammation, mastitis in the sheep (Dolan *et al.*, 2000).

Nitric oxide synthase (NOS) is expressed by spinal cord neurones, and its activation is calcium-dependent. There is much evidence that NO plays a role in nociceptive transmission, particularly after inflammation or after noxious stimuli sufficient to activate the NMDA receptor and thus generate sufficient increase in $[\text{Ca}^{2+}]_i$ (Chapman *et al.*, 1995; Gao & Qiao, 1998; Kawamata & Omote, 1999; Salter *et al.*, 1996; Stanfa *et al.*, 1996 and see Dickenson, 1997). Extended periods of inflammation and nerve injury can also cause an increased expression of both iNOS and nNOS in the rat but not in primates (Wu *et al.*, 1998; Zhang *et al.*, 1993). Newly generated NO has been demonstrated to evoke release of glutamate (Sorkin, 1993) and peptides (Aimar *et al.*, 1998; Garry *et al.*, 1994) in the dorsal

1.6 Inhibitory pharmacology of spinal processing

1.6.1 Inhibitory amino acids

GABA (γ -aminobutyric acid) is the major inhibitory neurotransmitter in the mammalian CNS and tonic GABA activity is essential for keeping neuronal activity in check. The effects of GABA are mediated via the GABA_A and GABA_C receptors which are ligand-gated chloride channels and the GABA_B receptor coupled via G-proteins to inhibition of Ca²⁺ influx. In the spinal cord, GABAergic terminals are concentrated in the superficial dorsal horn, and arise mainly from intrinsic interneurons with some contribution from descending tracts (Sorkin & Carlton, 1997). Glycine, galanin, 5-HT, somatostatin and enkephalin can all be found co-localised with GABA in the dorsal horn, and GABA is clearly localised to different populations of neurones than excitatory transmitters. An important drive for GABAergic neurones is afferent fibre input, and GABAergic terminals also make reciprocal synaptic contact with afferent fibre terminals (Dickenson *et al.*, 1997a; Merighi *et al.*, 1989). GABAergic control of afferent input is apparently activity dependent, and the spinal GABA systems appear to be near-maximally activated under normal conditions. GABA inhibition controls both C- and A-fibre input, normal controls on low threshold input appear to be particularly important, as inhibition of GABA receptor blockade can allow normally innocuous stimuli to evoke nociceptive responses (Dickenson *et al.*, 1997a). Loss of GABAergic control may be of particular importance in the development of neuropathic pain states (Kontinen & Dickenson, 2000), whereas enhanced GABA inhibitions after inflammation act to attenuate the NMDA-receptor mediated hyperexcitability (Dickenson *et al.*, 1997a; Green & Dickenson, 1997). Supraspinally, GABA continues to play a major role in tonic control of nociception, with a particular function in modulating OFF-cell activity (see later in this section), such that supraspinal GABA_A agonist application leads to hyperalgesia (Hammond & Graham, 1997).

Glycine-like immunoreactivity is localised in the dorsal horn, and most superficial glycinergic neurones also contain GABA. The effects of strychnine clearly demonstrate the importance of glycinergic inhibitory tone in the CNS. Spinal glycine inhibition in rats causes allodynia (Yaksh, 1989), and the role of glycine in nociceptive modulation is similar to that of GABA. Glycine is also required as a co-agonist at the NMDA receptor.

1.6.2 Opioids

The striking effectiveness of opioids as analgesics demonstrates the important role of this endogenous inhibitory system in the modulation of nociceptive information. Opioid receptors are divided into the μ , δ and κ opioid receptors (MOR, DOR and KOR respectively). The receptors have been cloned, and demonstrate a high degree of sequence homology. All three receptors have a seven transmembrane domain structure and couple via PTX sensitive (G_i/G_o) G-proteins to inhibit cAMP formation and to increase K⁺ channel

currents. Elevation of cAMP formation has also been observed for opioid receptors under some conditions (Shen & Crain, 1990), and coupling to PLC stimulation (Lee *et al.*, 1998) has also been reported.

Table 1.3 Opioid receptors, their ligands, and physiological effects

	μ (MOR)	δ (DOR)	κ (KOR)
Endogenous agonist	endorphins	enkephalins	dynorphins
Exogenous agonists	morphine DAMGO	DPDPE	enadoline
Antagonists	naloxone CTAP	naloxone naltrindole	naloxone nor-BNI
Physiological effects	analgesia, ↓GI motility, respiratory depression, sedation, nausea, euphoria	analgesia sedation	analgesia? dysphoria

DAMGO = [D-Ala², N-Me-Phe⁴, Gly-oI⁵]enkephalin; DPDPE = [D-Pen², D-Pen⁵]enkephalin;
CTAP = D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (cyclised between Cys and Pen)

Opioid receptors are distributed heterogeneously in the CNS and are particularly abundant in areas concerned with nociceptive processing (spinal cord, thalamus and PAG). In the spinal cord, opioid receptors are restricted mainly to the superficial dorsal horn (laminae I and II), with a smaller population in deeper layers (Besse *et al.*, 1990; Rahman *et al.*, 1998). The μ opioid receptor is most prevalent, representing about 70% of all opiate binding in the spinal cord, with the DOR making up 24% and the KOR 6% (Besse *et al.*, 1990). This distribution is maintained from the cervical to the lumbar spinal cord (Besse *et al.*, 1990). The majority (>70%) of opioid receptors are located presynaptically on the central terminals of primary afferent fibres and are only expressed by small diameter nociceptive primary afferents (C- and A δ -fibre). This selective expression means that both endogenous and exogenously applied opioids selectively suppress transmitter release from nociceptive afferents while innocuous-evoked activity is relatively little affected. Spinal application of morphine has been demonstrated to reduce SP and CGRP release after noxious stimulation (Go & Yaksh, 1987; Yaksh *et al.*, 1980; but see Morton *et al.*, 1990). More recently, recordings from spinal cord slices have demonstrated that opioids suppress excitatory but not inhibitory synaptic transmission in adult rat SG neurones and these actions are presynaptic in origin (Kohn *et al.*, 1999). Postsynaptic MORs have been demonstrated and visualised functionally as agonist-stimulated receptor internalisation (Trafton *et al.*, 2000), although it is generally accepted that opioid receptors on central terminals of nociceptive afferents constitutes the main site of action of spinal opioids (Lombard & Besson, 1989; Ossipov *et al.*, 1997).

Important supraspinal sites of opioid action are the midbrain and brainstem structures the PAG and the RVM (rostromedial medulla). Exogenous injection of morphine into either of these sites causes antinociception via increased activity in inhibitory descending controls terminating in the dorsal horn of the spinal cord (Heinricher, 1997; Yaksh, 1997 for reviews). Electrical stimulation or glutamate application to these sites also causes nociception, so morphine would appear to act through disinhibition to increase outflow from both sites. An influential hypothesis to explain this circuitry concerns the electrophysiological identification of two major populations of RVM output neurones: ON cells whose activity coincides with spinal nociceptive reflexes and OFF cells whose activity is associated with suppression of these reflexes. Infusion of morphine into the RVM is associated with a pronounced reduction in activity of ON cells and increased activity of OFF cells, supporting the pro- and anti-nociceptive labels assigned to ON and OFF cells respectively. ON cells are thought to express opioid receptors and are thus inhibited directly by morphine injection into the RVM, and when inhibitory PAG-RVM outputs are stimulated by morphine injection into the PAG. OFF cells are excited by PAG output and by opioid-mediated disinhibition. There are also important interactions between 5-HT and nitric oxide in the PAG. NO appears to be required for the 5-HT-mediated inhibition of PAG output and reversal of antinociception (Hamalainen & Lovick, 1997). The circuitry between the PAG and the RVM is complex, and is illustrated in a simple form in Figure 1.6. When combined with the ascending pathways discussed in section 1.4, a feedback loop in the modulation of nociceptive information becomes apparent.

Fibres descending from the RVM to the dorsal horn of the spinal cord are mostly serotonergic, enkephalinergic, glycinergic and GABAergic. The nucleus raphe magnus (NRM) and the noradrenergic nuclei (locus coeruleus, subcoeruleus, A5 and A7 cell groups) are major PAG relays for noradrenergic and serotonergic descending pathways, respectively, to the dorsal horn (Kwiat & Basbaum, 1992). Rather than the RVM being a homogeneous population of serotonergic neurones, GABA- (and glycine-) releasing neurones are now thought to constitute a significant proportion of spinally-projecting NRM fibres (Antal *et al.*, 1996). The pharmacology of noradrenergic and serotonergic modulation in the dorsal horn is complex. Receptor diversity leads to a large degree of diversity in the effects of applied 5-HT. Selective receptor expression determines the excitatory or inhibitory effect of released 5-HT in particular pathways.

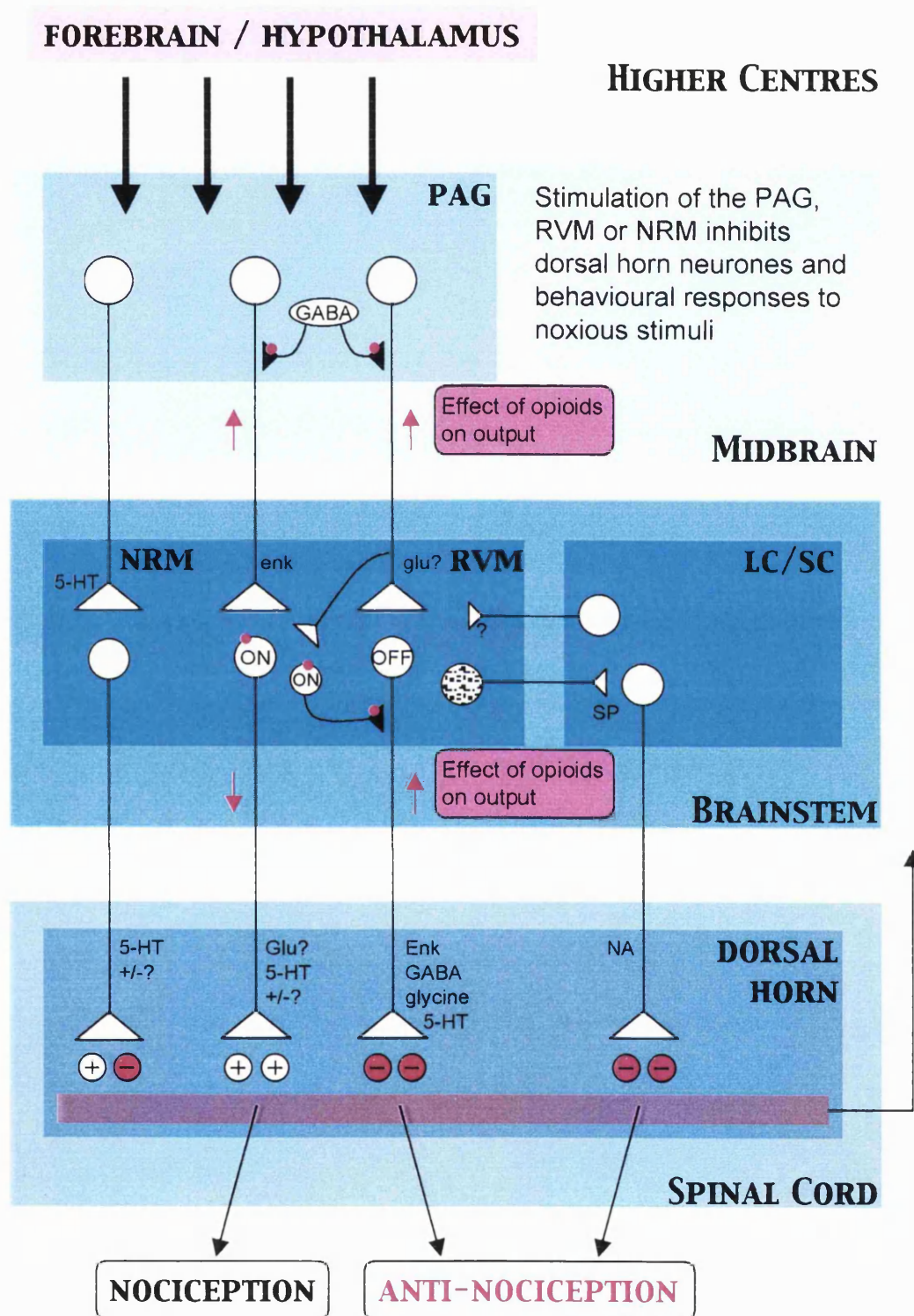


Figure 1.6 Schematic diagram of the descending circuits involved in PAG/RVM mediated antinociception. PAG, periaqueductal grey; RVM, rostroventromedial medulla; LC/SC, locus coeruleus/subcoeruleus and other noradrenergic cell groups; enk, enkephalin; glu, glutamate; NA, noradrenaline. pink circles, μ opioid receptors.

The ability of opiates to relieve pain has been exploited by man for at least 6000 years, as have their powerful euphoric and somnolent properties. Morphine, the main active component in crude opium, has some affinity for all three opioid receptors, with a pronounced preference for μ over δ and κ receptors. Attempts to separate the analgesic properties of opiates from their other CNS effects — the clinically problematic respiratory depression, nausea and sedation, or the perceived problems of dependence and euphoria — have been unsuccessful. All these effects, as well as the peripherally mediated constipatory effects of morphine are mediated via the μ opioid receptor. In an effort to avoid these unwanted central effects, agents selective for the δ and κ receptors have been investigated as potential analgesics, but with limited success. It was the search for other (hopefully more 'pain' specific) opioid receptors (or subtypes) that led to the discovery of the orphan opioid receptor-like (ORL1) receptor in the mid 1990s.

1.6.3 Nociceptin/orphanin FQ

Homology screening of the cloned DOR led to the identification of an 'orphan' opioid receptor (Wick *et al.*, 1994 and references in Meunier, 1997). Based on the high degree of structural homology between this receptor and the opioid receptors, it was termed the opioid receptor-like receptor 1 (ORL1). Since then various other names have been suggested, and IUPHAR guidelines now suggest the nomenclature OP4 be adopted (OP1, 2 and 3 refer to the μ , δ and κ receptors respectively). I will continue to use ORL1. The ORL1 is a seven-transmembrane spanning receptor, and couples via pertussis toxin-sensitive G-proteins to inhibit cAMP formation. Pharmacologically, the ORL1 is quite distinct from the opioid receptors, exhibiting low affinity for opioid receptor ligands including the endogenous opioids and the antagonist naloxone (Wang *et al.*, 1994; Wick *et al.*, 1994). ORL1 mRNA is present in the dorsal spinal cord and dorsal root ganglia (Neal *et al.*, 1999a) and functional receptors (activation of GTP γ S binding) have also been demonstrated in the superficial dorsal horn in mouse spinal cord (Narita *et al.*, 1999). Nociceptin/orphanin FQ (NC/OFQ) is the endogenous ligand for the ORL1 receptor (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), and is generated from the precursor peptide prepronociceptin. NC/OFQ-like immunoreactivity and prepronociceptin mRNA are detected in the superficial dorsal horn (Narita *et al.*, 1999; Neal *et al.*, 1999b) and to a lesser extent in the DRG (Andoh *et al.*, 1997). The spinal actions of exogenously applied NC/OFQ are predominantly inhibitory, similar to the opioids. A significant degree of postsynaptic activity has, however, been suggested. This is discussed in more detail in Chapter 6.

NC/OFQ and opioid peptides exhibit an overlapping but distinct distribution in areas concerned with modulation of nociception, but do not co-localise (Schulz *et al.*, 1996). Although many parallels between NC/OFQ and the endogenous opioids (particularly dynorphin) have been suggested, expression of these peptides is affected differently by peripheral insult (inflammation and nerve injury) (Rosen *et al.*, 2000). Together, these

suggest that NC/OFQ has a role distinct from that of the opioids in the modulation of nociceptive information. The distribution of both the peptide and its receptor in supraspinal and peripheral sites has also suggested a role for NC/OFQ in stress and anxiety, learning and memory, audition, circadian rhythm generation, feeding behaviour, gastrointestinal motility, the immune system and cardiovascular and renal effects.

1.6.4 Cannabinoids

Two cannabinoid receptors have been identified, CB₁ and CB₂, which are predominantly distributed in the CNS and periphery respectively. CB₁ receptors have been identified in the spinal cord and, in contrast with the opioids, cannabinoid binding sites appear to be evenly distributed between the central terminals of primary afferents and postsynaptic structures (Hohmann *et al.*, 1999). There is increasing pharmacological evidence that spinal CB₁ receptors contribute to endogenous inhibitory tone in the spinal cord (Chapman, 1999; Drew *et al.*, 2000; Fuentes *et al.*, 1999; Welch & Stevens, 1992) and that activity in this system is increased in response to injury (Martin *et al.*, 1999). Spinal antinociceptive effects of cannabinoids may be mediated in part via release of dynorphins (Houser *et al.*, 2000; Mason *et al.*, 1999). Central antinociceptive effects of endogenous cannabinoids appear to involve, as for opioids, the modulation of RVM neuronal activity (Meng *et al.*, 1998). The results obtained from studies of cannabinoid CB₁ receptor knockout mice do not shed much light on the role of endogenous cannabinoids in nociception. Baseline nociceptive thresholds have been reported as unchanged (Valverde *et al.*, 2000) or increased (Zimmer *et al.*, 1999) in CB₁^{-/-} mice. This latter study also reports a reduction in pain behaviours in the first phase of the formalin test, indicative of analgesia. This is contrary to what would be expected from the pharmacology, and may result from compensatory changes in CB₂ receptor expression or in other receptor systems, or result from the reduced mobility of these animals (Zimmer *et al.*, 1999). Other CNS changes in CB₁ knockout mice include enhancement of LTP (Bohme *et al.*, 2000), improved memory (Reibaud *et al.*, 1999) and an apparent reduction in opioid reward (Ledent *et al.*, 1999; Mascia *et al.*, 1999).

1.6.5 Galanin

Galanin-like immunoreactivity is dense in the superficial dorsal horn and has been localised to primary afferent terminals, interneurons and projection neurons. In afferent terminals, galanin can be co-localised with SP, CGRP, CCK and NOS. The galanin receptors, of which there are three, have not yet been fully characterized, but couple to PTX-sensitive G-proteins (G_i/G_o). Spinal administration of galanin has complex but predominantly inhibitory/antinociceptive effects, which are enhanced in models of inflammatory and neuropathic pain (Xu *et al.*, 2000c and Xu *et al.*, 2000b for review).

1.6.6 Purines

There has been much interest in the idea that the purines ATP and adenosine (produced via ATP metabolism) play a role in nociceptive processing. The role of peripherally released ATP at the sensory neurone specific P2X₃ receptor was discussed in this Chapter, section 1.2.8 and, although P2X receptors may contribute to enhanced nociceptive transmission after inflammation, their role in spinal processing in normal animals is minimal (Stanfa *et al.*, 2000b and references therein).

The involvement of adenosine in spinal processing is clearer, and clinical investigations are optimistic (Dickenson *et al.*, 2000). It is unclear whether adenosine is actually stored and released in a classical manner rather than ^{generated}by metabolism of ATP, but adenosine-like immunoreactivity has been detected in the SG as have the enzymes required for release of adenosine from ATP. Three receptors for adenosine have been characterised, A₁ couples to G_i/G_o intracellular processes whereas A₂ and A₃ couple to G_s. Of these, the A₁ receptor appears to be most important in mediating the antinociceptive effects of spinally applied adenosine and adenosine analogues (Honore *et al.*, 1998; Sawynok, 1998; and Dickenson *et al.*, 2000 for review). Interestingly, adenosine is able to inhibit NMDA receptor-mediated hyperexcitability and is effective in models of inflammatory and neuropathic pain (Dickenson *et al.*, 2000). Interactions between adenosine and serotonergic modulation are apparent (Sawynok & Reid, 1996).

1.6.7 Inflammation induces changes in inhibitory systems

Just as the excitatory systems of the spinal cord exhibit plasticity in the face of persistent noxious input, so do the inhibitory systems. The balance between the level of activity in excitatory and inhibitory systems determines the final 'amount' of information entering the ascending pathways. In general, activity in descending inhibitory control is increased by persistent, but 'normal' noxious input, such as that generated by tissue injury and inflammation. Either their activity or their effectiveness in balancing the excitatory processes is compromised in 'abnormal' persistent pain states like nerve injury.

Increased inhibitory tone from descending controls is a salient feature of the response to afferent-driven spinal hyperexcitability (Dubner & Ren, 1996). For example, increased release of 5-HT is detected in the spinal cord and PAG after carrageenan inflammation (Zhang *et al.*, 2000b). The interactions that occur between serotonergic, noradrenergic and purinergic controls will presumably be increased during inflammation.

The spinal potency of μ opioids — and to a lesser extent δ and κ — are enhanced rapidly after inflammation although no changes in receptor number or affinity have been described (Ossipov *et al.*, 1995; Stanfa & Dickenson, 1995; Stanfa *et al.*, 1992). Longer-term inflammation can lead to changes in expression of opioid peptides and their receptors (Calza *et al.*, 2000). Interactions between opioid and peptide systems are thought to be the main source of altered opioid potency in inflammation. The peptide cholecystokinin (CCK)

has anti-opioid effects and diminished levels of this peptide contribute to the enhanced effects of morphine after inflammation (Stanfa & Dickenson, 1993; Watkins *et al.*, 1985; Wiesenfeld-Hallin *et al.*, 1999). Co-localisation of CCK receptors and the μ opioid receptor has recently been demonstrated in lamina I and II neurones (Zhang *et al.*, 2000a), providing an anatomical opportunity for interaction. The potential role of another anti-opioid peptide, neuropeptide FF (NPFF), is less clear, although changes in the NPFF system have been observed after inflammation (Kontinen *et al.*, 1997; Panula *et al.*, 1999).

α_2 -Adrenoceptor agonists can augment the antinociceptive effects of spinal opioids in both behavioural and electrophysiological studies, and may contribute to the enhanced effects of spinal opioids after inflammation (Stanfa & Dickenson, 1995 for review). These systems interact, especially in persistent pain states, as shown by the synergistic effects between μ opioid receptor agonists and α_2 -adrenoceptor agonists, respectively (Honore *et al.*, 1996).

It is recognised in the clinic and in behavioural and electrophysiological studies that the efficacy of opioids is reduced after nerve injury. A reduction in the number of presynaptic receptors is one contributory factor to this situation, but it is highly complex (Dickenson & Suzuki 2000). In contrast, endomorphin-1 and -2 have recently been demonstrated to be effective against allodynia induced by neuropathy, (Przewlocka *et al.*, 1999) and NC/OFQ is also effective (potentially even more so) after nerve damage (Hao *et al.*, 1998; Xu *et al.*, 1999; reviewed in Moran *et al.*, 2000; Xu *et al.*, 2000a).

GDNF (glial cell-line-derived neurotrophic factor), although it cannot be considered a transmitter system, does appear to alleviate pain of nerve injury origin. Expression of GDNF and its receptor components are upregulated after nerve injury (Bar *et al.*, 1998; Bennett *et al.*, 2000; Hammarberg *et al.*, 1996; Hoke *et al.*, 2000). Exogenous spinal application of GDNF reduces the development of neuropathic behaviours after surgery (Boucher *et al.*, 2000). This does not appear to be achieved through direct effects on nociceptive transmission, but through trophic prevention of maladaptive changes that occur following nerve injury. Accordingly, a general protective role is seen for GDNF. Spinal administration prevents degeneration of motor neurones after dorsal and ventral root avulsion (Watabe *et al.*, 2000) and spinal cord ischaemia in the rabbit (Sakurai *et al.*, 2000). Direct application onto axotomised nerve causes some improvement of conduction velocity in sensory motor fibres (Munson & McMahon, 1997).

1.7 Aims of Study

The rich pharmacology of peripheral and central terminals of nociceptive afferent fibres provide the pharmacologist with many potential sites for manipulation. Morphine, the 'gold standard' analgesic for moderate to severe pain, exploits only one facet of this by augmenting the natural inhibitory opioid controls on spinal nociceptive processing. The analgesic potential of opioids is limited in some clinical situations, and their use is not without problems. Attenuating the activity-dependent enhancement of nociceptive transmission that occurs in the dorsal horn is a particularly attractive proposition. NMDA receptor antagonists are showing limited clinical success in neuropathic pain but extensive side effects are a major concern.

Several approaches to modulating spinal hyperexcitability are described in this thesis. Potential contributors to the processes of peripheral hyperalgesia are also studied.

The aims of this thesis were to investigate alternative strategies to attenuate enhanced processing of nociceptive information through:

- circumventing ubiquitous NMDA receptor blockade (NR2B subunit and glycine-site-selective compounds)
- combination of NMDA receptor blockade with opioid receptor activation
- manipulation of the endogenous NAAG neuropeptide system and investigation of potential changes in models of persistent pain
- study of the change occurring in the NC/OFQ system during inflammation
- manipulation of the environment around peripheral nociceptor terminals.

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Chapter 2. Methods

2.1 Anaesthesia and surgery

Male Sprague-Dawley rats (University College London Biological Services), weighing 200–225g were used in all experiments. Methods were approved by the Home Office and performed under a valid Project and Personal Licence, following IASP guidelines (International Association for the Study of Pain). These methods have previously been described in detail, including monitoring of blood pressure and end-expiratory carbon dioxide levels. These were seen to remain stable throughout experiments and during application of noxious stimuli (Dickenson & Le Bars, 1987).

For anaesthesia, the volatile anaesthetic halothane was used in a 66%:33% mixture of N₂O and O₂. To induce general anaesthesia, animals were placed in a sealed box with inflowing 3–4% halothane until the animal showed loss of the righting reflex. The animal was then removed from the box and placed on its back on a heating blanket and anaesthesia maintained through a nose-cone (2.5–3% halothane). Areflexia was confirmed, the trachea was exposed and cannulated, and halothane was thereafter administered through the cannula directly into the trachea (2–2.5% halothane). The animal was then transferred to a stereotaxic frame, and the head secured with ear bars and a tooth bar. Body temperature was monitored using a rectal probe, and maintained at 37°C with a heating blanket coupled to the probe. The location of lumbar vertebrae L1–L3 was determined from the position of the ribs, a clamp was secured to the vertebrae rostral to this and clamped tightly to the side bar of the frame. A thin strip of tissue and muscle was then removed to expose the vertebrae, and a laminectomy was performed to remove some of the dorsal arch of L1–L3 sufficient to expose the dorsal surface of spinal segments L4–L5. The membrane (dura mater) covering the exposed spinal cord was carefully removed. A clamp was then secured caudal to the laminectomy and secured tightly to the side bar of the frame. On completion of surgery, the halothane concentration was reduced to a level sufficient to maintain areflexia while the animals continued to breathe spontaneously (1–2% halothane).

2.2 Electrophysiological recordings

In summary, extracellular recordings were made from convergent dorsal horn neurones, and transcutaneous electrical stimulation was used to evoke repeatable and quantifiable neuronal responses. Tests consisted of a train of 16 electrical stimuli (0.5Hz, 3-times C-fibre threshold, applied to the centre of the receptive field), which is sufficient to evoke wind-up in most neurones tested. Tests were repeated every 10 minutes and pre-drug control values determined. Experimental drugs were then applied spinally or systemically and the effects of drug application on electrical evoked neuronal responses were recorded.

2.2.1 Data capture

Parylene-coated tungsten electrodes were used to record extracellular activity from dorsal horn neurones, and a NeuroLog (Digitimer) system used to record and analyse this signal. The electrode was held in a head stage that received signal via the electrode from the spinal cord (A) and also general electrical activity from the rat and surroundings (B) via a crocodile clip attached to a skin flap. The head stage was grounded via an earth lead to the stereotaxic frame and steel table-top. To minimise interference and non-neuronal electrical activity from the animal, the general activity (B) was subtracted from the signal received via the electrode (A). The resulting signal ($A - B$) was then amplified and filtered by a series of NeuroLog modules as shown in Figure 2.1. The resulting signal was fed through an audio amplifier to a speaker and through an oscilloscope to allow the neuronal activity to be monitored both visually and aurally.

Once a neurone had been isolated, the window discriminator (spike trigger module) was set so that neuronal action potentials could be counted over background activity. Action potentials above a certain height then triggered a counting pulse which was relayed to the oscilloscope (where it appeared as a dot over the spike), the latch counters (where a cumulative count was gated) and to the CED1401 for analysis. The frequency of stimulation (0.5Hz), pulse duration (2ms) and amplitude of applied current (3 times the C-fibre threshold current) were set using the period generator, digital width and pulse buffer modules respectively.

A train consists of 16 stimuli, triggered by the period generator, which for each sweep also sends a signal to the delay width and latch counter modules and to the CED, from which the latency is measured. The latch counters were set, via the delay width module, to count action potentials with a latency after electrical stimulation of 90–800msec, and so gave a cumulative count of C-fibre and post discharge activity evoked by each of the 16 stimuli. Spike2 software installed on the computer interpreted the output from the CED, and allowed the total electrical-evoked action potentials to be quantified on the basis of their latency from stimulus.

Rate recordings were also performed to record action potentials evoked by chemical, heat or mechanical stimuli. Data captured by the CED were compiled by the Spike2 software and shown as number of action potentials evoked per 1sec time bin.

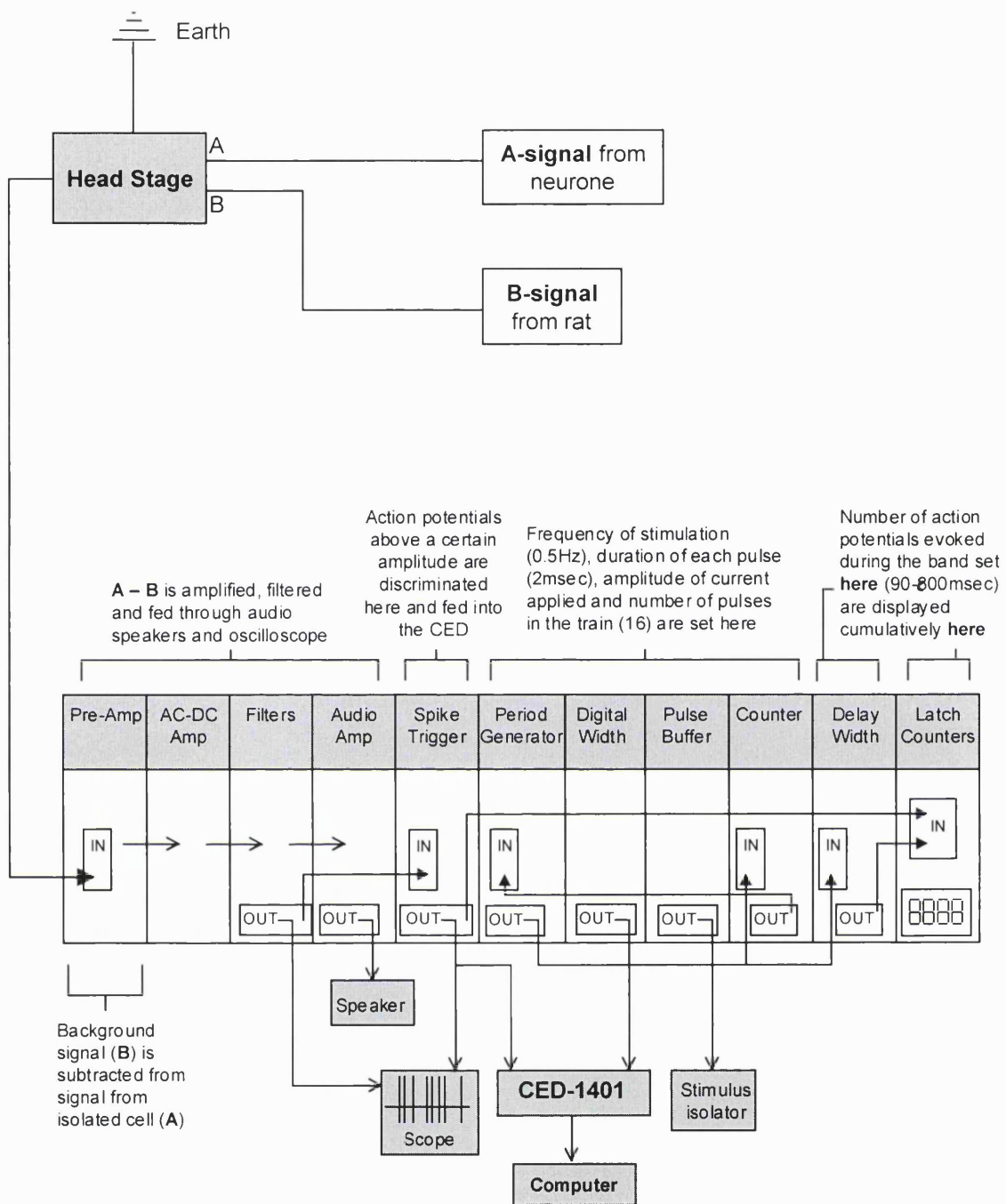


Figure 2.1 Schematic representation of the data capture procedure.

2.2.2 Isolating a neurone

The electrode in its holder was moved using a stepping microdrive with both gross manual micromanipulator and fine computer-aided controls. The electrode was moved laterally and rostro-caudally outside the cord using manual controls, and vertically (dorso-ventrally) through the cord in 10 μ m steps with an Epson Digital Stepper and a SCAT microdrive. The peripheral receptive field in the hindpaw was stimulated with the finger as the electrode was moved to identify an area receiving good input from the toe region. The electrode was then moved down from the cord surface while stimulating the toe region until one single neurone could be isolated over the background activity. A single convergent dorsal horn neurone was identified when action potentials of uniform amplitude were evoked by both innocuous (touch) and noxious (pinch) mechanical stimulation. Action potential amplitude had to be sufficient to allow discrimination from background activity or discharges of other nearby neurones with a signal to noise ratio of at least 5. The depth of the recording site from the dorsal surface of the cord was noted from the microdrive reading.

2.2.3 Characterisation of isolated neurone

Once a convergent dorsal horn neurone was isolated, electrically evoked neuronal responses were characterised. As electrical stimulation was simple to apply, accurately repeatable and the evoked responses were easily quantifiable, this forms the basis for most of the subsequent drug studies shown in this thesis. In addition, electrical stimulation bypasses the receptors on the primary afferent fibres, which may become sensitised during peripheral inflammation. Peripheral events of interest were studied separately.

Two fine stimulating needles were inserted subdermally into the centre of the receptive field, and the system allowed to rest for 5–10min. Single, transcutaneous electrical pulses of amplitude 0.1–3.3mA were applied in small incremental steps to determine the threshold current required to elicit a C-fibre evoked response. Subsequently, electrical stimuli were given at 3-times this C-fibre threshold. Maximum stimulus intensity was 9.9mA, and for the duration of the experiment (sometimes >10 hours) stimulation of this intensity was non-damaging to the hindpaw (visual observation).

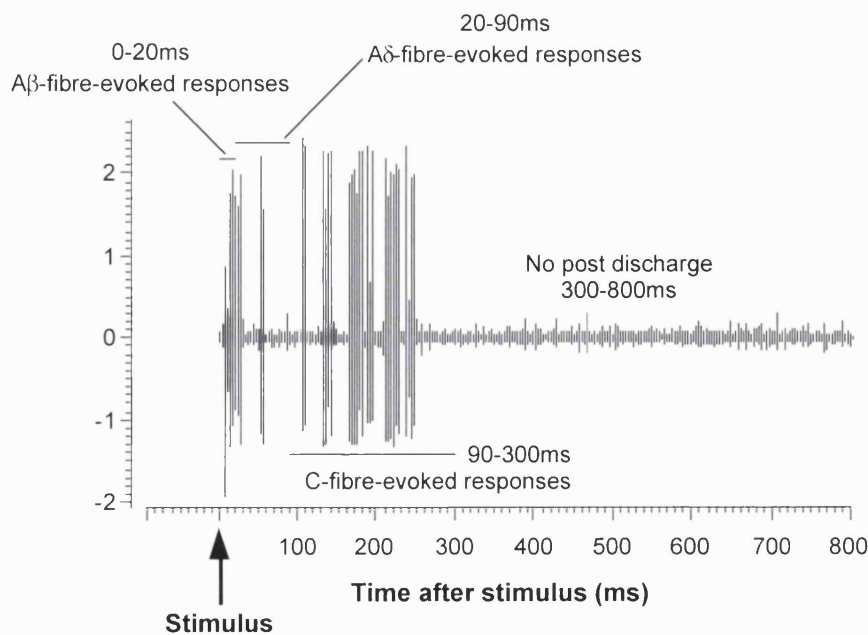


Figure 2.2 A series of action potentials is evoked from a convergent dorsal horn neurone after a single transcutaneous electrical pulse given in the receptive field.

A train of 16 stimuli was then given (0.5Hz, 2msec wide pulse at 3-times the C-fibre threshold). The action potentials evoked by each stimulus were superimposed and constructed into post-stimulus histograms by the Spike2 software. These were then separated, on the basis of latency, into total Aβ-fibre- (0–20msec); Aδ fibre- (20–90msec) and C-fibre-evoked (90–300msec) action potentials. The cumulative C-fibre (and post discharge) evoked response of the neurone were recorded from the latch counters. The response evoked by the first stimulus of the train was referred to as 'input' and consisted of the number of action potentials evoked by this stimulus recorded 90–800msec post-stimulus, i.e. the baseline C-fibre evoked response. Input is a measure of pre-synaptic activity and transmitter release, and baseline, resting post-synaptic activity.

Action potentials recorded after 300ms cannot have been evoked by even the slowest-conducting afferent fibre inputs, but instead are a result of hyper-excitability induced by the repeated stimulation. Such activity was termed post discharge, tended to appear only after repeated stimulation (not after the first stimulus in a train) and was recorded from 300–800msec post-stimulus. Activity-dependent hyper-excitability can also be quantified as 'excess spikes', a measure of the excess action potentials recorded above the predicted constant response. Excess spikes was calculated as the difference between the total number of action potentials evoked by the sixteen stimuli (from 90–800msec post-stimulus) and the input $\times 16$. Thus, if the number of C-fibre-evoked action potentials elicited by each stimulus increases during the train of 16 stimuli, the excess spikes will be greater than zero. Excess spikes and post discharge are therefore measures of post-synaptic activity and wind-up.

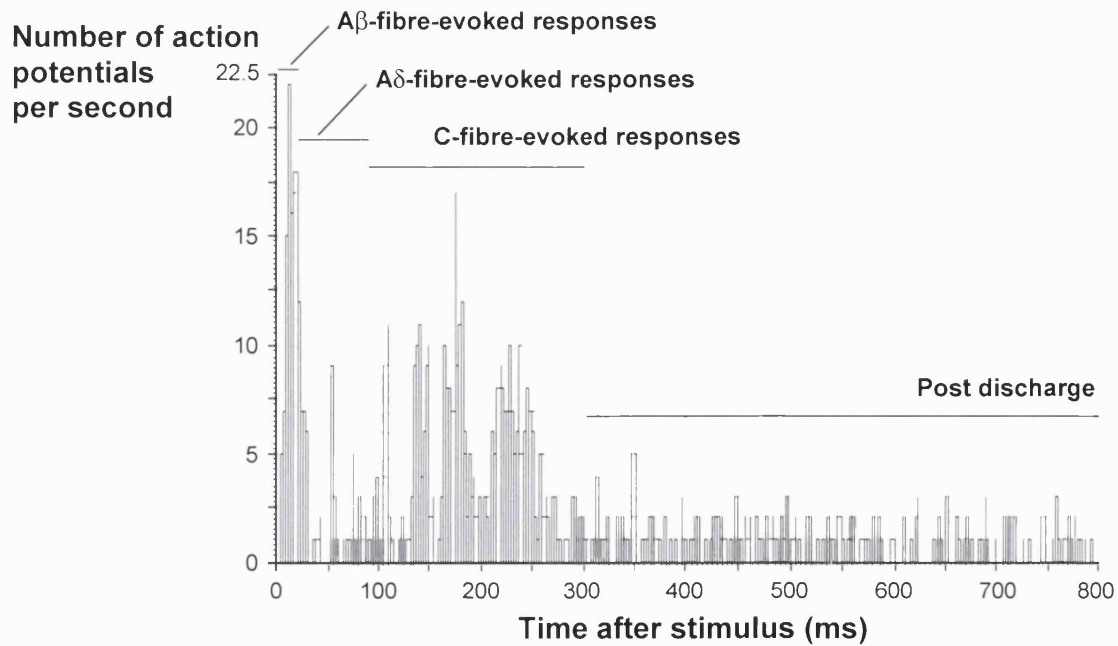


Figure 2.3 A typical post-stimulus histogram of responses evoked by a train of electrical stimuli. Latency bands for A β -, A δ - and C-fibre evoked responses and post discharge are indicated.

A drug that does not affect the input, but attenuates the wind-up of a neurone, will reduce the post discharge and excess spikes. However, if a drug inhibits the input and does not affect post-synaptic mechanisms of hyperexcitability, the post discharge will be unaffected but the excess spikes calculation will result in an apparent increase in the excess spikes value.

The wind-up of individual neurones was also monitored as the number of action potentials (90-800msec) evoked per stimulus and depicted graphically. Stimulus number was plotted on the X-axis, against the number of action potentials (90-800ms) evoked by each stimulus (Figure 2.4). As the input and wind-up displayed by individual neurones varies, only data from individual neurones could be presented in this manner.

Number of action potentials (90-300ms)

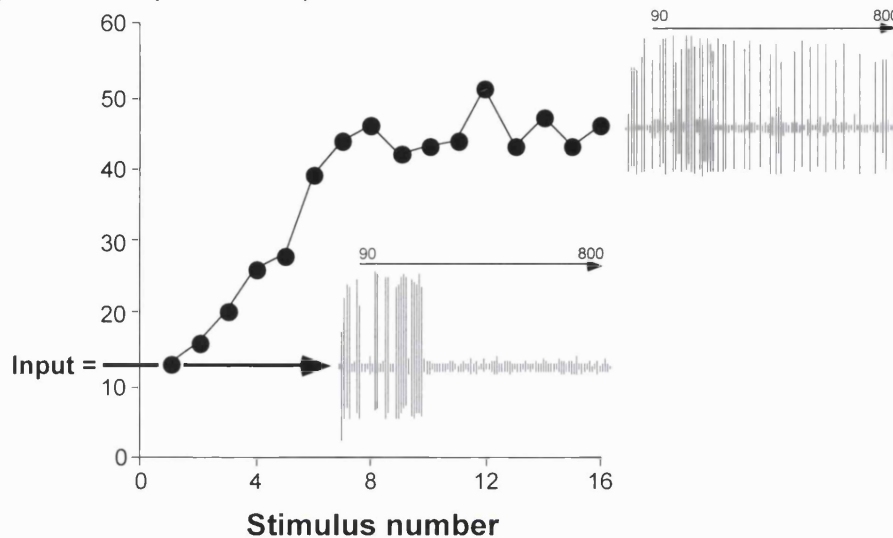


Figure 2.4 Repeated electrical stimulation evokes wind-up of convergent dorsal horn neurones. This is an example of the responses of one typical neurone evoked by a test comprising 16 electrical stimuli. The parameter quantified as 'input' is indicated. Individual recording sweeps are shown which correspond to the first and last stimulus of the train. Note how the post discharge increases – this measure is dependent on wind-up.

Electrical tests were repeated at 10-min intervals, the C-fibre threshold was re-checked before each train for the first few readings until it was stable. A β -, A δ - and C-fibre-evoked responses, input, post-discharge and excess spikes were recorded after each test. Once the responses evoked by subsequent tests were stable (variation within 10%) these responses were averaged to give pre-drug control values for each parameter. Drug application could then commence so that drug effects could be determined by comparing electrical-evoked responses to the relevant pre-drug control.

2.3 Drug application

2.3.1 Application of drugs for electrical studies

The routes of administration of drugs for electrical studies were either (i) spinal or (ii) systemic.

(i) Drugs were applied in a volume of 50 μ l directly onto the exposed spinal cord, into a 'well' formed by muscle and connective tissue around the laminectomy. The well was checked

after the surgery was complete to ensure it would hold this volume of saline and not leak. Steps could then be taken to stop the leak before recording began. Excess drug was carefully removed from the well (using absorbant tissue) after completion of the time-course before application of the next dose.

(ii) Sub-cutaneous administration was performed in volumes of 250µl into the scruff of the neck.

Electrical-evoked responses were followed, at 10-min intervals, for between 40–90 min after drug application, depending on the timecourse of action of the drug in use. The dose range to be used and the drug time-course were determined for each drug in preliminary experiments.

2.3.2 Peripheral administration of agents

In several studies, the agents of interest were administered peripherally, into the receptive field of the neurone. Nociceptin/OFQ and protons were administered in this manner. Detailed description of the protocol adopted in each case appears in the relevant chapter. Briefly, neurones were isolated and characterised as described in section 2.2 on the basis of their electrical-evoked activity. A qualitative measure of the responsiveness of the neurone to noxious heat was often determined. A gentle water jet at 45°C was applied to the plantar surface of the hindpaw, and the activity evoked was rated (scale 1–3; no response, moderate firing, high frequency sustained response). The system was left to rest for 5–10min. A 29G insulin needle was then inserted subdermally and left there for 5min before a small volume (20µl) of saline (neutral, or titrated to a range of pH) or solution of nociceptin was injected into the receptive field of the neurone. Injection-evoked action potentials were then counted for 60sec and corrected for any background activity.

After completion of injections, and cessation of any evoked responses, mechanical-evoked responses were quantified using von Frey filaments. Filaments (9g and 30g) were applied to bending force, and the static evoked responses counted over 10 second periods.

All evoked responses were recorded on Spike2 software in rate function.

2.4 Analysis of results

2.4.1 Graphical representation of results

Effects of drugs on electrically-evoked responses were expressed as a percentage of pre-drug control values (% control). The maximal effect (biggest increase or decrease from control) of each dose of drug in each experiment was determined and results from separate experiments compiled to give a mean % control $\pm$ standard error of the mean (s.e.m.) for each dose. Dose-response curves could then be constructed.

2.4.2 Statistical analysis of results

Due to the variable nature of recordings in a biological system, during statistical analysis raw values (action potentials) obtained after drug administration must be paired to the pre-drug control obtained for that neurone. Control responses and drug effects expressed as raw values (number of action potentials), were therefore compared using repeated measures ANOVA whenever possible. However, there were often instances of missing data — doses that could not be tested on that neurone for a range of reasons. Therefore to maintain the 'pairing' of drug effects to the pre-drug control values in these analyses, data were expressed as percentage of control, and analysed using simple (one-way) non-parametric ANOVA (Kruskal-Wallis).

If a significant change with increasing dose was observed ($P \leq 0.05$), individual dose effects were then compared with the the relevant pre-drug control values. This allowed the data to be paired to control again and was not affected by missing values, therefore raw data (not % control) were used. The maximal effect at each dose was compared to the pre-drug control recorded for that neurone using appropriate parametric or non-parametric post hoc tests.

In cases where groups of animals received different treatments, comparisons between the different groups of animals were preceded by comparing pre-drug control values to ensure that these baselines were not significantly different. Unpaired Student's *t*-tests were used to compare the control values (number of action potentials) for different groups of animals, and subsequently to compare drug effects (expressed as % control) between groups at each dose. As these data were from separate experiments, no correction was needed for repeated comparisons.

All statistical analysis of data were performed using the programme InStat (GraphPad) installed on iMac computers. Any additional analyses used for particular experiments are described in detail as they appear.

2.5 Models of chronic pain

2.5.1 Carrageenan-induced inflammation

Injection of carrageenan solution into the periphery was used as a model of peripheral inflammation, where the development of the inflammation could be followed during the time-course of the experiment.

Carrageenan powder (λ -carrageenan type IV from Sigma[®]) was dissolved in 0.9% saline to give a 2% solution. This was kept at room temperature and used for several months. Solution was administered in a volume of 100 μ l through a 27G needle inserted under the skin of the plantar surface of the hindpaw.

In some cases, carrageenan was injected as soon as the animal was secured with

the vertebral clamps, but in most cases, the carrageenan was injected only once a neurone had been isolated and several controls taken, allowing pre-carrageenan characterisation of the neurones. It was not deemed necessary to follow all neurones through the development of inflammation as this has already been studied in detail by Dr Louise Stanfa (Stanfa *et al.*, 1992). Repeating this would have made some experimental time courses prohibitively long.

Once a neurone was isolated, electrical tests were performed at 10 or 20min intervals until about 40min before the 3-h development time was reached. The C-fibre threshold of the neurone was then re-checked, the stimulus intensity altered if required and tests performed at 10min intervals. Three hours after the injection, if the tests were stable (evoked responses within 10% at subsequent tests) means were taken to give pre-drug control values, and drug application began. Occasionally, a neurone had not been found, or controls were not stable 3h after carrageenan injection. If this was the case, hunting continued until a suitable, stable neurone was found. Drug application always began within 4.5h of carrageenan injection.

2.5.3 Spinal nerve ligation

An established rat model of neuropathic pain is the tight ligation of spinal nerves L5 and L6 (Kim & Chung, 1992). Briefly, the left L5 and L6 spinal nerves were isolated and tightly ligated with 6-0 silk thread under halothane anaesthesia (50% O₂:50% N₂O). The surgical procedure for the sham operated group was identical to that of the SNL group, except that the L5/L6 nerves were not ligated.

Animals were kindly prepared by Miss Liz Matthews and Ms Sarah Flatters. Both are experienced in the surgical techniques and regularly prepare their own animals in this way. Behavioural responses and general well-being of the animals were monitored during the 2 weeks between preparation and use in the electrophysiological studies. If any motor impairments had been evident, or signs of autotomy seen, the animals would have been killed. However all SNL- and sham-operated animals prepared for studies remained healthy.

Animals were prepared for electrophysiological recordings as described above (sections 2.1 and 2.2), the only difference being that only neurones ipsilateral to the injury were used.

2.6 Drugs

All drugs used are listed below. The solvent used was physiological saline (0.9%) and solutions were stored at 4°C unless otherwise stated.

Mrz 2/571 Mrz 2/579 kindly provided by ^{Dr}Chris Parsons at Merz and Co. Made up in distilled water.

Ifenprodil kindly provided by Dr Alisdair Gibb.

ACEA-1244 IUPAC name 1-[2-(4-Hydroxy-phenoxy)-ethyl]-4-(4-methyl-benzyl)-piperidin-4-ol. Kindly provided by Parke Davis. Dissolved in ethanol, cremophor added and solution sonicated for 30 minutes before saline was added. Final proportions in the solution were 33% cremophor, 7% ethanol and 60% saline.

α -Methadone dissolved in conc HCl, saline added, then titrated with 1M NaOH to increase pH towards neutral (used at pH 6.5–7.5).

NC/OFQ Phe-Gly-Gly-Phe-Thr-Gly-Ala-Ala-Arg-Lys-Ser-Als-Arg-Lys-Leu-Ala-Asn-Gln. Purchased from Tocris. Separate aliquots stored at -20°C.

Naloxone

N-Phe full name [N-Phe¹]nociceptin(1-13)NH₂. Kindly provided by Dr Girolamo Calo, University of Ferrara, Italy and Dr Patrizia Romualdi, University of Bologna, Italy. Separate aliquots of required doses were stored at -20°C.

Phe¹ψN full name [Phe¹ψ(CH₂-NH)Gly²]nociceptin-(1-13)-NH₂. Purchased from Tocris. Separate aliquots stored at -20°C

J113397 chemical name 3-ethyl-1-[3*R*,4*R*]-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-1,3-dihydro-2*H*-benzimidazol-2-one hydrochloride. Kindly provided, under agreement, by Dr Satoshi Ozaki at the Banyu Tsukuba Research Foundation, Japan. Separate aliquots of required doses and a stock solution stored were at -20°C. Stock solution was thawed to make up subsequent aliquots, as the compound is known to be stable through several freeze/thaw procedures.

2-PMPA kindly provided by Dr Barbara Slusher, Guilford Pharmaceuticals, USA. Made up in saline, and then titrated with 5M NaOH to increase pH towards neutral (used at pH 6.5–7.5). Solutions were stored at 4°C for up to 2 months.

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Chapter 3. NMDA receptor blockade

3.1 Introduction

From the earliest studies on activity-dependent plasticity in the spinal cord, the crucial role of the NMDA receptor has been apparent. The critical events that lead to its activation have slowly been revealed and, since the cloning of the NMDA receptor subunits in the early 1990's (Ishii *et al.*, 1993; Moriyoshi *et al.*, 1991), the molecular basis for these controls is now understood. With this knowledge has also come an improved understanding of the many potential sites at which pharmacological manipulation of NMDA receptor function is possible. Drugs that block the NMDA receptor are extremely effective analgesics and antihyperalgesics but are compromised by severe side effects — selective receptor blockade exploiting these sites may improve the therapeutic profile NMDA receptor blocking agents. In this chapter I describe three potential approaches to circumventing the ubiquitous block of NMDA receptors that so compromises their therapeutic profile; experiments were carried out to assess the antihyperalgesic activity of two glycine site antagonists and two antagonists selective for NMDA receptors containing the NR2B subunit, and to investigate the potential contribution of NMDA receptor blockade to the therapeutic profile of the opioid methadone.

3.1.1 NMDA receptor structure

The NMDA receptor is a hetero-oligomer composed of two different polypeptide subunits, NR1 which forms the glycine co-agonist binding site (strychnine insensitive glycine_B site) and NR2 which forms the glutamate binding site. These subunits come together to form an integral ion channel which gates Na⁺, K⁺ and Ca²⁺. Figure 3.1 illustrates basic receptor structure, and indicates modulatory sites.

Four homologous rat brain NR2 cDNAs have been cloned, termed NR2A–D, which have 15–20% homology with the NR1 subunit, and are 50–70% homologous to each other (Ishii *et al.*, 1993; Monyer *et al.*, 1992). NR1 subunits can form functional channels when expressed in *Xenopus* oocytes (Moriyoshi *et al.*, 1991), but co-expression with NR2 subunits is required to generate whole cell currents comparable with native channels. In mammalian cell expression systems, however, NR1 subunits can not form functional homomeric channels (Boeckman & Aizenman, 1994), although receptor subunits can be detected immunologically (Chazot *et al.*, 1992), and bind glycine with high affinity (Grimwood *et al.*, 1995). It is now known that *Xenopus* oocytes express low levels of an endogenous glutamate receptor subunit (XenU1) that can assemble with NR1 (Soloviev & Barnard, 1997). It is possible that NR2 subunits are required for effective localisation of the receptor complex to the cell membrane. NR2 subunits do not form functional homomeric receptors in expression systems, but when NR1 is co-expression with NR2 channels with large whole cell currents are generated, which resemble native NMDA receptors (Ishii *et al.*, 1993). Combinations of NR2 subunits and NR1 splice variants gives a wide diversity of receptor types, which differ in single-channel properties, in sensitivity to glutamate and glycine-site

antagonists and, for example, sensitivity to ifenprodil antagonism (Grimwood *et al.*, 1996; Ishii *et al.*, 1993; Nankai *et al.*, 1996; and Sucher *et al.*, 1996 for review).

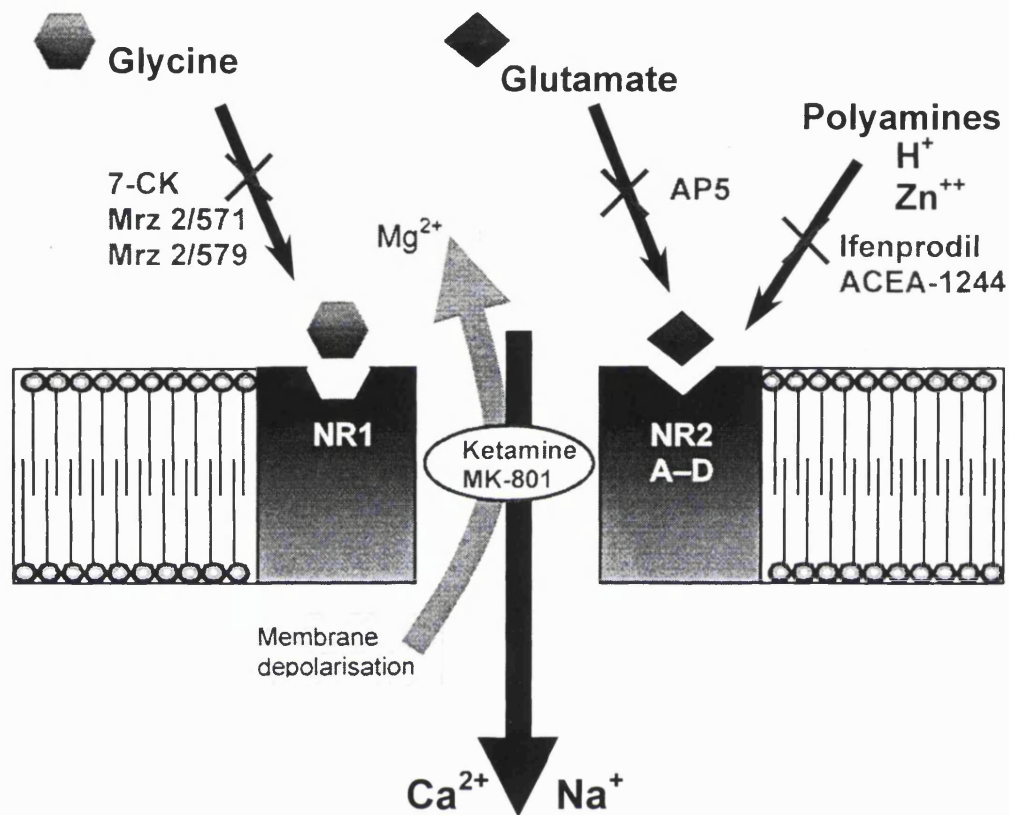


Figure 3.1 Simplified illustration of structure of the NMDA receptor for glutamate. Binding sites for glutamate, glycine and polyamines are indicated, along with drugs that block at these sites and in the channel pore. 7-CK = 7-chlorokynurenate; AP5 = 5 amino-phosphonovaleric acid

Each subunit of the NMDA receptor consists of four transmembrane regions. The second transmembrane region (TM2) forms a re-entrant membrane loop in all subunits (Bennett & Dingledine, 1995), such that the N-terminus is located extracellularly (Hollmann *et al.*, 1994) and the C-terminus intracellularly, where it has been shown to interact with various intracellular proteins (Ehlers *et al.*, 1996; and Sucher *et al.*, 1996 for review). TM2 is thought to contribute to the formation of the pore, as site-directed mutagenesis here alters permeability characteristics (Mori *et al.*, 1992). Alternative splicing of NR1 occurs, and at least eight different isoforms have been identified, with different functional properties (Zukin & Bennett, 1995).

The stoichiometry of the NR1–NR2 interaction has been in contention for some time (see Dingledine *et al.*, 1999 for a recent review). A pentameric structure was initially favoured, but more recently, Laube *et al.* (1998) presented evidence for a tetrameric structure for NMDA receptors, comprising two NR1 and any combination of two NR2 subunits. In a recent biochemical study, the co-association of three subunits in a single receptor has also been demonstrated (Hawkins *et al.*, 1999). This is supported by single channel work revealing a novel conductance, in addition to typical NR2A and NR2D currents, in *Xenopus* oocytes transfected with NR1/NR2A/N2D) (Cheffings & Colquhoun, 2000). These would suggest that a pentameric structure is possible, but whether this ever occurs in neurones *in vivo* is yet to be established.

A third NMDA receptor subunit is the NR3, which has an apparent regulatory function. NR3A (formerly termed NMDAR-L or χ -1), is expressed primarily during brain development and co-localises with receptor subunits NR1 and NR2. It reduces channel conductance when added to NR1 and NR2 and has thus been suggested to play a role in the development of synapse strength by modulating NMDA receptor activity (Das *et al.*, 1998). The 'epsilon' subunit (GluR ϵ ₁) is another possible 'regulatory' subunit that has been reported to attenuate nociceptive transmission (Inoue *et al.*, 2000).

Transgenic techniques have so far not been useful to investigate NMDA receptor function, apart from to highlight that the NR1 subunit is essential for receptor function. NR1 knockout mice die soon after birth, and as well as lacking NMDA-evoked Ca²⁺ currents, the expression of NR2B (but not 2A) was reduced, suggesting an interdependence of subunit expression (Forrest *et al.*, 1994). Conditional NR1 knockouts are an interesting prospect, and the strategy for the production of a conditional knockout of NR1 in the spinal cord was presented at the Society for Neuroscience meeting in New Orleans (South *et al.*, 2000).

The ability of glycine to augment NMDA receptor function was noted initially in cultured brain neurones (Johnson & Ascher, 1987) and subsequently also in spinal cord neurones *in vitro* (Brugger *et al.*, 1990) and *in vivo* (Budai *et al.*, 1992). The glycine site of the NMDA receptor is clearly distinguishable from the glycine receptor; the latter is blocked by strychnine whereas the glycine site on the NMDA receptor is strychnine-insensitive. The complex effects of glycine binding on NMDA receptor affinity for glutamate, for competitive antagonists and interactions with the polyamines is discussed in detail in Leeson & Iverson (1994). The glycine site is saturated and permits full activation of the NMDA receptor in brain slices *in vitro*. Physiological levels of glycine in the mammalian CNS are thought to be sufficient to permit glutamate activation of NMDA receptors. However, measurement of glycine in the CSF is not a good indicator of the true situation. Local glycine transport systems (Bergeron *et al.*, 1998) and variable sensitivity of different subunit combinations may serve to confer dependence on extracellular glycine in normal function. Since glycine site antagonists reliably attenuate NMDA-mediated events, this co-agonist site appears to be operative (unsaturated) under many conditions. D-Serine, an endogenous amino acid

may function as another ligand at the glycine site of the NMDA receptor, as it selectively binds to the glycine site, and can augment NMDA receptor function *in vitro* (Mothet *et al.*, 2000, and references therein) .

The voltage-dependence of NMDA receptor function is determined not by voltage-induced conformational changes, but by the simple occupancy of extracellular Mg^{2+} in the channel pore at resting membrane potentials and millimolar concentrations (Nowak *et al.*, 1984 and Dingledine *et al.*, 1999 for review) This block is highly voltage-dependent and is rapidly relieved on membrane depolarisation. The Mg^{2+} binding site is thought to be deep in the channel pore, and the subunit composition of the receptor alters sensitivity to Mg^{2+} blockade. Thus, receptors containing NR2A or NR2B subunits are blocked more strongly by Mg^{2+} than NR2C or 2D-containing receptors (Kuner & Schoepfer, 1996; and Dingledine, 1999 for review).

3.1.2 NMDA receptor function

The consequences of excess NMDA receptor activation can be severe; excitotoxicity in stroke and neurodegenerative diseases is testament to this (Coyle & Puttfarcken, 1993; Meldrum & Garthwaite, 1990). The molecular determinants of NMDA receptor function, as described above, restrict activation of the receptor; several events must coincide for it to open. To relieve the Mg^{2+} block, the membrane must be depolarised — this occurs after high frequency stimulation of the presynaptic neurone when excitatory postsynaptic potentials (EPSPs) summate sufficiently to depolarise the membrane. As AMPA excitatory postsynaptic currents (EPSCs) are so short-lived (5ms; Silver *et al.*, 1992), other neurotransmitters — especially peptides with slower EPSCs — are needed for this depolarisation to occur. The intracellular location of neuropeptide vesicles, at a distance from release sites, ensures that peptides are not released from the presynaptic terminal except in conditions of high frequency (see recent review by Nusbaum *et al.*, 2001).

The NMDA receptor is particularly important in the transmission, and particularly modulation, of nociceptive information. NMDA-dependent mechanisms are thought to underlie central hypersensitivity and the enhanced transmission of pain in both acute and chronic pain situations. The established dogma is that acute noxious stimuli, where no tissue damage occurs, are relayed through the spinal cord with little or no modulation and reach higher centres giving a 'faithful' report of the pain. This pathway is predominantly AMPA receptor mediated. Continued noxious stimulation and tissue damage leads to peripheral hyperalgesia and increased activity in nociceptive primary afferents and subsequent increased release of glutamate and of peptides such as substance P and CGRP from C-fibres. Excitatory post synaptic potentials generated by these peptides via their slow, G-protein linked receptors summate sufficiently to relieve the Mg^{2+} block of the NMDA receptor channel. Now the criteria for activation of the NMDA receptor are fulfilled and released glutamate can open the channel. The ensuing large Ca^{2+} influx activates many

intracellular pathways and signalling cascades generating a hyperexcited state in the postsynaptic neurone. Further stimuli arriving at the synapse now evoke an enhanced response from this dorsal horn neurone. This process is illustrated in Figure 3.2 and, when induced artificially, the hypersensitivity lasts in the order of minutes. Following repeated electrical stimulation of C-fibre afferents in the hindpaw of the rat, enhanced responses are then recorded from dorsal horn neurones although the intensity of the stimulus remains the same. This has been termed 'windup' and has been shown to be dependent on NMDA receptor activation (Davies & Lodge, 1987; Dickenson & Sullivan, 1987).

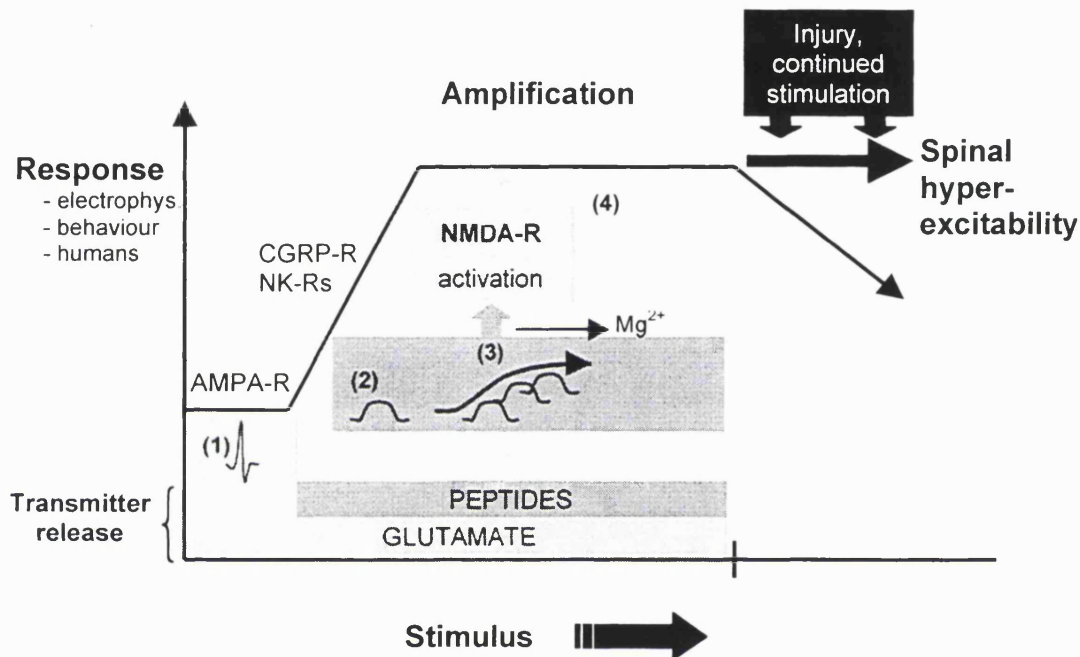


Figure 3.2 Involvement of the NMDA receptor in wind-up.

Repeated stimulation — of sufficient intensity to activate C-fibres — leads to an elevated level of response that can be measured in electrophysiology and behavioural experiments in animals, and psychophysical experiments in humans. The sequence of events during a train of stimuli can be explained as follows:

1. Released glutamate (light grey) acts via the AMPA receptor (AMPA-R) to produce a rapid excitatory postsynaptic potential (e.p.s.p) and action potential propagation.
2. Released peptides (dark grey) act via G-protein coupled receptors, e.g. the tachykinin receptors (NK-R) the CGRP receptor (CGRP-R), to give rise to longer-lasting e.p.s.p.s.
3. These summate to give a sustained membrane depolarisation, relieving the Mg^{2+} block from the pore of the NMDA receptor channel.
4. Released glutamate can now activate the NMDA receptor, and the ensuing Ca^{2+} influx, leads to a state of hyper-excitation of the postsynaptic neurone.

The NMDA receptor is required for the maintenance of hyperexcitability. Thus, sustained input — as occurs in persistent inflammatory pain or abnormal nerve injury pain — is required for true and lasting spinal sensitisation.

This phenomenon has been utilised as a model throughout the research forming this thesis. Although this is an experimentally-evoked and short-term phenomenon that is not equivalent to central sensitisation, similar processes underlie both phenomena. Continued C-fibre input is required to maintain the state of heightened excitability and, as discussed in Chapter 1 (section 1.4.2), many Ca^{2+} -dependent mechanisms are activated and contribute to central sensitisation. As such, windup has come to serve as a predictive model against which to validate agents that could be used to modulate central mechanisms of hyperalgesia.

The importance of NMDA receptor dependent windup in clinically relevant pain states has been demonstrated convincingly in many behavioural and electrophysiological animal studies that have revealed an antihyperalgesic rather than antinociceptive effect of NMDA receptor antagonists. NMDA receptor blockade is effective in attenuating the second phase of the formalin test (Chapman & Dickenson, 1995; Haley *et al.*, 1989), behaviour and neuronal activity indicative of neuropathic pain (Chaplan *et al.*, 1997; Mao *et al.*, 1993; Seltzer *et al.*, 1991; Suzuki *et al.*, 2001; Yamamoto & Yaksh, 1992), especially allodynia (Chapman & Dickenson, 1994; Yaksh, 1989). Results from the clinic are encouraging and NMDA receptor blockade appears to be an effective strategy to alleviate the notoriously complex and unresponsive pain arising from nerve injury, but the side effects preclude a wider use of these drugs (discussed recently in Sang, 2000).

3.1.3 Subunit composition as a pharmacological target

The complex controls governing activation of the NMDA receptor gives the pharmacologist a number of approaches to modulating its function. It has been postulated that as different subunit combinations generate NMDA receptor channels with different single-channel and whole-cell characteristics and the NR2 subunits have different distribution patterns, these relate to different functions and physiological roles. Analysis of NMDA receptor NR2 subunit composition in patch clamp recordings from synaptic and extrasynaptic membrane has revealed a different distribution for the NR2-subunits. In cerebellar slices from 2-week old rats, NR2D-containing receptors have been localised only to the extrasynaptic membrane, whereas NR2B-containing receptors were identified both in the immediate postsynaptic region and extrasynaptically (Misra *et al.*, 2000). No NR2A-containing receptors were identified.

Of particular interest, and of more immediate relevance to the work presented in this thesis, is an exceptional recent report from Momiyama (2000). Although most patch-clamping work is carried out in slices of neonatal rat brain or spinal cord, this study reports NMDA receptor mediated activity in slices of adult rat spinal cord. The age of the animal is an important consideration, as NR2 subunit populations are subject to pronounced developmental changes. Single channel recordings from somatic patches (extrasynaptic membrane) from substantia gelatinosa (SG) neurones, and analysis of the synaptic currents

evoked by dorsal root stimulation revealed three different populations of NMDA receptors. Currents underlying synaptic EPSPs had kinetics, Mg^{2+} -sensitivity and pharmacological profiles characteristic of NR2A-containing receptors; whereas extrasynaptic channels were either low conductance channels with sub-conductance states characteristic of NR2D-containing receptors or, more commonly, high conductance channels that were blocked by ifenprodil and designated NR2B-containing receptors. Thus, these data indicate that NR2A-containing receptors are mostly responsible for transmission between the primary afferent terminal and SG neurones, whereas NR2B-containing receptors form the majority of extrasynaptic NMDA receptors.

In addition to these differences in localisation, there are many characteristics of NMDA receptors that differ depending on the subunit makeup. For instance, the extent of glycosylation, the extent, sites and types of kinase phosphorylation, sensitivity to Mg^{2+} and Zn^{2+} blockade all affect current characteristics and vary according to subunit composition. The functional consequences of such differences are now beginning to emerge. For instance, NMDA receptors containing NR2A/2B appear to play a more predominant role in long term potentiation (LTP) than receptors containing NR2C/2D; the converse is true for long term depression (LTD; Hrabetova *et al.*, 2000). If similar functional significance can be determined elsewhere, then subunit selective drugs could be targeted to the relevant brain regions and receptor types, avoiding the side effects of ubiquitous block of this receptor.

Two *in vitro* studies have indicated that selective blockade of NMDA receptors containing the NR2B subunit may be an effective strategy to reduce the neurotoxic effects often reported with other NMDA receptor antagonists (Cudennec *et al.*, 1994; Duval *et al.*, 1992). Such agents also appear to have a reduced motor side-effect profile (Boyce *et al.*, 1999); this will be discussed in more detail later. Briefly, in the *in vivo* studies, NMDA receptor blockers and glycine-site antagonists (MK-801, L-687,414 and L-701,324) affected motor control at antinociceptive doses. In contrast, the selective NR2B antagonists, (+/-)-CP-101,606 and (+/-)-Ro 25-6981 caused no motor impairment or stimulation even at doses far in excess of those required to inhibit allodynia in neuropathic rats (Boyce *et al.*, 1999). These findings demonstrate that NR2B selective antagonists may have clinical utility for the treatment of pain conditions in man with a reduced side-effect profile compared with existing NMDA receptor antagonists.

The final aim of such research is to develop drugs that are effective in man and a potential problem in translating preclinical work is species differences in receptor. A single study has analysed the NMDA receptor subunits present in human spinal cord and could not detect the presence of NR2B subunits (Sundstrom *et al.*, 1997). However, as discussed later in section 3.4.2, although the majority of studies in the rat report localisation of NR2B in the spinal cord, some do not. A likely explanation is differences in antibodies and experimental conditions.

3.1.4 The glycine site as a pharmacological target

The requirement of glycine binding is another aspect of NMDA receptor function that can be manipulated using pharmacological tools (see Leeson & Iverson, 1994 for review). Agents that are selective for the glycine site of the NMDA receptor reduce receptor function *in vitro*. Functional studies have shown that this *in vitro* activity translates into a functional blockade of NMDA receptor mediated events. For example, glycine site antagonists are neuroprotective (Newell *et al.*, 1995). They are also antihyperalgesic, both electrophysiological and behavioural studies have revealed inhibition of wind-up and post discharge of dorsal horn neurones (Dickenson & Aydar, 1991) and reduced neuronal activity in the second phase of the formalin test (Chapman & Dickenson, 1995; Millan & Seguin, 1993; Millan & Seguin, 1994; Quartaroli *et al.*, 1999); however also see (Coderre, 1993) for contrasting results. An attractive feature of glycine site antagonists is the apparent lack of psychomimetic effects common with the channel blockers (Bristow *et al.*, 1996; Tricklebank *et al.*, 1994). These agents also appear to be free of the neuropathological changes associated with some competitive and non-competitive NMDA receptor antagonists (Hargreaves *et al.*, 1993). In line with other classes of NMDA receptor antagonists, glycine site blockers can interact synergistically with opioids and have also been reported to attenuate morphine tolerance (Quartaroli *et al.*, 1999). This class of antagonist has therefore received attention for further development of potential antihyperalgesic agents. With this aim in mind, Merz have developed a series of derivative agents with *in vitro* selectivity for the glycine site of the NMDA receptor (Parsons *et al.*, 1997). I tested two of these compounds, Mrz 2/571 and Mrz 2/579, for their effects on acute nociceptive processing in the dorsal horn.

3.1.5 NMDA receptor and opioid interactions

Methadone (a racemic mixture of *d*- and *l*-optical isomers) has long been recognised as a μ opioid receptor agonist and is used clinically both as a replacement for heroin in withdrawal therapy and in the treatment of pain. Due to factors such as a long and unpredictable half-life and variable pharmacokinetics, the dosage for each individual has to be carefully titrated to avoid overdose, especially in opioid naïve patients (Fainsinger *et al.*, 1993). The use of methadone in the treatment of pain is, therefore, usually limited to situations where the patient has become unresponsive to the opioids of choice. Methadone is atypical as an opioid in that tolerance to its analgesic effects are claimed to be slow to develop (Inturrisi *et al.*, 1990), and it has been reported to have incomplete cross-tolerance with other opioid agonists (Crews *et al.*, 1993; Thomas & Bruera, 1995). These are reports of individual cases, and not controlled trials. Recently, however, Doherty (2001) has used a controlled cross-over design to show complete cross-tolerance between morphine and methadone indicative of a common pharmacological action at therapeutic doses.

The pharmacological basis for the claimed differences between methadone and other opioids such as morphine is unclear, but has been attributed in part to the affinity of both the *d*- and *l*-isomers of methadone for the non-competitive site of the NMDA receptor in rat brain membranes (Gorman *et al.*, 1997) in common with some other opioids such as ketobemidone and dextropropoxyphene (Ebert *et al.*, 1995; Ebert *et al.*, 1998). Ebert *et al.* (1995) not only show that the racemic mixture of methadone binds to the channel at the MK-801 site, but that it is a non-competitive antagonist of the NMDA receptor and able to block responses to NMDA itself in both the cortex and spinal cord. Gorman *et al.*, (1997) show that the separate isomers bind to the non-competitive site and displace MK-801 — the inference is that both the isomers are non-competitive antagonists at the NMDA receptor and able to block the ion channel of the receptor.

In addition to its direct involvement in nociception, NMDA receptor activation can lead to a reduced sensitivity to opioids. This arises simply as a result of shifting the balance between excitatory and inhibitory pathways towards the excitatory; a higher dose of the opioid is now required to produce adequate analgesia (Dickenson, 1997; Price *et al.*, 2000). Hence, if methadone does have dual activity as an NMDA receptor antagonist and an opioid, this could explain the somewhat different functional profile of this drug compared with other opioids such as morphine. In support of this, several NMDA receptor antagonists have been shown to attenuate, but not abolish, the development of analgesic tolerance to morphine (Elliott *et al.*, 1994; Tiseo & Inturrisi, 1993). In addition, a marked synergy is observed between low doses of NMDA receptor antagonists and threshold doses of morphine (Chapman & Dickenson, 1992), the combination giving greater inhibition of neuronal responses than either drug alone. Consequently a combination of opioid receptor activation and block of the NMDA receptor would be a desirable property of an analgesic; this alliance may exist in methadone.

As the opioid activity of methadone is predominant in the *l*-isomer whereas the *d*-isomer is weaker as an opioid by approximately 40-fold (Scott *et al.*, 1948), it would be interesting to determine whether the *d*-isomer possesses *in vivo* activity at the NMDA receptor. A recent behavioural study has shown *d*-methadone to have naloxone insensitive antinociceptive effects in the rat (Shimoyama *et al.*, 1997). I studied the effects of *d*-methadone on electrically-evoked neuronal responses and compared these with the effects of racemic (*d/l*)-methadone in the same model. The sensitivity of these effects to naloxone reversal was used as a criterion to distinguish opioid from NMDA receptor mediated actions. The aim was to reveal any potential contribution of NMDA receptor blocking actions to the well documented analgesic activity of methadone and thus assess whether its reported affinity for NMDA receptors has physiological relevance *in vivo*.

3.2 Methods

Animals were anaesthetised and underwent surgery to expose the spinal cord, as previously described, and neuronal recordings began. Once a suitable neurone was isolated and control responses had stabilised, drugs were applied directly to the spinal cord, and electrically-evoked neuronal responses were followed for 40–60 min.

3.2.1 Glycine site antagonists

Mrz 2/571 and Mrz 2/579 were kindly provided by Chris Parsons at Merz and Co. Powder was dissolved in distilled water to give solutions of 1, 50 and 250µg (per 50µl saline), and stored at 4°C. Application of all three doses of Mrz 2/571 or 2/579 was achieved in each experiment, therefore repeated measures ANOVA was used to analyse the results for each drug. If a significant effect was revealed, individual doses were compared with pre-drug control values (Dunnett multiple comparison post hoc test).

3.2.2 NR2B-selective compounds

Ifenprodil, kindly provided by Dr Alisdair Gibb (Pharmacology Department, UCL) was dissolved in saline to give solutions of 1, 40 and 400µg (per 50µl saline) and stored at 4°C.

ACEA-1244, provided by Parke-Davis, is not water soluble and had to be dissolved first in ethanol, then cremophor was added and the solution sonicated for 30 minutes. Once it had dissolved, saline was added. Final proportions in the solution were 33% cremophor, 7% ethanol and 60% saline. Dilutions of this stock (500µg per 50µl vehicle) were made in saline to give solutions of 50, 100 and 250µg/50µl (Table 3.1). Vehicle alone, consisting of the same proportions of cremophor, ethanol and saline as the 500µg/50µl stock solution, was also prepared, and diluted in saline to give equivalent concentrations of cremophor, ethanol and saline as the 50, 100 and 500µg solutions of drug. Several experiments were then conducted with spinal application of these 3 'doses' of vehicle.

Table 3.1 Proportions of solvents present in each dose of ACEA-1244

	Cremophor	Ethanol	Saline
500µg/50µl	33%	7.0%	60%
250µg/50µl	16.5%	3.5%	80%
100µg/50µl	6.6%	1.4%	92%
50µg/50µl	3.3%	0.7%	96%

The 250 and 500µg/50µl dose of ACEA-1244 and the equivalent vehicle mixtures were extremely viscous, and application to the spinal cord was difficult. The fine-gauge Hamilton syringe that is normally used was too fine, and a Gilson pipette was used instead.

Probably as a consequence of this, and the ensuing de-stabilisation of the preparation, even neurones whose action potential activity was very clear above background activity at the start of the experiment, became increasingly difficult to discriminate from background as drug application progressed. Frequently recording from a neurone had to be abandoned and it was rare that the effect of all four doses could be studied on the same neurone. Two neurones were often used in the same animal to complete the drug application in each experiment (another neurone was isolated, controls were taken and the next dose was applied). This was only done when the previous dose applied was $\leq 100\mu\text{g}$, and the spinal cord was always washed with saline several times before continuing. A total of 18 animals were used, and the results from 2 were discarded as the recordings could not confidently be discriminated from background.

As a consequence of the incomplete dose-response curves for each neurone, repeated measures ANOVA could not be used to analyse the results. Instead the maximum effect of each dose on each neurone was expressed as a percentage of the pre-drug control recorded for that neurone and ordinary, unpaired Kruskal-Wallis was used to determine whether there was a significant change in the percentage of control data with increasing dose. If a significant change was revealed, then Student's *t*-tests were used to compare effects of individual doses with pre-drug control values (raw data again). As multiple comparisons were made, the Bonferroni correction for multiple comparisons was used.

Several experiments were also conducted applying vehicle alone to the spinal cord, to give equivalent proportions of solvents as present in the 50, 100 and $500\mu\text{g}/50\mu\text{l}$ solutions of ACEA-1244 itself. Cumulative 'dosing' was performed as for the drug itself. The effects of vehicle were studied on nine neurones in eight different animals. Results from one neurone were discarded, the mean control values being too small to give consistent results. Analysis of the data was performed as above (unpaired Kruskal-Wallis followed by paired *t*-tests). In addition, the maximal effect produced by ACEA-1244 was compared with the effect produced by the maximum 'dose' of vehicle alone (equivalent to $500\mu\text{g}/50\mu\text{l}$ ACEA-1244) using the unpaired Mann-Whitney test on the % control data.

In contrast, the application of ifenprodil was more straight-forward. Effects of each dose could be studied on each neurone, therefore repeated measures ANOVA could be used to analyse the results. If a significant effect with increasing dose was revealed, the effect of each individual dose was compared with the pre-drug control value for that neurone (Dunnett's multiple comparison post hoc test).

3.2.3 Methadone

In a previous set of experiments, performed under identical conditions by Dr Victoria Chapman, cumulative doses of *d*-methadone (5, 25, 50 and $250\mu\text{g}$) were applied spinally and the effects on electrically-evoked neuronal responses were followed for 40 min for each dose. I applied the same doses of *d*-methadone and also included a higher dose

(500 μ g/50 μ l). *d*-Methadone was dissolved in concentrated HCl, saline was added, then titrated with 1M NaOH to increase pH towards neutral (used at pH 6.5–7.5). This solution was then diluted in saline to give a stock solution of 500 μ g/50 μ l, from which the remaining doses were prepared. In both *d*- and *dl*-methadone experiments, application of all doses of drug was not possible in each animal studied, and on several occasions two neurones were studied in the same animal. As described previously for ACEA-1244, this was only done when the previous dose applied was $\leq$ 50 μ g.

For both methadone preparations, statistical analyses of overall drug effects were performed using the unpaired Kruskal-Wallis test on the % control data. If a significant effect was seen, the effects of individual doses were compared with control values (raw data) using multiple paired Student's *t*-test comparisons with the Bonferroni correction ($P \times 5$ for *d*-methadone, $P \times 4$ for *dl*-methadone). Comparisons between the groups (*d*-methadone vs. *dl*-methadone) were preceded by comparison of the control values recorded in each group of animals to ensure equal starting points. The effect of each dose of *d*-methadone (as % control) was then compared with the effect of *dl*-methadone (as % control) on the same measure. As the distribution of standard deviations was not always equal between the groups, non-parametric *t*-tests (Mann-Whitney) were used for all comparisons between *d*- and *dl*-methadone effects. As only a single comparison was made for each piece of data, no correction for multiple comparisons was needed.

The effects produced by the top dose of methadone were challenged at 40 minutes with 1 μ g naloxone ($n = 9$ following 500 μ g *d*-methadone, and $n = 6$ following 250 μ g *dl*-methadone) and/or 5 μ g naloxone ($n = 12$ following *d*-methadone and $n = 8$ following *dl*-methadone). Both doses of naloxone were tested where possible. The term "naloxone reversal" is used when the application of naloxone caused a significant increase in the number of action potentials from the maximum inhibition seen, using paired, two-tailed *t*-tests with the Bonferroni correction.

3.3 Results

3.3.1 Glycine site antagonists

Two glycine site antagonists were studied here (Mrz 2/571 and Mrz 2/579). Both were applied spinally (1, 50 and 250µg/50µl) and their effects on responses of dorsal horn neurones recorded. One neurone was used in each animal.

The effects of Mrz 2/571 were studied on four dorsal horn neurones (mean depth = $1093 \pm 122\mu\text{m}$, mean C-fibre threshold = $1.62 \pm 0.17\text{mA}$). Mrz 2/571 caused significant, dose-related inhibitions of C-fibre evoked responses, post discharge, input, excess spikes and Aδ-fibre evoked responses (repeated measures ANOVA, $^{\dagger}P = 0.0105$, $^{\dagger\dagger}P = 0.0089$, $^{\dagger}P = 0.0320$, $^{\dagger\dagger}P = 0.0050$, $^{\dagger\dagger}P = 0.0033$ respectively; Table 3.2, Figure 3.3A and B). Post hoc analysis shows that the inhibitions produced by each individual dose reached significance between 50 and 250µg (see Table 3.2). The Aβ-fibre evoked responses were not significantly affected by Mrz 2/571. Although it was not significant, there was a clear separation between the marked inhibition of post discharge and excess spikes, and the less pronounced inhibition of input at 50µg and 250µg.

Table 3.2 Effect of Mrz 2/571 on electrically-evoked neuronal responses

	Control	Drug effects (% control)		
		1µg	50µg	250µg
C-fibres[†]	312 ± 69 (n=4)	71.3 ± 9.1% (n=4)	43.9 ± 14% (n=4)*	37.1 ± 13% (n=4)**
Post discharge^{††}	240 ± 71 (n=4)	40.3 ± 11% (n=4)	17.4 ± 14% (n=4)**	13.5 ± 8.5% (n=4)**
Input[†]	13.6 ± 0.81 (n=4)	66.8 ± 19% (n=4)	55.5 ± 29% (n=4)	40.3 ± 9.3% (n=4)*
XS-spikes^{††}	425 ± 115 (n=4)	50.0 ± 8.9% (n=4)	21.8 ± 8.7% (n=4)**	19.7 ± 8.6% (n=4)**
Aδ-fibres^{††}	52.5 ± 15 (n=4)	34.1 ± 11.5% (n=4)*	20.0 ± 7.0% (n=4)**	12.6 ± 7.9% (n=4)**
Aβ-fibres	112 ± 16 (n=4)	92.2 ± 18% (n=4)	81.4 ± 18% (n=4)	85.8 ± 28% (n=4)

$^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, repeated measures ANOVA, significant effect with increasing dose;

* $P < 0.05$, ** $P < 0.01$, Dunnett's multiple comparison test, significant effect of individual dose compared with pre-drug control value.

The effects of spinal application of Mrz 2/579 was studied on four dorsal horn neurones (mean depth = $690 \pm 32\mu\text{m}$, mean C-fibre threshold = $2.33 \pm 0.19\text{mA}$). Mrz 2/579 caused significant, dose-related inhibitions of post discharge and excess spikes (repeated measures ANOVA, $^{**}P = 0.0071$ and $^{\dagger}P = 0.0191$). Significant inhibition was only reached by the top dose in both cases (see Table 3.3 and Figure 3.3C and D). No effect was seen on C-fibre-evoked responses, input, A δ - or A β -fibre-evoked responses, although a preference for inhibition of C- and A δ -fibre over A β -fibre-evoked responses was beginning to emerge at $250\mu\text{g}$.

Table 3.3 Effect of Mrz 2/579 on electrically-evoked neuronal responses

	Control	<i>Drug effects (% control)</i>		
		1 μg	50 μg	250 μg
C-fibres	440 $\pm$ 52 (n=4)	102 $\pm$ 2.8% (n=4)	76.0 $\pm$ 7.3% (n=4)	75.8 $\pm$ 9.3% (n=4)
Post discharge^{††}	290 $\pm$ 31 (n=4)	99.5 $\pm$ 11% (n=4)	87.5 $\pm$ 17% (n=4)	45.8 $\pm$ 11% (n=4) ^{**}
Input	20.5 $\pm$ 4.6 (n=4)	107 $\pm$ 14% (n=4)	97.5 $\pm$ 17% (n=4)	76.3 $\pm$ 18% (n=4)
XS-spikes[†]	441 $\pm$ 85 (n=4)	120 $\pm$ 24% (n=4)	89.8 $\pm$ 16% (n=4)	56.8 $\pm$ 2.9% (n=4) [*]
Aδ-fibres	46.8 $\pm$ 16 (n=4)	92.3 $\pm$ 7.5% (n=4)	71.5 $\pm$ 20% (n=4)	43.3 $\pm$ 16% (n=4)
Aβ-fibres	72.0 $\pm$ 18 (n=4)	115 $\pm$ 8.1% (n=4)	102 $\pm$ 21% (n=4)	104 $\pm$ 19% (n=4)

[†] $P < 0.05$, ^{††} $P < 0.01$, repeated measures ANOVA, significant effect with increasing dose;

^{*} $P < 0.05$, ^{**} $P < 0.01$, Dunnett's multiple comparison test, significant effect of individual dose compared with pre-drug control value.

Comparing the effects of these two glycine site antagonists, it is apparent that Mrz 2/571 is more potent than Mrz 2/579. It is evident from both compounds that the profile of the inhibitions produced is consistent with that expected and previously measured in this experimental paradigm for NMDA receptor blockade.

An example of the effects of Mrz 2/571 and Mrz 2/579 on the wind-up of individual neurones is shown in Figure 3.4. These particular neurones were selected as those with a clear windup, and the drug effects are consistent with the rest of the neurones tested. It is apparent that Mrz 2/571 reduces the windup in a dose-related manner, with a much less pronounced effect on the initial C-fibre input. Mrz 2/579 follows the same profile but is less

potent. C-fibre input is spared more by Mrz 2/571 than by 2/579, in comparison with wind-up and post discharge, an effect that is clearest at 50 μ g.

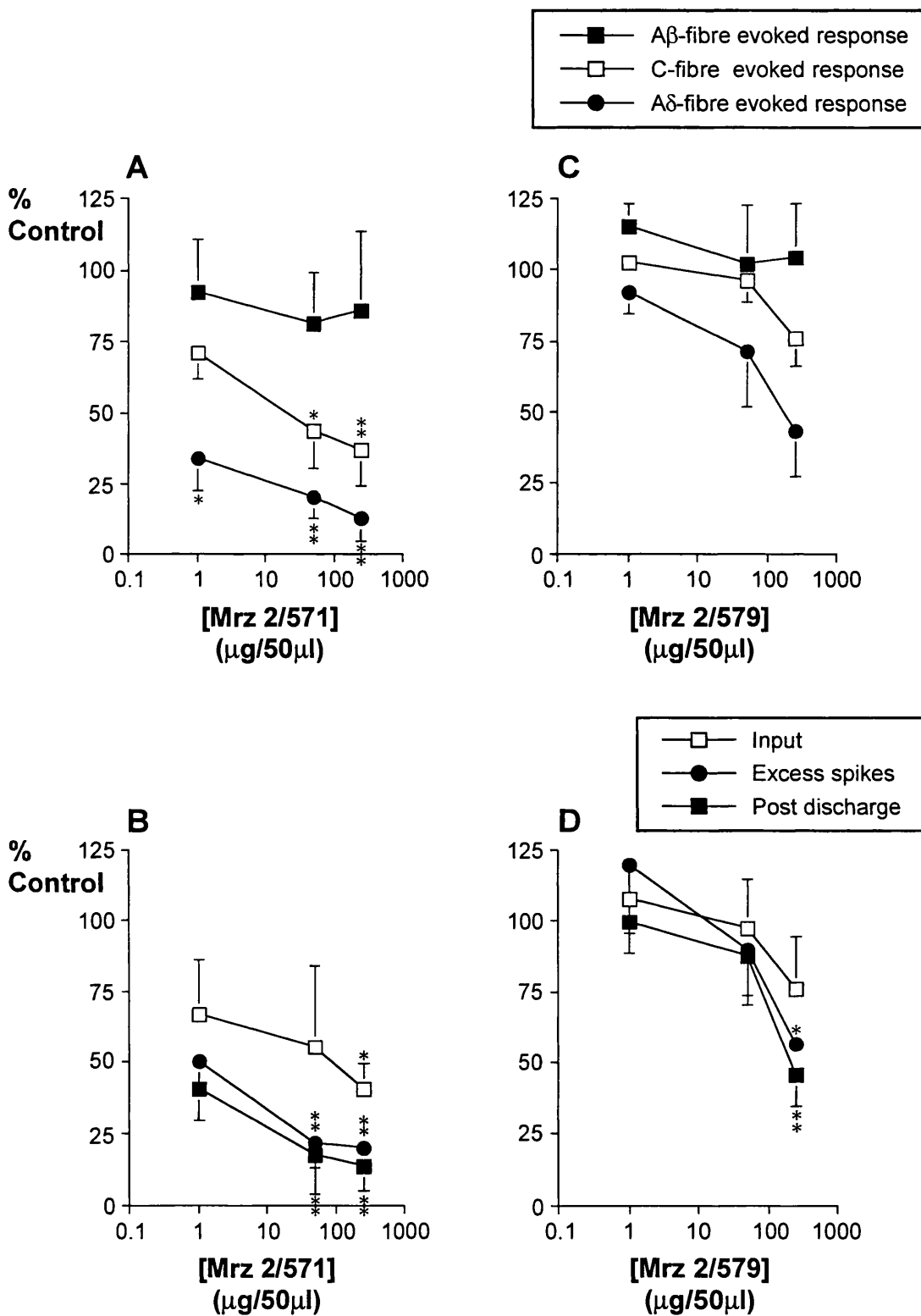


Figure 3.3 Effect of Mrz 2/571 (A and B) and Mrz 2/579 (C and D) on electrically evoked neuronal responses. Data are shown as mean $\pm$ s.e.m. Significant effects of individual doses as compared with pre-drug control are indicated (* P <0.05, ** P <0.01).

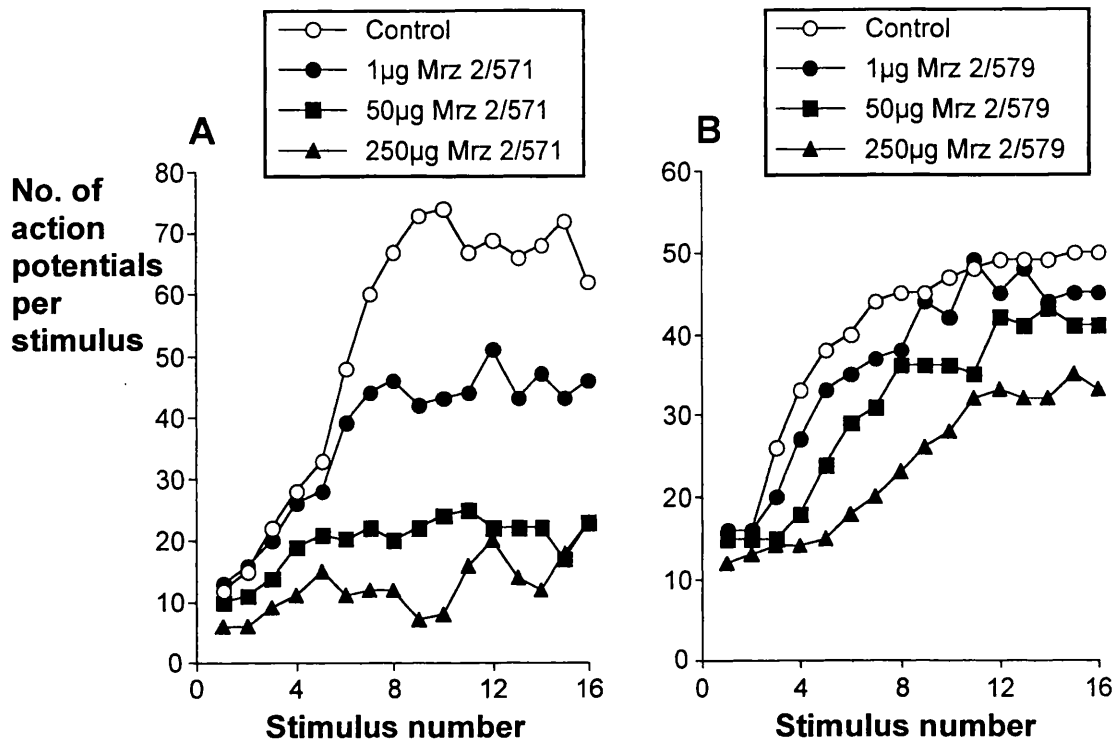


Figure 3.4 Effect of (A) Mrz 2/571 and (B) Mrz 2/579 on the windup of 2 individual neurones.

3.3.2 NR2B selective compounds

Two NR2B selective compounds were studied here; ifenprodil and ACEA-1244. Both were applied spinally and their effects on responses of dorsal horn neurones recorded.

The effects of spinal application of ifenprodil was studied on five dorsal horn neurones, one neurone was used in each animal (mean depth = $900 \pm 51 \mu\text{m}$, mean C-fibre threshold = $1.66 \pm 0.47 \text{mA}$). Ifenprodil caused significant, dose-related inhibitions of C-fibre-evoked response, post discharge, input and excess spikes (repeated measures ANOVA, $^{***}P = 0.0005$, $^*P = 0.0359$, $^{**}P = 0.0011$ and $^*P = 0.0495$, Table 3.4 and Figure 3.5). Post hoc analysis (Dunnett's multiple comparison test) revealed significant inhibitions of the C-fibre-evoked response, input and excess spikes with $400 \mu\text{g}$ (Table 3.4, $^{**}P < 0.01$, $^{**}P < 0.01$ and $^*P < 0.05$). The effect on A δ -fibre-evoked responses and post discharge was also pronounced at $400 \mu\text{g}$ (82% and 84% inhibition respectively), but surprisingly was not significant. The A β -fibre evoked responses were not significantly affected by ifenprodil. The pronounced inhibition of input seen here is in contrast with the effects of the glycine site antagonists, which tended to spare input (Figure 3.5B vs. Figure 3.3B,D).

Table 3.4 Effect of Ifenprodil on electrically-evoked neuronal responses

	Control	<i>Drug effects (% control)</i>		
		1 μ g	40 μ g	400 μ g
C-fibres^{†††}	394 $\pm$ 39 (n=5)	99.8 $\pm$ 12% (n=5)	91.2 $\pm$ 14% (n=5)	41.0 $\pm$ 7.7% (n=5)**
Post discharge[†]	137 $\pm$ 40 (n=5)	111 $\pm$ 25% (n=5)	79.2 $\pm$ 37% (n=5)	15.9 $\pm$ 7.4% (n=4)
Input^{††}	17.2 $\pm$ 4.6 (n=5)	106 $\pm$ 21% (n=5)	107 $\pm$ 29% (n=5)	28.4 $\pm$ 8.8% (n=5)**
XS-spikes[†]	279 $\pm$ 56 (n=5)	83.2 $\pm$ 22% (n=5)	80.0 $\pm$ 28% (n=5)	31.6 $\pm$ 12% (n=5)*
Aδ-fibres	43.9 $\pm$ 23 (n=5)	82.6 $\pm$ 16% (n=5)	82.0 $\pm$ 21% (n=5)	18.0 $\pm$ 8.2% (n=5)
Aβ-fibres	99.1 $\pm$ 17 (n=5)	80.4 $\pm$ 9.3% (n=5)	86.0 $\pm$ 7.8% (n=5)	108 $\pm$ 18% (n=5)

[†]P < 0.05, ^{††}P < 0.01, ^{†††}P < 0.001, repeated measures ANOVA, significant effect with increasing dose; *P < 0.05, **P < 0.01, Dunnett's multiple comparison test, significant effect of individual dose compared with pre-drug control value.

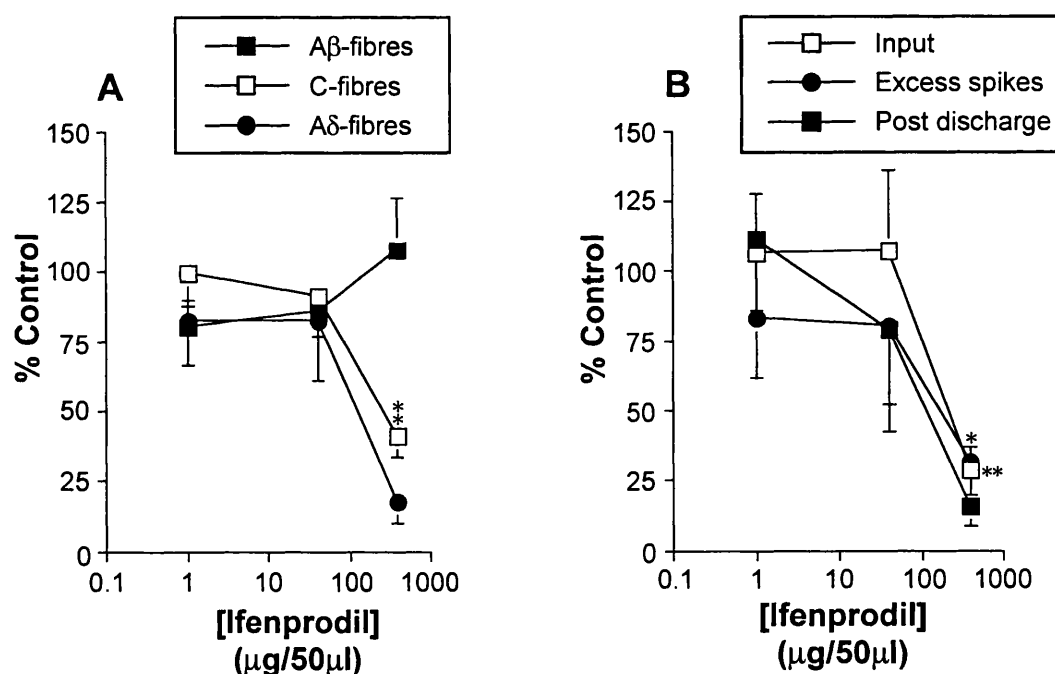


Figure 3.5 Effect of ifenprodil on (A) A β - A δ - and C-fibre evoked responses; and (B) on input, excess spikes and post discharge. Data are shown as mean $\pm$ s.e.m. Significant effects of individual doses as compared with pre-drug control are indicated (*P<0.05, **P<0.01).

The effects of ifenprodil on the wind-up of one individual neurone is shown in Figure 3.6A. In contrast to the situation illustrated in Figure 3.4A and B, doses of ifenprodil that reduced the slope of the line (i.e. inhibit wind-up) also reduced the initial input. Although this example is only one neurone, this difference in the profile of the glycine site antagonists and ifenprodil is also borne out in the dose response curves (Figure 3.1B and 3.5B).

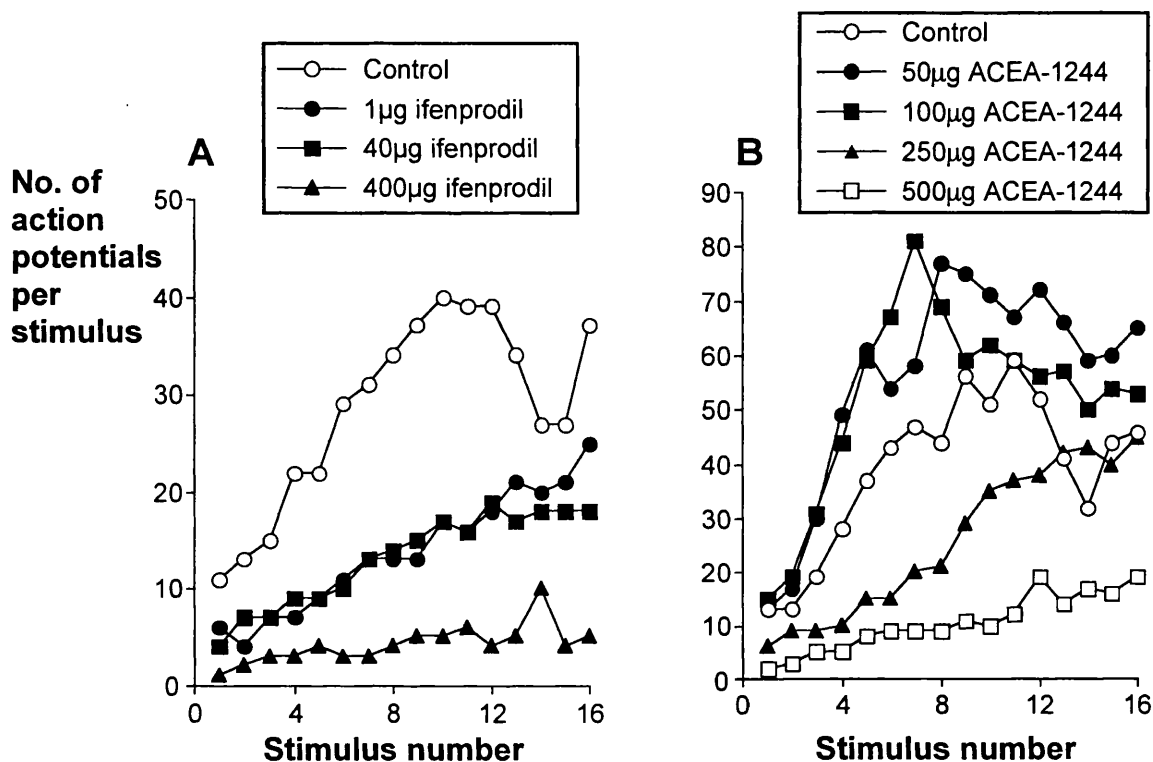


Figure 3.6 Effect of (A) ifenprodil and (B) ACEA-1244 on the windup of 2 individual neurones.

The effects of spinal application of ACEA-1244 were studied on 18 dorsal horn neurones and results from 16 could be used (mean depth = $891 \pm 47\mu\text{m}$, mean C-fibre threshold = $1.96 \pm 0.134\text{mA}$). Application of increasing doses (50, 100, 250 and $500\mu\text{g}$) resulted in significant, dose-related inhibitions of C- and A δ -fibre-evoked responses, post discharge and excess spikes (unpaired Kruskal-Wallis; $^{**}P = 0.0051$, $^{**}P = 0.0097$, $^{**}P = 0.0041$, $^{*}P = 0.0338$ and $^{**}P = 0.0035$; Table 3.5 and Figure 3.7A and C). Comparing the effect of individual doses of ACEA-1244 with control responses revealed a significant inhibition of A δ -fibre-evoked responses and excess spikes with $250\mu\text{g}$ and of C-fibre-evoked responses with $500\mu\text{g}$. No overall effect on A β -fibre evoked responses was observed, although a reduction is apparent at $500\mu\text{g}$ (reduced to 68.1% control).

Table 3.5 Effect of ACEA-1244 on electrically-evoked neuronal responses

	Control	<i>Drug effects (% control)</i>			
		50µg	100µg	250µg	500µg
C-fibres^{††}	393 ± 43 (n=16)	104 ± 5.9% (n=8)	87.7 ± 9.7% (n=11)	61.0 ± 8.5% (n=8)*	60.0 ± 13% (n=8)
Post discharge^{††}	241 ± 42 (n=16)	142 ± 15% (n=8)	110 ± 22% (n=11)	29.1 ± 10% (n=8)	50.0 ± 25% (n=7)
Input[†]	18.9 ± 3.4 (n=16)	122 ± 15% (n=8)	105 ± 16% (n=11)	58.0 ± 14% (n=8)	52.9 ± 20% (n=8)
XS-spikes^{††}	364 ± 45 (n=16)	117 ± 12% (n=8)	93.7 ± 15% (n=11)	48.4 ± 8.3% (n=8)*	50.5 ± 15% (n=8)
Aδ-fibres^{††}	76.6 ± 57 (n=16)	115 ± 14% (n=8)	87.4 ± 18% (n=11)	32.4 ± 7.1% (n=8)* *	54.8 ± 21% (n=8)
Aβ-fibres	100 ± 43 (n=16)	96.5 ± 8.8% (n=8)	78.0 ± 8.2% (n=11)*	90.9 ± 12% (n=8)	68.1 ± 12% (n=8)

[†]*P* < 0.05, ^{††}*P* < 0.01, Kruskal-Wallis non-parametric ANOVA, significant effect with increasing dose; **P* < 0.05, ***P* < 0.01, Student's *t*-test (with Bonferroni correction) significant effect of individual dose compared with pre-drug control value.

As viscous solvents were required to dissolve ACEA-1244 and keep it in solution, and no other drugs required such treatment, application of the vehicle alone was also performed. Eight neurones were studied, and the recordings for one could not be confidently discriminated from background. The remaining seven neurones had a mean depth of 917 ± 50µm, and a mean C-fibre threshold of 1.83 ± 0.31mA. Data were variable, but a general trend towards inhibition of neuronal responses was apparent. This was not statistically significant for any measure, but the trend is apparent from study of Figure 3.7B and D. Post discharge was inhibited most by vehicle alone, and Aβ-fibre-evoked responses were affected least. C- and Aδ-fibre-evoked responses, input and excess spikes were also reduced by the vehicle, albeit to a seemingly lesser extent than by ACEA-1244. However, comparing the effects of vehicle alone with the effects of ACEA-1244, no significant difference was found between the effect of the highest dose of ACEA-1244 and the top 'dose' of vehicle on any neuronal measure. I cannot therefore say that ACEA-1244 inhibits noxious-evoked neuronal responses, as the effects it produced were not significantly different from the effects of vehicle alone.

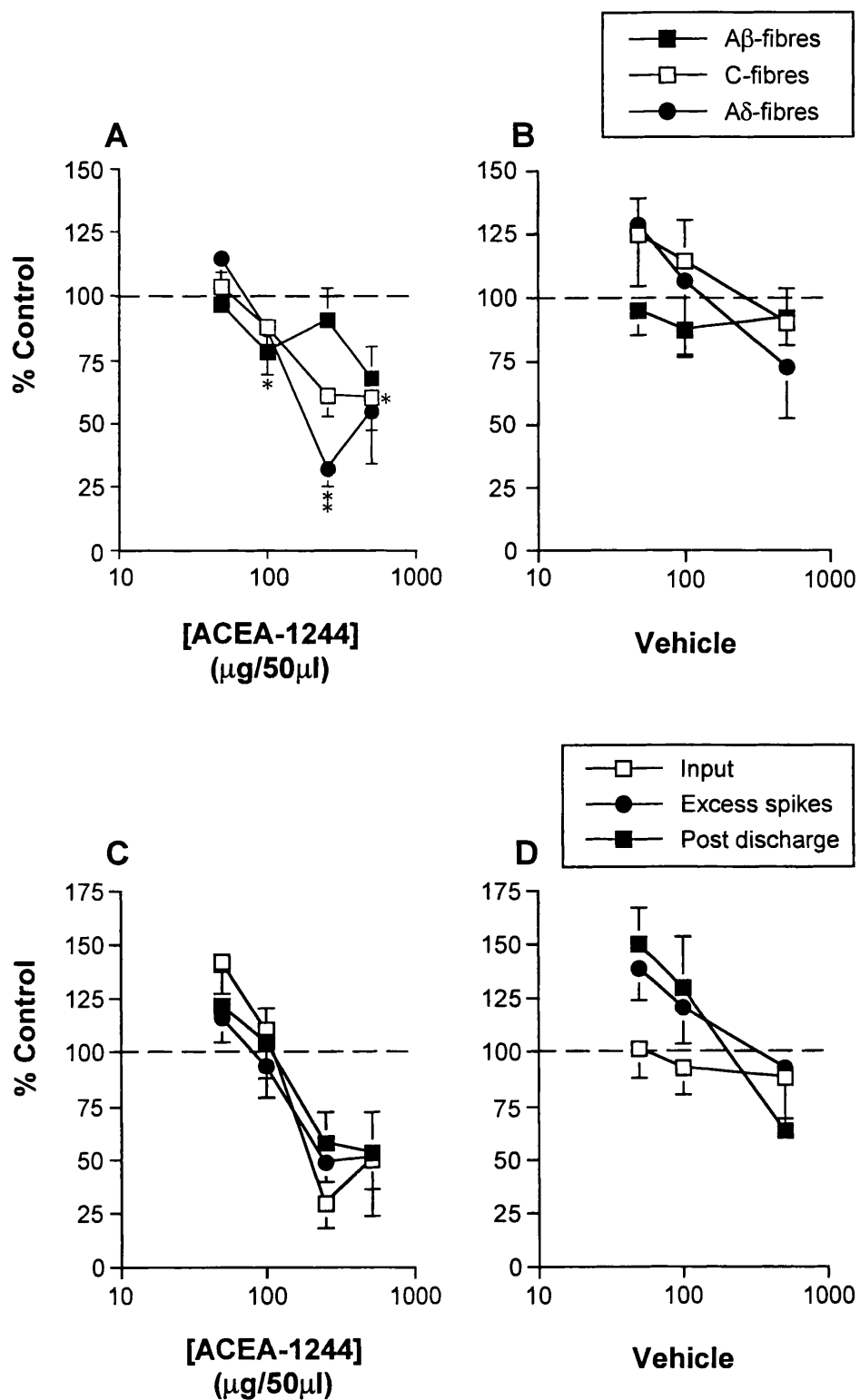


Figure 3.7 Effect of (A,C) ACEA-1244 and (B,D) vehicle alone, on electrically evoked responses of dorsal horn neurones. Data are shown as mean $\pm$ s.e.m. Significant effects of individual doses as compared with pre-drug control are indicated (* $P < 0.05$, ** $P < 0.01$).

3.3.3 Methadone

The effects of *d*-methadone, applied spinally, were studied on 18 dorsal horn neurones (mean depth = $684 \pm 42\mu\text{m}$, mean C-fibre threshold = $1.72 \pm 0.21\text{mA}$). *d*-Methadone was inhibitory, preferentially reducing the C-fibre evoked responses, post discharge, input and excess spikes (unpaired Kruskal-Wallis, $^{***}P < 0.0001$, $^{**}P = 0.0073$, $^{***}P = 0.0005$ and $^{**}P = 0.0073$ respectively), whereas the A β - and A δ -fibre evoked responses were not significantly affected (Figure 3.8). The C-fibre evoked responses were significantly inhibited by 250 μg *d*-methadone ($n = 9$, Student's *t*-tests with the Bonferroni-correction $^{***}P < 0.0005$), whereas 500 μg was required to inhibit post discharge, input and excess spikes ($n=14$, $^{***}P < 0.0005$, $^{**}P < 0.003$ and $^{***}P < 0.0005$, see Table 3.6 and Figure 3.8). Although it is quite clear that the A β -fibre-evoked responses were unaffected by *d*-methadone, some inhibition of the A δ -fibre evoked responses was evident (but not significant).

Table 3.6 Effect of *d*-methadone on electrically-evoked neuronal responses

	Control	<i>Drug effects (% control)</i>				
		5 μg	25 μg	50 μg	250 μg	500 μg
C-fibres^{***}	327 $\pm$ 35 (n=18)	91.5 $\pm$ 5.0% (n=13)	84.3 $\pm$ 6.8% (n=8)	94.2 $\pm$ 7.2% (n=14)	61.0 $\pm$ 8.7% (n=9) ^{***}	37.8 $\pm$ 5.6% (n=14) ^{***}
Post discharge^{**}	218 $\pm$ 32 (n=18)	92.5 $\pm$ 15% (n=13)	100 $\pm$ 27% (n=8)	100 $\pm$ 18% (n=14)	70.0 $\pm$ 20% (n=9)	25.8 $\pm$ 9.7% (n=14) ^{***}
Input^{***}	14.8 $\pm$ 2.5 (n=18)	110 $\pm$ 13% (n=13)	94.5 $\pm$ 19% (n=8)	118 $\pm$ 17% (n=14)	61.9 $\pm$ 19% (n=9)	30.6 $\pm$ 6.4% (n=14) ^{**}
XS-spikes^{**}	335 $\pm$ 75 (n=18)	80.4 $\pm$ 11% (n=13)	75.8 $\pm$ 19% (n=8)	90.0 $\pm$ 15% (n=14)	84.8 $\pm$ 22% (n=9)	36.8 $\pm$ 12% (n=14) ^{***}
Aδ-fibres	54.0 $\pm$ 7.1 (n=16)	76.9 $\pm$ 11% (n=12)	78.6 $\pm$ 15% (n=8)	67.4 $\pm$ 12% (n=12)	63.6 $\pm$ 13% (n=9)	44.8 $\pm$ 19% (n=14)
Aβ-fibres	98.0 $\pm$ 6.5 (n=18)	100 $\pm$ 6.9 (n=13)	90.6 $\pm$ 10% (n=8)	92.9 $\pm$ 9.2% (n=14)	98.2 $\pm$ 11% (n=9)	92.6 $\pm$ 10% (n=14)

[†] $P < 0.05$, ^{††} $P < 0.01$, ^{†††} $P < 0.001$, Kruskal-Wallis non-parametric ANOVA, significant effect with increasing dose; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ Student's *t*-test (with Bonferroni correction) significant effect of individual dose compared with pre-drug control value.

Naloxone (1 μg) reversed the effects of 500 μg *d*-methadone on C-fibre evoked responses and input (* $P = 0.0192$, * $P = 0.0354$), whereas 5 μg was required to reversed the inhibition of post discharge and excess spikes (* $P = 0.0104$, ** $P = 0.0062$, Figure 3.8).

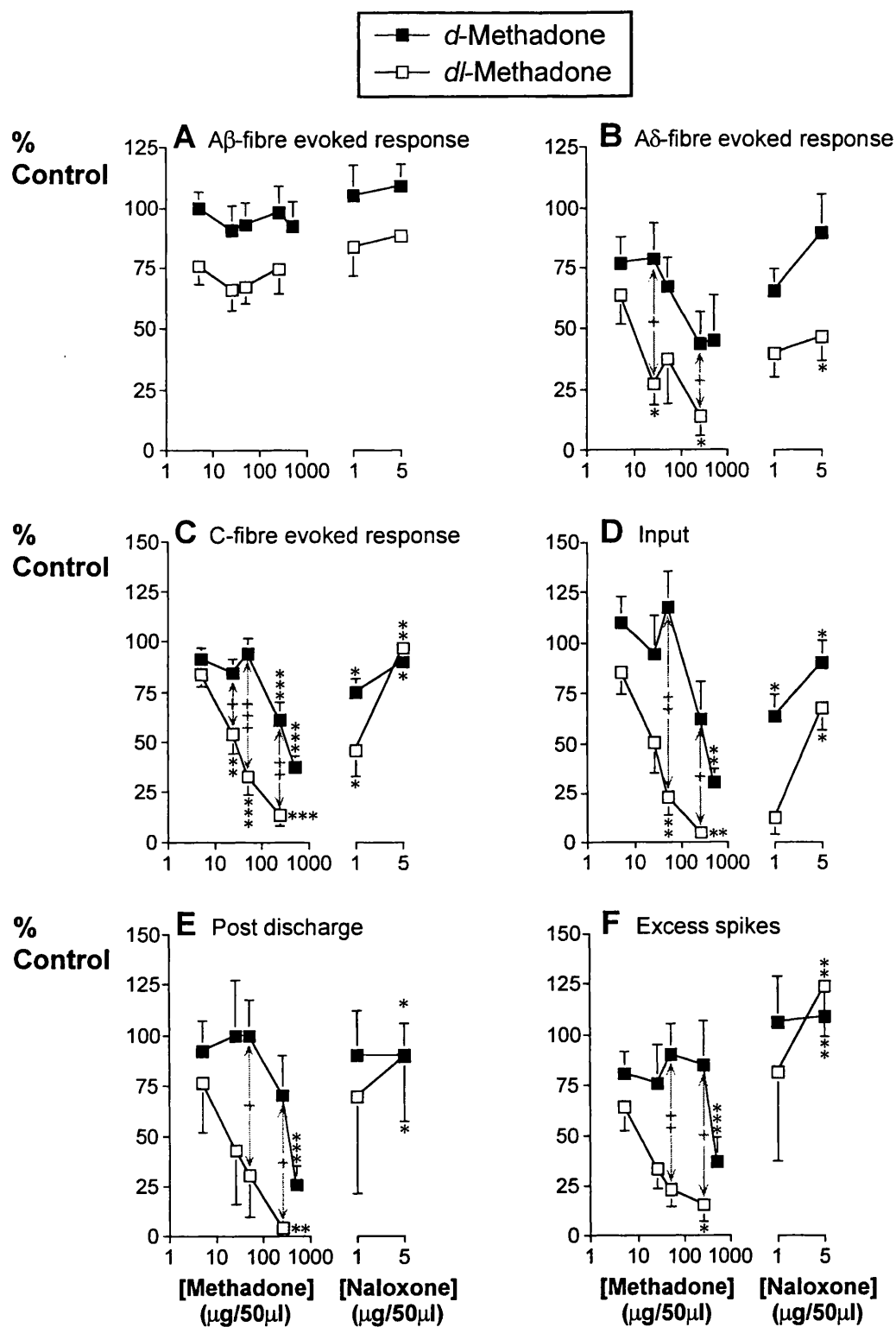


Figure 3.8 Effect of *d*- and *dl*-methadone on Aβ- Aδ- and C-fibre evoked responses (**A**, **B** and **C** respectively), input (**D**), post discharge (**E**) and excess spikes (**F**). Data are shown as mean $\pm$ s.e.m. Significant effect as compared with pre-drug control (*), significant naloxone reversal (*) and significant difference between effects of *d*- and *dl*-methadone (+) are indicated.

An example of the effect of *d*-methadone on the wind-up of an individual neurone is shown in Figure 3.9A. Here, dose-related reduction of input and wind-up was seen, although low doses that reduce the input had little effect on wind-up. Only the top dose of 500µg *d*-methadone began to reduce wind-up and somewhat reduce the slope of the curve. 5µg naloxone almost completely reversed the effect of spinal *d*-methadone on this neurone.

The effects of racemic (*dl*-) methadone (5, 25, 50 and 250µg) were studied on 10 dorsal horn neurones (mean depth = 639±74µm, mean C-fibre threshold = 1.48 ± 0.22mA). C- and Aδ-fibre evoked responses, post discharge, input and excess spikes were all significantly inhibited by increasing doses of *dl*-methadone (unpaired Kruskal-Wallis, ^{†††}*P* = 0.0002, [†]*P* = 0.0321, ^{††}*P* = 0.0092, ^{†††}*P* = 0.0003 and [†]*P* = 0.0255 respectively). Student's *t*-tests with the Bonferroni correction revealed that a significant inhibition was apparent at 25µg for C- and Aδ-fibre evoked responses (^{††}*P* = 0.0064 and ^{*}*P* = 0.0336) and at 50–250µg for other parameters; the maximal inhibition in each case was reached with 250µg *dl*-methadone (Figure 3.8, Table 3.7 for summary).

Table 3.7 Effect of *dl*-methadone on electrically-evoked neuronal responses

	Control	<i>Drug effects (% control)</i>			
		5µg	25µg	50µg	250µg
C-fibres^{†††}	307 ± 47 (n=10)	83.8 ± 6.1% (n=9)	54.3 ± 10% (n=8) ^{††}	32.9 ± 9.0% (n=9) ^{†††}	13.6 ± 5.2% (n=8) ^{††}
Post discharge^{††}	188 ± 75 (n=10)	76.4 ± 25% (n=9)	43.1 ± 27% (n=8)	30.6 ± 21% (n=9)	4.25 ± 2.3% (n=8) ^{††}
Input^{†††}	17.1 ± 4.8 (n=10)	85.6 ± 11% (n=9)	50.5 ± 15% (n=8)	22.9 ± 9.2% (n=9) ^{††}	5.12 ± 3.4% (n=8) ^{††}
XS-spikes[†]	231 ± 33 (n=9)	64.4 ± 12% (n=8)	33.4 ± 10% (n=7)	23.1 ± 8.6% (n=8)	15.6 ± 8.4% (n=7) [*]
Aδ-fibres[†]	48.6 ± 11 (n=10)	63.7 ± 12% (n=9)	27.1 ± 8.9% (n=8) [*]	37.2 ± 18% (n=9)	13.8 ± 7.7% (n=8) [*]
Aβ-fibres	92.1 ± 16 (n=10)	76.0 ± 7.6% (n=9)	66.0 ± 8.6% (n=8)	67.0 ± 6.6% (n=9)	74.4 ± 10% (n=8)

[†]*P* < 0.05, ^{††}*P* < 0.01, ^{†††}*P* < 0.001, Kruskal-Wallis non-parametric ANOVA, significant effect with increasing dose; ^{*}*P* < 0.05, ^{††}*P* < 0.01, ^{†††}*P* < 0.001, Student's *t*-test (with Bonferroni correction) significant effect of individual dose compared with pre-drug control value.

dl-Methadone-mediated inhibitions of C- and Aδ-fibre-evoked responses were significantly reversed by 1µg naloxone (^{*}*P* = 0.0358 and ^{*}*P* = 0.04), whereas 5µg naloxone was required to reverse the inhibitory effects of *dl*-methadone on post discharge, input and

excess spikes ($*P = 0.0236$, $*P = 0.0122$ and $**P = 0.0092$) and to fully reverse its effects on C-fibre evoked responses back to 100% of control (Figure 3.8).

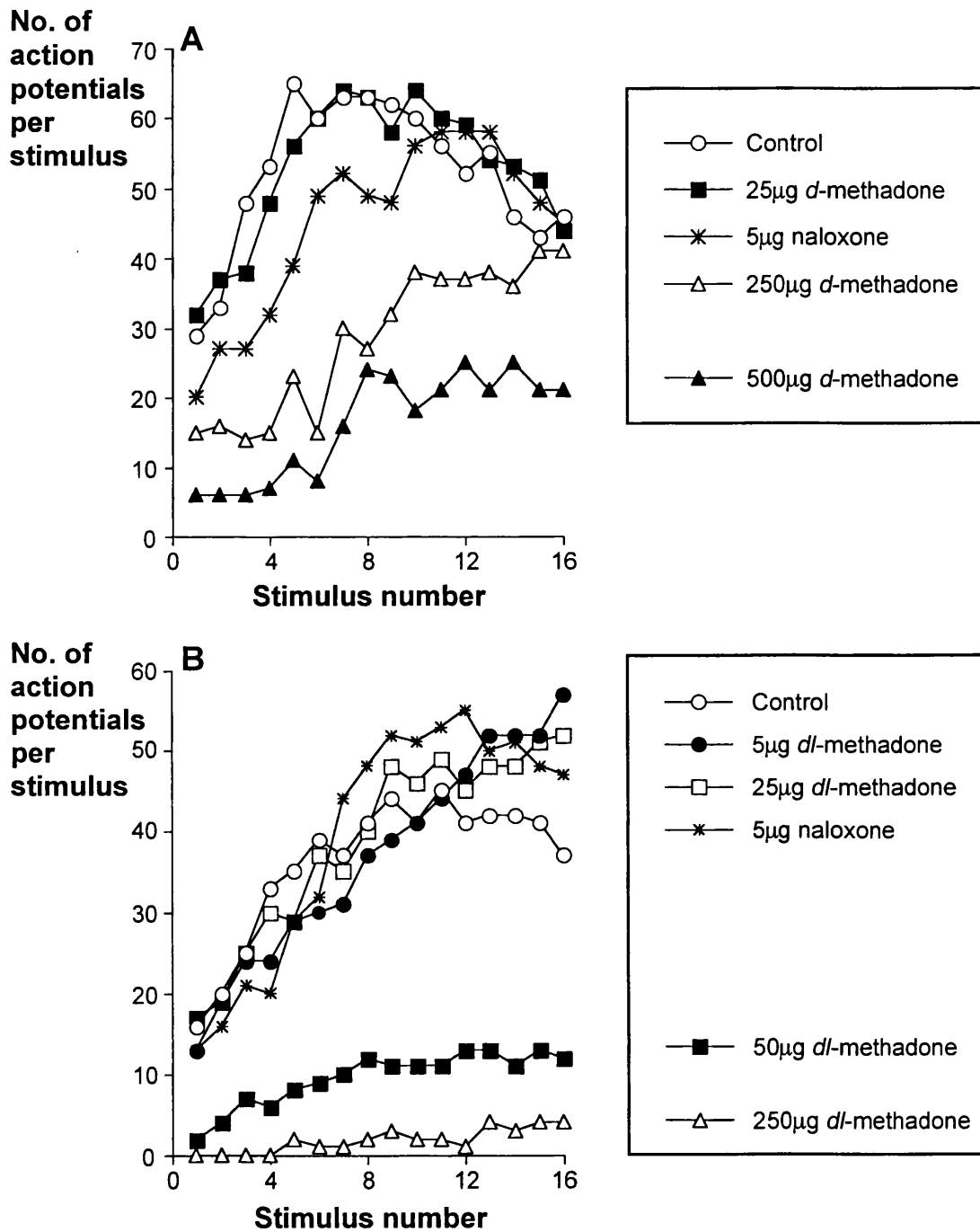


Figure 3.9 Effect of *d*-methadone (A) and *dl*-methadone (B) on the windup of 2 individual neurones. The reversal by 5µg naloxone is also indicated. Note that different doses are shown on each graph.

An example of the effect of *dl*-methadone on the wind-up of an individual neurone is shown in Figure 3.9B. Irrespective of the magnitude of the neuronal response, *dl*-methadone almost abolished the input at 50 μ g yet some wind-up still occurred from this baseline. The top dose of 250 μ g almost completely abolished the wind-up and a low dose of naloxone (5 μ g) completely reversed the effect of *dl*-methadone on both input and wind-up. Therefore, unlike NMDA receptor antagonists, effects on wind-up do not occur in the absence of inhibition of input.

Before comparisons were made between the effects of *d*- and *dl*-methadone, the mean control responses for all neuronal measures were compared and found not to be different between the groups (Mann-Whitney unpaired *t*-tests). Comparing the effects of the racemic mixture and the *d*-isomer of methadone alone on the different parameters showed that *dl*-methadone was more potent in all cases. At a dose of 250 μ g, *dl*-methadone produced a significantly greater inhibition than the same dose of *d*-methadone on C- and A δ -fibre evoked responses, input, post-discharge and excess spikes (unpaired Mann-Whitney using % control values, $^{**}P = 0.0025$, $^{*}P = 0.0104$, $^{*}P = 0.0111$, $^{*}P = 0.0206$ and $^{*}P = 0.0164$). The difference between the effects of the *d*-isomer and the racemic mixture was also significant at the lower dose of 50 μ g for the C-fibre evoked response, input, post discharge and excess spikes ($^{***}P = 0.0004$, $^{**}P = 0.0028$, $^{*}P = 0.0181$, $^{**}P = 0.0046$) and at 25 μ g for the C- and A δ -fibre evoked responses ($^{*}P = 0.0281$ and $^{*}P = 0.0140$). Drug effect (% control) values were re-plotted on log graph paper to enable the dose required to give 50% inhibition to be read accurately. *d*-Methadone was 13-times less potent than *dl*-methadone in inhibiting input and the C-fibre evoked response, 19-times less potent on post discharge, 42 times less potent in inhibiting wind-up and 48-times less potent on A δ -fibre evoked responses.

3.4 Discussion

3.4.1 Glycine-site antagonists

The two compounds tested here have been previously examined and their selective binding to the glycine site, antagonism of applied NMDA *in vitro* and functional antagonism of NMDA receptors in a behavioural convulsion model determined (Parsons *et al.*, 1997). References in this report also detail the reduced psychomotor and psychotomimetic effects of these antagonists observed in behavioural tests, and the lack of neurodegenerative changes consistent with the profile of other glycine site antagonists described above.

Here, Mrz 2/571 and 2/579 were inhibitory when applied directly to the spinal cord, with profiles characteristic of NMDA receptor antagonists. Both drugs spared the A β -fibre evoked responses, but significantly inhibited the post discharge and excess spikes, measures of postsynaptic, wind-up-induced activity. The effects of these drugs on wind-up is illustrated clearly in Figure 3.4A and C; the selectivity for inhibition of wind-up over input is characteristic for NMDA receptor antagonists. This lack of effect on the input is likely to reflect the post synaptic location of the NMDA receptor, and its minimal participation in 'normal' neuronal transmission. The measure I refer to as 'input' is the response of a dorsal horn neurone to the first stimulus in the train. This represents the excitatory effect of a single afferent volley on spinal neuronal circuitry, prior to which the system is at rest and therefore under conditions where the Mg²⁺ block of the NMDA receptor is presumed to be still intact. The established assumption is that the NMDA receptor has little or no participation in 'normal' activity, which implies that NMDA receptor antagonists should therefore have little or no effect on input. That these drugs do have some inhibitory effect on the input, especially at high doses, suggests that the input onto the neurone elicited by the first stimulus of a train is sufficient to recruit a small number of NMDA receptors.

The extent of inhibition of input is comparable to the inhibition of C-fibre-evoked responses — and less susceptible to inhibition than post discharge and excess spikes. Even when NMDA-receptor mediated wind-up is profoundly inhibited, C-fibre stimulation is still able to evoke a substantial amount of activity,. Thus, baseline C-fibre-mediated transmission occurs without reliance on the NMDA receptor, but when wind-up occurs the response to C-fibre input can be greatly enhanced

Interestingly, Mrz 2/571 produced a pronounced inhibition of A δ -fibre-evoked responses (87% inhibition) that is comparable to the inhibition of wind-up (86% and 80% inhibition of post discharge and excess spikes respectively). This suggests that, even more so than for C-fibres, wind-up is a major determinant of the neuronal response elicited by A δ -fibre inputs. Since recordings were made from convergent neurones, it is unlikely that this difference was due to distribution of NMDA receptors unless they are preferentially located on pathways associated with A δ -fibres. Rather, the smaller number of A δ -fibre afferents (in

comparison with numbers of C- and A β -fibre afferents) may mean that A δ -fibre inputs are not always sufficient to evoke action potentials from dorsal horn neurones — at least through the polysynaptic pathway to deep dorsal horn, WDR neurones. Inputs from A δ -fibre afferents may therefore contribute significantly to neuronal activity only when the system is in a hyperexcited state. This, in combination with the generally higher threshold of A δ -fibre nociceptors than C-fibre nociceptors, may form the basis for another mechanism underlying activity-dependent hyperalgesia and allodynia — the 'strengthening' of A δ -fibre–dorsal horn neurone synapses.

The data presented here add to the expanding volume of work showing that *in vitro* activity at the glycine site of the NMDA receptor translates into a functional blockade of NMDA receptor mediated events *in vivo* (Chapman & Dickenson, 1995; Davies & Lodge, 1987; Dickenson & Aydar, 1991; Millan & Seguin, 1993; Millan & Seguin, 1994; Quartaroli *et al.*, 1999). However, a recent report has suggested that the antinociceptive effect of a glycine site antagonist (ACEA-1021) in a rat model of post-operative pain was due, in part, to action at non-NMDA receptors (Brennan & Zahn, 2000).

Despite the reduced incidence of psychotomimetic side effects described for glycine site antagonists, they show poor separation between the dose required for anti-nociception and anti-hyperalgesia, and those that cause ataxia (for a thorough analysis see Boyce *et al.*, 1999 and also Danysz & Parsons, 1998). The potential clinical utility of antagonists selective for the glycine site of the NMDA receptor remains to be determined; drug development seems to have reached the bottle neck of improving toxicological and pharmacokinetic profiles before human testing. Preclinical studies with NMDA receptor antagonists have translated well into the clinic so far. Drugs developed from these agents may prove to be as efficacious antihyperalgesic agents as clinically approved agents such as ketamine, but have an improved CNS side effects profile, while retaining some degree of motor impairment. An approach to clinical use of this class of agent could be to limit the dose by co-administration with an opioid.

3.4.2 NR2B-selective antagonists

The distribution of NR2B-containing NMDA receptors in the rat spinal cord has been unclear for some time. The literature now agrees that NMDA receptors containing the NR2B subunit are present particularly in the superficial dorsal horn. Luque *et al.* (1994) report NR2B mRNA expression in lamina II (but not lamina I) of the dorsal horn, whereas Tölle *et al.* (1993) found no NR2B mRNA in the spinal cord. Development of subunit-specific antibodies has allowed detection of NR2B protein in lamina I and II of the dorsal horn (Boyce *et al.*, 1999; Yung, 1998). The latter study specifically aimed to address discrepancies in NR2B subunit localisation, and provides compelling evidence that NR2B-containing NMDA receptors are localised in lamina I and II of the dorsal horn. Specifically, NR2B protein was found in fibres, not cell bodies, in lamina I, which may indicate a presynaptic location for

NR2B-containing receptors. This could also explain the varied reports of NR2B mRNA expression in the spinal cord; but as yet NR2B expression has not been reported in primary afferents or the DRG. Functional presynaptic NMDA receptors have been demonstrated in immunocytochemical (Liu *et al.*, 1994), and behavioural (Liu *et al.*, 1997) studies. The localisation of NR2B subunits is unlikely to be exclusively presynaptic; single-channel recordings from SG neurones in adult rat spinal cord by (Momiya, 2000) have detected receptors thought to contain NR2B subunits on the extrasynaptic membrane of SG neurones. The binding of ifenprodil in adult rat spinal cord is another measure of NR2B subunit distribution; high affinity ifenprodil binding sites make up 18.6% of the total NMDA receptor population in adult rat spinal cord (Nankai *et al.*, 1996), and a much higher proportion in neonatal rat cord (Williams *et al.*, 1993).

In contrast with the discrete localisation of NR2B subunits, NR2A and NR2D subunit mRNA and protein is evenly distributed through dorsal and ventral horns, and NR2C mRNA is either absent, or present at low levels (Boyce *et al.*, 1999 and references therein; Luque *et al.*, 1994; Tölle *et al.*, 1993). In the brain, NR2B subunits are restricted mainly to the forebrain, whereas NR2A expression is widespread (Wenzel *et al.*, 1995). Here then is an anatomical correlate for the reduced motor side effects reported with ifenprodil and more selective NR2B-selective compounds; a lower expression in the ventral horn and in areas of the brain concerned with motor co-ordination. The concentration in the forebrain may, however, mean that NR2B selective antagonists may not circumvent the psychomimetic effects of the non-selective NMDA receptor antagonists.

It seems likely that NMDA receptors containing the NR2B subunit are present in the right place to play a role in the transmission of nociceptive information. The pressing question is whether blockade of this population of receptors is sufficient to attenuate NMDA receptor-mediated hyperexcitability and therefore prevent the development of hyperalgesia; or whether the more widespread distribution of NR2A-containing receptors will preclude any observable effect after blockade of NR2B-containing receptors. Once the involvement of NR2B-containing receptors in nociception is determined, then the therapeutic benefit of this selective blockade can be explored.

Ifenprodil is a noncompetitive NMDA receptor antagonist, exhibiting 400-fold preference for the NMDA NR2B subunit over NR2A in heteromeric NMDA receptors expressed in *Xenopus* oocytes (Priestley *et al.*, 1995; Williams, 1993). It is disputed whether ifenprodil and polyamines act at the same site, the polyamine site (Carter *et al.*, 1989), or at discrete sites on the NR2B subunit (Gallagher *et al.*, 1996). Whichever is the case, channel gating is altered such that the channel open time or gating frequency are reduced, and hence the total Ca^{2+} influx reduced *in vitro*. ACEA-1244 (also known as Co 101244, and PD174494), has greater than 2000-fold affinity for NR1A/NR2B over NR1A/NR2A receptors (Zhou *et al.*, 1998 and see Table 3.8). Ro 25-6981 and CP-101,606 are some of the newer, more selective compounds that are emerging from pharmaceutical companies. Both bind to

rat brain in patterns that correspond both to ifenprodil binding and NR2B distribution (Menniti *et al.*, 1997; Mutel *et al.*, 1998) and have functional neuroprotective activity via NMDA receptor blockade (Fischer *et al.*, 1997; Menniti *et al.*, 1997; Menniti *et al.*, 2000).

Table 3.8 Subunit selectivity of ifenprodil, ACEA-1244 and Ro 25-6981 at recombinant NMDA receptors expressed in *Xenopus* oocytes.

	IC ₅₀ (μM)		
	Ifenprodil	ACEA	Ro 25-6981
NR1/NR2B	0.34 ^a	0.043 ^b	0.009 ^c
NR1/NR2A	146 ^a	> 100 ^b	52 ^c

^a (Williams, 1993), ^b (Zhou *et al.*, 1999), ^c (Fischer *et al.*, 1997)

Here, spinal application of both ifenprodil and ACEA-1244 inhibited noxious- and windup-evoked activity of dorsal horn neurones. In contrast with the glycine site antagonists discussed in the previous section, inhibition of input was comparable to inhibition of wind-up activity for both ifenprodil and ACEA-1244 (Figure 3.5B, Figure 3.6 and Figure 3.7C). The maximal inhibition of input with both of these drugs is greater than with the glycine site antagonist. When the results obtained with ACEA-1244 are compared with the effects of the vehicle alone, the effects are less clear-cut. The drug certainly looks to cause a greater inhibition than the vehicle, but this was not significant. The unphysiological nature and viscous physical properties of the vehicle was unfortunate, and did complicate the experiments considerably. This problem had not been evident in previous experiments where it had been administered orally (behavioural experiments, Tony Carnell, personal communication), by subcutaneous injection (Blanchet *et al.*, 1999), or at very low concentrations *in vitro* (Zhou *et al.*, 1999). However, the similarities of the profiles of ifenprodil and ACEA-1244 suggest that they are working through the same mechanism.

The results obtained here, correlate well with other reports in the literature using ifenprodil (Bernardi *et al.*, 1996; Boyce *et al.*, 1999; Sakurada *et al.*, 1998; Song & Zhao, 1998; Tan-No *et al.*, 2000), and other NR2B selective compounds (Boyce *et al.*, 1999; Taniguchi *et al.*, 1997).

The most thorough examination of these NR2B selective compounds is certainly that by Boyce *et al.* (1999). In addition to determining the distribution of NR2B protein in the spinal cord of rats (as discussed earlier), a thorough behavioural study was performed using two models of clinically relevant pain states, and a thorough analysis of behavioural side effects as well as measures of windup in rabbit motor units. The two potent NR2B-selective compounds tested, (±)Ro25-6981 and (±)-CP-101,606, inhibited carrageenan-induced hyperalgesia and nerve injury-induced allodynia at doses that did not alter baseline pain behaviours and did not cause motor side effects. Approximately 25-fold higher doses are required to reduce spontaneous locomotor activity and impair motor coordination (rotarod test), compared with the ED₅₀ for inhibition of allodynia. Both (±)Ro25-6981 and (±)-CP-

101,606 also inhibited the wind-up evoked spinal reflex in the rabbit at similar doses. Ifenprodil was similarly effective in reversing the carrageenan-induced hyperalgesia, nerve injury-induced allodynia and the facilitated spinal reflex. However, ifenprodil also inhibited base-line behavioural responses, increasing the mechanical threshold for withdrawal in the contralateral (non-inflamed) paw. A reduction in spontaneous locomotor activity was seen within the therapeutic dose range, and only a fivefold increase in dose was required to cause rotarod impairment. MK-801 was also administered across the same range of tests, and no separation was seen between the dose required to inhibit carrageenan-induced hyperalgesia, nerve injury-induced allodynia and the facilitated spinal reflex and the dose that led to reduced spontaneous locomotor activity and impairment of rotarod performance. A similar selectivity of CP-101,606 for anti-nociceptive and anti-hyperalgesic effects over motor side effects has also been shown in the rat by Taniguchi *et al.* (1997).

Spinal application of ifenprodil in another electrophysiological study reports results comparable to those I obtained. This study in spinalised cats, saw an inhibitory effects of iontophoretically applied ifenprodil on NMDA- and noxious heat-evoked responses of WDR dorsal horn neurones (Song & Zhao, 1998). A pronounced synergy was reported between ifenprodil and the glycine site antagonist chlorokynurenate (7-CK), and ifenprodil and the glutamate competitive antagonist APV (AP5). Intrathecal administration of ifenprodil in the rat reduced the behavioural response to intraplantar injection of capsaicin (Sakurada *et al.*, 1998). In a different behavioural paradigm, intrathecal administration of ifenprodil in mice inhibited the pain behaviours (scratching, biting and licking) evoked by intrathecal injection of the endogenous polyamine spermine (a positive modulator of NMDA receptors). Effects on stimulus-evoked pain behaviours or hyperalgesia were not tested (Tan-No *et al.*, 2000).

Several other studies with ifenprodil, however, have produced contrasting results. Although Bernardi *et al.* (1996) found that systemic ifenprodil increased baseline acute nociceptive thresholds in the hot plate test in the mouse, pretreatment with naloxone prevented the effect of ifenprodil on nociceptive thresholds. This study, however, did not look at any models of hyperalgesia, where the mode of action of ifenprodil may be more dependent on NMDA receptor blockade. A study published early this year, (Chizh *et al.*, 2001), reported that ifenprodil (i.p.) reduced carrageenan-induced hyperalgesia, but i.v. ifenprodil had no effect on the activity of dorsal horn WDR neurones or on C-fibre-evoked wind-up of single motor units — measures that were reduced by the non-selective NMDA receptor antagonists MK-801 and APV. These animals were spinalised. When tested in the intact animal, ifenprodil was seen to inhibit wind-up of single motor unit activity, whereas the potency of MK-801 was unchanged. The authors conclude that behavioural effects of systemic ifenprodil are mediated through supra-spinal mechanisms, and that blockade of spinal NMDA receptors does not contribute to its anti-nociceptive profile. Coderre (1993) found ifenprodil to have no effect on pain behaviours in either phase of the formalin test; however, this same paper reported that the glycine-site antagonist 7-CK was unable to inhibit either phase of the formalin test, which is in contrast with many other studies with

intrathecal application glycine site antagonists in the formalin test (Chapman & Dickenson, 1995; Millan & Seguin, 1993; Millan & Seguin, 1994; Quartaroli *et al.*, 1999).

One potential explanation for these differences is the intensity of the stimuli applied in the tests. In acute nociceptive behavioural tests, NMDA receptor antagonists do not generally alter thresholds or responses (Nishiyama *et al.*, 1998). A sufficiently intense stimulus must be applied in order to recruit the NMDA receptor and generate spinal hypersensitivity; a level of stimulus that is not always possible in behavioural paradigms without the generation of a chronic pain state, for instance carrageenan inflammation or nerve ligation as used by Boyce *et al.* (1999).

It is, of course, necessary to bear in mind that ifenprodil is not completely selective for the NMDA receptor. It was initially developed as an α_1 -adrenoceptor antagonist and also acts as a noncompetitive antagonist at the 5-HT₃ receptor. The α_1 -adrenoceptor is expressed in vascular smooth muscle and does not participate in nociceptive processing in the spinal cord. The 5-HT₃ receptor is thought to participate in excitatory nociceptive transmission in conditions of inflammation but not in normal animals (Oyama *et al.*, 1996). As I tested animals in the absence of inflammation, the contribution of 5-HT₃ receptor blockade to the anti-hyperalgesic profile can be excluded. In chronic pain models, however, this action could contribute beneficially to the therapeutic antihyperalgesic potential of ifenprodil.

The effects of a novel NR2B-selective antagonist in animal models of acute and prolonged nociception were reported in two poster presentations at the 30th Annual meeting of the Society for Neuroscience (New Orleans, 4–9th November, 2000). Carter *et al.* (2000) reported reversal of carrageenan-induced hyperalgesia and neuropathy-induced hyperalgesia (but not allodynia) after systemic administration of CI-1041 (Co 200461, OD 196860) at doses that did not impair performance in the rotarod test. Fillhard *et al.* (2000) reported no effect on tests of acute nociception, but a dose-dependent reduction of both the first and second phase of the formalin test; effects were not reversed by treatment with naltrexone. Motor side effects were also much reduced when compared with MK-801. The ability of NR2B-selective compounds to attenuate both activity attributed to NMDA-receptor-mediated spinal hypersensitivity (wind-up in electrophysiological tests, second phase of the formalin test, carrageenan- and neuropathy-induced hyperalgesia) and acute nociceptive events (inhibition of input as presented earlier, inhibition of the first phase of the formalin test) may indicate an involvement of NR2B subunits in transmission of baseline nociceptive information.

The work described here with ifenprodil and ACEA-1244 adds to the emerging literature that NR2B subunits of the NMDA receptor are an attractive therapeutic target. This is interesting; as NR2B-containing NMDA receptors appear to make up a small proportion of the total NMDA receptor population in the rat spinal cord, and could imply that these receptors are not only functional in the spinal cord, but far more important in nociceptive

modulation than the more widespread NR2A subunit-containing receptors. NR2B selective NMDA receptor antagonists are also undergoing validation as an approach to stroke treatment. CP-101,606 has been shown to protect against CNS-injury- and focal ischaemia-induced cell death, without motor side effects in animal models (Menniti *et al.*, 1997; Menniti *et al.*, 2000) and is under phase I clinical trials to determine its suitability for further study as a treatment of stroke and traumatic CNS injury (Bullock *et al.*, 1999; Merchant *et al.*, 1999).

3.4.3 Methadone

After spinal application in the anaesthetised rat, the highest dose of *d*-methadone (500µg) was anti-nociceptive, showing selectivity for C-fibre and Aδ-fibre evoked neuronal responses, input, wind-up and post discharge over Aβ-fibre evoked responses. This broad antinociceptive action provides some clues as to the mechanism of action of this drug. NMDA receptors are thought to be located predominantly postsynaptically on spinal neurones. These receptors make a major contribution to the hyperexcitability produced by repeated C-fibre stimulation but play a minimal role in the underlying baseline responses of the neurones. Thus pure NMDA receptor antagonists have pronounced inhibitory effects on measures of hyperexcitability such as windup and post discharge and tend to spare the input, Aβ- and Aδ-fibre evoked responses (Dickenson, 1990). Conversely, the inhibitory µ opioid receptors are predominantly found on the terminals of afferent C-fibres with a much smaller postsynaptic population. Thus opioids have pronounced effects on C-fibre-evoked responses and input (the initial response of the neurone to the afferent volley) presumably as a result of their ability to reduce primary afferent transmitter release. At higher doses, however, opioids do cause a reduction in wind-up. Examination of wind-up responses (Figure 3.9) reveals that this inhibition of wind-up is secondary to the reduced baseline input responses of the cells. NMDA receptor antagonists will block wind-up without reducing the baseline (Dickenson, 1990). Another important difference between the actions of opioids and NMDA receptor antagonists is that only the former are sensitive to naloxone (Dickenson *et al.*, 1991).

The effects of *d*-methadone on neuronal responses did not resemble the profile expected of an NMDA receptor antagonist but rather mirrored the profile of the racemic mixture albeit with a lower potency (Figure 3.8). In addition, the inhibitory effects of this isomer were clearly and fully reversed by low doses of naloxone (1µg and 5µg). Overall, this could suggest that the *d*-methadone isomer has little or no NMDA receptor antagonist activity *in vivo*. We would attribute the antinociceptive effects seen, only at relatively high doses, to an agonist action at µ opioid receptors, based on the profile of the drug on the various neuronal measures and the full reversal by naloxone. Racemic (*dl*-) methadone was more potent than the *d*- isomer by a factor of between 10- and 50-fold, a similar order of magnitude to that previously reported for these optical isomers *in vitro* (Scott *et al.*, 1948) and in humans (Olsen *et al.*, 1976). Interestingly, the difference in potency between the two

drugs is greatest for excess spikes (a measure of wind-up) and A δ -fibre-evoked responses. If *d*-methadone had predominant NMDA-receptor blocking actions it would be expected to be more potent than *dl*-methadone on the measure of wind-up — an NMDA receptor dependent process. This is not the case, and the inhibition of wind-up by *dl*-methadone is probably a result of the profound inhibition of input.

Shimoyama *et al.* (1997) have reported on the opioid and NMDA receptor affinities of methadone and the isomers, and have studied extensively the antinociceptive actions of the *d*-isomer in behavioural studies. After spinal application, they have shown that the second phase of the formalin response is reduced by *d*-methadone and that this is not reversible by naloxone. Although this second phase is dependent on NMDA receptor mechanisms (Chapman & Dickenson, 1995; Haley *et al.*, 1989), it can also be inhibited by classical opioids such as morphine. In our hands, the effects of *d*-methadone, administered spinally and over a similar dose-range to that used by Shimoyama *et al.* (1997), were naloxone sensitive. This difference may well result from the different conditions that would be present in behavioural and electrophysiological approaches. Thus in a behavioural study where threshold responses are measured, the relative receptor profile of the drug may be different from that when suprathreshold, neuronal responses are measured. The model used in this thesis, where suprathreshold responses of sensory neurones are measured, is perhaps more relevant to the situation of moderate to severe clinical pain.

A more recently published study supports my own findings — that NMDA receptor blockade does not appear to contribute the antinociceptive effects of methadone (Chizh *et al.*, 2000). These authors showed that although *d*-methadone was anti-hyperalgesic in a behavioural model of inflammation, blocked motor neurone wind-up and NMDA-evoked activity of dorsal horn neurones, these effects were prevented by naloxone pretreatment. .

Synergy might contribute to the activity of methadone. Chapman & Dickenson, (1992) have shown that doses of an opioid agonist and an NMDA receptor antagonist which individually had little or no effect on either input (opioid-sensitive) or wind-up (NMDA receptor-dependent) showed profound synergy when applied together and abolished both input and wind-up. It follows that the inhibition of neuronal responses I saw with the highest dose of *d*-methadone could be caused by synergy between weak opioid and weak anti-NMDA receptor activity. In this case, naloxone would be expected to fully reverse the effects, as the final effect is dependent on both pharmacological components. Therefore the reversal of effects we saw with naloxone does not preclude an NMDA receptor antagonist activity for *d*-methadone.

Unlike methadone, ketobemidone, another opioid with NMDA receptor blocking effects, has a different profile in that it inhibits nociceptive responses of spinal neurones but, importantly, reduces wind-up in a non-naloxone-reversible manner in the same experimental model (Andersen *et al.*, 1996). This would suggest that NMDA receptor-blocking actions of ketobemidone can be dissociated from its opioid actions. Among other opioids with affinity

for the NMDA receptor is dextropropoxyphene (Ebert *et al.*, 1998), pointing toward a family of mixed opioid/NMDA receptor antagonist compounds, probably all with differing pharmacological profiles and, thus, different effectiveness in pain conditions.

Although a blend of desirable pharmacological actions in a single molecule is clearly advantageous, the same goals may be achieved by the administration of combinations of individual drugs. Morphine analgesia for example, can be enhanced by the co-administration of NMDA receptor antagonists (Chapman & Dickenson, 1992; Hoffmann & Wiesenfeld-Hallin, 1996). This synergy has also been seen in animal models of neuropathic pain, an experimental situation where the effectiveness of morphine is reduced (Advokat & Rhein, 1995; Nichols *et al.*, 1997; Yamamoto & Yaksh, 1992). This combination of opioids and NMDA receptor antagonists has been investigated in the clinic; ketamine as a licensed NMDA receptor antagonist is most often used. Javery *et al.* (1996) and Yang *et al.* (1996) both found that the combination of morphine and ketamine enhanced the analgesic effect of morphine against terminal cancer pain and post-operative pain respectively.

Combining opioid actions with NMDA receptor antagonism, either in a single molecule or by combination therapy, can provide superior pain relief with fewer side effects than could be achieved with either drug alone. I would conclude, however, that the potential NMDA receptor blocking component to the actions of methadone is minor under these experimental conditions and so may not contribute to the clinical profile of this drug (Fainsinger *et al.*, 1993). Interestingly, methadone may have other non-opioid activity apart from any NMDA-receptor antagonist actions. A recent study has pointed out that unlike morphine, methadone has effects on 5-HT uptake akin to tramadol (Giusti *et al.*, 1997). This is unlikely to contribute to the actions on methadone described here, as ongoing 5-HT-mediated controls are negligible in the spinal cord in acute pain conditions such as the present model (G.M. Green, personal communication) and all effects were naloxone reversible. However, a 5-HT component to the actions of methadone (or the *d*-isomer) may be present in models of more prolonged pain states such as the formalin test used by Shimoyama *et al.* (1997). Here the naloxone-insensitive effects of *d*-methadone may reflect inhibition of 5-HT uptake and thereby increased serotonergic control of spinal nociceptive transmission in this inflammatory state.

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Chapter 4. The NAAG system

4.1 Introduction

N-acetyl-aspartylglutamate (NAAG) is a neuropeptide first isolated from rabbit and horse brain by (Curatolo *et al.*, 1965), and its structure was confirmed soon afterwards by (Miyamoto *et al.*, 1966) after isolation from bovine brain. NAAG is recognised as one of the most abundant peptides in the mammalian brain and spinal cord.

NAAG is localised in many neuronal systems, including primary afferent sensory fibres. Evidence exists for the synthesis, storage release and breakdown of NAAG and an interesting pharmacological profile has been determined. NAAG is a potent agonist at the mGlu3 metabotropic glutamate receptor and a partial agonist at the NMDA receptor, a profile that affords a neuroprotective ability and is proving useful in models of stroke. In this chapter, I describe experiments carried out to determine whether the NAAG can attenuate hyperexcitability in the spinal cord. The intriguing receptor profile may be therapeutically useful and effectively circumvent the side effects generated by ubiquitous NMDA receptor blockade.

4.1.1 Neuronal Localisation of NAAG

Generation of antibodies to NAAG allowed the first immunohistochemical studies. These revealed NAAG-like immunoreactivity (LI) in selected areas of the rat brain (Anderson *et al.*, 1986), and initially appeared to show a localisation in predominantly glutamatergic systems. NAAG-LI has been detected in retinal ganglion cells of the rat (Anderson *et al.*, 1987), cat (Tieman *et al.*, 1987) and amphibians (Kowalski *et al.*, 1987); the rat lateral olfactory tract (French Mullen *et al.*, 1985); pyramidal neurones in motor and sensory cortices and the hippocampal formation of the rat forebrain (Tsai *et al.*, 1993) and putative glutamatergic pyramidal neurones of the cerebral cortex and hippocampus in human brain (Passani *et al.*, 1997).

Levels of NAAG, as determined by HPLC, are particularly high in the spinal cord, with a suggested partial localisation in long tract neurones, predicted by the effects of spinal cord transection (Koller *et al.*, 1984). Levels were markedly higher in the ventral cord than in the dorsal cord such that NAAG-LI is detected in cholinergic motoneurones in the rat ventral horn and in ventral roots (Forloni *et al.*, 1987; Ory Lavalley *et al.*, 1987), and in motor terminals at the neuromuscular junction (Berger *et al.*, 1995). Conversely, high levels of NAAG have also been detected in dorsal root ganglia indicating the peptide is likely to be present in primary afferent nerve fibres. Immuno-localisation of NAAG has revealed presence in large diameter (>35µm) neurones in amphibian and rodent spinal sensory ganglia (Cangro *et al.*, 1987; Kowalski *et al.*, 1987; Ory Lavalley *et al.*, 1987) HPLC of extracts from these large diameter sensory neurones has confirmed the presence of NAAG in primary afferent fibres (Cangro *et al.*, 1987).

The presence of NAAG in GABAergic interneurons was suggested by (Moffett & Namboodiri, 1995) after observing localisation of NAAG to areas of the brain that use GABA as a transmitter. However, comparing the distribution of NAAG with that of a marker for GABAergic neurons in the cerebellum shows that the distributions are not overlapping, but complementary (Moffett *et al.*, 1994). In the cerebellum, for example, the GABAergic Purkinje neurons show little or no NAAG-like immunoreactivity, and the densest staining was observed in the excitatory mossy fibre terminals. They suggested that release of NAAG may modulate granule cell input onto and the inhibitory output of the GABAergic Purkinje cells. A similar situation may occur in the cat visual system, where Xing & Tieman, (1993) have shown that relay neurons, not GABAergic interneurons, are immuno-positive for NAAG.

4.1.2 Synthesis, storage and release of NAAG

Relatively little literature exists on the biosynthesis of NAAG. In cultured spinal sensory ganglia cells, [³H]glutamate (but not [³H]aspartate) is accumulated to produce [³H]NAAG (Cangro *et al.*, 1987; Reichelt & Kvamme, 1973; Tyson & Sutherland, 1998). This process occurs in the presence of cycloheximide and anisomycin (protein synthesis inhibitors) and NAAG can therefore be considered a product of biosynthesis and not *de novo* synthesis (Cangro *et al.*, 1987). NAAG synthesis may, however, be inducible in non-neuronal cell types. Using HPLC to analyse NAAG levels in cell cultures, (Passani *et al.*, 1998) saw a several-fold increase in the levels of NAAG in microglia (previously trace levels) after incubation with lipopolysaccharide (LPS). This is interesting for two reasons; firstly, that NAAG can be found in, and thus may be released from glia and secondly, that the levels of NAAG can change in response to external stimulus.

The NAAG that is synthesised in neurons appears to be stored at suitable locations to be part of a releasable pool of transmitter. Ultrastructural visualisation with electron microscopy has identified NAAG-LI within synaptic vesicles at putative release sites at neuronal terminals in the amphibian visual pathway (Williamson & Neale, 1988b).

For NAAG to qualify as a neurotransmitter, calcium dependent release from nerve terminals on depolarisation must be demonstrated. The first experiments to demonstrate calcium dependent release were performed after preloading tissues with radiolabelled NAAG in the amphibian visual pathway (Tsai *et al.*, 1988; Williamson & Neale, 1988a) and in chick retinal cells (Williamson & Neale, 1992). The increased extracellular levels of [³H]NAAG seen after depolarisation may, however, represent release from compartments that do not normally sequester NAAG. Subsequently, *in vivo* microdialysis detected Ca²⁺ dependent release of endogenous NAAG from retinal ganglion cell terminals in the superior colliculus after electrical stimulation of the optic nerve (Tsai *et al.*, 1990). Calcium dependent release of endogenous NAAG has now been demonstrated in slices from rat neocortex, piriform cortex/amygdala, hippocampus, cerebellum, striatum, and spinal cord (Zollinger *et*

al., 1988; Zollinger *et al.*, 1994). In all cases, the process was largely calcium dependent. Interestingly, despite the high levels of NAAG in the spinal cord, the relative increase of NAAG efflux after depolarisation was smaller than for other areas, which may suggest a large presence of NAAG in compartments from which it cannot be released. The cell bodies of motoneurons, long axon tracts and astrocytes may represent such compartments. Additionally, 30% of the release of NAAG was calcium independent in the spinal cord. Whereas most of the Ca^{2+} -dependent releasable NAAG could be predicted to be located in terminals of descending or intrinsic pathways, or in subset of primary afferent terminals, the sources of the Ca^{2+} independent release are not clear.

4.1.3 NAAG binds to the NMDA receptor

The first steps towards understanding the functional role of NAAG began in 1983, when the peptide was found to displace labelled L-[^3H]-glutamate from brain membranes, with a high affinity and selectivity (Zaczek *et al.*, 1983). An excitatory action of NAAG has been demonstrated in various systems including the rat lateral olfactory tract (French Mullen *et al.*, 1985), spinal cord neurones (Westbrook *et al.*, 1986) and on guinea pig cerebellar Purkinje cells (Sekiguchi *et al.*, 1987). However, several groups found NAAG to be without any excitatory activity in brain (Luini *et al.*, 1984; Riveros & Orrego, 1984) or on dorsal horn neurones (Schneider & Perl, 1988) preparations. One explanation of this inconsistent activity of NAAG, put forward by Whittemore & Koerner, (1989), suggested that the excitations normally seen were due to potassium salts of the dipeptide present in solution. The possibility of glutamate release from NAAG degradation could also not be discounted in some of the studies.

More satisfactory explanations have been put forward recently, as the pharmacology of NAAG has become clearer. When assayed for interactions with ionotropic glutamate receptors, NAAG showed selectivity for the NMDA receptor over AMPA and kainate receptors *in vitro* (IC_{50} for NMDA = $8.8\mu\text{M}$ versus $790\mu\text{M}$ and $500\mu\text{M}$ respectively) (Valivullah *et al.*, 1994). However, this high affinity for the NMDA receptor is countered by a low efficacy, conferring the profile of a partial agonist. Hence, in unphysiological situations when the NMDA receptor is challenged with NAAG alone at high concentrations, a response can be measured. In *Xenopus* oocytes transiently transfected with NMDA NR1 mRNA, the maximum current amplitude induced by NAAG is around 70% of that induced by L-glutamate and NAAG is 115 times less potent in producing the response (Sekiguchi *et al.*, 1992). In guinea pig cerebellar slices, NAAG was able to depolarise the Purkinje cell soma when superfused directly into the slice (Sekiguchi *et al.*, 1987) but subsequently, in a similar paradigm, NAAG was shown to inhibit the Purkinje cell response to glutamate released from climbing fibre stimulation (Sekiguchi *et al.*, 1989). Several other *in vitro* NMDA receptor containing preparations reveal these contrasting effects of NAAG. Excitatory actions in lateral geniculate relay neurones (Harata *et al.*, 1999), pyramidal neurones (Bernstein *et al.*, 1985; French Mullen *et al.*, 1985) and cultured spinal cord (Westbrook *et al.*, 1986) and

forebrain (Koenig *et al.*, 1994) neurones contrast with attenuation of normal glutamatergic transmission (Sekiguchi *et al.*, 1989) and antagonism of the effects of application of NMDA (Burlina *et al.*, 1994; Puttfarcken *et al.*, 1993).

Some studies have reported no excitatory action nor antagonism of excitatory effects of glutamate (Luini *et al.*, 1984; Riveros & Orrego, 1984; Schneider & Perl, 1988).

4.1.4 NAAG binds to the mGlu3 receptor

In cultured cerebellar granule cells, NAAG inhibits forskolin-stimulated cAMP formation via a pertussis-toxin-sensitive metabotropic glutamate receptor and has no effect at mGlu receptors coupled to phosphoinositol turnover (Wroblewska *et al.*, 1993). This classifies the receptor targeted by NAAG as a group II mGlu receptor, a class that includes the mGlu2 and the mGlu3 receptors. The precise receptor specificity of NAAG was determined by Wroblewska *et al.*, (1997) using cultured cells transfected with cloned rat cDNAs for mGlu receptors 1–6. NAAG was found to be a full agonist only in cells expressing the mGlu3 receptor (previously referred to as the mGluR3), with no activity at the closely related mGlu2 receptor, or any of mGlu1 receptor, 4–6. Furthermore, at chimeric receptors, NAAG activated only receptors bearing the extracellular domain of mGlu3. Cultured cerebellar glial cells (mainly astrocytes) express mGlu receptors 1 and 3–8, and in these cultures NAAG is able to inhibit forskolin-induced cAMP formation via activation of mGlu3 receptor (Wroblewska *et al.*, 1998). NAAG is a potent, full agonist of the group II mGlu3 receptor, which is present on neurones and glia.

4.1.5 NAALADase breaks down NAAG

Termination of action of a neurotransmitter is vital. Early radiolabelling experiments indicated that NAAG is broken down before uptake of the products, as is evident from accumulation of [³H]glutamate in synaptosomes after incubation with NAA[³H]G (Blakely *et al.*, 1986). A specific transport mechanism for the product NAA has been demonstrated in astrocyte cultures (Sager *et al.*, 1999). Peptidase activity was linked to the extracellular membrane in cultures of both neuronal and glial cultures, and led to steady accumulation of extracellular glutamate (Cassidy & Neale, 1993a). This indicates that breakdown of NAAG on the extracellular plasma membrane was not closely linked to glutamate uptake and, therefore, that NAAG could act as a significant source of extracellular glutamate. However, the situation may be different *in vivo*, where closer links between neurones and astrocytes are maintained.

In primary cultures of mouse brain, high NAAG levels are found in both neurones and in glia. High affinity transport of NAAG into glia was observed, suggesting that an alternative route for terminating the actions of NAAG exists (Cassidy & Neale, 1993b). This phenomenon of uptake of the intact peptide is also observed in avian retina (Williamson & Neale, 1992).

4.1.6 Localisation of NAALADase

The peptidase responsible for the cleavage of NAAG was characterised from rat brain membrane preparations and designated N-acetylated α -linked acidic dipeptidase (Robinson *et al.*, 1987; Robinson *et al.*, 1986) and several approaches have been used to map the distribution of NAALADase in the CNS. Fuhrman *et al.*, (1994) assayed 50 discrete rat brain regions for peptidase activity against NAAG and found a linear relationship between NAALADase activity and NAAG concentration. This suggests that NAALADase is indeed responsible for breakdown of NAAG after release. Interestingly the level of peptidase activity in the spinal cord was low in comparison to the high concentration of NAAG (64nmol/mg protein, the highest in the CNS, Fuhrman *et al.*, (1994). This may indicate a prolonged activity of NAAG in the spinal cord.

Using a different approach, Slusher *et al.*, (1992) visualised NAALADase distribution using antisera against the purified rat brain protein. NAALADase-LI was found throughout the brain and spinal cord and correlated well with NAAG-LI. In the dorsal spinal cord, dense NAALADase staining was detected in Laminae I and II, with some in Lamina X. Some NAALADase-LI was seen in areas that show no significant NAAG staining, suggesting that NAALADase is responsible for catabolism of other peptides (Slusher *et al.*, 1992).

A rat brain cDNA encoding NAALADase was cloned simultaneously by two independent groups. Both cDNAs were able to confer NAAG-hydrolysing ability on transfection into cell lines (Bzdega *et al.*, 1997; Luthi Carter *et al.*, 1998). Structural analysis of the peptidase has led to the more accurate name glutamate carboxypeptidase II (GCP II). *In situ* hybridisation was now possible, and revealed an uneven regional distribution in rat brain that was consistent with the distribution as revealed by assays of peptidase activity and immunostaining (Bzdega *et al.*, 1997; Luthi Carter *et al.*, 1998). Earlier indications that the peptidase activity was present both in neurones and glia (Cassidy & Neale, 1993a) were re-investigated, and revealed expression of NAALADase mRNA predominantly by astrocytes. (Luthi Carter *et al.*, 1998). Looking at different glial cell cultures, Berger *et al.*, (1999) saw NAALADase expression solely by and by virtually all astrocytic glial cells including Bergmann glial cells in the cerebellum and satellite cells in dorsal root ganglia. Tying in with this, NAA released from NAAG is transported into astrocytes by an astrocyte-specific transporter (Sager *et al.*, 1999).

Another partial cDNA capable of conferring NAAG-hydrolysing ability has been isolated that closely resembles a human protein PSMA (prostate-specific membrane antigen, a marker for prostate cancer) (Carter *et al.*, 1996). Although these two proteins are remarkably similar, there are subtle differences between the prostate (PSM) and brain (NAALADase) enzymes in terms of their substrate specificity and kinetics (Tiffany *et al.*, 1999). The importance of these differences remains unclear, and homology screening has begun to reveal different degrees of crossover in their substrates and localisation (Pangalos *et al.*, 1999).

4.1.7 Function of NAAG

As for the functional consequences of NAAG release, the activity of NAAG was initially thought to be purely excitatory, as predicted by its high affinity for a subset of glutamate binding sites in rat brain. Accordingly, injection of NAAG into rat hippocampus caused quisqualic acid-like convulsions (Zaczek *et al.*, 1983), supporting an excitatory transmitter action. This is contrary to more recent reports of NAAG in integrated systems, where the peptide interferes with normal glutamatergic transmission at the NMDA receptor.

The functional significance of mGlu3 receptor activation could be many-fold. Via activation of this receptor, NAAG is able to regulate GABA_A subunit expression (Ghose *et al.*, 1997) and induce long lasting depression in the dentate gyrus of the hippocampus (Huang *et al.*, 1999). mGlu3 receptor agonists are neuroprotective (Bruno *et al.*, 1995; Bruno *et al.*, 1994) and accordingly, NAAG is able to protect against rat striatal quinolinic acid lesions *in vivo* (Orlando *et al.*, 1997) and *in vitro* (Bruno *et al.*, 1998b) via the mGlu3 receptor. The effect seen by Bruno *et al.* may involve the release of a neurotrophic agent (Bruno *et al.*, 1998a; Bruno *et al.*, 1997). mGlu3 receptor agonists are also able to reduce cell death and improve recovery after traumatic neuronal injury *in vivo* and *in vitro*, via reducing cAMP levels and glutamate release (Allen *et al.*, 1999).

Therefore, the ability of NAAG to protect against excitotoxic lesions seems to be a result of its specific combination of actions; partial blockade of the NMDA receptor, and activation of the inhibitory mGlu3 receptor on neurones and glia. However, there has been a contradictory report of toxicity associated with application of NAAG (Pai & Ravindranath, 1991).

NAAG levels and NAALADase function have been implicated in the pathology of several disease states other than stroke. In the genetically-epilepsy-prone rat, an increase in NAALADase activity is observed, consistent with increased turnover of NAAG and liberation of glutamate, potentially contributing to this epileptic phenotype (Meyerhoff *et al.*, 1992). Amyotrophic lateral sclerosis (ALS) is a motor neurone disease in which motor nerves appear to prematurely age and degenerate, and the muscles they innervate progressively waste. Glutamate excitotoxicity is one potential cause of the neuronal death. NAAG is present in high levels in the ventral horn of the spinal cord in rats, and may have a developmental role in motor neurone axon myelination (Berger *et al.*, 1995; Berger & Schwab, 1996). Post mortem studies of ALS patients have revealed reduced levels of NAAG in the ventral horn and motor cortices, with concurrent increase in NAALADase activity in motor roots and the motor cortex (Tsai *et al.*, 1991). NAAG may therefore be required to prevent glutamate excitotoxic damage to motor neurones, and ALS may be associated with increased catabolism of this peptide leading to raised glutamate levels.

Many symptoms of schizophrenia can be mimicked by NMDA receptor antagonists, leading to the hypothesis that hypofunction of the NMDA receptor and glutamatergic systems underlies this condition. Fittingly, NAAG levels are elevated and NAALADase activity is

reduced in post-mortem brain tissue from schizophrenic subjects (Tsai *et al.*, 1995), suggesting that NAAG may be partly responsible for hypoactivity of glutamatergic systems in schizophrenia.

The prospect of manipulating the NAAG system with a view to therapeutic function in stroke, epilepsy, dementia, schizophrenia or ALS must consider the involvement of this peptide in these and undoubtedly many other physiological processes. It may be a neurotransmitter in the visual pathway, a modulator of cerebellar output and may be important in the development of the peripheral nervous system (Berger & Schwab, 1996).

NAAG can be protected from breakdown by 2-PMPA

Investigation of the physiological function of NAAG is limited due to its peptide nature. Protecting NAAG from breakdown by inhibition of NAALADase is an effective approach to manipulate levels of endogenous NAAG. Injections of quisqualate, phosphate, dithiothreitol and EGTA increase the half-life of radiolabelled NAAG *in vitro* (Stauch *et al.*, 1989), but these tools are rather non-specific. The first specific inhibitor of NAALADase was described in 1996 by (Jackson *et al.*, 1996). 2-PMPA is over 1000 times more selective for NAALADase inhibition than the tools used previously and, as such, can be used to manipulate NAAG levels in order to study the physiological role of this dipeptide. Inhibition of NAALADase activity with 2-PMPA increases brain levels of NAAG and protects against ischaemic injury *in vitro* (Slusher *et al.*, 1999) and *in vivo* (Vornov *et al.*, 1999).

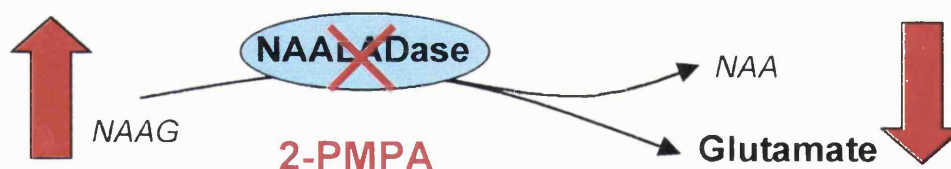


Figure 4.1 Manipulation of endogenous NAAG levels with the NAALADase inhibitor, 2-PMPA.

4.1.8 NAAG — a role in nociception?

Pharmacological strategies that are effective in neuroprotection paradigms often translate into effective analgesic strategies, as an underlying factor in each condition is enhanced glutamatergic activity. As raising the levels of endogenous NAAG appears to be effective in neuroprotection, it could be interesting to see what effect this has on the spinal processing of nociceptive information. NAAG and NAALADase are certainly present in the right places, primary afferent fibres and in the superficial laminae of the dorsal horn. As already discussed NMDA receptor blockade is effective in attenuating spinal hyperexcitability, but global block of the receptor is associated with problematic side effects. NAAG-mediated partial agonism at the NMDA receptor may be an effective strategy to

circumvent ubiquitous NMDA receptor blockade, and target only areas where NMDA receptor activation is excessive.

As for the mGlu3 receptor, the literature on its role in nociception is growing, as more selective compounds are generated. The discrete expression of mGlu3 receptor in the dorsal horn (laminae II–V) is enhanced after peripheral inflammation, paralleling the development of hyperalgesia (Boxall *et al.*, 1998). It may function here as an autoregulatory control on the excessive glutamate release seen in inflammation. This is supported by evidence that whereas group I mGlu receptor agonists are pro-nociceptive, group II mGlu receptor agonists are anti-nociceptive in tests of acute nociception after intrathecal administration (Dolan & Nolan, 2000). In addition, activation of group II mGlu receptors with L-CCG-I inhibits pre-synaptic voltage-dependent calcium channels in rat brain slices (Glaum & Miller, 1995). Increasing levels of NAAG may therefore be an effective strategy to control hyperexcitability in spinal nociceptive processing. This approach may also elucidate the role of the mGlu3 receptor in the spinal processing of nociceptive information, as few useful tools exist for the study of this receptor.

4.2 Methods

Electrophysiology was carried out on four groups of rats; normal animals, animals subjected to carrageenan inflammation of the hindpaw and animals subjected to either spinal nerve ligation surgery or sham surgery 14 days prior to experiments.

4.2.1 Normal animals

Animals were anaesthetised and underwent surgery to expose the spinal cord as usual and neuronal recordings begun. 2-PMPA was applied directly to the spinal cord, as cumulative doses 50, 100, 500 and 1000 μ g in 50 μ l saline. After each application, evoked neuronal responses were followed for 90 min, the drug removed from the cord and the next dose applied.

4.2.2 Carrageenan inflammation

In this group of animals, peripheral inflammation was generated by injection of carrageenan into the hindpaw. 100 μ l carrageenan (2% solution in saline) was injected into the plantar skin of the hindpaw. In a few cases, the carrageenan was administered as soon as the anaesthetised animal was secured with the vertebral clamps, but in most cases the carrageenan was injected only once a neurone had been isolated and several controls taken. Evoked neuronal responses were followed while the inflammation developed, and pre-drug controls were re-established after 3 h. Once these controls were stable, the experiments then proceeded as described above for normal animals (4.2.1). Occasionally, a neurone had not been found, or controls were not stable at 3 h and drug application was therefore delayed, but pharmacology always began within 4 1/2 h of carrageenan injection.

4.2.3 Spinal nerve ligation

Two weeks prior to electrophysiology animals underwent ligation or sham surgery, as described in Chapter 2 (2.5.3). Surgery was kindly performed by Miss Elizabeth Matthews and Ms Sarah Flatters. Animals were prepared for electrophysiology and a neurone was isolated on the left hand side of the spinal cord (i.e. ipsilateral to the injury). The experiments then proceeded as described above for normal animals (4.2.1).

4.2.4 Data analysis

Data were collected as before: cell depth, C-fibre threshold and pre-drug control values were recorded for C-, A β - and A δ -fibre-evoked responses, input, post discharge and excess spikes and drug effects on these neuronal responses quantified.

Each group of animals and each neuronal measure were first analysed in isolation. The maximum effects for each dose (expressed as % of pre-drug control) were analysed using unpaired nonparametric ANOVA (Kruskal-Wallis). Using the % control data allowed

'pairing' of drug effects to the pre-drug control value, as missing values made repeated measures ANOVA impossible. Individual dose effects were then compared with the pre-drug control recorded for that neurone using paired Student's *t*-tests. As pairing for each neurone was now possible, the raw data (number of action potentials) were used. The Bonferroni correction for multiple comparisons was used.

Comparisons between the different groups of animals were preceded by comparing pre-drug control values to ensure that these baselines were not significantly different in these four groups of animals (unpaired Student's *t*-tests). Following this, drug effects (expressed as % control) were compared between all four groups at each dose using unpaired Student's *t*-tests. As the data were from separate experiments, no correction was needed for repeated comparisons.

4.3 Results

The effect of 2-PMPA application was studied on eight normal animals, 12 carrageenan-injected animals, seven sham-operated animals and 11 spinal-nerve-ligated animals. One neurone was used in each animal. In most cases, application of 2-PMPA produced a dose-related inhibition of electrical-evoked responses. In the remaining cases, neurones were excluded from further analysis because no drug-related effects were seen. Reasons for this could include problems of access due to blood on the surface of the cord. Results were also excluded for a particular parameter if the control values were very small.

The number of animals in each group, the mean neuronal depth and mean C-fibre threshold are shown in Table 4.1 for all groups of animals studied. These characteristics of the neurones were not different between any of the experimental groups.

Table 4.1 Characteristics of neurones on which the effects of 2-PMPA were studied

	<i>Cell characteristics</i>	
	Depth	C-fibre threshold
Normal (n=5)	789 ± 153µm	1.38 ± 0.35mA
Inflammation (n=10)	876 ± 61µm	1.55 ± 0.23mA
Sham (n=6)	845 ± 95µm	1.77 ± 0.18mA
SNL (n=10)	857 ± 52µm	2.15 ± 0.22mA

No difference in these characteristics were apparent between the groups (unpaired Student's t-tests).

4.3.1 Normal animals

In normal animals, the spinal application of 2-PMPA caused significant, dose-related inhibitions of C-fibre evoked responses, post discharge, input and Aδ-fibre evoked responses (unpaired Kruskal-Wallis, $^{**}P \leq 0.01$, $^*P \leq 0.05$, $^*P \leq 0.05$, $^*P \leq 0.05$ respectively). The inhibitions produced by each individual dose do not reach significance until the top dose was applied in all cases ($^*P \leq 0.05$, $^*P \leq 0.01$), apart from post discharge, which was markedly inhibited with 500µg 2-PMPA ($^*P \leq 0.01$, $n = 5$, Student's *t*-tests). The Aβ-fibre evoked responses were not significantly affected by 2-PMPA (see Table 4.2 and Figure 4.2, open squares).

Table 4.2 Effects of 2-PMPA on electrically-evoked responses in normal animals

	Control	<i>Drug effects (% control)</i>			
		50µg	100µg	500µg	1000µg
C-fibres^{††}	411 ± 78.5 (n=5)	—	103 ± 8.65% (n=5)	81.4 ± 6.4% (n=5)	56.3 ± 6.6% (n=5)*
Post discharge[†]	257 ± 64 (n=5)	—	117 ± 18% (n=5)	46.7 ± 8.5% (n=5)**	29.1 ± 13% (n=5)*
Input[†]	24.7 ± 6.64 (n=5)	—	115 ± 13% (n=5)	81.1 ± 21% (n=5)	40.9 ± 11% (n=5)*
Excess-spikes[†]	306 ± 71.8 (n=5)	—	110 ± 29% (n=5)	82.0 ± 25% (n=5)	36.2 ± 11% (n=5)
Aδ-fibres[†]	75.6 ± 22.5 (n=5)	—	104 ± 11% (n=5)	80.4 ± 13% (n=5)	52.8 ± 4.4% (n=5)*
Aβ-fibres	98.0 ± 17.4 (n=5)	—	89.1 ± 7.1% (n=5)	94.6 ± 14% (n=5)	73.8 ± 16% (n=5)

[†] $P \leq 0.05$, ^{††} $P \leq 0.01$, significant overall effect with drug applications (unpaired Kruskal-Wallis,); * $P \leq 0.05$, ** $P \leq 0.01$, significant effect of individual dose compared with pre-drug control value (paired Student's *t*-test with the Bonferroni correction).

4.3.2 Carrageenan inflammation

Three hours after peripheral injection of carrageenan and development of inflammation and oedema, pre-drug control values were not significantly different from control values recorded in normal animals. From this stable baseline, spinal application of 2-PMPA produced small but non-significant reduction of the C-fibre evoked responses and significant inhibition of post discharge and input (unpaired Kruskal-Wallis [†] $P \leq 0.05$ and ^{††} $P \leq 0.01$ respectively). Now the lower dose of 100µg 2-PMPA was able to elicit a significant inhibition of the input (* $P \leq 0.05$, $n = 8$). A comparable but non-significant reduction of post discharge was produced by this dose, the effect was significant with the application of the next dose (500µg, * $P \leq 0.05$, $n = 9$). These data are shown below in Table 4.3 and in Figure 4.2 in red-filled squares. An example of the effect of 2-PMPA on the wind-up of one individual neurone (after inflammation) is shown in Figure 4.3. The effect on input is pronounced at doses that do not noticeably affect wind-up (i.e. do not alter the slope of the line).

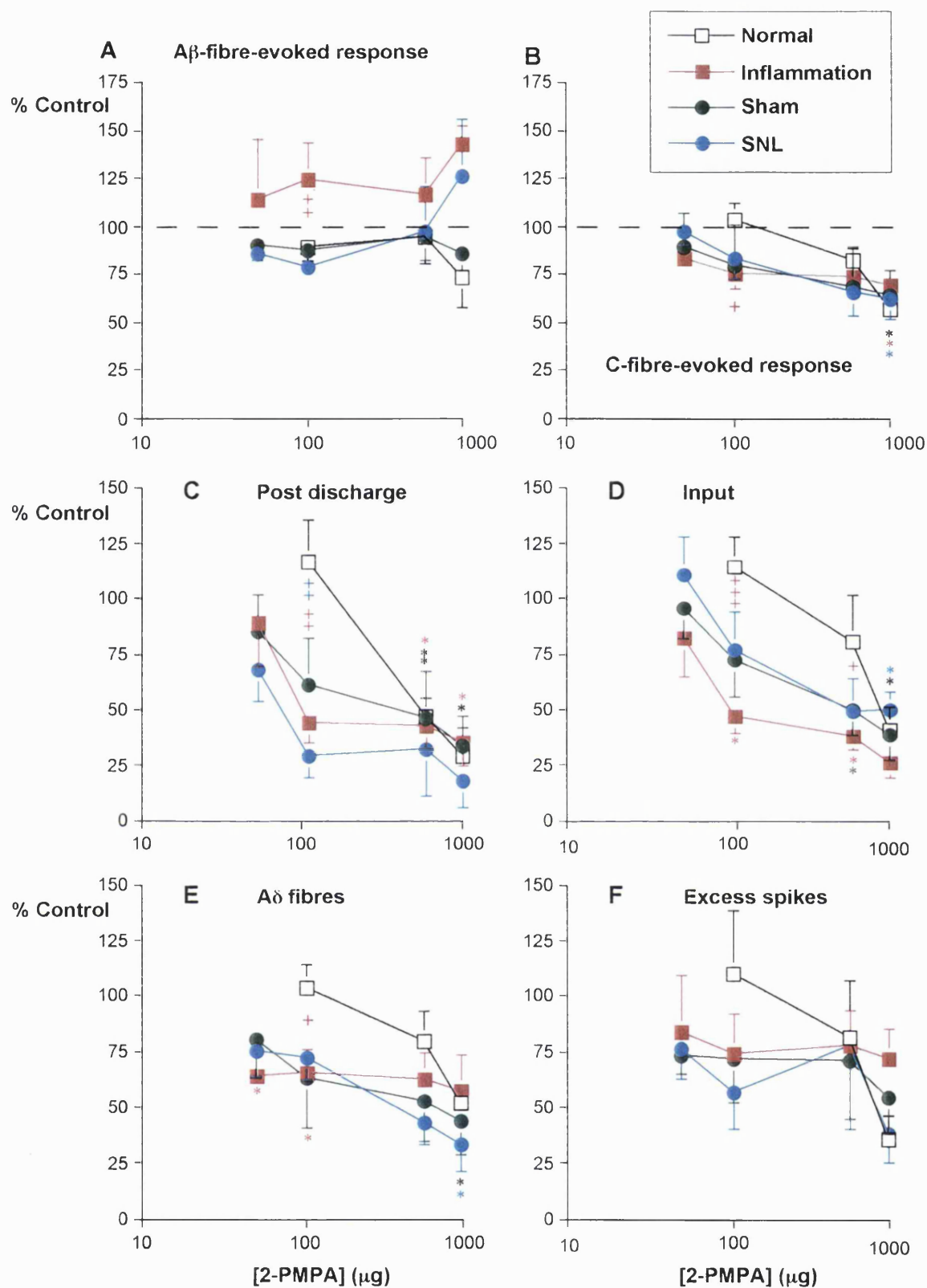


Figure 4.2 Effects of 2-PMPA on (A) A β -fibre-evoked responses, (B) C-fibre-evoked responses, (C) post discharge, (D) input, (E) A δ -fibre-evoked responses and (F) excess spikes recorded in four different groups of animals. Significant inhibition compared with pre-drug control * $P \leq 0.05$. and significant difference compared with normal animals + $P \leq 0.05$, ** $P \leq 0.01$) are indicated.

Table 4.3 Effects of 2-PMPA on electrically-evoked responses after inflammation

	Control	<i>Drug effects (% control)</i>			
		50µg	100µg	500µg	1000µg
C-fibres	457 ± 59% (n=10)	82.6 ± 2.5% (n=5)	74.7 ± 7.8% (n=8)	72.9 ± 6.5% (n=9)	69.0 ± 9.1% (n=6)*
Post discharge	327 ± 61% (n=10)	89.2 ± 20% (n=5)	43.6 ± 8.7% (n=8)	42.6 ± 9.7% (n=9)*	34.7 ± 10% (n=6)*
Input[†]	27.1 ± 5.4% (n=10)	82.6 ± 17% (n=5)	47.2 ± 7.3% (n=8)*	38.3 ± 6.0% (n=9)*	26.2 ± 6.4% (n=6)
XS-spikes	424 ± 86% (n=9)	84.0 ± 25% (n=5)	74.4 ± 18% (n=7)	77.7 ± 16% (n=8)	72.2 ± 13% (n=5)
Aδ-fibres	101 ± 13% (n=10)	64.6 ± 3.8% (n=5)*	65.8 ± 11% (n=8)*	62.7 ± 11% (n=9)	57.9 ± 17% (n=6)
Aβ-fibres	79.2 ± 16.8 (n=10)	114 ± 31% (n=5)	125 ± 19% (n=8)	117 ± 19% (n=9)	143 ± 9.8% (n=6)

[†] $P \leq 0.05$, significant overall effect with drug applications (unpaired Kruskal-Wallis);

* $P \leq 0.05$, , significant effect of individual dose compared with pre-drug control value (paired Student's t-test with the Bonferroni correction).

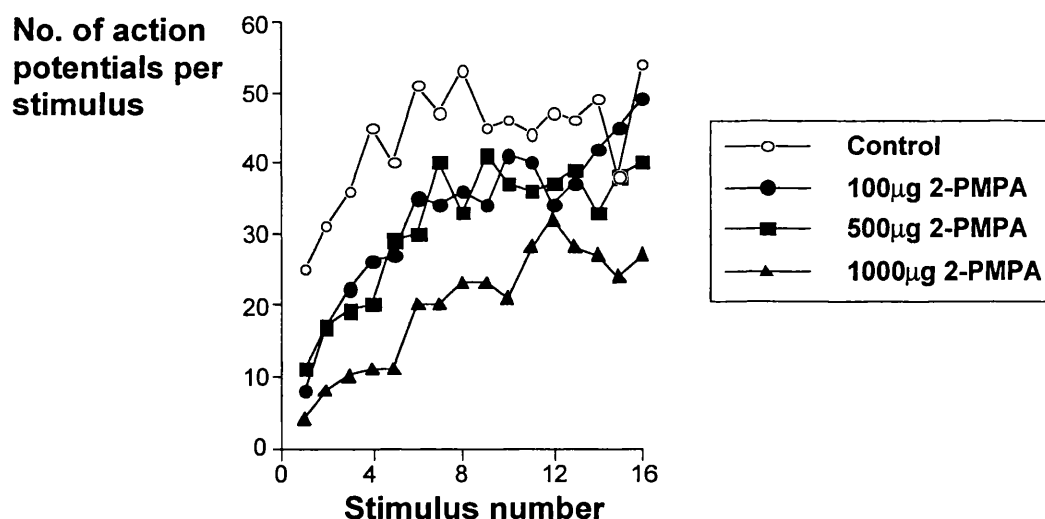


Figure 4.3 An example of the effect of 2-PMPA on the wind-up of an individual neurone, after carrageenan inflammation.

4.3.3 Sham operated animals

Two weeks after sham surgery to expose, but not ligate, spinal nerves L5–L6, pre-drug control values were not significantly different from control values recorded in normal animals. Spinal 2-PMPA application caused a significant inhibition of input (unpaired Kruskal-Wallis $^{\dagger}P \leq 0.05$). A tendency towards inhibition of the C-fibre evoked responses, post discharge, input and A δ -fibre evoked responses was observed, but did not reach significance (Table 4.4, Figure 4.2, green-filled circles).

Table 4.4 Effects of 2-PMPA on electrically-evoked responses in sham operated animals

	Control	<i>Drug effects (% control)</i>			
		50 μ g	100 μ g	500 μ g	1000 μ g
C-fibres	453 $\pm$ 70' (n=6)	89.1 $\pm$ 7.9% (n=6)	79.3 $\pm$ 12% (n=6)	68.2 $\pm$ 10% (n=6)	63.6 $\pm$ 13% (n=5)
Post discharge	247 $\pm$ 66 (n=6)	84.8 $\pm$ 17% (n=6)	61.4 $\pm$ 21% (n=6)	46.0 $\pm$ 21% (n=6)	33.3 $\pm$ 13% (n=5)
Input[†]	20.5 $\pm$ 4.9 (n=6)	96.0 $\pm$ 13% (n=6)	72.8 $\pm$ 17% (n=6)	50.1 $\pm$ 11% (n=6)*	38.9 $\pm$ 11% (n=5)
XS-spikes	431 $\pm$ 105 (n=6)	73.6 $\pm$ 8.1% (n=6)	72.3 $\pm$ 20% (n=6)	71.0 $\pm$ 26% (n=6)	54.9 $\pm$ 16% (n=5)
Aδ-fibres	49.2 $\pm$ 9.1 (n=6)	80.7 $\pm$ 17% (n=6)	63.5 $\pm$ 22% (n=6)	53.2 $\pm$ 18% (n=6)	44.3 $\pm$ 15% (n=5)
Aβ-fibres	113 $\pm$ 17 (n=6)	89.7 $\pm$ 7.6% (n=6)	87.9 $\pm$ 6.4% (n=6)	94.2 $\pm$ 12% (n=6)	85.6 $\pm$ 11% (n=5)

[†] $P \leq 0.05$ unpaired Kruskal-Wallis, significant overall effect with drug applications; * $P \leq 0.05$.

4.3.4 Spinal nerve ligated animals

Two weeks after spinal nerve ligation surgery, the pre-drug control responses were no different to control values in normal animals. Application of increasing doses of 2-PMPA caused a significant inhibition of input (unpaired Kruskal-Wallis $^{\dagger}P \leq 0.05$). Despite a trend towards reduction for C-fibre evoked responses, post discharge and A δ -fibre evoked responses, no overall significant effects were seen. Analysis of individual doses showed that C-fibre evoked responses and input were inhibited by 1000 μ g 2-PMPA (* $P \leq 0.05$ in both cases) and A δ -fibre evoked responses were inhibited by 500 μ g 2-PMPA (** $P \leq 0.01$). A β -fibre evoked responses were unaffected (Table 4.5, Figure 4.2 blue-filled circles).

Table 4.5 Effects of 2-PMPA on electrically-evoked responses in SNL animals

	Control	<i>Drug effects (% control)</i>			
		50µg	100µg	500µg	1000µg
C-fibres	320 ± 28 (n=10)	96.9 ± 76% (n=6)	83.0 ± 11% (n=6)	64.9 ± 12% (n=7)	61.7 ± 11% (n=6)*
Post discharge	143 ± 39 (n=10)	67.8 ± 14% (n=6)	28.8 ± 9.5% (n=6)	32.0 ± 21% (n=6)	17.9 ± 12% (n=6)
Input[†]	14.3 ± 1.7 (n=10)	111 ± 17% (n=6)	77.5 ± 17% (n=6)	49.4 ± 15% (n=7)	50.1 ± 8.4% (n=6)*
XS-spikes	254 ± 63 (n=10)	76.3 ± 13% (n=6)	57.3 ± 17% (n=6)	77.8 ± 37% (n=7)	38.4 ± 13% (n=6)
Aδ-fibres	69.2 ± 16 (n=10)	75.5 ± 11% (n=6)	73.0 ± 10% (n=6)	43.6 ± 10% (n=7)**	33.5 ± 12% (n=6)
Aβ-fibres	77.9 ± 8.4 (n=10)	86.0 ± 6.8% (n=6)	78.8 ± 7.7% (n=6)	97.4 ± 23% (n=7)	126 ± 30% (n=6)

[†]*P* ≤ 0.05 unpaired Kruskal-Wallis, significant overall effect with drug applications; **P* ≤ 0.05,

***P* ≤ 0.01 paired Student's *t*-test with the Bonferroni correction, significant effect of individual dose compared with pre-drug control value.

4.3.5 Comparison of drug effects between groups

Although the baseline (pre-drug control) responses recorded in the four groups of animals tested were not different, pronounced differences in the pharmacology of 2-PMPA became apparent when the groups were compared. The potency of 2-PMPA in inhibiting C-fibre evoked responses, post discharge and input is greatly enhanced after peripheral inflammation. This can be seen as a significant difference in the degree of inhibition of several measures by 100µg 2-PMPA in the carrageenan inflammation group compared with normal animals (C-fibres [†]*P* ≤ 0.05, post discharge [†]*P* ≤ 0.01, input ^{†††}*P* ≤ 0.001 and Aδ-fibre evoked responses [†]*P* ≤ 0.05). At 500µg 2-PMPA still causes a greater inhibition of input in carrageenan animals than in normal animals ([†]*P* ≤ 0.05). These differences can be seen clearly in Figure 4.2. Although the Aβ-fibre evoked responses are not significantly affected even by the top dose of 2-PMPA when groups are analysed individually, there is a significant difference between the effect of the top dose on normal and carrageenan-inflamed animals. After sham surgery, the extent of inhibition evoked by 2-PMPA is not significantly different from that seen in normal animals, for any of the neuronal measures recorded. After spinal nerve ligation, post discharge and Aδ-fibre evoked responses are inhibited to a significantly greater extent by 100µg 2-PMPA than in normal animals (^{††}*P* ≤ 0.01 and [†]*P* ≤ 0.05 respectively). A similar degree of inhibition in the SNL group — with a similar separation from the results in normal animals — is also evident for input. This separation is not significant.

4.4 Discussion

For discussion of these results, we must first be able to assume that spinal application of 2-PMPA elevates spinal levels of NAAG. This has been demonstrated in the brain (Slusher *et al.*, 1999) and I will therefore assume it to be the same here. Thus, elevating spinal levels of NAAG produced a selective inhibition of noxious-evoked neuronal responses. These effects were markedly enhanced after generation of peripheral inflammation, and tended to be enhanced after spinal nerve ligation surgery, but not significantly so after sham surgery.

To explain the nature of the effects of 2-PMPA, the effects in normal animals will be examined first, with reference to the distribution of NAAG, the NMDA receptor and the mGlu3 metabotropic glutamate receptor. The distribution of the NMDA receptor and its involvement in nociception has already been discussed, the mGlu3 receptor has a more ambiguous involvement in nociception.

4.4.1 mGlu3 receptor localisation

mRNA encoding the mGlu3 metabotropic glutamate receptor has been detected in the dorsal horn of the spinal cord (Berthele *et al.*, 1999; Ohishi *et al.*, 1993a; Silva *et al.*, 1999; Valerio *et al.*, 1997). In the dorsal spinal cord of young rats (P11-13), expression of the mGlu3 receptor is restricted to lamina II–V, with the highest density localised to inner layer of lamina II and lamina III (Boxall *et al.*, 1998). Immunohistochemistry can not yet be targeted to the mGlu3 receptor alone, but to group II mGlu receptors (mGlu2 and 3) — as mGlu2 expression appears to be very low in the spinal cord (Ohishi *et al.*, 1993b), group II immunoreactivity will closely (if not exactly) relate to mGlu3 receptor distribution. The established group II mGlu-receptor-selective antibody reveals localisation of the protein in lamina I through to III. Most reports agree that the group II mGlu receptor immunoreactivity is strongest in inner lamina II and dorsal lamina III (Azkue *et al.*, 2000; Jia *et al.*, 1999; Petralia *et al.*, 1996; Tang & Sim, 1999). Strong group II mGlu receptor immunoreactivity is also detected in cultured astrocytes (Silva *et al.*, 1999) and using electron microscopy of cord sections, immunoreactive astrocytes are seen to wrap closely around neuronal cell bodies and synapses (Petralia *et al.*, 1996; Tang & Sim, 1999).

Electron microscopy has so far only revealed mGlu2/3 receptor staining on presumed post-synaptic and extra-synaptic structures in the dorsal horn (Jia *et al.*, 1999; Petralia *et al.*, 1996; Tang & Sim, 1999). This seems unusual, as group II receptors are detected pre-synaptically in other areas of the central nervous system (Ohishi *et al.*, 1994; Testa *et al.*, 1998) and other classes of mGlu receptors are found in rat and human DRG (Li *et al.*, 1996; Valerio *et al.*, 1997) and on primary afferent nociceptor terminals in the dorsal horn (Li *et al.*, 1997; Ohishi *et al.*, 1995). Functional evidence also indicates a pre-synaptic role for mGlu3 receptors elsewhere in the CNS (for example (Glaum & Miller, 1995).

The pharmacology of mGlu receptors is highly complex, but some evidence exists for a functional pre-synaptic role of mGlu3 as auto- or hetero-receptors regulating glutamatergic transmission pre-synaptically in the spinal cord. In the lamprey spinal cord, (Cochilla & Alford, 1998) saw pre-synaptic enhancement of outward K⁺ currents and could not rule out contribution of group II mGlu receptors; in neonatal rat spinal cord, group II mGlu receptors were determined to be possibly (Cao *et al.*, 1997; Kemp *et al.*, 1994) and probably (Jane *et al.*, 1996) involved in pre-synaptically mediated depression of dorsal-root-evoked motoneurone currents. In the latter study, the potent group II antagonist EGLU blocked pre-synaptically mediated (1S,3S)-ACPD (group II agonist)-evoked depression of the dorsal root–ventral root potential, but could not block I-AP4 (group III agonist)-evoked depression. As mRNA for the mGluR2 is very low in the adult rat spinal cord (Ohishi *et al.*, 1993b), the authors conclude that “the (1S,3S)-ACPD-activated receptor in the neonatal rat spinal cord may therefore be mGluR3 or an as yet uncharacterised receptor”. These pharmacological tools are listed in Table 1.2 in Chapter 1.

Whether or not they are present pre-synaptically in the dorsal horn (and small terminals may not always be visualised), the mGlu3 receptor is certainly expressed in the termination areas of primary afferent C- and A δ -fibres.

4.4.2 Inhibition of input

Spinal application of 2-PMPA in normal animals caused most pronounced inhibitions of input and post discharge. Non-selective NMDA receptor antagonists, as discussed in Chapter 3, do not produce such marked inhibitions of input, and certainly inhibit post discharge to a greater extent than input at a given dose. This is not the case here, so the inhibition of input must be mediated, at least in part, by the action of NAAG at the mGlu3 receptor. To inhibit the measure I refer to as input, an agent must interfere with normal, fast, faithful, predominantly glutamatergic/AMPA receptor mediated transmission in the absence of any prior synaptic activity. As NAAG has no activity at the AMPA receptor (Sekiguchi *et al.*, 1992; Valivullah *et al.*, 1994) it must either reduce transmitter release from the pre-synaptic terminal, or attenuate the ability of the post-synaptic terminal to be depolarised. The mGluR3 is perfectly situated to do either — but released NAAG is unlikely to produce this effect. Firstly, as a peptide it may not be released by the first stimulus in the train. Secondly, even if NAAG were released, it is debatable whether it would reach the peri-synaptic mGlu3 receptors and produce the slow G-protein coupled response to reduce transmitter release or hyperpolarise the post-synaptic membrane before synaptic AMPA-receptor-mediated transmission has finished. It is more likely that protection of NAAG breakdown with 2-PMPA is sufficient to maintain NAAG levels around the synapse between tests (10 min intervals between tests). In fact, as basal release of NAAG is relatively high (and not exclusively dependent on Ca²⁺) in the spinal cord, and NAALADase activity is low (Fuhrman *et al.*, 1994; Zollinger *et al.*, 1994), this seems very likely that the effects on input that we see are a

manifestation of constitutive modulation of the excitability of dorsal horn neurones by NAAG via the mGlu3 receptor.

Inhibition of A δ -fibre evoked responses was also observed. *Jia et al.*, (1999) have determined that the mGlu3 receptor was present post-synaptically to thinly myelinated primary afferent fibres terminating in the superficial dorsal horn. These probably represent A δ -fibre terminals. Presence of the mGlu3 receptor here would explain, assuming the presence and release of NAAG from A δ -fibres, the inhibition of the A δ -fibre evoked response that is seen. In fact, NAAG-LI has been located in large diameter (<35 μ m) cells in the DRG of rats that may well correspond to a thinly myelinated, nociceptive subset of A δ -afferent fibres.

4.4.3 Attenuation of hyperexcitability

Any significant interaction with the NMDA receptor will attenuated the wind-up and associated post discharge of dorsal horn neurones. The pronounced effects on input made the calculation of wind-up (as excess spikes) unreliable, and 2-PPA had little consistent effect on this calculated measure. Looking at examples of wind-up curves for individual neurones, the gradient of these curves (an indication of wind-up) was not noticeably affected. So although the baseline C-fibre afferent input onto these neurones is markedly reduced, the processes of wind-up can still occur to some extent (see Figure 4.3). This could imply that interfering with glutamatergic activation of the NMDA receptor does not play a major part in the mechanism of action of NAAG.

There is, however, a pronounced inhibition of post discharge — the action potentials recorded after synaptic transmission evoked by each stimulus of the train has ceased. Electronmicroscopic localisation of mGlu3 receptors in brain neurones show that they are not located in the immediate post-synaptic density (Ottersen & Landsend, 1997) but peri-synaptically. It is feasible that the activation of peri-synaptic mGlu3 receptors by released glutamate and accumulating NAAG is insufficient to prevent action potential generation, sustained membrane depolarisation and activation of NMDA receptors in the immediate post-synaptic area and thus not significantly attenuate wind-up mechanisms in the course of events immediately following a stimulus; but can sufficiently reduce neuronal excitability between stimuli to prevent full expression of NMDA receptor-mediated wind-up and to attenuate post discharge.

4.4.4 The mGlu3 receptor — a role in prolonged pain?

The work in normal animals detailed here is exciting as it reveals a potential role for the mGlu3 receptor, and for NAAG, in noxious-activity-dependent protection of neurones from the consequences of excessive glutamatergic activation. This may be the normal function of NAAG, revealed here by NAALADase inhibition. Knowing that NAALADase activity is modulated in several pathological conditions, it is tempting to speculate that by

inhibiting NAALADase activity we are simply recreating a situation that may exist if continued noxious stimulation were to continue. The studies in animals subjected to inflammation and SNL seem to back this up, as a greater degree of inhibition was observed after 2-PMPA application in these different groups of animals.

The inhibitory effects of 2-PMPA were enhanced following the development of peripheral inflammation. This could either result from increased levels of NAAG, or by increased levels of the target for NAAG (i.e. the mGlu3 receptor and maybe the NMDA receptor). Expression of the mGlu3 receptor in the dorsal horn is markedly enhanced following peripheral insult (UV irradiation) (Boxall *et al.*, 1998). The authors could not conclude whether the mGlu3 receptor contributed to or compensated for the hyperalgesia. In light of the inhibitory nature of the mGlu3 receptor and the discussions above, it seems almost certain that this receptor functions to attenuate spinal hyperexcitability, pre- and/or post-synaptically.

It is also tempting to predict that changes can occur in the expression of NAALADase. As 2-PMPA inhibits enzyme activity, reduced levels of the target for 2-PMPA (i.e. NAALADase) would allow the same dose of 2-PMPA to inhibit a greater number of sites, and thus allow higher levels of NAAG to persist. Thus, the enhanced inhibitory effects resulting from 2-PMPA application after inflammation, could result from reduced NAALADase expression.

Upregulation of the mGlu3 receptor or downregulation of NAALADase does not have to fully account for the enhanced effects seen, as elevated glutamate (and presumably also NAAG) in the dorsal horn has been observed in similar experimental models of inflammation (Sluka & Westlund, 1992; Sorkin *et al.*, 1992), and there is a precedent for enhanced activation of mGlu receptors in conditions of increased glutamate availability (Scanziani *et al.*, 1997).

The finding that the effects of 2-PMPA are enhanced following nerve injury was exciting. This established animal model of neuropathic pain has proved extremely useful in predicting the clinical effectiveness of novel approaches to pain control (e.g. memantine, Parsons *et al.*, 1999; gabapentin, Chapman *et al.*, 1998; and adenosine, Suzuki *et al.*, 2000). The effects of 2-PMPA after SNL are not as consistent as in the carrageenan model of inflammation, but the extent of the inhibitions resemble those in inflamed animals rather than normal animals. The inhibition of post discharge is significantly greater in spinal nerve ligated animals than in normal animals.

The effects of 2-PMPA in sham-operated animals is not significantly different from the effects in normal animals, or SNL-operated animals, although the trend does seem to be towards greater-than-normal inhibition in the sham-operated group. This raises the question of what animals should be used as a control group with which to compare the effects of spinal nerve ligation. Sham operated animals have undergone fairly extensive surgery, which will surely generate some inflammatory processes. As 2-PMPA is altered to such an

extent after peripheral inflammation, perhaps a sham-operated group is not the best 'control' for this drug.

The lack of difference between sham- and SNL-operated animals would suggest that the nerve injury induced by the SNL surgery plays little or no part in enhancing the effects of 2-PMPA, but that inflammatory component of the SNL and sham surgery is sufficient to cause changes in spinal processing. Also the lack of difference between naïve animals and sham-operated animals indicates that no changes occur in the NAAG/NAALADase system or mGlu3 following sham surgery.

To the best of my knowledge, no study has yet been published investigating the effects of SNL and sham surgery on the expression of the mGlu3 receptor. In the study by (Boxall *et al.*, 1998), injury in the hindpaw receptive field enhanced expression of the mGlu3 receptor only in the lumbar segment, but both ipsilateral and contralateral to the site of injury. This suggests diffuse changes, but not beyond the segment receiving input from the damaged area. The area injured by sham surgery is distant from the receptive field of L4–L5 dorsal horn neurones. Over a longer period of time (14 days vs. 1–3 days) more diffuse changes in mGlu3 receptor expression may occur. It is also possible that changes in NAALADase expression could occur and contribute to the enhanced effects of 2-PMPA.

Elucidation of the role of the mGlu3 metabotropic glutamate receptor

The work presented in this chapter goes some way to elucidating the functional role of the mGlu3 receptor, which was previously little studied with relation to nociception. It appears to contrast with the role of the group I mGlu receptors. Most reports agree that group I mGlu receptors contribute to nociceptive transmission (Chen *et al.*, 2000; Dolan & Nolan, 2000; Fundytus *et al.*, 1998; Young *et al.*, 1998; Young *et al.*, 1997) and are pro-neurodegenerative (Pellegrini Giampietro *et al.*, 1999). However, inhibitory effects in nociception (Chen *et al.*, 2000; Stanfa & Dickenson, 1998) and neuroprotective effects (Pizzi *et al.*, 2000) have also been reported.

The mGlu3 receptor is also expressed in astrocytes in the spinal cord, and expression increases from birth to adulthood (Berthele *et al.*, 1999). The mGlu3 receptor may have a role in the maturation of the sensory nervous system, and in mature spinal cord a role in neuronal–glial signalling. Such signalling is an important function of astrocytes, and in cerebral ischaemia paradigms, the release of trophic factors from astrocytes after glutamate activation contributes to the neuroprotective profile of group II mGlu receptor agonists (Bruno *et al.*, 1998a; Bruno *et al.*, 1997). However, not all groups agree that release of factors from astrocytes occurs, or that the neuroprotective effects are mediated via group II mGlu receptors (Behrens *et al.*, 1999). Astrocytes play an important role in the response to physical stress in the brain, Ca²⁺-dependent activation of astrocytes and the ensuing loss of their vital physiological functions has been suggested as a contributory mechanism to excitotoxic cell death. (need ref. All this said in Schubert abstract).

In the spinal cord, mGlu3 receptor-expressing astrocytes closely appose synapses, and may be vital to regulating glutamate levels via specific transporters and activity of NAALADase. The mGlu3 receptor could play a vital role as the sensor for changes in glutamate levels and it would not be surprising if NAALADase activity were in some way regulated by astrocyte mGlu3 receptor activation.

4.4.5 Conclusions

It certainly seems that enhancing the normal levels of NAAG by NAALADase inhibition is an effective strategy to attenuate both basal and augmented nociceptive transmission. In inhibiting NAALADase activity we may be recreating a situation that exists during persistent pain states, where synaptic levels of NAAG are elevated by reduced NAALADase activity. The enhanced effect of spinally applied 2-PMPA in a model of short-term inflammation and longer-term neuropathy supports this theory. The NAAG affinity of the NMDA receptor appears to contribute little to the profile of this interesting peptide in modulation of nociceptive transmission at the level of the spinal cord *in vivo*.

The possible synaptic actions of NAAG are summarised over the page in Figure 4.4.

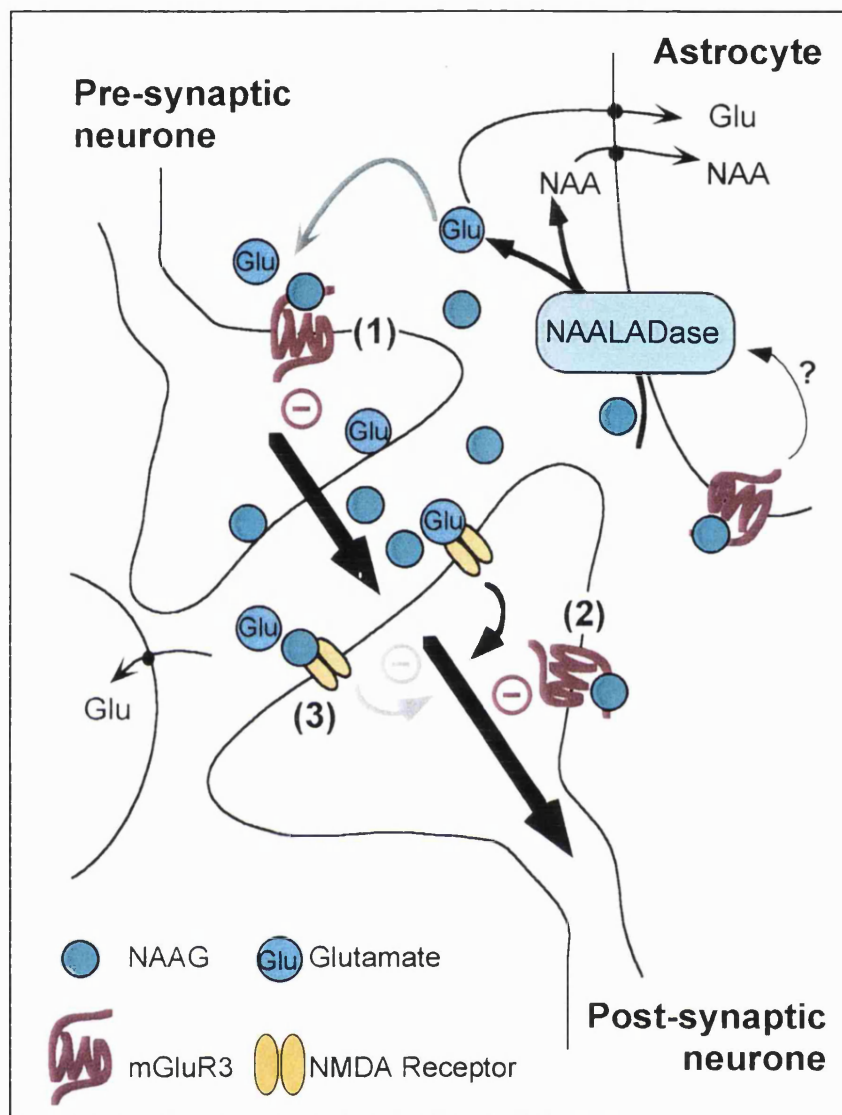


Figure 4.4 Illustration of the neurotransmitter actions of NAAG. After release, NAAG (1) activates pre-synaptic inhibitory mGluR3 to attenuate further release of neurotransmitters, including NAAG and glutamate. At the post-synaptic membrane, NAAG attenuates excitability by (2) the activation of post-synaptic mGluR3 and possibly (3) by interfering with binding of released glutamate at the NMDA receptor. Inhibition of NAALADase with 2-PMPA can reveal these effects.

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Chapter 5. Peripheral proton sensing

5.1 Introduction

Following several reports of the existence of pre-synaptic NMDA receptors (for example see Carlton *et al.*, 1995; and Carlton, 2001 for review), the potential role of peripheral NMDA receptors in initiation of nociceptive processes has been studied. Some evidence, using peripheral administration of NMDA receptor antagonists in the formalin test, has suggested that the NMDA receptor does indeed contribute to nociceptive behaviours in this particular model of inflammatory pain (Davidson *et al.*, 1997). We therefore set out to examine whether peripheral administration of solutions of NMDA could activate peripheral nociceptors and be recorded as activity in dorsal horn neurones.

A few preliminary experiments ($n = 3$) revealed that administration of low concentrations NMDA ($1\text{--}10\mu\text{g}/20\mu\text{l}$) generated non-dose-dependent dorsal horn neuronal activity. The solutions of NMDA were acidic (pH 2.5) and saline buffered to pH 2.5 evoked greater responses than administration of neutralised NMDA solution. The decision was then made to investigate peripheral administration of a range of pH solutions, rather than to pursue investigation into the peripheral NMDA receptor.

As described previously (Chapter 1, section 1.2.5), there is now fairly conclusive evidence for a role of protons in nociception, as evident from the generation of pain sensations on infusing acidic buffer into the skin of human volunteers (Steen & Reeh, 1993). This persistent pain that continues for the duration of the infusion can be attributed to the persistent proton-evoked currents recorded in sensory neurones. Some examples of the characteristics of currents recorded in sensory nerve are shown in Table 5.1.

Table 5.1 Characteristics of various H^+ -sensitive preparations*

Tissue	Cell type	Current	pH-sensitivity	Selectivity
Rat DRG ^a	< 30 μm and caps-sensitive	Biphasic: - transient - sustained	< 6.8 < 6.2	Na^+ selective $\text{K}^+ > \text{Cs}^+ > \text{Na}^+$
Rat spinal and trigeminal ganglia neurones ^b	All cells < 26 μm responded (47% of total)	- 84% monophasic - 16% biphasic	Threshold 7.0–6.5 Max at 5.4	$\text{pNa}^+ > \text{pK}^+$ (also Ca^{2+} permeable)
Chick DRG ^c	1-2 day old cultured cells	Mean $\tau_i = 0.3\text{s}$	< 6.7	Na^+ selective
Frog DRG ^d	All cells < 20 μm responded	Mostly $\tau_i = 2\text{--}15\text{ s}$ (full range 0.5s–1min)	Threshold 7.0 Max at 5.2	Na^+ selective
Rat skin-nerve preparation ^e	38% of polymodal C-fibres were sensitive	Sustained non-adapting excitation	Threshold 6.1–6.9 Max at 5.2	—

*Voltage clamp channel characteristics and *in vitro* single-unit recordings. ^a(Bevan & Yates, 1991); ^b(Krishtal & Pidoplichko, 1980; Krishtal & Pidoplichko, 1981; Kovalchuk *et al.*, 1990); ^c(Davies *et al.*, 1988); ^d(Akaike *et al.*, 1990); ^e(Steen *et al.*, 1992). Caps, capsaicin.

Comparing these characteristics with the properties of cloned ASICs listed in Table 5.2 shows that it is not possible to correlate exactly the native currents in sensory nerves with the current characteristics of any one of the ASICs cloned and studied so far. The left-hand column of this table lists the names of each channel given as they were cloned. For simplicity I will use the ASIC nomenclature shown in the table in bold.

Table 5.2 Properties of cloned rat ASICs

Channel*	Localisation	Current	pH-sensitivity	Selectivity
ASIC1 ^{a,b}	Brain, DRG + SC	Monophasic transient current	Threshold < 7.0	Na ⁺ > Ca ²⁺ > K ⁺
ASICβ ^c (splice variant of ASIC)	DRG	Monophasic transient current	Threshold < 7.0	Na ⁺ > K ⁺
ASIC2 (also ASIC2a, MDEG ^{d,e} , BNC1(a) ^{f,g} , BNaC1(a) ^h)	Brain, DRG + lanceolate nerve endings ^g	Monophasic, slower inactivation than ASIC	Threshold < 5.5	Na ⁺ selective
ASIC2b (splice variant of ASIC2. Also, MDEG2 ^e , BNaC2 ^h or BNaC1b ^f)	Brain + DRG	Does not form functional channels on it's own. See Table 5.3 for properties of ASIC2b-containing heteromers (with ASIC2 and ASIC3)		
ASIC3 (DRASIC) ⁱ	Mainly DRG reported in brainstem + SC	Biphasic: - transient - sustained	1/2max pH 6.5 threshold <4.0	Na ⁺ selective Na ⁺ selective
ASIC4 (SPASIC) ^j	Brain, SC, brainstem, cerebellum	Does not form functional channels on it's own. No data yet on heteromeric combinations.		

*The cloned channels have been given many different names. I will use **ASIC** nomenclature, alternative names are listed here for reference. ^a(Waldmann et al., 1997b), ^b(Olson et al., 1998), ^c(Chen et al., 1998), ^d(Waldmann et al., 1996), ^e(Lingueglia et al., 1997), ^f(Price et al., 1996), ^g(Price et al., 2000), ^h(Garcia Anoveros et al., 1997), ⁱ(Waldmann et al., 1997a), ^j(Akopian et al., 2000). SC, spinal cord; DRG, dorsal root ganglion.

As no channel expressed alone in various expression systems can match exactly or entirely account for native currents, it is more likely that the native currents result from the association of different subunits. For example, ASIC1 and ASIC2 co-immunoprecipitate from rat brain, indicating the existence of heteromultimeric channels (Bassilana et al., 1997). When co-expressed, these two ASICs form channels with novel characteristics; however, as ASIC2 expression is restricted to the brain, this has little direct relevance to nociception apart from setting a precedent. Following this precedent, several other ASIC genes have been co-expressed and found to form channels with novel characteristics. Of particular interest is ASIC2b, which cannot form functional channels on its own, but combines with ASIC2 and ASIC3 to alter their properties. When co-expressed with ASIC1 however, no novel characteristics are generated. Table 5.3 shows the characteristics that results from subunit co-expression.

Table 5.3 Properties of combinations of rat ASIC subunits

Combination	Areas of overlap	Novel properties?	Current characteristics	pH-sensitivity	Na ⁺ / K ⁺ selectivity
ASIC1 + ASIC2 ^a	Brain	Yes	Monophasic transient	Intermediate between subunits	Less selective than either subunit
ASIC + ASIC2b ^b	Brain + DRG	No	No different from those recorded with ASIC alone		
ASIC2 + ASIC2b ^b	Brain	Yes	Biphasic: - transient - sustained	Doesn't saturate at low pH	- Na ⁺ selective - Poor Na ⁺ / K ⁺ selectivity
ASIC2b + ASIC3 ^c	DRG	Yes	Biphasic: - transient - sustained	- As for ASIC3 alone - Current at pH 4.0	- Na ⁺ selective - pNa ⁺ = pK ⁺
ASIC2a + ASIC3 ^c	Brain (rat) Brain, sensory ganglia and testis (human)	Yes	Biphasic: - transient: unchanged - sustained: enhanced, little desensitisation	Both present at pH 4.0	Na ⁺ selective

^a(Bassilana *et al.*, 1997), ^b(Lingueglia *et al.*, 1997), ^c(Babinski *et al.*, 2000).

Although expressing combinations of these channels gives rise to currents that share more similarities with currents recorded from sensory nerves, no combination has as yet accounted for all facets of native H⁺-evoked currents. The ASICs cloned so far are likely to represent no more than potential subunits of functional acid-sensing channels; however, post-translational modifications and the presence of as-yet unidentified channel genes must be considered.

Conversely, although sensitive to low pH, the channels cloned so far may not function as acid-sensors in their physiological setting. In fact, ASIC2 (BNC) has recently been shown to be necessary for normal touch sensation (Price *et al.*, 2000). BNC^{-/-} mice showed normal pH-evoked responses in both whole-cell voltage clamp and skin-nerve preparations, yet rapidly adapting (A-fibre) mechanosensory fibres could not code normally for the intensity of the stimulus (small membrane displacements). Current injection determined that the abnormal mechanotransduction resulted not from the second step of the mechanotransduction process (turning currents into action potentials) but, therefore, from the first step of the process, turning membrane displacement into currents. Peripheral localisation of both the transcript and BNC1 (ASIC2) protein was seen in lanceolate nerve endings adjacent to the hair follicle, an important site for light touch and brush sensation in hairy skin (Garcia-Anoveros *et al.*, 2000; Price *et al.*, 2000). A channel necessary for normal mechanotransduction in *Drosophila melanogaster* has also recently been identified (Walker *et al.*, 2000).

The cytoskeleton proteins with which these putative mechanosensory channels interact are likely to be important in the mechanism of transduction. Of the proton-sensing ion channels, ASIC1, ASIC2 and ASIC2b have novel PDZ binding domains on their N-

terminals, not found on other ASICs, which appear to interact with the PDZ domain-containing protein PICK1 (protein interacting with C kinase) (Hruska-Hageman *et al.*, 2000). PICK1 has recently been shown to interact, via its PDZ domain, with the extreme C-terminus of the AMPA receptor subunit GluR2, resulting in the clustering of this subunit into intracellular membrane compartments (Dev *et al.*, 1999). Such proteins may be important in regulating localisation of these channels and in regulating their sensory function, and the novel PDZ interactions of ASIC1 and ASIC2 may suggest a different function for these channels from others in the family.

Previous studies into acid-sensing ion channels have focused on the dynamics (whole cell voltage clamp and single channel activity) of cloned channels, application of low pH directly onto dissected nerves *in vitro*, or behavioural and human psychophysical studies. The consequences of activation of peripheral ASICs on activity of dorsal horn neurones *in vivo* is as yet unknown. The spinal cord, as the site for integration, relay and modulation of nociceptive information from primary afferents is an important area to investigate.

Here I describe the effects of administration of bolus injections of saline buffered to a range of pH, into the hindpaw of the anaesthetised whole animal. This approach allows recording of neuronal activity and quantification of acid-evoked single unit responses, and avoids damaging dissection of the receptive field. The effect of low pH on punctate mechanical-evoked responses were also studied.

5.2 Methods

Animals were prepared for electrophysiology and a neurone isolated as previously described. A suitable neurone was isolated in each experimental animal on the basis of the ability to respond to both pinch and touch. Electrical stimulation was applied briefly to determine the C-fibre threshold, and one train of stimuli at 3-times C-fibre threshold was given. This confirmed the convergent nature of each neurone and allowed characterisation of the neurone on the basis of total C-, A β - and A δ -fibre-evoked responses, input, post-discharge and excess spikes. A qualitative measure of the responsiveness of the neurone to noxious heat was also determined. A gentle water jet at 45°C was applied to the plantar surface of the hindpaw, and the activity evoked was rated (scale 1–3; no response, moderate firing, high frequency sustained response).

Physiological saline (0.9%) was titrated to a range of pH using concentrated HCl. Five different pH solutions were used: control of pH 7.4, followed by pH 5.85, 5.0, 4.0 and finally pH 2.5 (the same acidity as the original NMDA solution).

After the neurone had been characterised and allowed recovery time (10 min), a 29G insulin syringe needle was inserted subdermally into a toe in the centre of the receptive field. Recovery time was allowed for any activity evoked by insertion of the needle to return to baseline levels (2–10 min). Leaving the needle in place throughout, four separate injections of 20 μ l saline (pH 7.4) were made and the neuronal activity evoked by each one recorded. The interval between injections varied depending on the duration of injection-evoked activity, and ranged from 2–5 min. Baseline activity was always recorded for 60 s prior to each injection.

The syringe was then removed and, once firing had returned to baseline, von Frey filaments (9g and 30g) were applied to the centre of the receptive field. Application lasted for 10 s once bending force was reached, and the number of action potentials evoked during the static 10 s was recorded for each von Frey filament. The next needle was then inserted, with syringe containing saline buffered to pH 5.85 and, once any spontaneous activity had returned to baseline, the same protocol of injections and von Frey filament application was repeated (and again for pH 5.0, 4.0 and 2.5).

This protocol is illustrated in Figure 5.1, which shows a trace recorded at the start of one particular experiment. A Spike 2 data capture programme was used in the rate function to continuously record the activity of each neurone for the duration of the experiment.

No lasting damage to the area was caused; needle-stick injury was kept to a minimum by leaving the needle in place for the duration of the four injections of each pH solution. For each neurone and receptive field, a needle was introduced five times.

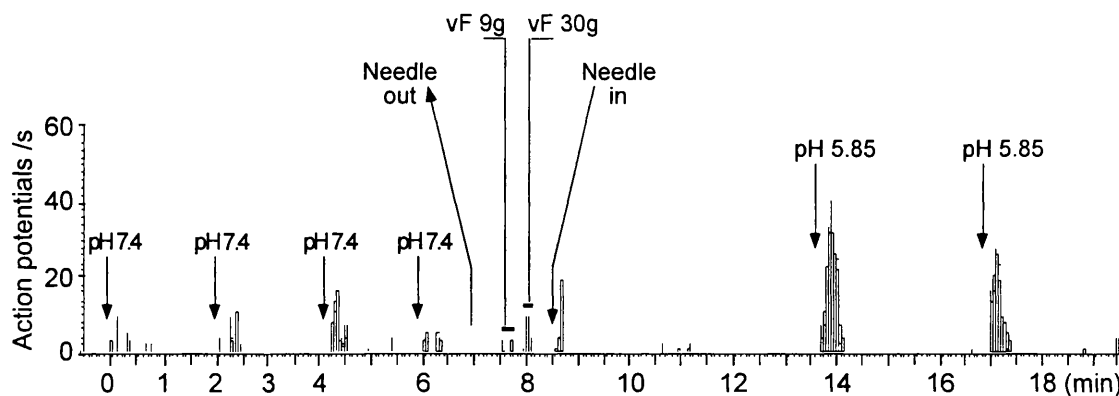


Figure 5.1 A section of a trace recorded during a typical experiment, to illustrate the protocol adopted for peripheral administration of low pH solutions and application of mechanical stimuli. The number of action potentials evoked per second is shown on the vertical axis, and the time (in minutes) is shown on the horizontal axis.

Neutral saline (20 μ l, pH 7.4) was administered four times; activity evoked by each administration was allowed to subside before the next injection. Next, the needle was removed and von Frey filaments (9g and 30g) were applied to bending force for 10 seconds each. A second syringe needle was inserted and activity allowed to subside. Saline titrated to pH 5.85 (20 μ l) was then administered (just the first two injections are shown).

This protocol would have been followed by two more administrations of pH 5.85, followed by pH 4.0 (x4), followed by pH 2.5 (x4). Von Frey filaments were applied after administration of each solution.

5.2.1 Data analysis

Injection of each test solution evoked a short-lived period of firing that tended to have subsided within 60 s. Injection-evoked responses were, therefore, counted over a period of 60 seconds from completion of injection. Any background spontaneous activity of the neurone was recorded over 60 s prior to each administration and subtracted from the injection-evoked count. For each neurone, counts/60 s (corrected for background) were recorded for four injections of pH 7.4 solution; counts/60 s for 4 injections of pH 5.85 and so on for pH 5.0, pH 4.0 and pH 2.5. The activity evoked by each of the 4 injections of the same pH solution was fairly consistent for each individual neurone and therefore these 4 counts were averaged to give, for each neurone, one single value (counts/60 s) for each pH solution administered. These values were then averaged across the 21 neurones, giving a final value, mean $\pm$ s.e.m for each pH solution ($n = 21$ in all cases).

Von Frey filaments of weights 9g and 30g were applied to bending force and counts/10 s were recorded; any spontaneous background activity (recorded over 10 s prior

to application) was subtracted. One application was made after each pH administration, and the mean $\pm$ s.e.m. of these determined across the neurones tested. If, after completion of the whole experiment, a neurone had not responded to application of 9g and/or 30g, results were excluded for that weight of hair for that neurone.

Repeated measures ANOVA was used to determine any effect (injection evoked responses and mechanical evoked activity) as the experiment progressed. Post hoc analysis with the Dunnett multiple comparison test revealed any significant effects of each individual pH solution administered on evoked counts or on mechanical evoked activity.

5.3 Results

A total of ²² neurones were studied in 12 different animals; when more than one neurone was used in an animal it was ensured that the receptive fields did not overlap. All but one of the neurones studied responded to administration of solutions of decreasing pH with a short-lived period of firing. The one non-responding neurone was excluded from all further calculations. The mean depth of the remaining 21 neurones was $871 \pm 49.5\mu\text{m}$, and the mean C-fibre threshold was $1.38 \pm 0.21\text{mA}$.

5.3.1 Injection-evoked responses

The duration of evoked activity increased as the pH of the solution decreased (Table 5.4, repeated measures ANOVA, $P \leq 0.0001$), but activity tended to have subsided and returned to baseline levels within one minute. The mean injection-evoked counts/60 s are shown $\pm$ s.e.m. in Figure 5.2 and Table 5.4. The increase in counts (per 60 s) with administration of solutions of decreasing pH was significant (Repeated measures ANOVA, $F = 7.5481$, $^{+++}P \leq 0.0001$) and pH-dependent ($P \leq 0.0001$,). Post-hoc analysis showed that the response evoked by pH 2.5 was significantly greater than control saline-evoked responses (Dunnett multiple comparisons test, $^{**}P \leq 0.01$).

Table 5.4 Effect of injections of lowering pH on responses of dorsal horn neurones

	<i>pH of solution administered</i>				
	pH 7.4	pH 5.85	pH 5.0	pH 4.0	pH 2.5
Duration of activity (s) ⁺⁺⁺	$22.7 \pm 3.0\text{s}$	$25.3 \pm 4.0\text{s}$	$28.4 \pm 5.1\text{s}$	$31.4 \pm 5.2\text{s}$	$59.0 \pm 11\text{s}^{**}$
Injection-evoked counts (/60 s) ⁺⁺⁺	86.3 ± 29	171 ± 82	196 ± 61	332 ± 95	$648 \pm 181^{**}$
von Frey 9g [†] (/10 s)	38.1 ± 21	13.7 ± 8.1	23.9 ± 12	43.8 ± 24	65 ± 32
von Frey 30g ^{††} (/10 s)	190 ± 84	215 ± 80	228 ± 82	284 ± 102	$371 \pm 115^{**}$

[†] $P \leq 0.05$ ^{††} $P \leq 0.01$ ⁺⁺⁺ $P \leq 0.001$, repeated measures ANOVA, significant effect with lowering pH; $^{**}P \leq 0.01$, Dunnett multiple comparison test, significant effect of individual 'doses' compared with pH 7.4 control.

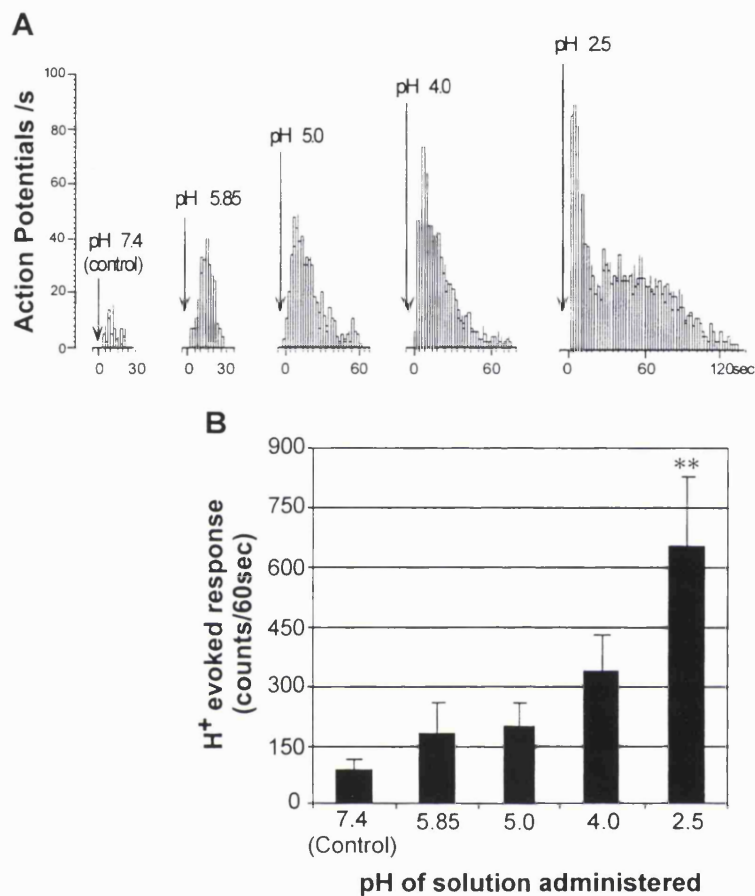


Figure 5.2 Neuronal activity evoked by injection of solutions of progressively lower pH. Panel A shows sections of a trace recorded from one individual neurone as an example. Panel B shows the data obtained from 21 neurones (mean + s.e.m). Significant effect of pH 2.5, as compared with neutral saline (pH 7.4) control, is indicated (** $P \leq 0.01$, Dunnett multiple comparison test).

5.3.2 Mechanically evoked responses

Of the 22 neurones studied, 11 responded to application of von Frey 30g, and 9 neurones responded to von Frey 9g. With progressive lowering of the pH, mechanical responses evoked with both 9g and 30g von Frey filaments showed a significant increase from control action potentials evoked after saline (Table 5.4, Figure 5.2, repeated measures ANOVA, $^*P = 0.0377$ and $^{**}P = 0.0076$ respectively). Maximum mechanical evoked response was achieved after administration of pH 2.5 in both cases, and this increase was significant for von Frey 30g after pH 2.5 (Dunnett Multiple Comparisons Test $^{**}P \leq 0.01$).

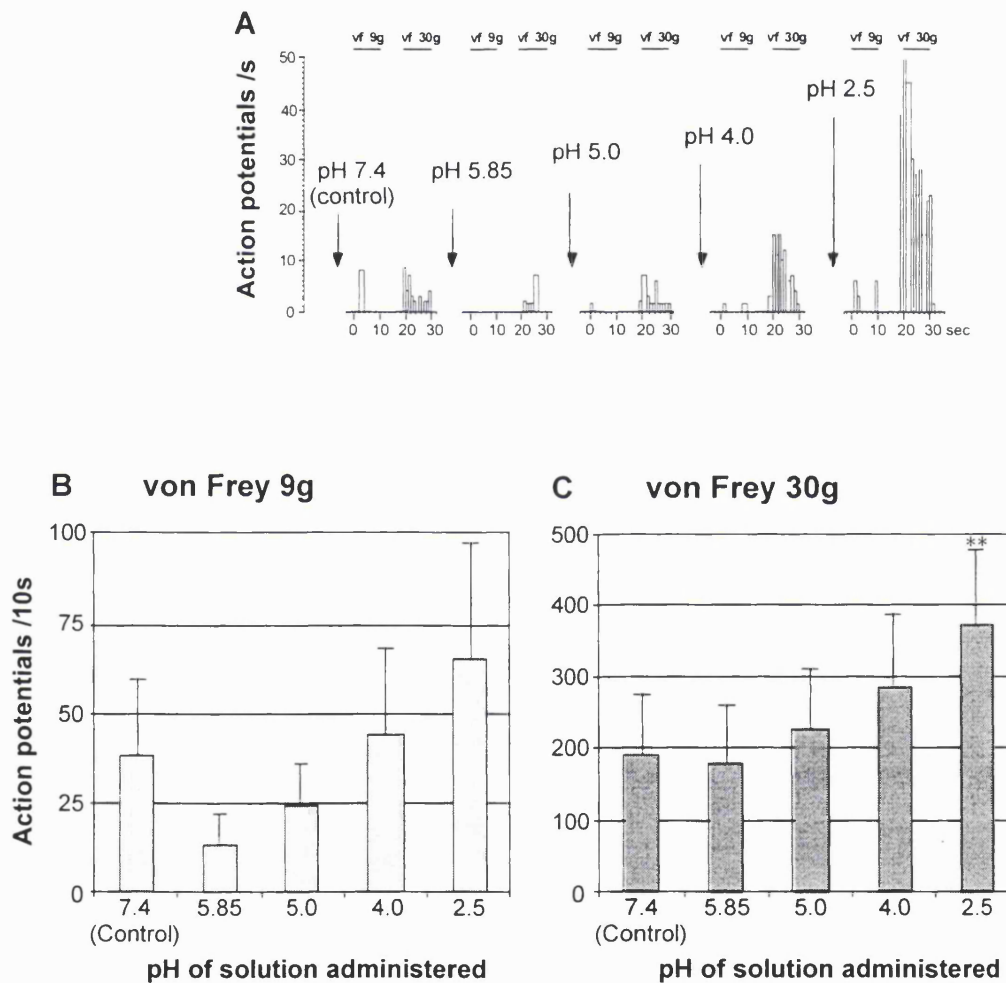


Figure 5.3 Effect of lowering pH on mechanically evoked responses of dorsal horn neurones. Panel A shows sections of a trace recorded for one individual neurone as an example. The lower panel shows mean data (+ s.e.m.) from B, application of von Frey 9g ($n = 9$); and C, application of von Frey 30g ($n = 11$). Significant difference in responses evoked after individual administration of pH compared with response evoked after neutral saline (pH 7.4) control, is indicated ($^{**}P \leq 0.01$, Dunnett multiple comparison test).

5.4 Discussion

Administration of low pH solutions, subdermally, into the hind-paw receptive field of convergent dorsal horn neurones evoked a series of action potentials. Significant increase in the number of action potentials evoked was not observed until a solution of pH 2.5 was administered; however, increases in the responses of individual neurones were observed after pH 4.0. Direct comparisons with *in vitro* experiments would indicate that a much lower pH is required to activate primary afferents in this paradigm. As the intact hind-paw is used, and constant infusion was not possible, the buffering capacity of the hind-paw would ensure that the proton concentration reaching the site of action would be less.

In fact, the only other study to investigate hind-paw injections of low pH in the intact rat has actually recorded the intraplantar pH in response to administration of a range of pH from 7.4 to 3.0. The pH recorded in the hindpaw was never as low as, but was linearly correlated to, the pH administered, but had a linear correlation to the pH administered (Hamamoto *et al.*, 1998). Although the authors administered 50µl of solution subcutaneously into the plantar surface of the hindpaw, and I administered 20µl subdermally into the toe, their work can be used as a rough guide to the approximate pH at the nociceptor after administration of each pH solution. These are shown in Table 5.5.

Table 5.5 Predicted pH at peripheral nerve endings after administration

	<i>pH of solution administered</i>				
	7.4	5.85	5.0	4.0	2.5
Predicted actual pH at nociceptor	7.0	6.2	5.6	5.0	4.2

Values predicted from graphs shown in Hamamoto et al., 1998.

Hamamoto *et al.* (1998) also investigated the withdrawal responses to a range of von Frey filaments after administering of a range of pH solutions. Although this study has great relevance to my work, interpretation of their findings are almost impossible for several reasons. First, the solutions of hyaluronic acid were made up in distilled water, before being adjusted to the correct pH with 0.1M HCl or 0.1M NaOH. Previous work by Haley in this laboratory has shown that distilled water, even at a neutral pH, evokes large responses from dorsal horn neurones, and so would presumably elicit a painful sensation, and perhaps a degree of hyperalgesia, in these awake animals. Secondly, a different weight von Frey filament was used in each animal (ranging from 1.7–21.3g) chosen on the basis of the lowest weight filament that could evoke “one or two withdrawals out of 10 applications”. The results show that frequency of paw withdrawal (withdrawals per 10 applications) was significantly greater after administration of pH 6.0, as compared to just needle-stick. Close examination of the methods and results reveals that the mode von Frey filament used after

needle-stick was 9g, whereas the mode filament for the pH 6.0 group was 11g. Thirdly, results (frequency of withdrawals) were always compared with the needle-stick group, rather than to the pH 7.4 group. Introduction of even a small volume of fluid can alter mechanosensation, so pH 7.4 should be used as the control value for comparisons. This brings us back to point 1; their pH 7.4 values are not useable as a control as neutral distilled water activates primary afferents. Overall although this study is useful for the intraplantar recordings of pH the other work, that would appear to parallel my own methodologically, cannot be compared to my work presented here.

Desensitisation of the sustained component of the proton-evoked currents is characteristic in voltage clamp recordings of sensory nerve. Interestingly, my results show no desensitisation; the magnitude and the duration of the responses continues to increase, over a period of several hours, with the administration of progressively lower pH solutions. This indicates that in a physiological setting where a group of nociceptors is present to mediate the response to protons, no desensitisation occurs. This is in keeping with human studies, where volunteers report sensations of constant pain for the duration of infusion of acidic buffer (Steen & Reeh, 1993).

Hyperalgesia was evident in the human studies undertaken by Steen & Reeh (1993); a reduction was apparent in the punctate mechanical threshold required to evoke pain sensations. Although no such definitions can be applied to our work, a significant increase in the magnitude of responses evoked by both low (9g) and high (30g) intensity stimuli was observed. This could be said to correlate with the behavioural measures of allodynia and hyperalgesia, respectively.

5.4.1 A correlation with heat sensitivity?

The one neurone that did not respond to low pH could not be differentiated from those that were sensitive to pH on the basis of C-fibre threshold, total C- or A-fibre counts, input, post discharge or excess spikes. This non-responding neurone was not sensitive to noxious heat (application of water at 45°C). Conversely, one neurone that did not respond to heat had a maximum pH-evoked response of 1911 action potentials (/60 s). If this neurone is excluded, then there is a significant correlation between the sensitivity of each neurone to noxious heat (arbitrary scale 1–3, no response–sustained high-frequency response) and the maximum pH-evoked response (action potentials/60 s) shown by that neurone (Pearson correlation, $P = 0.0124$).

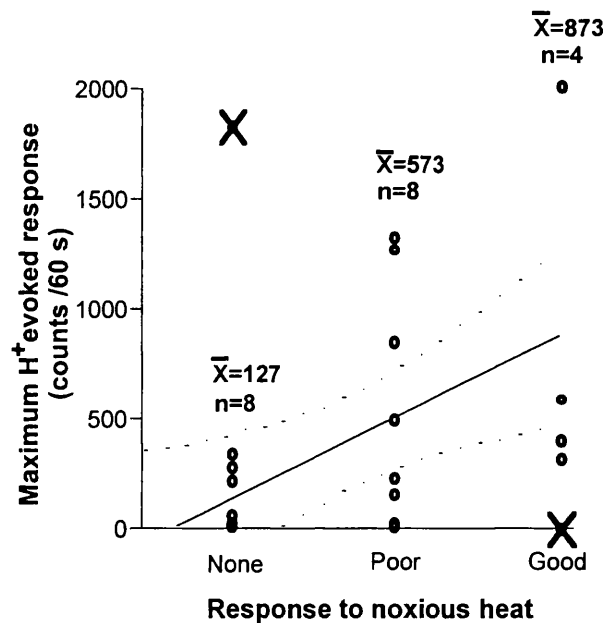


Figure 5.4 Correlation of proton-evoked responses with sensitivity to noxious heat. The maximum proton-evoked response for each neurone is plotted on the y-axis, against its response to noxious heat (none, poor, good) on the x-axis. The mean proton-evoked response is also shown for each heat classification. Two neurones had anomalous response to heat (marked with a cross); if these are excluded then the correlation is significant ($P = 0.0124$).

This indicates that primary afferent nociceptors sensitive to noxious heat and to protons may tend to converge on the same dorsal horn neurones. In fact, it has been suggested that capsaicin and protons might activate the same ion channels, namely the VR1 receptor for capsaicin and the suggested endogenous sensor for noxious heat (Bevan & Geppetti, 1994). The currents evoked by protons and by heat do share many biophysical properties. Also, the VR1 receptor antagonist capsazepine is able to attenuate proton-evoked cough reflexes (Lalloo *et al.*, 1995) and currents (Bevan *et al.*, 1992). There are however, important objections; proton activated channels are less permeable to Ca^{2+} than capsaicin-activated channels (Zeilhofer *et al.*, 1997), and show much slower (or no) inactivation (Bevan & Yeats, 1991; Caterina *et al.*, 1997).

In conclusion, despite the buffering capacity of the intact rat hindpaw, administration of low pH solutions in the receptive field caused excitation of convergent dorsal horn neurones and sensitisation of these neurones to both low and high intensity punctate mechanical stimuli. I also observed a tendency for heat-activated and pH-sensitive primary afferents to converge on the same dorsal horn neurones. Resting half-way between the physiological nature of human studies and single-unit-recordings in skin-nerve preparations, the series of experiments described here support the findings of both.

5.5 References

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Chapter 6. Nociceptin/orphanin FQ

6.1 Introduction

6.1.1 The 'orphan' receptor

With the application of molecular biology techniques to the characterisation of receptors, many new receptor genes were identified through homology screening of related and previously cloned receptors. The pharmacological characterisation of these novel receptors often lags behind the structural characterisation and in many cases the endogenous ligands for the receptors remain unidentified. One such 'orphan' receptor was the opioid receptor-like (ORL1) receptor, identified following the cloning of the opioid receptors (μ , δ and κ) (Mollereau *et al.*, 1994; Wang *et al.*, 1994; Wick *et al.*, 1994). Like the opioid receptors the ORL1 receptor is a seven transmembrane spanning receptor coupled to inhibitory G-proteins (G_i) and inhibition of forskolin-induced cAMP accumulation. Receptor activation has been demonstrated to activate inwardly rectifying potassium channels (Allen *et al.*, 1999; Amano *et al.*, 2000; Chiou, 2000; Madamba *et al.*, 1999; Vaughan & Christie, 1996) and inhibit voltage-gated calcium channels (Connor *et al.*, 1996; Reinscheid *et al.*, 1995; for review see Hawes *et al.*, 2000; Meunier *et al.*, 1995).

More recently, in a similar manner to that of opioid receptors (Crain & Shen, 1990; Joshi *et al.*, 1999; Olanas & Onali, 1999), the ORL1 receptor has also been shown to also stimulate cAMP accumulation (Onali & Olanas, 2000). A pertussis toxin insensitive component to NC/OFQ-mediated reduction in forskolin-induced cAMP formation has been seen. This may involve G_z and G_{16} G-proteins, with a possible involvement of phospholipase C and c-Jun N-terminus kinases (JNKs) (Chan & Wong, 2000; Chan *et al.*, 1998; Hawes *et al.*, 2000). In addition, chronic activation of endogenous ORL1 receptors can lead to G_i/G_o -mediated adenylyl cyclase supersensitization, a hallmark of cellular tolerance to opioids (Chan & Wong, 1999). Cellular consequences of receptor activation are reviewed in Hawes *et al.* (2000) and Moran *et al.* (2000). Interestingly, co-expression of NC/OFQ and the ORL1 receptor has been detected in the same neuronal populations in multiple areas in rat brain including the hippocampus, arcuate nucleus in the hypothalamus, ventrolateral periaqueductal gray (PAG) and raphe nuclei in brain stem (Wang *et al.*, 2000). The expression of ORL1 mRNA in nociceptinergic neurons may suggest a presynaptic autoreceptor function.

6.1.2 The endogenous ligand, NC/OFQ

Despite a marked structural homology between the ORL1 receptor and opioid receptors, pharmacologically they are quite distinct. The ORL1 receptor exhibits low affinity for the endogenous opioids, opioid receptor agonists such as morphine and for opioid receptor antagonists such as naloxone (Mollereau *et al.*, 1994; Wang *et al.*, 1994; Wick *et al.*, 1994). However, a partial agonist profile for buprenorphine at the cloned ORL1 has been suggested (Bloms-Funke *et al.*, 2000; Wnendt *et al.*, 1999) and some similarity between

receptor recognition sites of opioid and NC/OFQ receptor ligands is apparent (Hawkinson *et al.*, 2000). This low affinity for the opioid peptides suggested the existence of a previously unknown endogenous opioid. Nociceptin, also known as orphanin FQ, (hereafter referred to as NC/OFQ) a heptadecapeptide (FGGFTGARKSARKLANQ) was subsequently isolated simultaneously by two groups in 1995. It exhibits a high affinity for the ORL1 receptor and is now widely accepted to be the endogenous ligand for this receptor (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995).

6.1.3 Additional functional peptides encoded by the prepronociceptin gene

NC/OFQ may not be the only peptide released from the precursor polypeptide whose sequence contains one copy of NC/OFQ; Lys-Arg proteolytic excision motifs flank the NC/OFQ sequence but are also found at other points in the precursor sequence (Nothacker *et al.*, 1996; and Meunier, 1997 for review). Several peptides may therefore also be generated from the precursor protein (prepronociceptin), including the bioactive peptides nocistatin (Okuda-Ashitaka & Ito, 2000) and OFQ2 (Amodeo *et al.*, 2000). Nocistatin has variously been reported to block inhibitory neurotransmission in superficial dorsal horn and increase nociceptive behaviour in the formalin test (Zeilhofer *et al.*, 2000), to inhibit the first phase of the formalin test in the rat (Yamamoto & Sakashita, 1999), to inhibit both phases of the formalin test in mice with no effect on NC/OFQ responses (Nakano *et al.*, 2000) and to block i.t. NC/OFQ-induced hyperalgesia (Okuda-Ashitaka & Ito, 2000). The nature of the effects reported for nocistatin depends to a large amount on the animal, model, route of administration and dose of NC/OFQ given. Its function remains unclear.

6.1.4 Localisation of NC/OFQ and the ORL1 receptor

Functionally, NC/OFQ has been implicated in many biological processes as the receptor and prepronociceptin transcript, the receptor and NC/OFQ protein, and NC/OFQ binding have been detected throughout the CNS and in immune cells in rodents (for review see Meunier, 1997; Mollereau & Mouledous, 2000) and in humans (Peluso *et al.*, 1998).

More specifically, NC/OFQ-like immunoreactivity has been detected in superficial layers of mouse and rat spinal cord (Lai *et al.*, 1997; Narita *et al.*, 1999; Neal *et al.*, 1999a; Schulz *et al.*, 1996; Williams *et al.*, 1998;) and in rodent brain (Florin *et al.*, 2000; Houtani *et al.*, 2000; Schulz *et al.*, 1996; Wang *et al.*, 2000). Prepronociceptin mRNA has been detected in low levels in rodent DRG (Andoh *et al.*, 1997) and spinal cord (Houtani *et al.*, 2000) and throughout the forebrain, limbic circuits and the brainstem (Neal *et al.*, 1999a; Nothacker *et al.*, 1996; Houtani *et al.*, 2000). In general, the distribution of NC/OFQ and endogenous opioid peptides parallel each other, but there is little co-existence within the same neurones (for example Riedl *et al.*, 1996).

The ORL1 receptor, as detected both by *in situ* hybridisation to the transcript and by autoradiography of NC/OFQ binding, has been reported throughout rat, mouse and human

brain (Florin *et al.*, 2000; Houtani *et al.*, 2000; Peluso *et al.*, 1998; Wang *et al.*, 2000), and in both dorsal (Florin *et al.*, 2000; Jia *et al.*, 1998; Pettersson *et al.*, 2000) and ventral (Florin *et al.*, 2000; Pettersson *et al.*, 2000) spinal cord. Localisation of the receptor in the DRG is less well documented — radioligand NC/OFQ binding and immunohistochemistry have not detected the receptor protein in the DRG whereas receptor mRNA has been detected (Neal *et al.*, 1999b; Wick *et al.*, 1994). The receptor may not be functional or at the cell surface until it reaches the terminals. Other mismatches between detection of ORL1 receptor mRNA and NC/OFQ binding may be explained by the finding that alternative splicing of ORL1 occurs, generating non-functional receptors (Xie *et al.*, 2000) or by the existence of other receptors for NC/OFQ.

6.1.5 Function

Of the many proposed function of NC/OFQ, an involvement in learning and memory has received particular attention. Mice lacking the ORL1 receptor or NC/OFQ have been reported as showing improved learning and memory and enhanced LTP suggesting that NC/OFQ has inhibitory functions (Mamiya *et al.*, 1998; Manabe *et al.*, 1998; Nabeshima *et al.*, 1999; reviewed in Goda & Mutneja, 1998; Noda *et al.*, 2000). NC/OFQ induces outward K^+ currents in hippocampus (Amano *et al.*, 2000; Madamba *et al.*, 1999), and interferes with NMDA receptor mediated LTP and LTD (Wei & Xie, 1999). The role of NC/OFQ in stress and anxiety has also received much attention. Mice with disruption of the NC/OFQ receptor or peptide are characterised by increased sensitivity to or reduced adaptation to stressful stimuli (Koster *et al.*, 1999; Reinscheid *et al.*, 2000). A recently described non-peptide NC/OFQ receptor agonist was shown to have an anxiolytic profile after systemic administration in rats (Jenck *et al.*, 2000).

Various studies also suggest roles in circadian rhythm generation, (Allen *et al.*, 1999), audition (Nishi *et al.*, 1997; Sulaiman *et al.*, 1999), feeding behaviour (Polidori & Massi, 2000), gastrointestinal motility (Krowicki *et al.*, 2001; Osinski & Brown, 2000) and cardiovascular and renal effects (Champion *et al.*, 1999; Kapusta *et al.*, 1999; Lai *et al.*, 2000; Larsson *et al.*, 2000; reviewed in Kapusta, 2000; Salis *et al.*, 2000).

6.1.6 Role in nociception — supraspinal

Although the ORL1 receptor seems to couple to similar effector mechanisms as the more extensively studied classical opioid receptors, functionally NC/OFQ has effects that differ from and even oppose those of opioids. In initial behavioural studies, the intracerebroventricular (i.c.v.) injection of NC/OFQ was found to induce hyperalgesia in mice using the hot-plate (Meunier *et al.* 1995) and tail-flick (Reinscheid *et al.* 1995) tests. This is where the name 'nociceptin' originated. On closer inspection, the i.c.v. injection of NC/OFQ was found to induce a biphasic response, comprising a brief period of hyperalgesia followed by a prolonged period of naloxone-sensitive analgesia (Rossi *et al.*, 1996). This has been attributed to the fact that the i.c.v. injection route of administration may be stressful enough

to induce an opioid-mediated stress-induced analgesia (SIA) which outlasts the transient reversal and hyperalgesia caused by NC/OFQ (King *et al.*, 1998; Mogil *et al.*, 1996a; Mogil *et al.*, 1996b). Another issue in interpreting results from i.c.v. administration of NC/OFQ in mice is the finding that the magnitude of SIA produced by i.c.v. injections, and the ability of NC/OFQ to antagonize this opioid-mediated SIA, are strain-dependent (Mogil *et al.*, 1999).

Functional anti-opioid effects of NC/OFQ may occur at the site of the periaqueductal grey (PAG), as terminals in the PAG have been shown to contain both the NC/OFQ peptide and ORL1 receptors. Furthermore, Morgan *et al.* (1997) have demonstrated an inhibition of PAG output neurones by NC/OFQ — a mechanism that could explain reversal of opioid-mediated stress-induced analgesia by NC/OFQ. Modulation of GABAergic control (Citterio *et al.*, 2000), and inhibition of opioid-induced histamine release in the hypothalamus (Eriksson *et al.*, 2000) have also been suggested. Thus, despite the hyperalgesia that is evident after i.c.v. administration of NC/OFQ, an inhibitory mechanism of action is preserved. In fact, (Chiou, 1999; Chiou, 2000) have demonstrated that the ORL1 mediates the activation of inwardly rectifying K⁺ channels in ventrolateral neurons of rat PAG. Furthermore, microinjection of NC/OFQ into the PAG has been demonstrated to selectively facilitate the noxious-evoked activity of wide dynamic range spinal cord neurones (Yang *et al.*, 2001). The opioid-modulating properties of NC/OFQ have been recently reviewed (Harrison & Grandy, 2000). Methodological inconsistencies, different experimental paradigms, strain differences in mice and species differences between rats and mice may explain some of the contradictions in the literature. A recent paper suggests a mechanism for the apparent bidirectional modulation of nociception by NC/OFQ, describing two populations of brainstem neurones, each being affected differently by NC/OFQ (Pan *et al.*, 2000).

Supraspinal NC/OFQ does not have anti-opioid effects in motivational paradigms. Although abundant in the locus coeruleus, NC/OFQ does not precipitate a withdrawal syndrome when injected into the lateral ventricle in morphine-dependent rats (Tian *et al.*, 1997). In fact, i.c.v. injection of NC/OFQ was found to be motivationally neutral in the rat place preference/aversion test (Devine *et al.*, 1996) indicating that the peptide lacks abuse liability. In fact, NC/OFQ has been shown to abolish the rewarding properties of ethanol and morphine in several paradigms, including stress-induced ethanol seeking behaviour. As such, manipulation of this system could have potential in the treatment of addiction (Ciccocioppo *et al.*, 2000; Fardon *et al.*, 2000; Martin-Fardon *et al.*, 2000; Pieretti, 2000). In contrast, ORL1 receptor knock-out mice exhibit a reduced liability to develop physical dependence to morphine and tolerance to morphine analgesia, a phenotype that can be reproduced by spinal or systemic administration of the antagonist J-113397 (Ueda *et al.*, 1997; Ueda *et al.*, 2000).

6.1.7 Role in nociception — spinal

At the level of the spinal cord, NC/OFQ is predominantly antinociceptive. Behavioural studies show that spinal NC/OFQ is antinociceptive in tests of acute nociception in rats (Hao *et al.*, 1998; Jhamandas *et al.*, 1998), whereas acute nociceptive tests in mice reveal antinociception (Kamei *et al.*, 1999), hyperalgesia (Inoue *et al.*, 1999; Minami *et al.*, 2000; Sakurada *et al.*, 1999) or no effect (Reinscheid *et al.*, 1995). In models of more clinically relevant pain states, intrathecal administration of NC/OFQ can inhibit the first and second phase of the formalin test in rats (Wang *et al.*, 1999a) and mice (Kamei *et al.*, 1999; Nakano *et al.*, 2000), inhibit formalin-induced expression of Fos-LI in the superficial dorsal horn (Yamamoto *et al.*, 2000) and is antihyperalgesic in models of inflammatory and neuropathic pain (Hao *et al.*, 1998; Yamamoto *et al.*, 1997; for review see Xu *et al.*, 2000). In almost all cases, a biphasic effect was seen. At low (picomolar) doses, hyperalgesia was observed and at higher (micromolar) doses, pronounced antinociceptive and antihyperalgesic effects were revealed. A situation akin to that for the opioids may again exist, where low dose of an inhibitory agent predominantly reduces inhibitory controls, leading to disinhibition and apparent hyperalgesia.

The mechanisms by which NC/OFQ has its spinal inhibitory effects are now fairly well established. Electrophysiological studies from our group in the intact anaesthetised rat have shown that spinal administration of NC/OFQ inhibits the C-fibre- dependent wind-up and post discharge of dorsal horn neurones, while sparing the A β -fibre-evoked responses (Stanfa *et al.*, 1996). This is supported by studies in the rat neonatal hemisectioned spinal cord, where C-fibre-mediated responses are inhibited in preference to A-fibre-evoked responses (Faber *et al.* 1996), demonstrating a tendency towards selective effects on noxious evoked activity and hyperalgesia that is consistent with the behavioural reports described above. Examining the spinal mechanism of action of NC/OFQ further, NC/OFQ can be shown to inhibit transmitter release. Recording from superficial dorsal horn neurones in spinal cord slices, reductions in magnitude of electrically stimulated EPSCs and EPSPs (excitatory post-synaptic currents/potentials) and frequency of resting mini-EPSCs (mEPSCs) are reduced. In contrast, glutamate-evoked EPSCs are not affected (Lai *et al.*, 1997; Liebel *et al.*, 1997). This is in accordance with reports that NC/OFQ attenuates transmitter release throughout the CNS (for example Nelson *et al.*, 2000 and see Schlicker & Morari, 2000 for review) and from peripheral nerve terminals in the airways (for example Helyes *et al.*, 1997; and see Giuliani *et al.*, 2000 for review)

The situation is of course not that simple. Although most reports do point to a predominantly pre-synaptic site of action in the superficial spinal cord, there is also evidence for a post-synaptic component. For example, the work by Stanfa *et al.*, (1996) revealed a difference in the profile of the inhibitory effects of NC/OFQ with those of the classical opioids. The ability of NC/OFQ to inhibit events that are known to be dependent on postsynaptic mechanisms (wind-up, and the associated post discharge) is a functional

indication of postsynaptic effects. *In vitro* work also supports a post-synaptic component (Jennings, 2001).

When all these findings are combined with the localisation studies for both peptide and receptor, it is apparent that the receptor may be present at sites postsynaptic to primary afferent fibre inputs into the spinal cord as well as at presynaptic locations. Thus the ability of NC/OFQ to modulate both transmitter release and neuronal activity is consistent with the location of the receptor.

6.1.8 Use of relevant models

The role of the NC/OFQ system in nociception has not so far been aided by the generation of mice lacking the ORL1 receptor and/or NC/OFQ peptide. The increased levels of anxiety seen in peptide and receptor knockout mice may complicate tests of acute nociception. Additionally, most investigation of nociceptive transmission so far has been based on cursory tests of acute nociception (tail-flick, hot plate etc.). Acute pain rarely presents as a clinical problem. In order to approach clinically relevant studies, it is necessary to employ models of more persistent inflammatory-mediated pain states as well as those with a neuropathic component. It is now fairly well accepted that there is plasticity within the NC/OFQ system following peripheral inflammation and nerve injury. Both increased levels of the NC/OFQ gene transcript in the DRG (Andoh *et al.* 1997) and increased NC/OFQ binding in the superficial laminae of the spinal cord (Jia *et al.*, 1998; Rosen *et al.*, 2000) have been demonstrated. Accordingly, alterations in the potency of NC/OFQ administered after development of inflammation or neuropathy have recently been reported (Hao *et al.*, 1998 and reviewed in Xu *et al.*, 2000).

I studied the effects of spinal application of NC/OFQ on neuronal responses after induction of carrageenan inflammation. These were compared with the effects of NC/OFQ in normal animals using the same experimental set-up, performed in this laboratory by Stanfa *et al.* 1996). This should reveal any plasticity in the NC/OFQ system in a model of clinically relevant pain.

6.1.9 Role in nociception — peripheral

The ORL1 receptor is detected in the DRG (Wick *et al.*, 1994) so is thought to be synthesised in the cell body of primary afferent fibres. Receptors can be transported from here not only to central terminals in the spinal cord, but also to the peripheral terminals. There is mounting evidence that NC/OFQ modulates transmitter release from peripheral terminals of sensory nerves (Giuliani *et al.*, 2000 for review). A role in peripheral inflammation via a non-neuronal site has been suggested, as intradermal NC/OFQ increases vascular permeability, an effect that may involve histamine release from mast cells (Kimura *et al.*, 2000). To determine whether NC/OFQ receptors are present and functional on peripheral terminals of primary afferent nerve fibres, NC/OFQ was

administered either alone or with the putative antagonist J-113397, into the peripheral receptive field of dorsal horn neurones in both normal and in carrageenan-injected animals.

6.1.10 Putative NC/OFQ antagonists

To elucidate the role of endogenous NC/OFQ, a selective ORL1 antagonist is vital. Three compounds in particular have been synthesised in recent years and proposed as selective antagonists at the ORL1 receptor. I investigated the potential of these compounds to antagonise NC/OFQ applied spinal and peripherally. These and other ligands are reviewed fully in Calo *et al.* (2000a).

6.1.10.1 [$\text{Phe}^1\psi(\text{CH}_2\text{-NH})\text{Gly}^2$]nociceptin-(1-13)- NH_2

This pseudopeptide analogue of NC/OFQ (hereafter referred to as Phe ψ) was first characterised as a competitive antagonist of NC/OFQ in guinea-pig ileum and mouse vas deferens (Guerrini *et al.*, 1998). More recently, several other studies have revealed species-dependent and receptor-density-dependent differences in the agonist, partial agonist or antagonist profiles that have been reported for this pseudopeptide (Burnside *et al.*, 2000). Phe ψ antagonises the effects of NC/OFQ in rat hypothalamic (Amano *et al.*, 2000) and vestibular neurones (Sulaiman *et al.*, 1999), in the airways (Peiser *et al.*, 2000) and NO/OFQ-stimulated GTP γ S binding in mouse spinal cord neurones (Narita *et al.*, 1999). In contrast, Phe ψ is a full agonist *in vitro* in Chinese hamster ovary (CHO) cells expressing the human ORL1 receptor, (Butour *et al.*, 1998) and in an ORL1 reporter-gene assay (Wnendt *et al.*, 1999). Phe ψ can partly mimic the effects of NC/OFQ but still antagonises (i.e. it is a partial agonist) *in vitro* in rabbit ileum (Pheng *et al.*, 2000), in rat suprachiasmatic (Allen *et al.*, 1999) and PAG ventrolateral (Chiou, 1999; Chiou, 2000) neurones and in rat cerebral cortex membranes (Berger *et al.*, 2000) and hippocampal slices (Madamba *et al.*, 1999). *In vivo* most reports agree on an agonist profile for Phe ψ as it mimics the hyperalgesic and antinociceptive effects of NC/OFQ after i.c.v. and i.t. administration respectively in the rat, as reported in this thesis (and see Candeletti *et al.*, 2000; Carpenter & Dickenson, 1998; Wang *et al.*, 1999b; Xu *et al.*, 1998), and in feeding behaviour (Olszewski *et al.*, 2000), gastric motor activity (Krowicki *et al.*, 2000) and cardiovascular and renal effects (Kapusta *et al.*, 1999).

To explain these inconsistent reports, it has been suggested that Phe ψ is a partial agonist and so, depending on the receptor density of the system in which it was tested, appears to function as a full agonist, low potency agonist or an antagonist. This idea is challenged by the work of Burnside *et al.* (2000). Here, the authors examine the effect of Phe ψ on GTP γ S binding in CHO cells expressing low or high levels of human or mouse ORL1. There appears to be a prominent species difference. Maximal effect of Phe ψ ranged from 76% to 90% of the NC/OFQ maximal effect at the human receptor, whereas maximal effect at the mouse receptor was around 38% of the NC/OFQ maximal effect. These effects

were similar in low expressing and in high expressing clones, suggesting receptor number is not a factor in the profile of Phe ψ . A problem with expression systems is the availability of the effector machinery for a transiently expressed receptor. Poor linkage of the ORL1 receptor in transfected cell lines to adenylyl cyclase may also explain some of the inconsistencies.

It is tempting to postulate on the existence of receptor subtypes or splice variants in different tissues to explain the diverse effects reported. Indeed splice variants have been identified and heterogeneity (curvi-linear Schatchard plots and a shallow Hill slope) has been reported for ORL1 receptors in mouse brain (Mathis *et al.*, 1997).

6.1.10.2 [N-Phe¹]nociceptin(1-13)NH₂

NPhe is a derivative of NC/OFQ which behaves as a competitive antagonist at the recombinant human receptor (Calo *et al.*, 2000b; Hashimoto *et al.*, 2000), blocks the effects of NC/OFQ on ischaemia-stimulated glutamate release from rat cortical slices (Nelson *et al.*, 2000), K⁺ currents in hippocampus (Amano *et al.*, 2000 but see below), and NC/OFQ effects in rabbit ileum (Pheng *et al.*, 2000). NPhe also enhances morphine analgesia when both are given i.c.v. (Rizzi *et al.*, 2000). Although the hyperalgesic effects of i.c.v. NC/OFQ are fairly well established, there are marked differences between mouse strains and experimental technique and as Rizzi *et al.*, (2000) did not repeat i.c.v. NC/OFQ administration, this cannot be taken as conclusive proof of antagonism of NC/OFQ. In contrast, NC/OFQ-like (agonist or partial agonist) effects of NPhe have been demonstrated *in vivo* after i.c.v. and i.t. administration, (brief reduction and increase respectively in tail flick latency) (Candeletti *et al.*, 2000), and *in vitro* on K⁺ currents in hippocampus (Madamba *et al.*, 1999).

As the antagonist effects of NPhe have been reported in many of the same assays that originally showed Phe ψ to be antagonist (e.g. Hashimoto *et al.*, 2000) results from other systems are needed before we can confidently describe NPhe as a useful ORL1 receptor antagonist.

6.1.10.3 J-113397

In vitro J-113397 shows high affinity for both the cloned human ORL1 receptor and mouse brain ORL1 receptor, inhibition of NC/OFQ stimulation of GTP γ S binding, and *in vivo*, hyperalgesia evoked by i.c.v. injection of NC/OFQ is attenuated (Ozaki *et al.*, 2000).

We obtained a sample of J-113397 (kindly provided by Dr Satoshi Ozaki, Banyou Pharmaceuticals, Japan). As a non-peptide putative antagonist, this seemed a potential best candidate for determining *in vivo* the involvement of the NC/OFQ system in nociceptive transmission. As such, this compound was also co-administered with NC/OFQ peripherally to determine whether the peripheral effects of NC/OFQ were mediated by the ORL1 receptor.

6.2 Methods

6.2.1 Spinal administration of NC/OFQ

Electrophysiology was carried out on a groups of rats that had been subjected to carrageenan inflammation of the hindpaw. The effects of spinal NC/OFQ were then compared with the effects in normal animals previously determined by Stanfa *et al.* (1996) under identical conditions in this laboratory.

Animals were anaesthetised and underwent surgery to expose the spinal cord, as previously described, and neuronal recordings began. As spinal NC/OFQ had already been studied in normal animals, only animals in which peripheral inflammation had been induced were used. Peripheral inflammation was generated by injection of carrageenan into the hindpaw. 100µl carrageenan (2% solution in saline) was injected into the plantar skin of the hindpaw. In a few cases, the carrageenan was administered once the animal was secured with the vertebral clamps, but in most cases, the carrageenan was injected only once a neurone had been isolated and several controls taken. Electrically evoked neuronal responses were followed while the inflammation developed.

Three hours after carrageenan injection, once results from subsequent electrical tests were constant, control values were re-established and drug application begun. NC/OFQ was applied directly to the spinal cord as cumulative doses of 5, 50, 125 and 225µg in 50µl saline. Evoked neuronal responses were followed for 40–60 min per dose. After the top dose had been applied, the opioid receptor antagonist naloxone (10 and/or 50µg in 50µl saline) was applied spinally in an attempt to reverse the effect of NC/OFQ. All neuronal responses were expressed as % of pre-drug control responses, and statistical analyses performed using the Kruskal-Wallis (unpaired non-parametric ANOVA) test. Effects of individual doses were determined using paired, 2-tailed Student's *t*-tests with the Bonferroni correction. The effects of NC/OFQ on neuronal responses after induction of carrageenan inflammation were compared with the effects of NC/OFQ in normal animals, using unpaired, 2-tailed Student's *t*-tests (or the alternate Welch test, when the populations had unequal standard deviations) to compare the % control for each dose in each experimental group.

6.2.2 Peripheral administration of NC/OFQ

Animals were prepared for electrophysiology and neurones isolated as previously described. Convergent dorsal horn neurones were isolated and initially characterised on the basis of their electrical-evoked activity. Neurones with large C-fibre-evoked responses (>200 action potentials) and post discharge (>100 action potentials) were selected. Stimulating electrodes were removed from the hindpaw, and the system left to rest for 10 min. A 29G insulin needle containing 40µl of 0.9% saline was then inserted intradermally into a toe in the

centre of the receptive field of the neurone, and left for 5 min for any activity evoked by the procedure to return to base-line levels. The plunger was then depressed, 20 μ l of saline was administered and the number of action potentials evoked in 60 s was recorded. Leaving the needle in place the injection was repeated after 5 min and the response counted and recorded. The needle was then removed, and after activity had returned to baseline levels, von Frey filaments (9g and 30g) were applied to bending force for 10 s and the static mechanically evoked responses counted over the 10 s period.

A new needle and syringe containing 40 μ l (two 20 μ l 'doses') of 0.01 μ g/20 μ l NC/OFQ solution was then inserted and, as before, at 5 min intervals two injections were made and responses recorded. Application von Frey filaments followed. The saline administrations and von Frey application were repeated before each successive dose of NC/OFQ (0.05, 0.1, 2.0 μ g per 20 μ l saline) and served as a control readings. The protocol is illustrated in Figure 6.1, which shows part of a trace recorded in one of the experiments.

The average of the two administrations of a dose of NC/OFQ or saline was taken, and this figure was averaged across all experiments to give the mean NC/OFQ-evoked responses (action potentials evoked in 60s) $\pm$ s.e.m. for each dose of NC/OFQ and the intervening saline controls. Paired, two-tailed Student's *t*-tests were used to test for difference between the saline and NC/OFQ-evoked responses. Unpaired Kruskal-Wallis tests were used to test for any overall effect of either saline or NC/OFQ. Values of $P \leq 0.05$ were taken as significant, and all data are expressed as mean $\pm$ s.e.m.

6.2.3 Co-administration of NC/OFQ and J-113397

The same protocol as described above was followed, but NC/OFQ was co-administered with the putative antagonist J-113397, still in a total volume of 20 μ l. Just prior to the first test injection, 20 μ l of NC/OFQ (0.01 μ g/10 μ l saline) was mixed with 20 μ l of J13397 (1 μ g/10 μ l saline). When mixed, these gave a final volume of 40 μ l allowing two injections containing final concentrations of 0.01 μ g NC/OFQ in 20 μ l and 1 μ g J-113397 in 20 μ l. Saline injections and von Frey filament application were repeated between subsequent 'test' injections containing 0.05 μ g NC/OFQ and 1 μ g J-113397, and 0.1 μ g NC/OFQ and 1 μ g J-113397. Analysis of the results previously obtained after administering of NC/OFQ alone showed that maximal responses were achieved with 0.1 μ g NC/OFQ; therefore, in order to conserve stocks of NC/OFQ and J-113397, the 2 μ g dose of NC/OFQ was not administered.

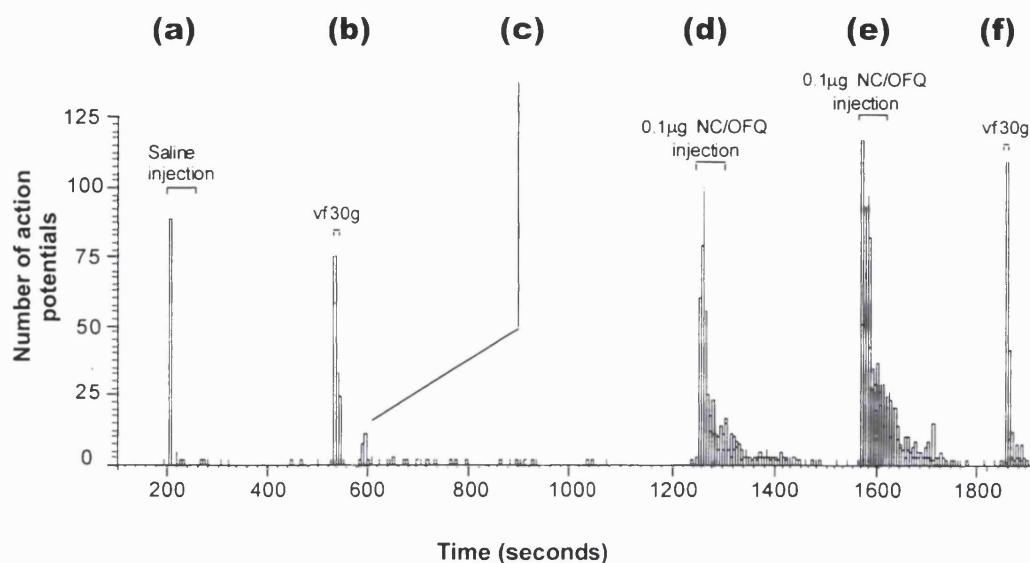


Figure 6.1 A trace of the neuronal activity evoked during a typical experiment, to illustrate the protocol adopted for administration of peripheral NC/OFQ and application of mechanical stimuli. The number of action potentials evoked per second is shown on the vertical axis, and the time in seconds is shown on the horizontal axis. This particular neurone did not respond to application of 9g von Frey filament.

The recording shows: **(a)** 20µl of saline was administered through a subdermal needle. After activity had subsided the needle was removed. **(b)** Five min after the injection, a 30g von Frey filament was applied to bending force for 10 s. **(c)** A second syringe needle was inserted and activity allowed to subside. **(d)** 20µl of solution containing 0.1µg of NC/OFQ was administered and **(e)** this was repeated 5 min later. Once activity after the second injection had subsided (and the 60 s recording period was over), the needle was removed. **(f)** Five min after the last injection, the von Frey filament (30g) was applied again.

This would have been preceded by administration of saline followed by 0.01µg NC/OFQ and saline followed by 0.05µg NC/OFQ; and followed by saline then the final dose of NC/OFQ (2.0µg). Von Frey filaments are applied after administration of each solution.

6.2.4 Putative NC/OFQ antagonists

6.2.4.1 [Phe¹ψ(CH₂-NH)Gly²]nociceptin-(1-13)-NH₂

Animals were prepared for electrophysiology and neurones isolated as previously described. Initially, Pheψ was administered alone, with the aim of determining any basal NC/OFQ activity in the spinal cord. Surprisingly, after these preliminary experiments, pronounced inhibitions were seen. It was decided, therefore, to adopt the same protocol for

administration of NC/OFQ. Thus, after control responses stabilised, cumulative doses of Phe ψ (1 μ g, 25 μ g, 75 μ g and 125 μ g) in 50 μ l saline, were applied to the spinal cord and neuronal responses were followed for 40 min. After maximum inhibition was reached, either 5 μ g (n = 3) or 10 μ g (n = 4) naloxone was applied intrathecally, followed by a high dose of naloxone (50 μ g). Again, each dose was followed for 40 min. Statistical analysis of drug effects used unpaired Kruskal-Wallis on the % control data to test for any overall effect of drug application. $P \leq 0.05$ was considered significant, and in this case paired Student's *t*-tests were performed (with the Bonferroni correction) to test for significance of individual dose effects compared with pre-drug control.

6.2.4.2 [N-Phe¹]nociceptin(1-13)NH₂

Animals were prepared for electrophysiology and neurones isolated as previously described. In a few preliminary experiments, NPhe was applied alone, to determine any basal NC/OFQ activity. High doses of NPhe (100 and 200 μ g) appeared to inhibit neuronal responses. A lower dose of NPhe was then chosen (50 μ g in 50 μ l saline) and two sets of experiments were performed. In one set of experiments, NPhe was applied to the spinal cord for 30 min. Following this, without removal of the drug, two doses of NC/OFQ were applied (125 and 225 μ g in 50 μ l saline) and electrical-evoked responses followed for 50 min for each dose. Naloxone (10 and 50 μ g in 50 μ l saline) was then applied and followed for 30 min each dose. The other set of experiments was carried out to determine whether NPhe alone had any significant effects on neuronal responses over the same timecourse as the first experiment. NPhe (50 μ g in 50 μ l) was applied spinally and electrically evoked responses were followed for 140 min, before morphine (1 and 5 μ g in 50 μ l saline) was applied.

The electrically evoked neuronal responses (C- A β - and A δ -fibre-evoked responses, post discharge, input and excess spikes) were recorded every 10 min, and the mean at each timepoint was taken across all the experiments to give a timecourse. The effect on dorsal horn responses of NC/OFQ alone, NC/OFQ in the presence of NPhe, and NPhe alone could then be compared. The effect of NC/OFQ in the presence of NPhe was determined using repeated measures ANOVA, followed by Dunnett's multiple comparison test to test for the significance of individual dose effects compared with control. The effects (% control) of 125 μ g and 225 μ g of NC/OFQ alone (i.e. no antagonist present) were compared with the effects (% control) of the same doses of NC/OFQ in the presence of 50 μ g NPhe using unpaired Student's *t*-tests. Control values for all neuronal measures were also compared.

6.2.4.3 J-113397

Animals were prepared for electrophysiology and neurones isolated as previously described. In a few preliminary experiments, J-113397 was applied alone to determine any basal NC/OFQ activity. High doses of J-113397 (100 μ g) appeared to have no effect on neuronal responses. Therefore, a different experimental design was used than for NPhe.

Cumulative doses of NC/OFQ were applied to the spinal cord (50, 125 and 225µg in 50µl saline) and electrically evoked neuronal responses followed for 40 min for each dose. 40 min after application of 225µg NC/OFQ (or after maximal inhibition with this dose had been observed) 100µg of J-113397 (in 50µl saline) was then applied, in an attempt to reverse the inhibition produced by NC/OFQ. The effect of J-113397 on the electrically evoked responses was followed for 60 min. The maximum effects of each drug administration were determined and averaged across all the experiments. For NC/OFQ, the maximum effect was taken as the largest deviation from control recorded during the timecourse for each dose; it was usually an inhibition. The mean maximum effect of 225µg NC/OFQ was compared with the pre-drug control to determine whether NC/OFQ caused an inhibition (Student's paired, 2-tailed *t*-tests).

The maximum effect for the antagonist was taken as the greatest deviation from the level of inhibition produced by NC/OFQ; this was always an increase and was, therefore, effectively the recording that was closest to, or greater than, 100% of control. The reversal produced by J-113397 was considered significant if mean maximum effect of J-113397 was significantly different to the maximum effect of 225µg NC/OFQ (Student's paired *t*-tests). The reversal was considered a full reversal when the effect of J-113397 was no different to pre-drug control values (Student's paired *t*-tests).

In some cases, after the drug applications were completed, morphine was applied spinally after J-113397. This was to gauge whether the antagonist interfered with the inhibitory effects of morphine on dorsal horn neuronal responses and therefore as a measure of any putative µ opioid receptor effects.

6.3 Results

6.3.1 Spinal NC/OFQ

The effect of spinally applied NC/OFQ (5, 50, 125 and 225 μ g) was studied on nine dorsal horn neurones 3 h after the induction of carrageenan inflammation in the ipsilateral hindpaw. No effect, or non-dose-dependent facilitations were observed in two neurones — these were excluded from subsequent analyses.

In the remaining seven neurones (mean depth = $746 \pm 73\mu\text{m}$, mean C-fibre threshold = 1.29 ± 0.24), the application of NC/OFQ caused a significant inhibition of the C-fibre-evoked responses, post discharge, input and excess spikes ($^{\dagger}P < 0.05$ in all cases, unpaired Kruskal-Wallis, Table 6.1, Figure 6.2, Figure 6.3A,B). Student's *t*-tests revealed significant inhibition of C-fibre-evoked responses, input and excess spikes with 125 μ g ($^{**}P \leq 0.01$, $^{*}P \leq 0.05$ and $^{*}P \leq 0.05$ respectively). The effect on C-fibre-evoked responses was also evident at the other doses (5 μ g $^{**}P < 0.01$; 50 μ g $^{*}P < 0.05$; 225 μ g $^{***}P < 0.001$). The application of 10 and 50 μ g naloxone tended to return all responses towards the pre-drug control values but never reached 100% (maximal reversals: C-fibre evoked responses to 63.5% control; post discharge to 50.0% control; input to 40% control and excess spikes to 62.0% control; $n = 2$ for top dose of naloxone in all cases, so s.e.m. cannot be calculated). Because of the small number of neurones where naloxone could be tested, the significance of this 'reversal' could not be gauged. Neither the A δ - or A β -fibre evoked responses were significantly affected by spinal application of NC/OFQ showing a selective action on noxious-evoked activity, agreeing with the effects of NC/OFQ previously seen in normal animals (Stanfa *et al.* 1996).

Comparing these effects of NC/OFQ in animals in which peripheral inflammation had been induced, with the effects of NC/OFQ in normal animals revealed several differences (Figure 6.2). The inhibitory effect of NC/OFQ on the C-fibre evoked response was greater in carrageenan-injected rats (closed boxes) than in normal animals (open boxes) and the dose-response curve for NC/OFQ in carrageenan-injected rats lies to the left of that obtained from normal animals (Figure 6.2A, approx. 4.5-fold shift). The difference between the effects of doses was significant at 5 μ g ($^{\dagger}P = 0.0317$ and $^{*}P = 0.0474$ respectively, unpaired, 2-tailed Welch test). Therefore, whereas 225 μ g NC/OFQ reduced C-fibre evoked responses to $62 \pm 15\%$ control ($n = 8$) in normal animals; in inflamed animals the C-fibre evoked responses were reduced to $39 \pm 15\%$ control ($n = 3$). The difference was even more marked for inhibition of input, a parameter that was not significantly affected by NC/OFQ in normal animals (Fig. 6.2, approx. 20-fold leftward shift). The difference between the dose-points was significant at 5 μ g and 50 μ g ($^{*}P = 0.0462$ and $^{**}P = 0.0028$ respectively, unpaired, 2-tailed Student's *t*-test). Whereas 225 μ g NC/OFQ

reduced the input to $58.25 \pm 13.3\%$ control in normal animals (open boxes), after inflammation the same dose reduced input to $18.2 \pm 14.6\%$ control (closed boxes).

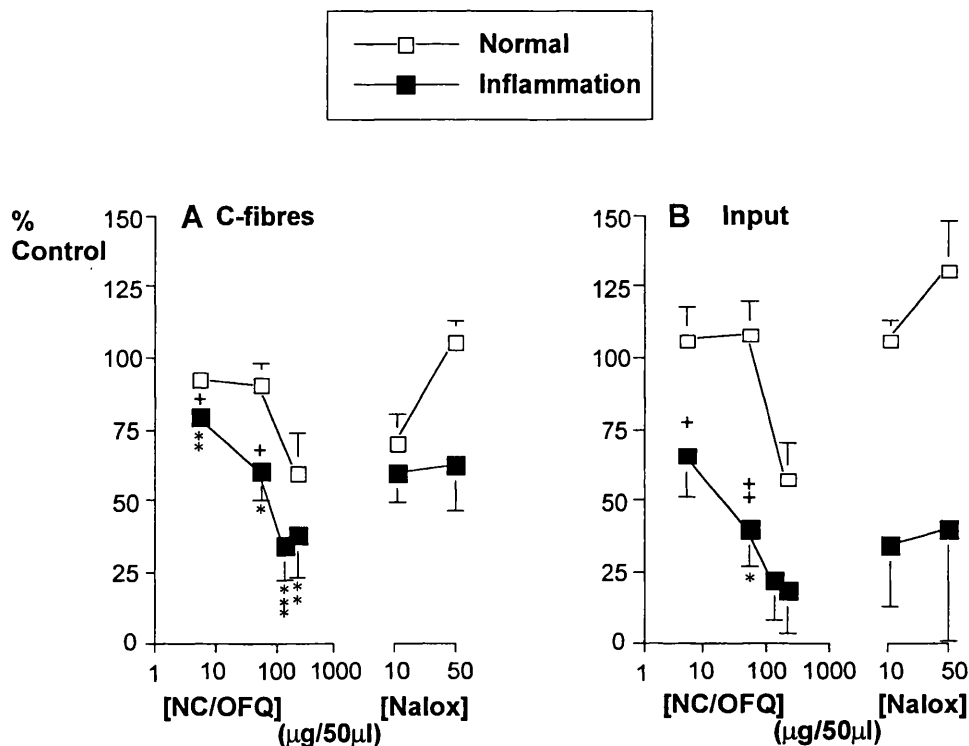


Figure 6.2 The effect of spinal application of NC/OFQ on electrically evoked neuronal responses in normal animals (open squares) and after carrageenan inflammation (filled squares). Data are shown as mean $\pm$ s.e.m. Significant effect of individual doses, as compared with pre-drug control (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$) and significant difference between the effect of individual doses in normal and inflamed animals (* $P \leq 0.05$, ** $P \leq 0.01$) are indicated. The ability of naloxone (10 μ g and 50 μ g) to reverse the effects is shown.

In contrast, the inhibitory effects of NC/OFQ on wind-up and post discharge were not different after inflammation. Figure 6.3 shows that the dose-response curves correspond almost exactly. Thus, although NC/OFQ still inhibited wind-up and post discharge, the effect was no greater in carrageenan-injected animals than in normal animals. There was some trend towards a greater inhibition of A δ -fibre-evoked responses after inflammation, but this was not significant. The lack of effect on A- β -fibre evoked responses was evident in both experimental groups (Figure 6.3).

Interestingly, the inhibitory effects of NC/OFQ on all parameters seem to be less sensitive to naloxone reversal after inflammation (Figures 6.2 and 6.3); however, only a limited series of neurones were tested with naloxone — both in normal animals and after

inflammation. The major aim was to study the putative ORL1 antagonists rather than naloxone.

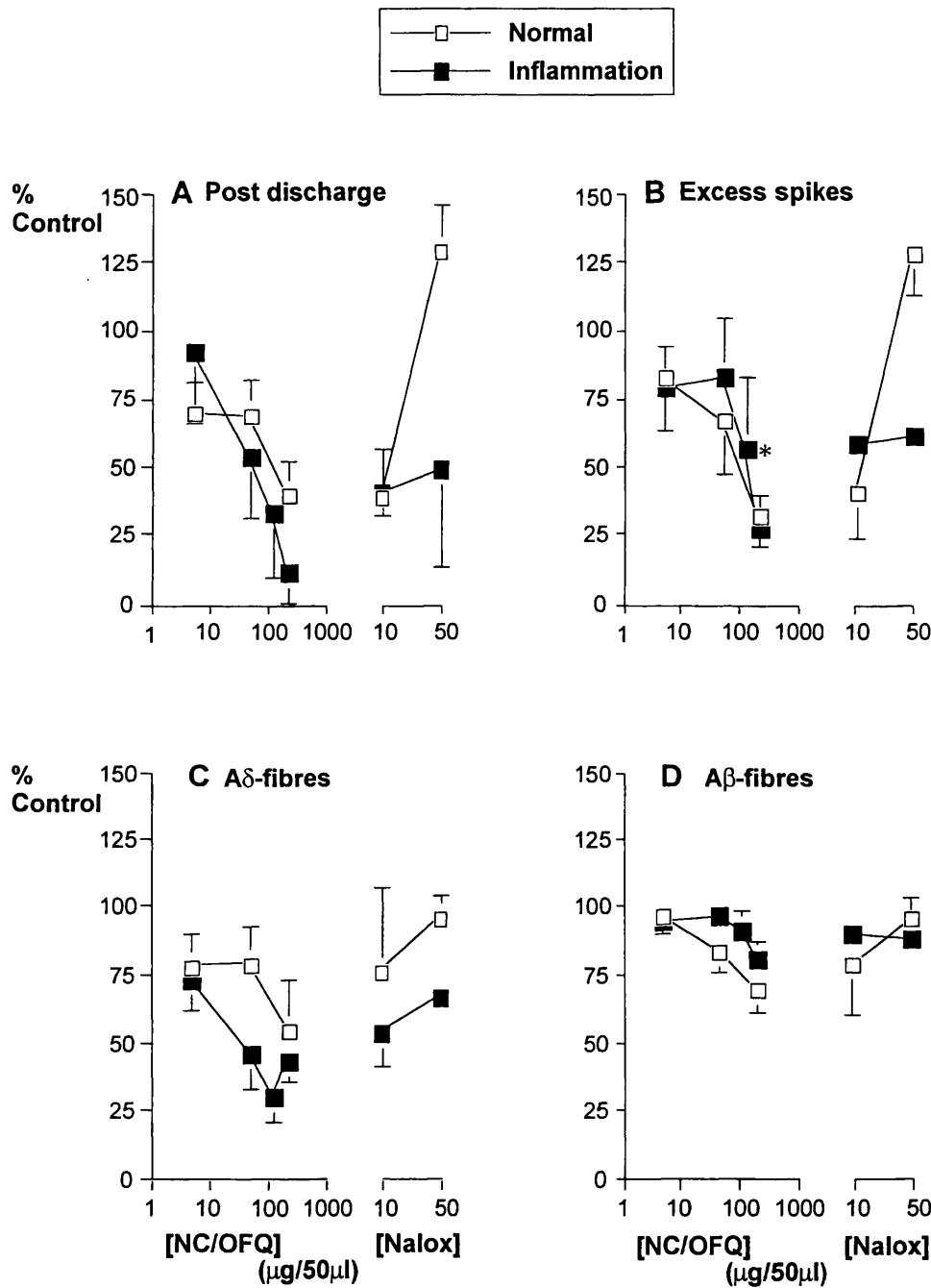


Figure 6.3 The effect of spinal application of NC/OFQ on electrically evoked neuronal responses in normal animals (open squares) and after carrageenan inflammation (filled squares). Data are shown as mean $\pm$ s.e.m. Significant effect of individual doses, as compared to pre-drug control ($*P \leq 0.05$) is indicated. The ability of naloxone (10µg and 50µg) to reverse the effects is shown.

Table 6.1 Effect of NC/OFQ on electrically-evoked neuronal responses after inflammation

	Control	<i>Drug effects (% control)</i>			
		5 μ g	50 μ g	125 μ g	225 μ g
C-fibres[†]	314 $\pm$ 43 (n=7)	80.3 $\pm$ 2.7% (n=6)**	61.5 $\pm$ 11% (n=6)*	35.4 $\pm$ 13% (n=6)**	39.0 $\pm$ 15% (n=3)***
Post discharge[†]	190 $\pm$ 60 (n=7)	92.4 $\pm$ 26% (n=6)	54.0 $\pm$ 22% (n=6)	33.6 $\pm$ 23% (n=6)	12.1 $\pm$ 11% (n=3)
Input[†]	14.9 $\pm$ 2.3 (n=7)	66.6 $\pm$ 14% (n=6)	40.2 $\pm$ 13% (n=6)	22.5 $\pm$ 14% (n=6)*	18.2 $\pm$ 15% (n=3)
XS-spikes[†]	298 $\pm$ 89 (n=7)	79.7 $\pm$ 15% (n=6)	83.2 $\pm$ 21% (n=6)	56.8 $\pm$ 27% (n=6)*	28.3 $\pm$ 12% (n=3)
Aδ-fibres	59.1 $\pm$ 14 (n=7)	73.9 $\pm$ 11% (n=6)	46.8 $\pm$ 13% (n=6)	30.5 $\pm$ 9.4% (n=6)	43.7 $\pm$ 7.0% (n=3)
Aβ-fibres	108 $\pm$ 9.8 (n=7)	93.0 $\pm$ 5.4% (n=6)	94.3 $\pm$ 4.4% (n=6)	89.3 $\pm$ 6.8% (n=6)	79.0 $\pm$ 6.1% (n=3)

[†]*P* < 0.05, unpaired Kruskal-Wallis test, significant effect with increasing dose; **P* < 0.05,

P* < 0.01, *P* < 0.01, Student's *t*-test (with Bonferroni correction) significant effect of individual dose compared to pre-drug control value.

6.3.2 Peripheral NC/OFQ

When injected into the receptive field of 18 dorsal horn neurones in normal animals, NC/OFQ evoked marked responses from 16 of these neurones. The two neurones that did not respond to NC/OFQ were discarded from all subsequent analysis. Saline (0.9%) injected in the same manner, also evoked a small amount of activity in most cases. For the remaining 16 neurones (mean depth 876 $\pm$ 45 μ m, mean C-fibre threshold = 1.07 $\pm$ 0.14mA) the number of action potentials evoked within 60s of injection was recorded, and the mean action potentials per 60 s for all cells used is shown in Table 6.2 and in Figure 6.4A.

In normal animals, the mean number of action potentials evoked over 60 s by 0.05, 0.1 and 2.0 μ g of NC/OFQ was significantly greater than the corresponding saline-evoked response (paired 2-tailed Student's *t*-test; see Table 6.2 and Figure 6.4A). As the experiment progressed, there was no increase in the magnitude of responses evoked by the saline injections (unpaired Kruskal-Wallis).

In carrageenan-injected animals, NC/OFQ evoked responses from all 10 neurones tested (mean depth 932 $\pm$ 48 μ m, mean C-fibre threshold = 1.56 $\pm$ 0.29mA). These responses were significantly greater than the preceding saline evoked responses for 0.01 and 0.05 μ g NC/OFQ. The smaller population size by the time of application of the higher doses led to a degree of variability in these later results and, although not significant, the

saline-evoked responses appear larger in these animals than in normal animals, with a tendency to increase in magnitude as the experiment progresses indicative of hyperalgesia induced by the inflammation. (Table 6.2, Figure 6.4B). There was no significant difference in the magnitude of the saline-evoked responses or the NC/OFQ-evoked responses between normal animals and carrageenan-injected animals at any stage.

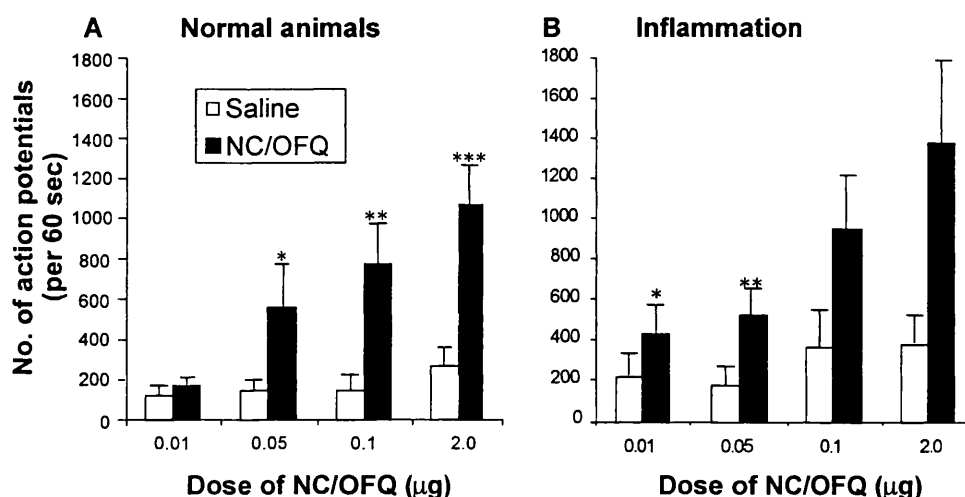


Figure 6.4 Responses evoked from dorsal horn neurones by injection of saline (white bars) and increasing doses of NC/OFQ (black bars) in normal animals, and after induction of carrageenan inflammation. Results are shown as mean + s.e.m. Significant difference between NC/OFQ-evoked response and the preceding saline-evoked response is indicated (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

Table 6.2 Action potentials evoked by peripheral injection of saline and NC/OFQ

	Normal		Inflammation	
	Control (counts/60s)	Test (counts/60s)	Control (counts/60s)	Test (counts/60s)
Saline	125.7 $\pm$ 36.7 (n=11)		225.4 $\pm$ 110 (n=8)	
0.01 μg NC/OFQ		175.0 $\pm$ 49.6 (n=11)		435.6 $\pm$ 149* (n=8)
Saline	145.2 $\pm$ 53.4 (n=11)		178.9 $\pm$ 84.8 (n=9)	
0.05 μg NC/OFQ		566.7 $\pm$ 213* (n=14)		524.9 $\pm$ 129** (n=9)
Saline	154.3 $\pm$ 65.0 (n=14)		358.9 $\pm$ 189 (n=7)	
0.1 μg NC/OFQ		781.7 $\pm$ 193** (n=13)		951.6 $\pm$ 270 (n=7)
Saline	267.6 $\pm$ 92.6 (n=13)		375.8 $\pm$ 138 (n=7)	
2.0 μg NC/OFQ		1068 $\pm$ 198*** (n=9)		1374 $\pm$ 416 (n=4)

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant effect of individual dose compared with corresponding (preceding) saline injection (Student's *t*-test).

In normal animals, responses evoked by the 9g von Frey filament after 0.01 μ g and 0.1 μ g NC/OFQ were significantly greater than the responses evoked after saline controls (Table 6.3, Figure 6.5A; $P = 0.0323$, $n = 6$; $P = 0.0457$, $n = 5$ respectively; paired 2-tailed Student's t -test). Although the mechanically evoked responses after saline do tend to increase as the experiment progressed, this effect was not significant (unpaired Kruskal-Wallis). After inflammation, the responses evoked by the 9g von Frey filament after NC/OFQ were not significantly different to responses evoked after the saline controls (Table 6.3 and Figure 6.5B). Comparing responses evoked by the 9g von Frey stimulus in normal with inflamed animals (Figure 6.5A compared with 6.5B) there was no significant difference in the magnitude of these responses — either after saline or after NC/OFQ. Thus, inflammation did not alter the neuronal responses to a low threshold mechanical stimulus in either the control situation or after NC/OFQ injection.

Table 6.3 Effect of saline and NC/OFQ on mechanically evoked responses of dorsal horn neurones — von Frey 9g

	<i>Normal</i>		<i>Inflammation</i>	
	Control (counts/10s)	Test (counts/10s)	Control (counts/10s)	Test (counts/10s)
Saline	20.5 $\pm$ 10.6 (n=6)		79.9 $\pm$ 45.9 (n=7)	
0.01 μ g NC/OFQ		41.7 $\pm$ 12.8* (n=6)		71.4 $\pm$ 36.8 (n=7)
Saline	20.7 $\pm$ 7.78 (n=7)		104.5 $\pm$ 50.1 (n=8)	
0.05 μ g NC/OFQ		66.2 $\pm$ 20.8 (n=6)		120.5 $\pm$ 42.5 (n=8)
Saline	39.8 $\pm$ 20 (n=5)		114.6 $\pm$ 54.1 (n=7)	
0.1 μ g NC/OFQ		99.6 $\pm$ 26.9* (n=5)		138.6 $\pm$ 55.5 (n=7)
Saline	83.8 $\pm$ 28.8 (n=5)		130.1 $\pm$ 65.4 (n=7)	
2.0 μ g NC/OFQ		106.6 $\pm$ 36.0 (n=5)		137.0 $\pm$ 62.9 (n=4)

* $P < 0.05$, ** $P < 0.01$, significant effect of individual dose compared with corresponding saline injection (Student's t -test).

In normal animals, the response evoked by von Frey 30g after 0.05 μ g NC/OFQ was significantly greater than the response evoked after saline injection (Table 6.4 and Figure 6.5C). No significant difference was observed after the remaining doses of NC/OFQ. After inflammation there was no difference between the magnitude of responses evoked after saline injection and after NC/OFQ injection (Figure 6.5D). The tendency of mechanically evoked responses, after either injection, to increase as the experiment progressed was not significant.

Comparing responses evoked by von Frey 30g in normal and inflamed animals (Figure 6.5C and 6.5D) there was no significant difference in the magnitude of these responses either after saline or after NC/OFQ — thus inflammation did not increase the neuronal responses to the stimulus in either the control situation or after NC/OFQ.

A general increase in both low intensity (9g) and high intensity (30g) mechanically evoked responses was evident as the experiment progressed in both groups, but did not reach significance in either case (unpaired Kruskal-Wallis). Thus, overall, NC/OFQ increased the responses of neurones to low intensity mechanical stimuli in normal animals. After inflammation, this effect may be masked by the increased baseline responses, as is evident from the augmented responses to applied stimulus after saline alone.

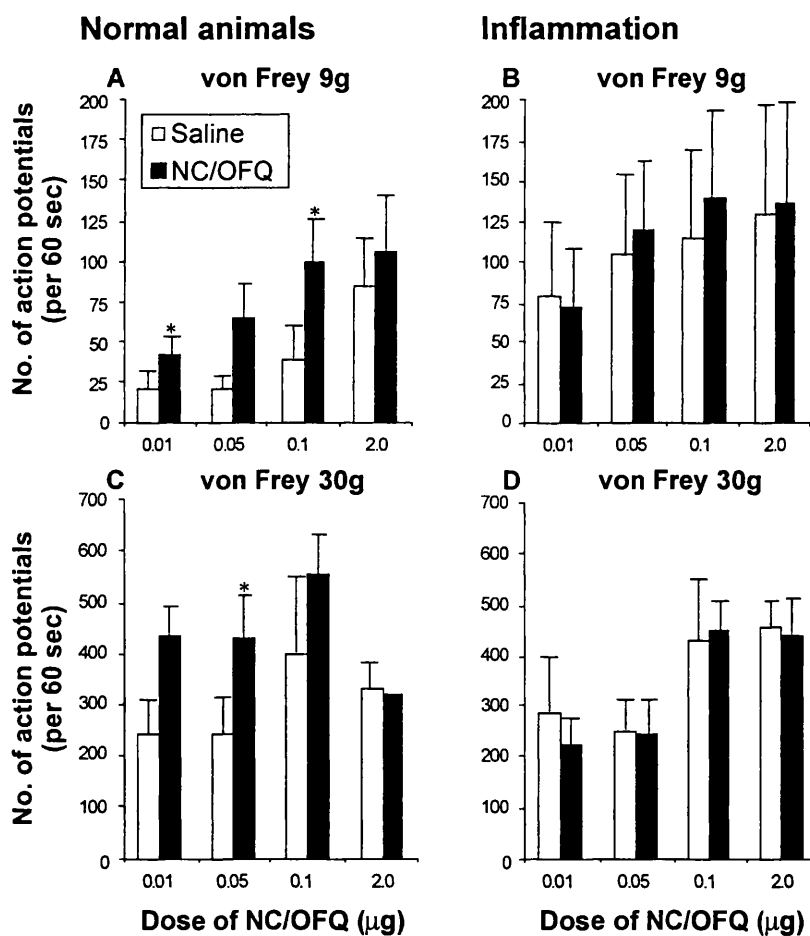


Figure 6.5 Responses evoked from dorsal horn neurones by application of innocuous (9g) and noxious (30g) von Frey filaments, after injection of saline (white bars) and after increasing doses of NC/OFQ (black bars). The figures on the left show results in normal animals, and on the right after induction of carrageenan inflammation. Results are shown as mean + s.e.m. Significant difference between mechanically evoked responses obtained after NC/OFQ and after the preceding saline injection is indicated (* $P \leq 0.05$).

Table 6.4 Effect of saline and NC/OFQ on mechanically evoked responses of dorsal horn neurones — von Frey 30g

	<i>Normal</i>		<i>Inflammation</i>	
	Control (counts/10s)	Test (counts/10s)	Control (counts/10s)	Test (counts/10s)
Saline	243 ± 78.7 (n=8)		290 ± 102 (n=8)	
0.01µg NC/OFQ		433 ± 107 (n=8)		221 ± 60.5 (n=8)
Saline	242 ± 52.1 (n=9)		253 ± 58.5 (n=9)	
0.05µg NC/OFQ		428 ± 82.8 (n=9)*		245 ± 69.5 (n=9)
Saline	400 ± 91.7 (n=9)		429 ± 114 (n=7)	
0.1µg NC/OFQ		554 ± 124 (n=9)		447 ± 62.9 (n=7)
Saline	331 ± 94.6 (n=8)		453 ± 54.4 (n=7)	
2.0µg NC/OFQ		321 ± 67.8 (n=7)		441 ± 76.2 (n=4)

**P* < 0.05, significant effect of individual dose compared with corresponding saline injection (Student's *t*-test).

6.3.3 Co-administration of NC/OFQ and J-113397

In a separate group of animals, 0.01µg, 0.05µg and 0.1µg NC/OFQ were administered in the presence of 1µg of the nonpeptide putative NC/OFQ antagonist J-113397. Five neurones were studied (mean depth = 702 ± 30.2µm, mean C-fibre threshold = 1.14 ± 0.24mA) and the responses evoked by saline alone and the NC/OFQ evoked responses (0.01, 0.05 and 0.1µg) in the presence of 1µg J-113397 (total volume of each injection was still 20µl) were recorded (counts/60s).

When this antagonist was included in the injection solution, the NC/OFQ + J-113397 injection did not evoke significantly larger responses than the corresponding saline injection (Table 6.5 and Figure 6.6a). The saline evoked responses were no different in this group of animals from the previous group. The NC/OFQ-evoked responses were much lower in the presence of J-113397, although this observed difference was not significant when compared with NC/OFQ alone. This was surprising, and may result from the smaller number of animals used in the co-administration study. The difference was considered pronounced enough not to justify the use of any more animals.

The responses evoked by low (9g) and high intensity (30g) mechanical stimuli were also determined (Table 6.6 and Figure 6.6B and C). When von Frey stimuli were applied after NC/OFQ in the presence of 1µg J-113397, the mechanically evoked responses were not enhanced compared with application after the saline controls; this is in contrast to administration of NC/OFQ alone, which sensitised neurones to the 9g von Frey stimulus (Figure 6.5A).

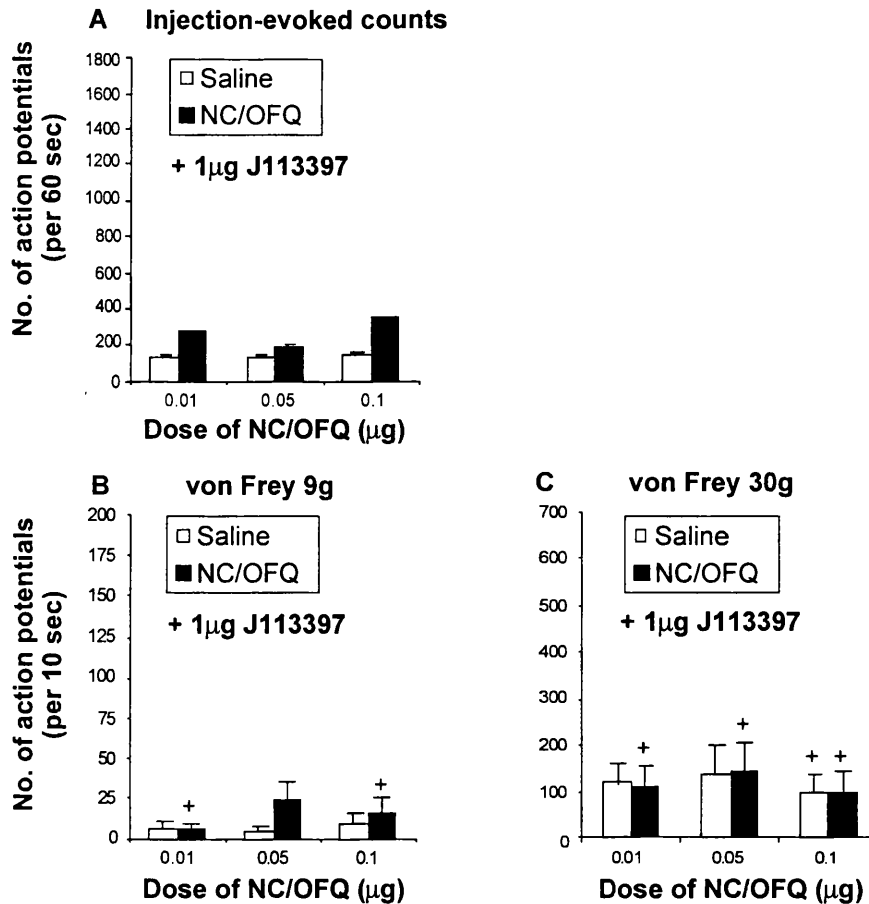


Figure 6.6 The top panel (A) shows responses evoked from dorsal horn neurones by injection of saline (white bars) and increasing doses of NC/OFQ in the presence of 1 µg J-113397 (black bars). The lower panel shows responses evoked by mechanical stimulation with (B) innocuous (9g) and (C) noxious (30g) von Frey filaments after injection of saline (white bars) or after increasing doses of NC/OFQ in the presence of J-113397 (black bars). Results are shown as mean $\pm$ s.e.m. Significant difference between magnitude of the response evoked here, and the corresponding response when NC/OFQ was administered alone (Figure 6.4) is indicated (* $P \leq 0.05$).

The mechanically evoked responses after administration of NC/OFQ in the presence of 1 µg J-113397 are significantly smaller than the mechanically evoked responses after administration of NC/OFQ alone for von Frey 9g (0.01 µg, * $P = 0.0374$; 0.1 µg, * $P = 0.0196$, unpaired Student's t -tests) and after von Frey 30g (unpaired Student's t -tests, 0.01 µg, * $P = 0.046$; 0.05 µg, * $P = 0.0391$; 0.1 µg, * $P = 0.022$). The noticeable (but non-significant) increase in the baseline von Frey evoked responses (applied after saline administration) through the course of the experiments with NC/OFQ alone was not apparent in this set of studies.

Table 6.5 Action potentials evoked by peripheral injection of saline, and by NC/OFQ in the presence or absence of 1µg J-113397

	NC/OFQ + J-113397		NC/OFQ alone	
	Control (counts/60s)	Test (counts/60s)	Control (counts/60s)	Test (counts/60s)
Saline	126.8 ± 20.6 (n=5)		125.7 ± 36.7 (n=11)	
0.01µg NC/OFQ		276.3 ± 97.0 (n=5)		175.0 ± 49.6 (n=11)
Saline	125.3 ± 40.5 (n=5)		145.2 ± 53.4 (n=11)	
0.05µg NC/OFQ		191.2 ± 56.1 (n=5)		566.7 ± 213* (n=14)
Saline	150.6 ± 48.6 (n=5)		154.3 ± 65.0 (n=14)	
0.1µg NC/OFQ		354.6 ± 134 (n=5)		781.7 ± 193** (n=13)

*The data for NC/OFQ alone are repeated from Table 6.2. *P < 0.05, **P < 0.01, significant effect of individual dose compared with corresponding saline injection (Student's t-test).*

Table 6.6 Effect of saline and NC/OFQ (in the presence of 1µg J-113397) on mechanically evoked responses of dorsal horn neurones

	von Frey 9g		von Frey 30g	
	Control (counts/10s)	Test (counts/10s)	Control (counts/10s)	Test (counts/10s)
Saline	5.6 ± 5.1 (n=5)		121 ± 47 (n=5)	
0.01µg NC/OFQ		5.8 ± 4.7 (n=5) ⁺		113 ± 47 (n=5) ⁺
Saline	4.4 ± 3.7 (n=5)		140 ± 66 (n=5)	
0.05µg NC/OFQ		24.0 ± 12 (n=5)		147 ± 62* (n=5) ⁺
Saline	9.0 ± 6.6 (n=5)		98.0 ± 43 (n=5) ⁺	
0.1µg NC/OFQ		16.0 ± 10 (n=5) ⁺		101 ± 43 (n=5) ⁺

⁺P < 0.05, significant difference from the equivalent measure in animals administered saline and NC/OFQ alone (unpaired Student's t-test).

Thus, in the experiments where NC/OFQ was administered alone, the mechanically evoked responses (9g and 30g) after saline increase slightly but nonsignificantly, the response evoked by von Frey 9g was elevated after NC/OFQ and that evoked by von Frey 30g was slightly but not significantly increased as compared with responses evoked after saline. None of these changes occurred when J-113397 was administered with the NC/OFQ, and the mechanically evoked responses after NC/OFQ in the presence of 1µg J-113397 are significantly smaller than the responses evoked after NC/OFQ alone.

6.3.4 Putative NC/OFQ antagonists

6.3.4.1 [Phe¹ψ(CH₂-NH)Gly²]nociceptin-(1-13)-NH₂

The effect of [Phe¹ψ(CH₂-NH)Gly²]nociceptin-(1-13)-NH₂ (hereafter referred to as Pheψ) was studied on 17 dorsal horn neurones. On three of these, the application of Pheψ had either no effect on the responses, or produced a dose-independent facilitation of the responses. These neurones were not included in subsequent analyses. The remaining 14 neurones had a mean depth from the cord surface of 669 ± 48.8µm from the cord surface and mean C-fibre threshold of 1.28 ± 0.14mA. One control value for post discharge was too low for drug effects to be reliably calculated;[†] this neurone was excluded from the post discharge calculations (therefore n = 13).

Table 6.7 Effect of Pheψ on electrically evoked neuronal responses

	Control	<i>Drug effects (% control)</i>			
		1µg	25µg	75µg	125µg
C-fibres[†]	302 ± 39.7 (n=14)	95.4 ± 8.4% (n=7)	73.7 ± 10% (n=16)	63.0 ± 6.9% (n=6)***	58.6 ± 5.9% (n=6)*
Post discharge[†]	155 ± 33.1 (n=13)	131 ± 24% (n=6)	69.0 ± 19% (n=6)	41.7 ± 19% (n=6)	46.4 ± 3.0% (n=6)
Input[†]	13.6 ± 2.16 (n=14)	115 ± 15% (n=7)	80.8 ± 19% (n=6)	50.5 ± 16% (n=6)	64.8 ± 20% (n=6)
XS-spikes	252 ± 55.6 (n=14)	108 ± 18% (n=7)	93.3 ± 25% (n=6)	65.3 ± 22% (n=6)	68.4 ± 21% (n=6)
Aδ-fibres[†]	45.9 ± 7.66 (n=14)	94.9 ± 13% (n=7)	94.2 ± 14% (n=6)	54.7 ± 16% (n=6)	44.3 ± 9.9% (n=6)*
Aβ-fibres	104 ± 8.35 (n=14)	99.6 ± 14% (n=7)	79.3 ± 3.7% (n=6)	94.7 ± 8.8% (n=6)	97.5 ± 13% (n=6)

[†]P < 0.05, unpaired Kruskal-Wallis, significant effect with increasing dose; *P < 0.05, **P < 0.01, ***P < 0.001, Student's t-test (with Bonferroni correction) significant effect of individual dose compared to pre-drug control value.

C- and Aδ-fibre evoked responses, input and post discharge were preferentially inhibited by Pheψ (*P ≤ 0.05 in all cases, unpaired Kruskal-Wallis) whereas the Aβ fibre evoked responses were not altered, indicating selective actions on noxious evoked activity (Table 6.7, Figure 6.7). C-fibre evoked responses were significantly inhibited by 75 and 125µg Pheψ, and Aδ-fibre evoked responses with 125µg Pheψ (***P = 0.0004, *P = 0.0116 and *P = 0.0268 respectively, Student's paired t-test with the Bonferroni correction).

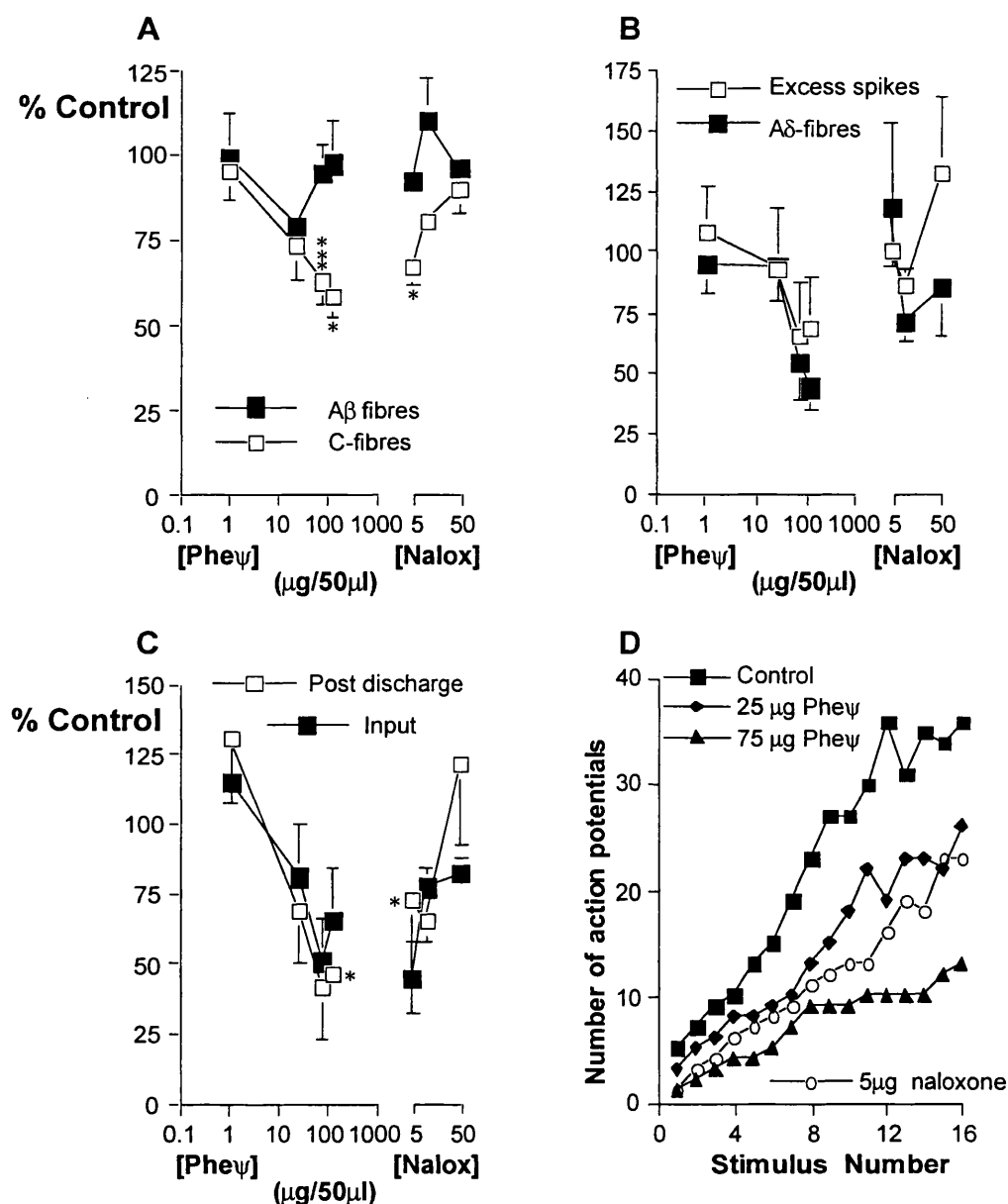


Figure 6.7 The effect of Pheψ on electrically evoked neuronal responses (**A–C**). Data are shown as mean ± s.e.m. Significant effects of individual doses as compared with pre-drug control and significant naloxone reversal are indicated (* $P \leq 0.05$, *** $P \leq 0.001$). **D** shows the effect of Pheψ and naloxone on the wind-up of an individual neurone.

The input and post discharge were significantly inhibited by Pheψ, but individual dose effects did not reach significance. Excess spikes were not significantly inhibited, although some tendency towards inhibition was apparent (Figure 6.7B). Figure 6.7D shows the effect of increasing doses of Pheψ on the wind-up of one individual neurone. The effects were not altogether typical of opioids; there was a marked inhibition of wind-up (i.e. a flattening of the curve) before a marked inhibition of the input (initial value) was apparent. This is consistent with the effects of NC/OFQ reported by Stanfa *et al.*, (1996).

The ability of naloxone to reverse the effects of Phe ψ is apparent in Figure 6.7. Significant naloxone reversal of C-fibre-evoked responses and post discharge was apparent with 5 μ g naloxone. Less neurones were tested with the higher doses, which meant that significance could not be determined, but a marked effect is evident (Figure 6.7). Maximum reversal of C-fibre evoked responses was to $90 \pm 7\%$; A δ -fibre-evoked responses to $118 \pm 24\%$; post discharge to $121 \pm 29\%$; input to $82.5 \pm 6\%$; and wind-up to $133 \pm 32\%$ of pre-drug control values.

These effects of Phe ψ parallel almost exactly those of NC/OFQ in normal animals. The examples shown in Figure 6.8 (input and C-fibre-evoked response) illustrate that the same neuronal measures are inhibited to the same extent.

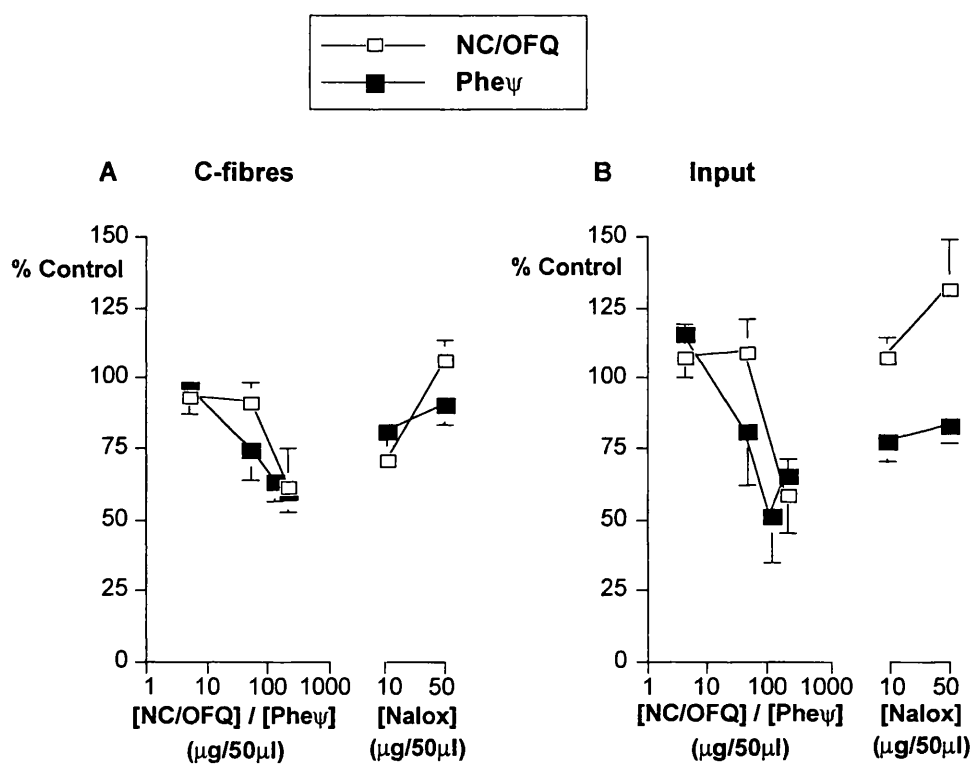


Figure 6.8 The effects NC/OFQ and Phe ψ on C-fibre-evoked response and the input are chosen to illustrate that the inhibitory effects of Phe ψ closely parallel the inhibitory effects of NC/OFQ. Results are shown as mean $\pm$ s.e.m.

6.3.4.2 [N-Phe¹]nociceptin(1-13)NH₂

The effect of NPhe on the responses of dorsal horn neurones, and its effect on the inhibitions produced by NC/OFQ was studied in six animals. In one animal, NPhe and NC/OFQ application had no effect on responses, and neither did subsequent application of morphine. This neurone was excluded from further analyses. The mean depth of the remaining neurones was $730 \pm 67\mu\text{m}$ and the mean C-fibre threshold was $1.32 \pm 0.24\text{mA}$.

Table 6.8 Effect of NPhe on electrically-evoked neuronal responses, and on subsequent application of NC/OFQ

	Control	<i>Drug effects (% control)</i>		
		50 μg NPhe	125 μg NC/OFQ	225 μg NC/OFQ
C-fibres^{††}	340 $\pm$ 58 (n=5)	93.10 $\pm$ 5.6% (n=5)	50.96 $\pm$ 11% (n=5)*	39.44 $\pm$ 14% (n=5)**
Post discharge	209 $\pm$ 94 (n=5)	93.97 $\pm$ 26% (n=5)	59.26 $\pm$ 27% (n=5)	42.52 $\pm$ 25% (n=5)
Input[†]	20.6 $\pm$ 2.0 (n=5)	124.7 $\pm$ 36% (n=5)	34.32 $\pm$ 9.6% (n=5)	20.42 $\pm$ 11% (n=5)
XS-spikes[†]	299 $\pm$ 137 (n=5)	89.26 $\pm$ 19% (n=5)	60.74 $\pm$ 22% (n=5)	25.88 $\pm$ 11% (n=5)
Aδ-fibres	80.6 $\pm$ 25 (n=5)	76.96 $\pm$ 12% (n=5)	68.64 $\pm$ 15% (n=5)	51.38 $\pm$ 3.0% (n=5)
Aβ-fibres	76.2 $\pm$ 17 (n=5)	97.36 $\pm$ 13% (n=5)	95.08 $\pm$ 14% (n=5)	77.42 $\pm$ 5.4% (n=5)

[†] $P < 0.05$, ^{††} $P < 0.01$, repeated measures ANOVA, significant overall effect with drug applications; * $P < 0.05$, ** $P < 0.01$, Dunnett's multiple comparisons test, significant effect of individual dose compared with pre-drug control value.

When applied spinally, 50 μg NPhe (in 50 μl saline) had no effect on electrically evoked neuronal responses over 30 min (Dunnett's multiple comparison test; see Table 6.8). Subsequent application of 125 μg and 225 μg NC/OFQ caused inhibitions of the C-fibre evoked responses, input and excess spikes ($P = 0.002$, $P = 0.0142$, and $P = 0.0231$, respectively, repeated measures ANOVA). Inhibition of C-fibre-evoked responses was significant with application of both 125 μg and 225 μg NC/OFQ (Dunnett multiple comparison test, * $P \leq 0.05$ and ** $P \leq 0.01$ respectively). This can be seen in Figures 6.9A and B and Figure 6.10B (open circles), where the effect of drug applications on neuronal responses (% pre-drug control values) is shown as a function of time. As I now go on to compare several experimental conditions, timecourse graphs such as these show the data more clearly.

The data on the effect of spinally applied NC/OFQ (50µg, 125µg and 225µg) on responses of dorsal horn neurones before application of J-113397 (as described in the following section, 6.3.4.3), was pooled with the data obtained and published by Stanfa *et al* (1996). This is shown in Figure 6.9 and 6.10 as the timecourse for effects of NC/OFQ alone (grey-filled circles). The inhibition produced by NC/OFQ alone was no more pronounced than when it was preceded by application of 50µg NPhe (open circles). This was confirmed by comparing the maximum effect data (% control) for 125µg and 225µg NC/OFQ alone, and after NPhe pretreatment. There was no difference between the two groups for any neuronal measure (Mann-Whitney unpaired, nonparametric *t*-test, for data with unequal standard deviations). Pre-drug controls were also not different.

The effect of application of 50µg NPhe alone was studied on six dorsal horn neurones (mean depth = $637 \pm 114\mu\text{m}$, mean C-fibre threshold = $1.36 \pm 0.14\text{mA}$). This was followed by the application of 1µg and 5µg morphine, to determine whether NPhe had any activity at classical opioid receptors. Figure 6.9 and 6.10 show that when compared with the effects of NPhe + NC/OFQ, or with NC/OFQ alone, it is evident that NPhe (50µg, black-filled circles) had no effect on the electrically evoked neuronal responses. Application of 1µg and 5µg morphine, 140 min after 50µg NPhe, still caused a pronounced and typical inhibition of neuronal responses.

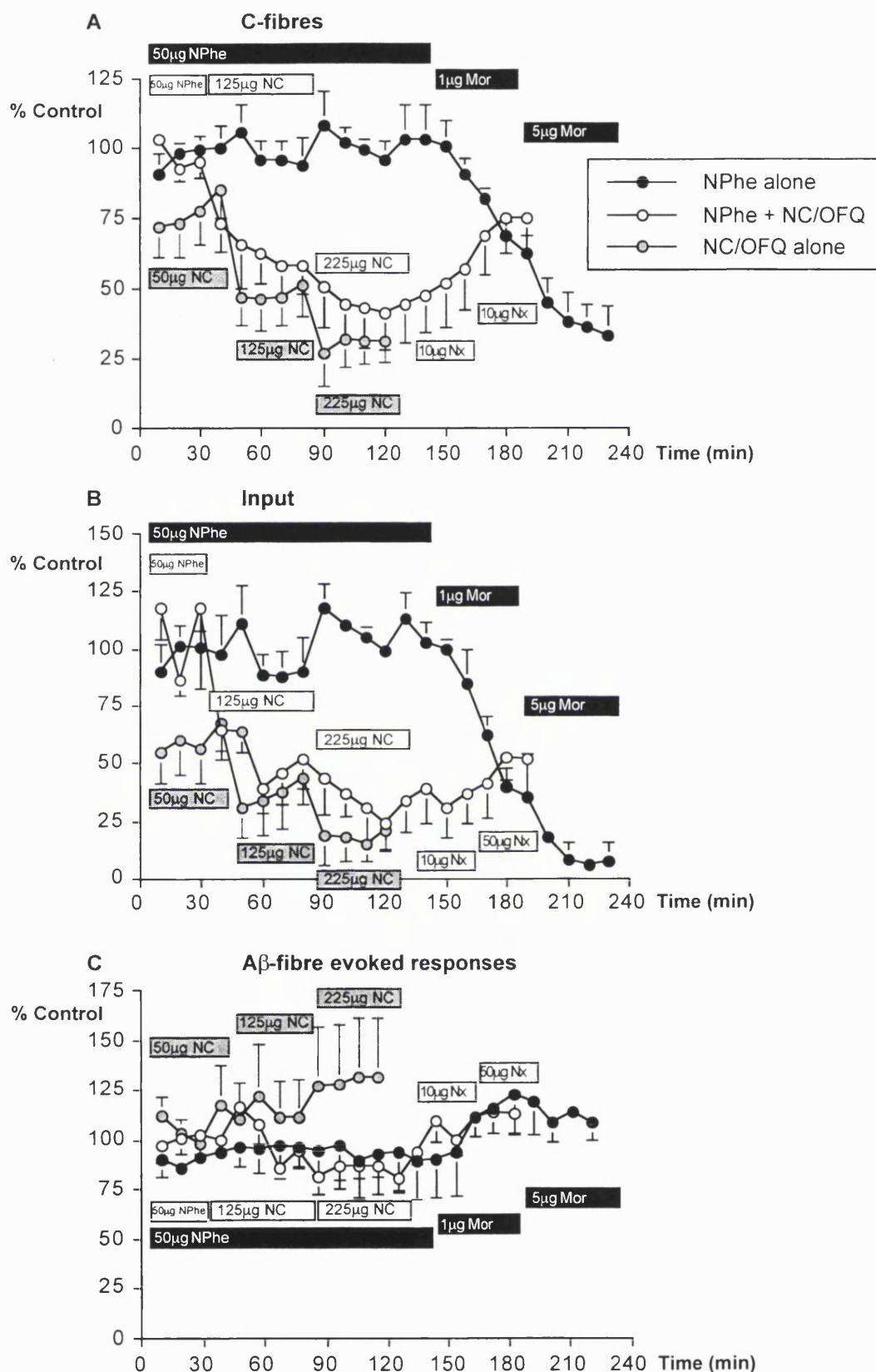


Figure 6.9 Timecourse of the effects of NPhe alone (black-filled circles), NC/OFQ alone (grey-filled circles) or NC/OFQ after NPhe pretreatment (open circles) on electrically evoked neuronal responses. Data are shown as mean $\pm$ s.e.m. for each time point.

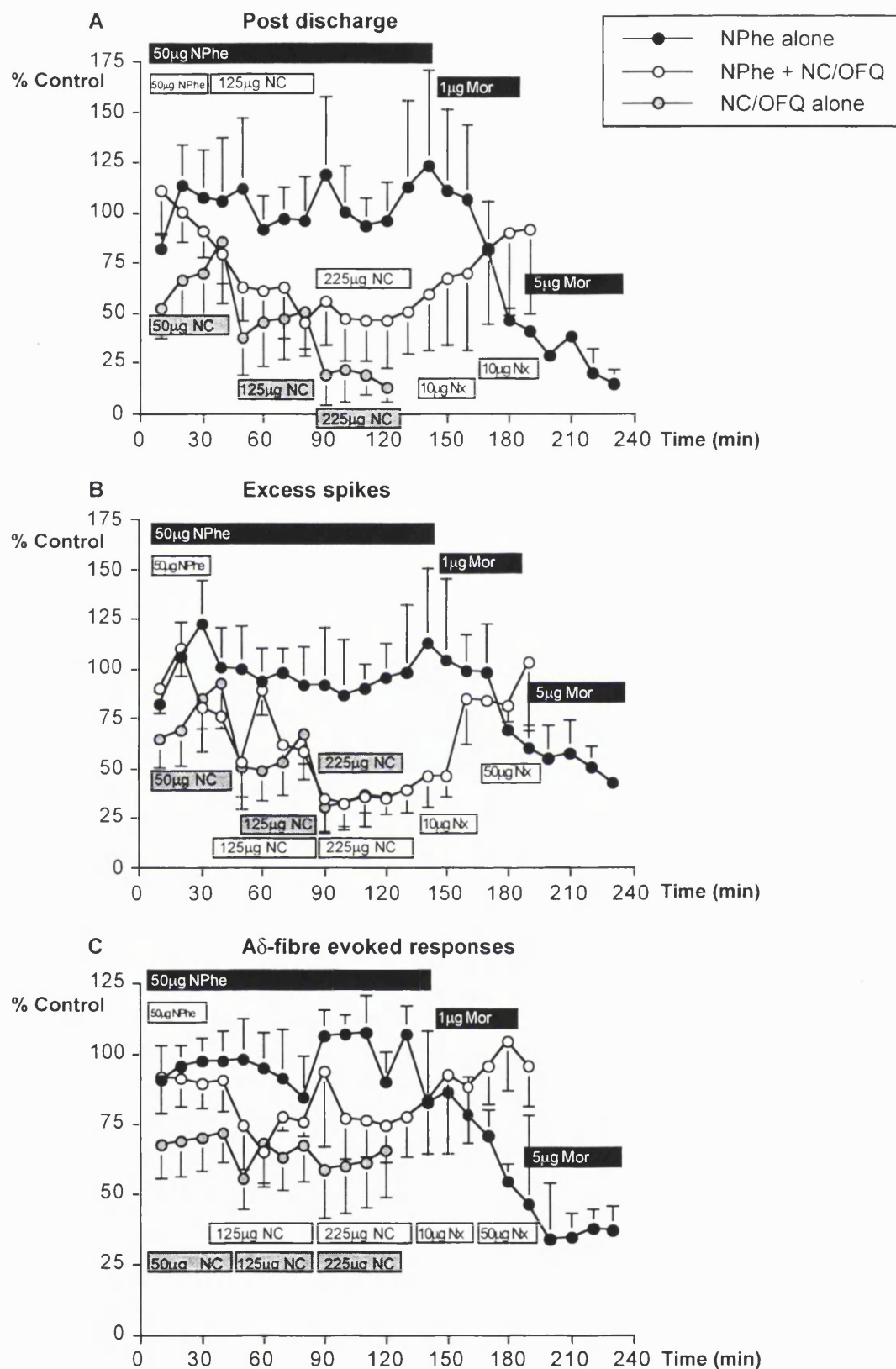


Figure 6.10 Timecourse of the effects of NPhe alone (black-filled circles), NC/OFQ alone (grey-filled circles) or NC/OFQ after NPhe pretreatment (open circles) on electrically evoked neuronal responses. Data are shown as mean $\pm$ s.e.m. for each time point.

6.3.4.3 J-113397

The effect of J-113397 on NC/OFQ-evoked inhibition of neuronal responses was studied in nine animals. In three cases, neither NC/OFQ nor morphine had any effect on the electrically evoked neuronal responses. J-113397 was, therefore, not applied to these neurones, and the data are not included here. The reasons for the lack of effect of NC/OFQ and morphine in these instances could be problems with drug access (e.g. the well was leaking) or, more likely, that these neurones did not express or receive input from pathways that expressed opioid or NC/OFQ receptors. The effects of J-113397 were therefore studied on six neurones, mean depth = $735 \pm 36\mu\text{m}$, mean C-fibre threshold = $1.63 \pm 0.23\text{mA}$.

As previously reported, spinal application of NC/OFQ caused inhibition of neuronal responses. In contrast with the study by Stanfa *et al.*, (1996), a dose of $125\mu\text{g}$ NC/OFQ was included here meaning that the final cumulative dose was higher than in the earlier study and consequently the extent of the inhibition produced by the top dose of $225\mu\text{g}$ was slightly greater. Significant inhibition of the C-fibre evoked responses, post discharge, input, excess spikes and A δ -fibre evoked responses was observed (Table 6.9).

Table 6.9 Effect of NC/OFQ on electrically-evoked neuronal responses, and reversal by J-113397

	Control	<i>Drug effects (% control)</i>			
		50 μg	125 μg	225 μg	100 μg J-11
C-fibres^{†††}	331.3 $\pm$ 44 (n=6)	68.9 $\pm$ 15% (n=6)	37.8 $\pm$ 11% (n=6)	27.6 $\pm$ 7.4% (n=6)**	80.2 $\pm$ 6.9% (n=6) ^{††}
Post discharge^{††}	160.9 $\pm$ 26 (n=6)	61.6 $\pm$ 28% (n=6)	30.3 $\pm$ 22% (n=6)	10.8 $\pm$ 8% (n=6)**	89.7 $\pm$ 16% (n=6) ^{††}
Input^{††}	14.23 $\pm$ 2.1 (n=6)	55.7 $\pm$ 21% (n=6)	30.3 $\pm$ 22% (n=6)	10.8 $\pm$ 8.8% (n=6)**	72.8 $\pm$ 12% (n=6) ^{††}
XS-spikes^{††}	285.2 $\pm$ 56 (n=6)	80.0 $\pm$ 25% (n=6)	56.0 $\pm$ 26% (n=6)	25.9 $\pm$ 7.8% (n=6)**	107 $\pm$ 19% (n=6) ^{††}
Aδ-fibres[†]	59.17 $\pm$ 17 (n=6)	66.5 $\pm$ 16% (n=6)	70.9 $\pm$ 20% (n=6)	62.9 $\pm$ 18% (n=6)	71.5 $\pm$ 10% (n=6)
Aβ-fibres	84.30 $\pm$ 15 (n=6)	118 $\pm$ 25% (n=6)	124 $\pm$ 31% (n=6)	126 $\pm$ 30% (n=6)	132 $\pm$ 31% (n=6)

[†] $P < 0.05$, ^{††} $P < 0.01$, repeated measures ANOVA, significant effect with increasing dose;

** $P < 0.01$, Dunnett multiple comparisons test, significant effect of individual dose compared to pre-drug control value. ^{††} $P < 0.01$, Student's *t*-test, significant reversal by J-113397.

For simplicity, just the effect of the top dose of NC/OFQ (the product of cumulative doses of 50µg, 125µg and 225µg) will be discussed. This dose produced a significant inhibition of all of the neuronal measures, except for the Aβ- and the Aδ-fibre evoked responses. In all these cases, the inhibition produced by 225µg NC/OFQ was significantly reversed by the application of J-113397 (Student's paired *t*-tests: C-fibres $^{**}P = 0.0055$; post discharge $^{**}P = 0.0064$; input $^{**}P = 0.012$; excess spikes $^{**}P = 0.0035$) and is illustrated clearly in Figure 6.11. The reversal produced was full in all cases (i.e. not significantly different from pre-drug control) except for the input (Figure 6.11B), which was returned to $72.8 \pm 12\%$ control.

In four experiments, J-113397 reversal was followed by application of morphine (1µg in 50µl saline). This low dose of morphine produced pronounced inhibition of the C-fibre evoked responses, post discharge, input and excess spikes ($P = 0.0288$, $P = 0.0058$, $P = 0.0188$ and $P = 0.0365$ respectively, Student's *t*-test, comparison with pre-drug control) without affecting the Aβ- or Aδ-fibre evoked responses. J-113397 clearly had selective effects on NC/OFQ mediated events and not on μ opioid receptor mediated inhibition.

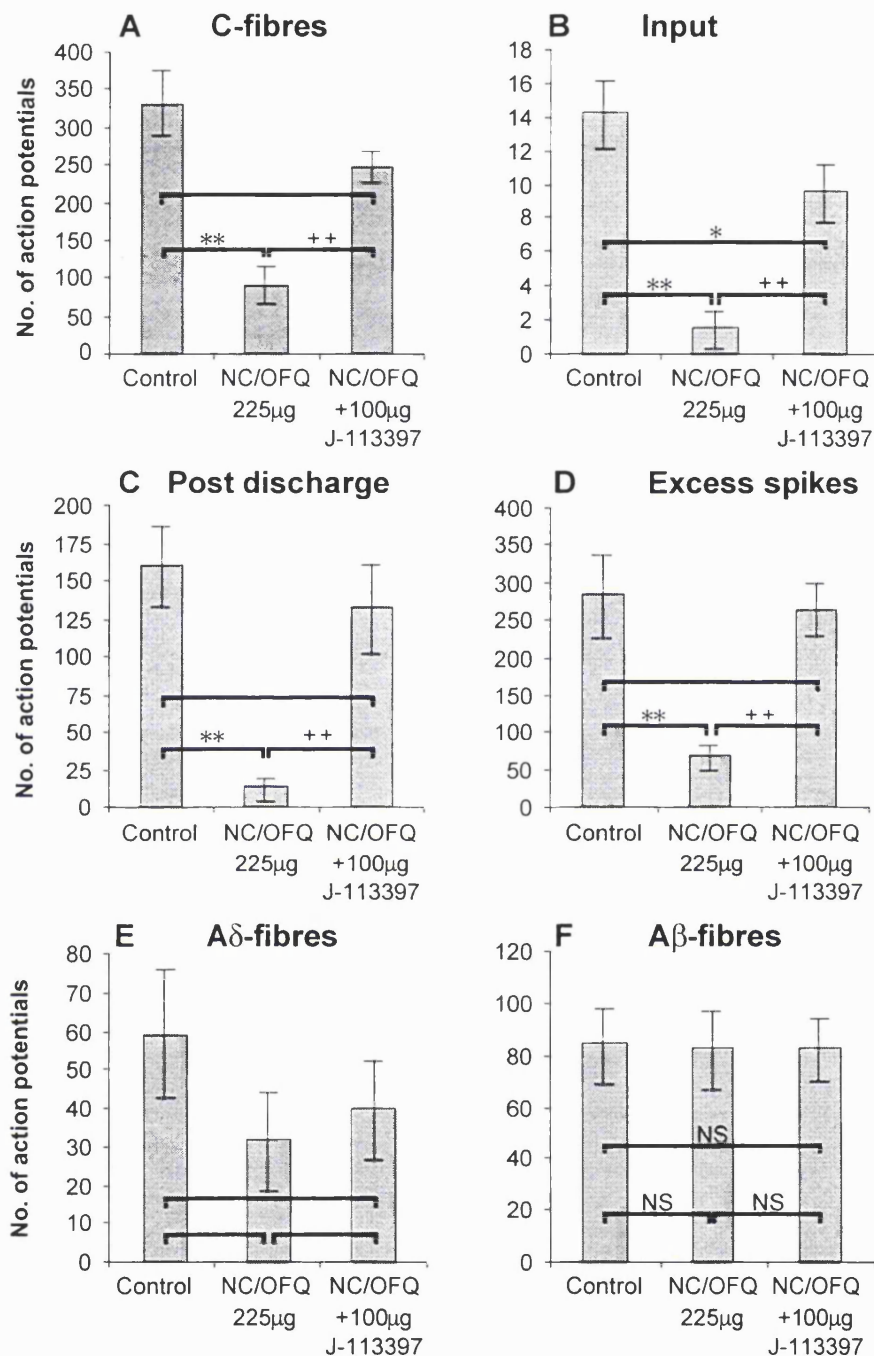


Figure 6.11 Maximal effect of NC/OFQ (after cumulative doses of 50, 125 and 225µg) on responses of dorsal horn neurones, and the reversal by application of 100µg J-113397. Data are shown as mean $\pm$ s.e.m. (number of action potentials). Significant inhibition compared with pre-drug control ($*P \leq 0.05$, $**P \leq 0.01$) and significant reversal by J-113397 ($++P \leq 0.01$) are indicated. NS, not significant.

6.4 Discussion

6.4.1 Spinal NC/OFQ

Any discussion of the spinal effects of the NC/OFQ peptide must be preceded by evidence that it persists in the spinal cord for long enough to have effects. It is likely to do so since the peptide is thought to be stable *in vivo* (Wnendt, personal communication), and although metabolic products can be produced, they appear to have higher affinity for non-ORL1 receptors (Suder *et al.*, 1999), and one fragment retains the ability to inhibit SP release in the dorsal horn (Terenius *et al.*, 2000)

From this study it is evident that exogenous NC/OFQ applied to the spinal cord elicits antinociceptive effects, and that this inhibitory effect on spinal nociceptive transmission is increased in the presence of inflammation. The most marked action of NC/OFQ after inflammation was inhibition of C-fibre-evoked responses and input, which were affected to a much greater extent than in normal animals (Figure 6.2). This suggests an enhanced action of NC/OFQ at predominantly pre-synaptic sites, which is not surprising as the receptor is synthesised in the DRG (Neal *et al.*, 1999b; Wick *et al.*, 1994). A pre-synaptic mechanism of action is supported by receptor and peptide localisation studies, by *in vitro*, *in vivo* and behavioural studies (evidence discussed in this Chapter, sections 6.1.1–6.1.7). There is also previous evidence that the NC/OFQ system exhibits plasticity in models of clinically relevant pain states. Jia *et al.*, (1998) saw increased binding of NC/OFQ in superficial laminae of the spinal cord four days after generation of persistent inflammation with complete Freud's adjuvant. Increases in receptor number after inflammation would explain our findings of increased potency of NC/OFQ after inflammation. An increase in the number of pre-synaptic receptors following inflammation would allow the same dose of NC/OFQ to bind to more receptors — leading to a more profound inhibition of transmitter release than in normal animals.

The enhanced effects of NC/OFQ seen here were very rapid in onset (3 hours), whereas Jia *et al.* (1998) did not see increases in receptor number until four days after CFA-generated inflammation. Although carrageenan inflammation develops more rapidly than that induced with CFA, Andoh *et al.* (1997) have described increased levels of mRNA message for the NC/OFQ peptide precursor in the DRG as early as 3 h after carrageenan inflammation, suggesting that changes in the system can occur rapidly.

This increased expression of prepronociceptin in the DRG after inflammation was suggested by Andoh *et al.* to contribute to the development of hyperalgesia during inflammation, but I believe it is more likely that the peptide acts to attenuate the increased activity in spinal nociceptive circuits. This concept is now supported by a large body of evidence from behavioural studies (Hao *et al.*, 1998; Jhamandas *et al.*, 1998; Kamei *et al.*, 1999; Nakano *et al.*, 2000; Wang *et al.*, 1999a; Xu *et al.*, 2000; Yamamoto *et al.*, 1997) and

electrophysiological studies (Faber *et al.*, 1996; Lai *et al.*, 1997; Liebel *et al.*, 1997; Stanfa *et al.*, 1996). As shown here and discussed in the introduction the peptide clearly inhibits sensory transmission at the spinal level. In fact, the increased presence of the peptide in central terminals after inflammation (Andoh *et al.*, 1997) suggests that NC/OFQ may begin to be released by primary afferents as a negative feedback mechanism and act at the increased number of receptor sites in the superficial dorsal horn in order to return spinal nociceptive transmission to baseline. An autoreceptor function for NC/OFQ has also been suggested in the brainstem (Wang *et al.*, 2000).

The presence of NC/OFQ in primary afferents after inflammation is clear, but there is debate as to whether NC/OFQ is present in primary afferents in normal animals. Andoh *et al.* (1997) detected no expression in the DRG in normal animals and dorsal rhizotomy does not detectably affect NC/OFQ-like immunoreactivity in the spinal cord (Riedl *et al.*, 1996). NC/OFQ release has been detected after stimulation of the dorsal root entry zone (Williams *et al.*, 1998) and a putative NC/OFQ precursor peptide has been detected in small diameter primary afferent neurones in dorsal root ganglion of adult mice (Saito *et al.*, 1996). It remains unclear therefore whether NC/OFQ is present in primary afferent fibres in normal animals.

NC/OFQ originating from intrinsic spinal neurones is a more likely source of the peptide in the absence of persistent pain conditions. Prepronociceptin mRNA (Houtani *et al.*, 2000) and NC/OFQ-like immunoreactivity is detected in both terminals and fibres in the superficial layers of mouse and rat spinal cord (Lai *et al.*, 1997; Narita *et al.*, 1999; Neal *et al.*, 1999a; Schulz *et al.*, 1996; Williams *et al.*, 1998;) and release of NC/OFQ-like substance has been detected in rat dorsal horn (Williams *et al.*, 1998). This presence in and release from intrinsic spinal cord neurones adds support to the idea that NC/OFQ has some basal modulatory role in sensory transmission.

In addition to these reports of plasticity in the expression of the NC/OFQ peptide and the ORL1 receptor several studies, and the work presented here, now report functional evidence of this plasticity. Alterations in the effectiveness of spinally administered NC/OFQ have been reported in behavioural studies after inflammation and after nerve injury (Hao *et al.*, 1998; Yamamoto *et al.*, 1997). There is a discrepancy in the above studies over whether NC/OFQ has increased or decreased effectiveness after carrageenan. Here I clearly found the former. Effects of spinal NC/OFQ under normal and pathological conditions is reviewed in Xu *et al.*, (2000).

An increased anti-nociceptive effect of opioids has been reported after inflammation in both behavioural and electrophysiological studies (Stanfa & Dickenson, 1995 for review). This altered potency of opioids may occur not through a receptor level interaction, but possibly via functional anti-opioid activity of the peptide CCK (cholecystokinin), which is downregulated during inflammation. It is possible therefore, that the increased potency of spinal NC/OFQ that we see here may be mediated not only by increased receptor number but also through interaction with other spinal systems.

Interestingly, I saw that the ability of naloxone to reverse NC/OFQ-mediated inhibitions was greater in the normal animals than in the group of animals studied after inflammation. This was true whether the responses were inhibited to a greater extent by NC/OFQ after inflammation (C-fibre evoked responses and input), or were inhibited to the same extent (post discharge and wind-up). Thus, this differential naloxone effectiveness was not dependent on the degree of inhibition produced by NC/OFQ. Whether this differential reversal reflects receptor changes after inflammation remains to be determined, and the small number of neurones studied in each case means that this difference may be purely coincidental. Significant differences could certainly not be determined.

6.4.2 Peripheral NC/OFQ

As the ORL1 receptor is synthesised in the DRG (Neal *et al.*, 1999b), it follows that these bipolar primary afferent fibres may have functional receptors not only at their central terminals, but also at the terminals in the periphery. Work presented here shows that receptors are present and functional. Although NC/OFQ is inhibitory in the spinal cord, administration in the periphery evoked clear excitatory responses from dorsal horn neurones. Not only was neuronal firing elicited by peripheral injection of NC/OFQ but the peptide sensitised responses to low intensity mechanical stimuli. This is not the only study to find peripheral pro-nociceptive effects of NC/OFQ. Inoue *et al.*, (1998) recently found that injection of extremely low doses of NC/OFQ (in the region of 10^{-9} g) into the hindpaw evoked nociceptive responses in awake mice and that these flexor responses were dependent on release of substance P. The doses of NC/OFQ I found to evoke responses were equivalent to 5 pmole to 1 nmole, which equates to 10^3 to 10^6 times more than the amounts used by Inoue *et al.* (1998) to elicit behavioural responses in mice. This may be explained by the different paradigms used; behavioural tests record the threshold responses of the complete system to stimuli whereas the powerful excitatory responses we recorded from neurones probably represent what would be behaviourally suprathreshold stimulation. Additionally, the difference in size between mice (as used in the original study) and rats (as we use here) is likely to be a factor.

This excitatory action of NC/OFQ was unexpected as the ORL1 receptor has generally been thought to couple to inhibitory intracellular processes. Even the apparently pro-nociceptive activity of NC/OFQ supraspinally has been explained by a functional inhibition of analgesia — the receptor simply inhibits neurones that subserve inhibitory (antinociceptive) functions. Direct inhibitory effects of activation of the receptor are mostly via inhibition of adenylyl cyclase (and possibly PLC), and via actions on ion channels to hyperpolarise and reduce excitability in cells expressing the receptor (see Meunier, 1997 for review, and this chapter, section 6.1.1). The peripheral excitatory actions of the peptide must be through different mechanisms. There is a precedent for bidirectional effects of opioids, in that μ opioid receptors have been reported to couple to both excitatory and inhibitory intracellular processes (Crain & Shen, 1990); opioid receptors on DRG neurones have been

shown to couple via both G_s and G_i proteins (Shen & Crain, 1990) and the same could be true for the ORL1 receptor on primary afferent fibres. The flexor-reflex responses evoked by NC/OFQ in the study by Inoue *et al.*, (1998) were blocked by pertussis toxin, showing that the receptor is still coupled to G_i — exactly how this leads to the reported substance P release is unclear.

Another possibility is that the ORL1 receptor is not a single entity and that different subtypes are present in the periphery. Support for this premise may be provided by the observations that [Phe¹ψ(CH₂-NH)Gly²]nociceptin-(1-13)-NH₂, characterised as an ORL1 receptor antagonist in peripheral tissues, behaves as an agonist in the spinal cord (Carpenter & Dickenson, 1998; Xu *et al.*, 1999; and see this chapter, section 6.1.10.1). This may suggest the existence of different receptor subtypes — either originating from different genes or as a result of splice variants — that can couple to different intracellular mechanisms.

Classical opioids, when administered in the periphery, are inhibitory. These actions of opioids are only revealed in inflammation and are observed before increased peripheral receptor density is evident. This effectiveness after inflammation has been suggested to result from changes around the peripheral nerve endings that facilitate access of the opioid to the receptor (Stein, 1994; Stein *et al.*, 2001 for review). Here, however, the excitatory effects of NC/OFQ are evident in normal animals, which implies that this large peptide is able to reach its receptor without difficulty. That we observed no difference in the effect of NC/OFQ 3 h after carrageenan inflammation is not surprising — if access to the receptor is not an issue, unlike the case with other opioids, then any change in peripheral activity will depend on a change in receptor density and this will not be apparent until some days after increased synthesis in the DRG. If transport of the NC/OFQ receptor is analogous to the opioid receptors, it will take 3–4 days after inflammation for peripherally directed axonal transport to convey these newly synthesised receptors to peripheral endings of sensory afferents (Hassan *et al.*, 1993). The sensitisation to low intensity mechanically evoked responses was also unaffected after inflammation.

6.4.2.1 Effects are mediated by the ORL1 receptor

It is possible that the peripheral administration of NC/OFQ elicits the excitation of primary afferent fibres reported here and the pronociceptive effects reported by Inoue *et al.*, (1998) by physical mechanisms that bypass the ORL1 receptor. This issue was addressed by Inoue *et al.* (1998) and antisense oligonucleotides to the ORL1 receptor were applied to the spinal cord three times over the course of a week before testing. This treatment abolished NC/OFQ-evoked flexor responses. Bearing in mind the questions over the effectiveness of oligonucleotide application *in vivo*, this does go some way to demonstrate a receptor-mediated effect. The involvement of ORL1 receptors on mast cells, macrophages and vascular cells has been suggested. As these receptors would be untouched by

antisense treatment, it would appear that they do not contribute to the peripheral pronociceptive effects of NC/OFQ on flexor behaviour in mice. We aimed to further clarify whether the effects were receptor-mediated by co-administration of NC/OFQ with the ORL1 receptor antagonist J-113397. This compound was chosen as it is the only nonpeptide antagonist available and for which most evidence points to real antagonist activity. When NC/OFQ and this antagonist were injected together (in the same volume and protocol as for NC/OFQ alone), the excitatory effects of NC/OFQ were abolished. Injection of 0.01 µg, 0.05 µg and 0.1 µg NC/OFQ evoked responses no larger than the saline control responses. The sensitising effect on low threshold mechanical stimulation (von Frey 9g) was also abolished. It must, however, be noted that this study group was small (five neurones).

6.4.3 Putative NC/OFQ antagonists

6.4.3.1 [$\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2$]nociceptin-(1-13)-NH₂

The work described here (section 6.3.4.1) was published soon after the study was completed (Carpenter & Dickenson, 1998). Since the work was published, other reports have emerged and most are in agreement (see section 6.1.10.1) — Pheψ is not a full antagonist of the ORL1 receptor. Rather, it mimics the effects of NC/OFQ in many systems and is probably a partial agonist at the ORL1 receptor. In most peripheral preparations — for example the guinea pig ileum and the mouse vas deferens — Pheψ blocks the effects of NC/OFQ, but is an agonist at most CNS sites and at recombinant human receptors.

One explanation for this would be that Pheψ is indeed a partial agonist. Its low efficacy in expression systems is exacerbated by the low linkage ratios to effector mechanisms. However, as the differences are so dramatic, and Pheψ is a full agonist at recombinant human ORL1 receptors, there may be alternative explanations. These tissue-dependent effects could suggest that the cloned human receptor and the receptor that is functional in rat spinal cord differ from the receptors found in the guinea-pig ileum and mouse vas deferens. In support of this premise of receptor diversity, Mathis *et al.*, (1997) have presented biochemical evidence for heterogeneity of the ORL1 receptor in mouse brain. Splice variants of the receptor have also been reported. These lines of evidence could be taken to indicate the presence NC/OFQ receptor subtypes — an exciting prospect.

6.4.3.2 [N-Phe^1]nociceptin(1-13)NH₂

NPhe was developed soon after it appeared that Pheψ was not going to be useful in dissecting the NC/OFQ system, and promised to be a full antagonist. Here, pretreatment with 50 µg NPhe prior to application of NC/OFQ had no effect on neuronal inhibition produced by NC/OFQ, nor did it have any effect on neuronal responses when applied alone. Several possibilities arise from these observations. First, NPhe was not applied at a high enough concentration; second, that the NC/OFQ effects we observed are not mediated via

the ORL1 receptor; and third, that this peptide is active as an antagonist of NC/OFQ when tested in several *in vitro* and *in vivo* systems ^{subtly} its activity here. Determining the correct dose to use is always a problem for *in vivo* electrophysiologists; effective concentrations in *in vitro* assays cannot be easily translated into effective concentrations in our assays. This study was preceded by a preliminary study to investigate the NPhe at concentrations ranging from 5µg/50µl to 150µg/50µl. At the highest dose, some inhibition of neuronal responses was observed (n = 2); therefore the lower dose of 50µg was chosen. Perhaps this dose was too low to reveal antagonism of NC/OFQ effects; however, a higher dose may have produced an inhibition of responses itself.

The fact that many of the assays in which NPhe is full antagonist of NC/OFQ are the same ones in which Pheψ appeared so promising, also casts doubt on the usefulness of this peptide to investigate the NC/OFQ system *in vivo*.

6.4.4.3 J-113397

When applied spinally after cumulative doses of NC/OFQ had caused a pronounced and selective inhibition of noxious-evoked neuronal responses, 100µg J-113397 reversed these effects. Subsequent application of morphine produced normal inhibition in all the neurones that were tested — a qualitative indication that µ opioid receptors were not affected by J-113397 application. As described earlier (section 6.4.2.1) the inclusion of this agent with peripheral administration of NC/OFQ blocked the appearance of NC/OFQ-evoked responses and sensitisation to low threshold mechanical stimuli.

Of the three putative NC/OFQ receptor antagonists I tested during my research, this is the only one to convincingly block (in peripheral injection) and reverse (after spinal administration) the effects of NC/OFQ. It is probably not a co-incidence that J-113397 is not a peptide. This could well be the factor that has been holding back, and perhaps confusing, research into the many functions of NC/OFQ. Without stable small-molecule inhibitors or agonists, the functional roles of this intriguing peptide cannot be investigated in the whole animal with any degree of certainty. I have relied upon the integrity of the NC/OFQ peptide for several studies in this thesis, but for relatively short periods of time and in a system containing relatively few proteases. Adding peptide antagonist into the mix will certainly compound the problems.

6.4.5 Conclusions

It is rapidly becoming clear that NC/OFQ and the ORL1 receptor genuinely comprise a novel opioid-like transmitter system. Aspects of the function of this system resemble those of classical opioids in that spinally the consensus is that this peptide elicits antinociceptive effects. The supraspinal anti-opioid actions of NC/OFQ are not without precedent — there is a literature on the ability of κ opioids to overcome the effects of µ opioids by functional antagonism both spinally (Dickenson & Knox, 1987) and supraspinally (Pan *et al.*, 1997).

The peripheral pro-nociceptive actions of this peptide are surprising, novel and have not been reported for any other opioid.

The various and contrasting central and peripheral effects of the NC/OFQ peptide on pain processing raise the question of the relative importance and balance of these diverse effects on nociception. Only a systemically active and stable agonist at the NC/OFQ receptor, able to target all sites simultaneously, will reveal the analgesic potential of drugs based on NC/OFQ. Along the same lines, further advances in the design of antagonists will be required to dissect out the true physiological role of this peptide. The production of a non-peptide agonist with the ability to penetrate the CNS after systemic application will be just as crucial — only a compound that accesses the supraspinal, spinal and peripheral receptors with equivalent concentrations will reveal which of the anti- or pro-nociceptive effects of ORL1 receptor activation prevail under normal conditions and in clinically relevant pain states.

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Chapter 7. General discussion

Drugs with roots in the opium poppy *Papaver somniferum* have been Man's main tools for the control of pain for the past 6000 years. A long history and a natural source does not necessarily make for a crude drug. On the contrary, opium derivatives are exceptionally well designed for providing pain relief. In hijacking the body's own provision for pain control they interact with a sophisticated system that can faithfully sense and transmit information about damaging or potentially damaging stimuli, a system that can also augment or attenuate this signal for, on the most part, good reason.

Opioids, however, are not without their problems and are not always effective — this can be a particular problem for pain arising from nerve damage, where opioid inhibitory pathways seem less able to control nociceptive transmission.

In the search for new targets for the development of analgesics, understanding the circuitry underlying the spinal transmission and modulation of nociceptive information has provided the pharmacologist with rich pickings. The low-hanging fruit like the NMDA receptor and the tachykinin NK₁ receptor have been the focus of much research over the past decade. Unlike the unsuccessful trials with NK₁ receptor antagonists, drugs that block the NMDA receptor are analgesic in humans. The side effects associated with ubiquitous block of this receptor are severe and preclude the wide use of non-selective agents like ketamine, although memantine, dextromethorphan and amantadine have had some success. NMDA receptor antagonists have many other potential applications. Patients with epilepsy, ischaemic brain damage, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis may all benefit from selective NMDA receptor blockade. NMDA receptor antagonists even have the potential to treat osteoporosis as they have been shown to slow bone resorption.

In Chapter 3 of this thesis I demonstrated that two different approaches to improving the side effect profile of NMDA receptor antagonists retain the ability to attenuate NMDA receptor mediated events *in vivo*. The glycine site antagonists Mrz 2/571 and Mrz 2/579 inhibited noxious-evoked neuronal activity with a profile characteristic of NMDA receptor blockade. Although glycine site antagonists have been known to attenuate NMDA receptor function *in vivo* for the past decade, and to lack the psychomimetic effects common with the channel blockers (Bristow *et al.*, 1996; Tricklebank *et al.*, 1994) and neuropathological changes associated with some competitive and non-competitive NMDA receptor antagonists (Hargreaves *et al.*, 1993), none have successfully made it into clinical use. Several glycine site antagonists have reached clinical trials. For example, Gavestinel (GV 150526, Glaxo Wellcome), progressed through phase II to phase III trial, but did not improve outcome after acute stroke (Lees *et al.*, 2000). That such agents are well tolerated is encouraging, and it would be interesting to see whether it will be tested for treatment of pain. Glaxo Wellcome do have an analogue of this (GV 194771A) under Phase I safety testing trial for treatment of chronic pain (see Danysz & Parsons, 1998).

The NR2B-selective NMDA receptor antagonists ifenprodil and ACEA-1244 were also effective in inhibiting noxious-evoked activity and wind-up of dorsal horn neurones. Their profile was slightly different, and the pronounced inhibition of input is interesting. This indicates an involvement of NR2B-containing NMDA receptors in normal, baseline nociceptive transmission. This raises two questions: how do they do it, and why?

The idea that some degree of prolonged stimulation is required to recruit NMDA receptors at a synapse is well founded. For example, the requirement for tetanic stimulation for the generation of LTP, kindling in epilepsy and repeated afferent fibre stimulation for wind-up. It also makes sense, as the consequences of NMDA receptor activation can cause, in the short-term, dramatic increases in cell excitability and, in the long-term (in the brain at least), permanent enhancement of synaptic efficacy. For inhibition of input to be seen, NR2B-containing NMDA receptors must be involved synaptic transmission in the absence of any prior synaptic activity.

It is difficult to see how enough activity could have been generated by the first stimulus in the train to sufficiently depolarise the membrane to remove Mg^{2+} block. A logical step would be that Mg^{2+} has a lower affinity for blockade of NR2B-containing NMDA receptors. There is great potential for diversity in NMDA receptor composition, generated by NR1 subunit splice variants and the four different NR2B subunits. It would be surprising indeed if there were not functional consequences of this diversity. Another key factor is anatomical diversity. Sub-types of the NMDA receptor have different distributions in the CNS lending hope for more selective targeting of disorders and diseases. Receptors with different subunit composition can indeed be affected differently by the numerous modulators of NMDA receptor function. These are reviewed and discussed in detail by (Dingledine *et al.*, 1999). For example, phosphorylation by protein kinase C enhances NMDA receptor currents. However, PKC phosphorylation is extensive in resting membranes and seemingly non-selective for subunit make-up. In contrast, the NR2B subunit seems to be more susceptible to tyrosine kinase phosphorylation than NR2A, C or D subunits. Specifically, brain-derived neurotrophic factor (BDNF) has been shown to selectively increase the tyrosine phosphorylation of NR1 and NR2B (but not NR2A) subunits (see Dingledine, 1999 and references therein).

Zn^{2+} can also block the NMDA receptor, and NR2A containing receptors are blocked by lower concentrations of ambient Zn^{2+} than receptors containing other NR2 subunits (see Dingledine, 1999). One effect of Src tyrosine kinase phosphorylation of NMDA receptors may be to decrease sensitivity to Zn^{2+} blockade. This could have the effect of relieving Zn^{2+} block for other subunits while the NR2A subunit is still blocked. Polyamines are other endogenous modulators of NMDA receptor function, and have been seen to enhance NMDA receptor currents independently of glycine only in receptors containing the NR2B subunit.

In addition to altering the sensitivity of a channel to Mg^{2+} block, the voltage-dependent block may be removed more easily if the receptors were effectively clustered

close to other excitatory receptors in the post-synaptic membrane. It is likely that the NMDA receptor subunits have binding sites for different membrane-localising intracellular proteins, determined by sites in the C-terminus region, and could thus be positioned differently in the membrane. The recent work by Momiyama, (2000) has, however, suggested that NR2B-containing receptors are not clustered in the post-synaptic density, but localised to extra-synaptic areas of dorsal horn neurones in adult rat spinal cord. Although it is debatable whether any one of these mechanisms would be sufficient to allow NR2B-containing NMDA receptors to participate in baseline transmission, a combination of these effects, and perhaps many more, could underlie its 'easier' activation.

Why would an NMDA receptor be involved in baseline transmission of noxious activity? It is possible that Ca^{2+} currents through calcium permeable AMPA, kainate or NMDA receptors could act as a 'primer' system. These NMDA receptors could be activated by minimal afferent activity, thereby serving to help recruit higher 'threshold' NMDA receptors elsewhere on the neurone if the afferent drive increases. If expression of NR2B-containing receptors were increased or reduced in different conditions, this would provide a means of enhancing or limiting NMDA receptor activation in response to afferent input.

Optimisation of currently available analgesics is an important area of research. Such drugs do not need the extensive investment (both financially, and of time) that is required of novel agents, as they are already approved for clinical use. The reportedly atypical profile of the opioid methadone has been suggested to arise from combined opioid agonist and NMDA receptor antagonist actions. In the work presented in Chapter 3, no NMDA receptor component to the inhibition of noxious-evoked responses could be seen. This does not mean that methadone has no future in the management of pain. I cannot rule out synergy between weak NMDA receptor blockade and opioid analgesia. Evidence-based medicine should prevail. Better reporting, more controlled analyses of the available clinical reports and perhaps a few well-designed trials should determine whether methadone analgesia could have advantages over classical opioids. Surprisingly, the simple test of whether naloxone fully reverses the effects of methadone in patients has not been reported.

The work described in Chapter 4 began with the aim of using elevation of NAAG, as a partial agonist at the NMDA receptor, as another means to attenuate NMDA receptor function while avoiding the effects of ubiquitous blockade. As a partial agonist, NAAG was predicted to display some degree of 'activity-dependent block' and only significantly interfere with glutamatergic transmission at the NMDA receptor in the presence of substantial activity. It is not entirely clear from our results whether there is an NMDA receptor blocking component to the effects of NAAG elevation in the spinal cord. In activating the mGlu3 metabotropic glutamate receptor, NAAG appears to have a very interesting profile that suggests the potential for analgesia.

It is possible that in normal animals, release of NAAG from C-fibre (and possibly

A δ -fibre) afferents in the spinal cord and subsequent activation of pre- and/or post-synaptic mGlu3 receptors provides a feedback mechanism for controlling excitability. If afferent drive were to continue and increase and a state of spinal hyperexcitability develop, NAALADase levels may be reduced such that NAAG persists for longer in the synapse and can sustain a basal inhibitory control of both transmitter release and post-synaptic excitability. mGlu3 receptors on the astrocytes that are closely associated with synapses in the spinal cord may serve to detect the increased activity and alter NAALADase expression accordingly.

As a first step towards validating this theory, it would be very interesting to determine whether the effects of spinal 2-PMPA application can be reduced or completely blocked by a selective mGlu3 receptor antagonist. Application of such agents alone, in normal animals and in models of chronic pain, could clarify any involvement of the elusive mGlu3 receptor in control of spinal hyperexcitability. The mGlu3 agonist (1S,3S)-ACPD has been studied in the same experimental conditions (Stanfa & Dickenson, 1998). In normal animals, the drug had mixed effects, producing inhibition of noxious-evoked activity in some cases, and no effect or facilitation in others. After carrageenan inflammation, inhibitions were consistently seen. When the effects of 2-PMPA application are compared with this work, the effects appear to be consistent with the profile of an mGlu3 receptor agonist. As pointed out in Chapter 4, not all neurones responded to application of 2-PMPA, which suggests that not all dorsal horn neurones are under control by NAAG under normal conditions.

The work described in Chapter 5 may seem to be rather different from the ideas pursued in the preceding chapters, but really approaches similar problems from a different angle. The environment around a nociceptor changes dramatically after tissue damage. Released protons are just one component of the inflammatory soup that begins the process of modulating nociceptive transmission by generating primary hyperalgesia. It is now clear that protons do contribute to this process, rather than just being a by-product of cellular damage. This has been demonstrated in a range of systems, which vary in their relevance to the *in vivo* situation. Patch-clamp recordings of cloned channels in expression systems and of pH sensitive sensory nerve preparations have revealed much about the current characteristics (see Chapter 5, section 5.1). Single-unit recording in skin–nerve preparations demonstrate that proton-sensing channels are functional *in situ*, but the experiments do involve damaging dissection of the receptive field and application of pH in an unphysiological manner. Injection of low pH solutions into the hindpaw of the intact rat, as reported here, is also an unphysiological way to administer protons, but probably leaves sensory nerves relatively undamaged. For as long as administered protons surmount the buffering capacity of the intact hindpaw, activation of primary afferents and subsequent activity in dorsal horn neurones could be recorded. There was no desensitisation of the same area to repeated administration over the course of several hours. This is in contrast with the situation in *in vitro* studies, and indicates that in the physiological setting where a

population of nociceptors is present to mediate the response to protons, no desensitisation occurs. This is consistent with the results from human studies, where reports of burning pain remain undiminished for the duration of the infusion of acidic buffer.

Although by no means conclusive, work presented here also indicates that nociceptors sensitive to protons and to noxious heat tend to converge on similar populations of dorsal horn neurone. Whether this would still be true in the setting of chronic inflammation or nerve injury is an interesting question.

The analgesic effects of classical opioids at the level of the spinal cord are mediated mostly by inhibition of transmitter release from C-fibre terminals. In this regard, opioids effectively target nociceptive pathways, but post-synaptic mechanisms remain intact. Wind-up of neuronal responses can still be observed in the presence of sufficient afferent drive. This situation is not so apparent for the newest member of the opioid family, NC/OFQ. In normal animals, NC/OFQ not only inhibits input and C-fibre evoked responses as a mu opioid receptor agonist would, but also attenuates post-synaptic activity to a certain extent (see Chapter 6 and Jennings, 2001; Stanfa *et al.*, 1996). This post-synaptic component could mean that agents targeting the ORL1 receptor could be effective in situations where the efficacy of opioids is reduced.

The plasticity reported in expression of both the ORL1 receptor and NC/OFQ was confirmed here. After inflammation, the potency of spinally applied NC/OFQ was enhanced, but only on the C-fibre-evoked response and the input. This is consistent with an upregulation of the ORL1 receptor at pre-synaptic sites. Whether expression of post-synaptic receptors is altered after longer-term inflammation or after nerve injury remains to be determined.

The peripheral excitatory action of NC/OFQ is interesting, not least because the physiological reason for such an effect is unclear. It is possible that NC/OFQ is never released in the periphery, so the ORL1 receptors presumed present on nociceptor terminals are not normally activated. Although the work presented here does demonstrate that the effects are mediated by the ORL1 receptor, it was not possible to show that the receptor was expressed on the afferent nerve fibres. It would be interesting to apply the peptide to the skin-nerve preparation, where inflammatory cells that express the receptor are not likely to be present.

The aims of this thesis, which are set out in Chapter 1, have been met. I have managed to shed some light on one of the fundamental questions in neurobiology in describing both peripheral and central mechanisms that underlie normal transmission of nociceptive information and aspects of plasticity in pain pathways.

In conclusion, the work that I have described here could have important therapeutic

implications, but a warning note is necessary. For the agents and approaches studied, I can only determine their efficacy, and only in one system. The price to pay for being able to record so precisely the activity in this complex pathway is that effects at other sites cannot be determined. Indications of unwanted effects in other systems can be monitored to a certain extent in behavioural work, but then we must wait for pharmacokinetic and safety testing before the benefit to patients can be known. However, gauging efficacy is still of prime importance. Given a selective agent, its ability to differentially control and modulate various neuronal responses strongly supports the idea that these *in vivo* approaches have both sensitivity and predictive value. The validation of pharmacological targets in models such as this is the first step on the road to alleviating the pains suffered by an estimated 355 million people in the world today.

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Chapter 8. Publications



SPECIAL REPORT

Evidence that $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$, a peripheral ORL-1 receptor antagonist, acts as an agonist in the rat spinal cord

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$[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$, a pseudopeptide analogue of nociceptin is an antagonist in peripheral assays. Here, using *in vivo* electrophysiological recordings of dorsal horn neurones, $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ appears to have agonist activity after spinal administration. The noxious evoked activity of the neurones was inhibited by $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$, which was as potent as nociceptin itself.

Keywords: nociceptin; spinal cord; ORL-1 receptor; antinociception

Introduction Nociceptin (orphanin FQ), the endogenous ligand for the opioid receptor-like orphan receptor (ORL-1), is implicated in many biological processes including pain (see Meunier, 1997 for review). The ORL-1 transcript (Wick *et al.*, 1994) and ORL-1-like immunoreactivity (Anton *et al.*, 1996) have been detected in rat spinal cord and human central nervous system (CNS) (Peluso *et al.*, 1998). Williams *et al.* (1998) have demonstrated basal and an electrically evoked release of nociceptin-like material from the rat dorsal horn *in vitro*. Pharmacological studies *in vivo* show spinally applied nociceptin inhibits nociceptive neuronal transmission in the dorsal horn (Stanfa *et al.*, 1996). In contrast, intracerebroventricular nociceptin produces hyperalgesia and can also reduce morphine analgesia (Reinscheid *et al.*, 1995; Meunier, 1997 and references therein).

To elucidate the role of endogenous nociceptin, a selective ORL-1 antagonist is vital. $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$, a pseudopeptide analogue of nociceptin, is a competitive antagonist in guinea-pig ileum and mouse vas deferens (Guerrini *et al.*, 1998). However, in Chinese hamster ovary (CHO) cells expressing the human ORL-1 receptor, $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ is an agonist (Butor *et al.*, 1998). Here we investigate the effects of intrathecal $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ on spinal neurones in the anaesthetized rat, an *in vivo* model where nociceptin is inhibitory.

Methods Male Sprague-Dawley rats (200–250 g) were anaesthetized with halothane and maintained at 1.8% (see Dickenson *et al.*, 1987 for full methods) and extracellular recordings made from single dorsal horn neurones responding to noxious and innocuous stimuli in L4–L5 segments. Responses were elicited from ipsilateral hindpaw receptive fields by transcutaneous electrical stimulation, at three times the C-fibre threshold. At 10-min intervals, a train of 16 stimuli (0.5 Hz) was given and post-stimulus histograms constructed. These evoked responses were separated by latency into A β -fibre evoked activity (0–20 ms), A δ -fibre (20–90 ms), C-fibre (90–300 ms) and post-discharge (300–800 ms) and quantified. Responses evoked by the first stimulus of the train are referred to as 'input' and consist of the number of action

potentials (90–300 ms) evoked by this stimulus. Wind-up, a measure of the enhanced neuronal response elicited by repetitive stimulation, was quantified as the difference between the total number of action potentials produced by the 16 stimuli (90–800 ms), and the input $\times$ 16.

After control responses stabilized, cumulative doses of $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ (1 μg , 25 μg , 75 μg and 125 μg) in 50 μl saline, were applied to the spinal cord and neuronal responses followed for 40 min. After maximum inhibition was reached, either 5 μg ($n=3$) or 10 μg ($n=4$) naloxone was applied intrathecally, followed by the highest dose of naloxone (50 μg). Each dose was followed for 40 min.

Statistical analysis of drug effects used one-way analysis of variance (ANOVA) and Fisher's protected least significant difference test. $P < 0.05$ was considered significant.

Results The effect of $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ was studied on 14 dorsal horn neurones ($665 \pm 42 \mu\text{m}$ depth from the cord surface). C- and A δ -fibre evoked responses, input, wind-up and post discharge were preferentially inhibited by $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$, in a dose-related manner, whereas the A β -fibre evoked responses were not altered, suggesting selective actions on noxious evoked activity.

$[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ significantly inhibited the C-fibre evoked response ($P=0.009$, control = 266 ± 19 action potentials, Figure 1A). Inhibition by 25 μg of the drug was significant with the maximum effect seen with 75 μg of drug ($n=5$, $P < 0.05$ for both). In contrast, the A β -fibre evoked response (control = 97 ± 8 action potentials) was not significantly affected by intrathecal $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ (Figure 1A).

The input (control = 13 ± 8 action potentials) and wind-up (control = 190 ± 25) responses were consistently reduced by $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ (Figure 1B) but these effects did not reach significance. Post discharge, another measure of wind-up, was significantly reduced by $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ ($P < 0.05$, control = 124 ± 25 action potentials) after an initial facilitation with the lowest dose (Figure 1B). There was a trend towards inhibition of A δ -fibre evoked responses (control = 42 ± 7 action potentials).

In the example shown in Figure 2, 5 μg naloxone partly reversed the inhibitory effect of 75 μg intrathecal $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ on wind-up. Seven of the 14

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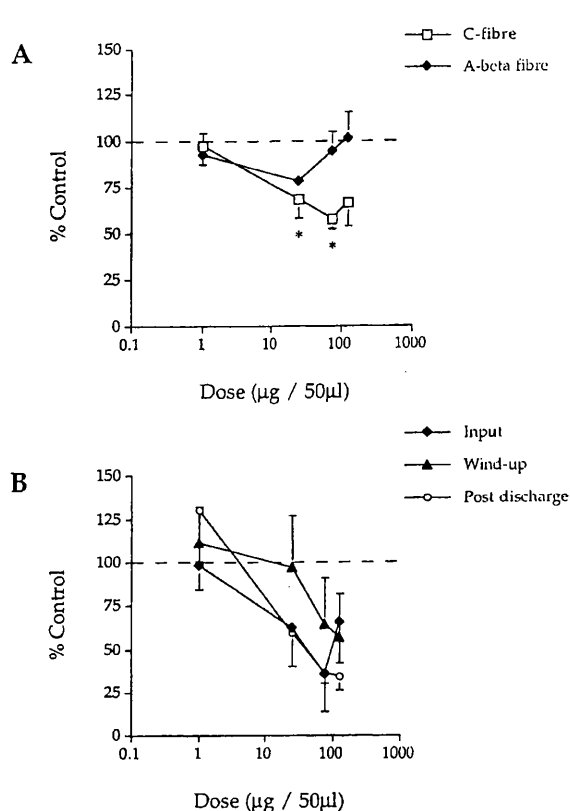


Figure 1 The effect of intrathecal $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ on (A) C-fibre and $\text{A}\beta$ -fibre evoked responses and (B) input, wind-up and post discharge. Data are shown as mean percentage control and vertical lines show s.e.mean. * $P < 0.05$.

neurons studied were tested with a range of doses of naloxone (5 µg, 10 µg and 50 µg), which partially reversed the inhibitory effects of $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$. The highest dose of naloxone produced the greatest reversal. C-fibre evoked responses were reversed, on average, to $84 \pm 6\%$ control; post discharge to $94 \pm 21\%$; wind-up to $108 \pm 22\%$ and input to $68 \pm 10\%$ of pre-drug control values.

Discussion This is the first study of $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$, a putative antagonist at the ORL-1 receptor *in vivo*. Overall, $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ had inhibitory actions, significantly inhibiting C-fibre evoked responses and post discharge but not $\text{A}\beta$ -fibre evoked responses. This selectivity of action mirrored that of spinal nociceptin; a comparison of Figure 1 with Figure 1 in Stanfa *et al.* (1996) shows the similarity between the potency of the two drugs.

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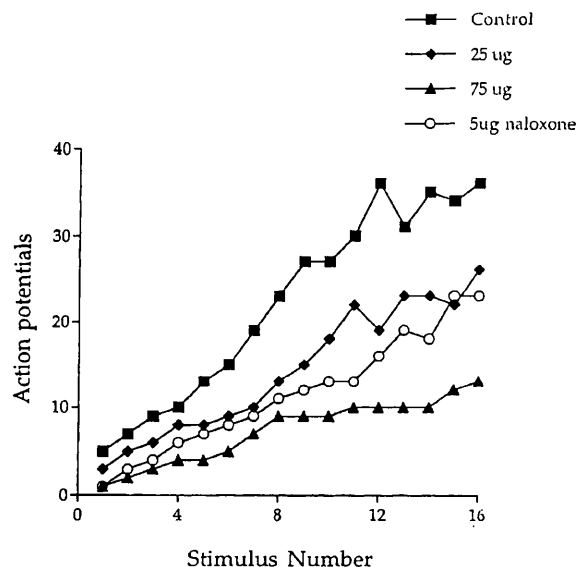


Figure 2 The effect of intrathecal $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ (25 µg and 75 µg) on an individual neurone exhibiting wind-up. 5 µg naloxone, applied intrathecally, partially reversed the effect.

In contrast to nociceptin, $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ tended to reduce the C-fibre input (related to the baseline C-fibre evoked response recorded for nociceptin), an effect seen with intrathecal opioids (Dickenson *et al.*, 1987). The inhibitory effects of $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ were more susceptible to naloxone reversal than those of nociceptin. $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ may therefore have some activity at spinal μ - or δ -opioid receptors although it has been thought to be selective for the peripheral ORL-1 receptor (Guerrini *et al.*, 1998).

In two peripheral preparations—the guinea-pig ileum and the mouse vas deferens— $[\text{Phe}^1 \psi(\text{CH}_2\text{-NH})\text{Gly}^2]\text{nociceptin-(1-13)-NH}_2$ is an antagonist, blocking the effects of nociceptin. We have shown, in the rat spinal cord, that this pseudopeptide mimics nociceptin, and so is likely to be an agonist at the rat spinal ORL-1 receptor. Butor *et al.* (1998) have also shown agonist actions of this pseudopeptide on recombinant human ORL-1 receptors. These tissue dependent effects suggest that the cloned human receptor and that in rat spinal cord could differ from the receptors found in the guinea-pig ileum and mouse vas deferens. In support of this premise of receptor diversity, Mathis *et al.* (1997) have biochemical evidence for heterogeneity of the ORL-1 receptor in mouse brain.

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Pain relief

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This meeting contained a considerable body of data on both basic science and clinical studies dedicated to the alleviation of pain. Presentations covered a number of exciting areas, where both novel disclosed compounds and clinical data were covered. From these we have selected excitability blockers acting on sodium and calcium channels, progress in drugs acting at glutamate receptors, cannabinoid receptors, capsaicin analogs, novel opioids acting at receptors other than the μ receptor for morphine, substance P antagonists and cyclooxygenase (COX)-2 inhibitors as being of particular interest.

Introduction

This, the ninth triennial congress organized by the International Association for the Study of Pain was aimed at persons working in, and interested in, the fields of pain research and treatment. There was a record attendance and the nearly 6000 delegates from 70 countries included basic scientists, physicians, psychologists, dentists, nurses, physiotherapists, pharmacists and other health professionals. The overall theme of the congress was "Pain: From Molecule to Mind" and the program comprised a choice of 20 refresher courses, 20 plenary sessions, about 100 topical workshops and, during the 5 days of the congress, approximately 1500 papers were presented as posters. This multidisciplinary meeting aimed to provide delegates with a state-of-the-art overview of a wide range of topics in the areas of acute, chronic, and cancer pain; to provide practical reviews of current research and therapies in these areas; and to enable delegates to participate in formal and informal discussions with international experts.

Excitability blockers as analgesics

Based on the parallels between epilepsy and pain, in that both disorders involve an excess of activity in CNS circuitry, a number of anticonvulsants have been employed in the treatment of pain. There is ample clinical evidence for effectiveness of the anti-epileptic and sodium channel blocker carbamazepine in the treatment of pain from nerve injury.

More recently, gabapentin, an adjunct anticonvulsant, has also been proven as effective in two types of neuropathic pain, including post-herpetic neuralgia, based on large-scale controlled clinical trials. These clinical studies were landmarks because of the large numbers of patients included. Further trial results were presented at the meeting supporting the use of gabapentin in different neuropathic pain syndromes and phantom limb pain, although side effects were reported in some patients (drowsiness,

dizziness, tremor). The mode of action of this drug, despite its name, is poorly understood but it appears to interact with calcium channels (the gabapentin binding protein appears to be associated with the $\alpha_2\delta$ subunit), where it probably acts as an antagonist. Mention was also made of pregabalin (Warner-Lambert Co), a next-generation drug based on gabapentin, which is efficacious in animal models.

There were a number of studies reporting that lamotrigine (Glaxo Wellcome plc) has analgesic properties – this agent blocks sodium channels in a manner akin to carbamazepine. In this regard, despite a lack of selective agents at the present time, there was much discussion of the potential of sodium channel blockers, selective for the peripheral C-fiber tetrodotoxin-insensitive sodium channels. No drugs with selectivity for these channels were reported, but this area has enormous potential, since blocking sodium channels in peripheral nociceptive fibers would be expected to have high efficacy in pain management. Regulation of the channel in different models of pain in animals was reported with implications for the treatment of pain from both neuropathic and inflammatory origins. Complex changes occur following nerve injury, and more selective drugs will be required before the exact role of this channel in nerve injury pain can be fully interpreted.

Glutamate

NMDA-receptors

There has been a growing interest in the use of NMDA receptor antagonists for the treatment of persistent pain states, particularly neuropathic pain. NMDA receptors have been implicated in the events underlying spinal hyperexcitability and much attention has focused on the potential use of NMDA receptor antagonists for the treatment of neuropathic pain states, due to their potential ability to suppress the excess excitability and wind-up associated with this chronic pain state. Amongst the NMDA receptor antagonists currently available for clinical use, ketamine and dextromethorphan have been shown to be effective in relieving some of the symptoms of neuropathic pain. One limitation in the use of these agents, however, is the occurrence of pronounced side effects, which often make them unsuitable for clinical use. Memantine, on the other hand, is a 1-amino-3,5-dimethyladamantane derivative which possesses antinociceptive effects similar to those of ketamine, but with a better side effect profile. Memantine may therefore offer an alternative approach in the absence of side effects. A series of clinical studies on NMDA and non-NMDA receptor antagonists formed the basis for a workshop; there was consensus that the clinically available agents are effective but this is only reached by doses that produce unpleasant side effects. Further dose escalation is therefore impossible and so complete pain control is hard to achieve.

The problem of side effects can be partly overcome through the use of NMDA receptor antagonists in conjunction with another agent, such as morphine. Combination therapy has

the advantage of producing fewer side effects, and may result in a synergistic interaction between two drugs, thus greatly enhancing the therapeutic effect. In this regard, the ability of methadone to bind to both opioid and NMDA receptors attracted much attention although there is no evidence as yet to suggest that the latter action contributes to the clinical action of this drug. Interestingly, tramadol (Grünenthal GmbH), by merit of the fact that it has multiple pharmacological actions (weak opioid receptor binding together with uptake block of norepinephrine and 5-HT) is now the best selling opioid. These multiple actions have been shown to contribute to its analgesic properties in humans.

Combination therapy was widely discussed as a potential future approach of the treatment of 'difficult' pains, particularly neuropathic pain.

Another approach to limiting the side effects of ubiquitous NMDA receptor antagonists is to target agents to different receptor subtypes, by their subunit makeup. Drugs selective for NMDA receptors containing the NR2B subunit appear to lack side effects (particularly motor problems) in behavioral studies, whilst still maintaining good analgesic profiles. Ifenprodil (Synthelabo) is one such selective drug, but more selective compounds have recently been described. These include conantokin G (toxin isolated from cone snail venom; Annovis Inc), ($\pm$)-CP-101606 (Pfizer Inc), and ($\pm$)-Ro-25-6981 (Hoffmann-La Roche AG) which show a greater margin between doses needed for analgesia and doses at which motor impairment becomes apparent in behavioral rodent studies.

Non-NMDA glutamate receptors

In recent years, non-NMDA glutamate receptors, such as kainate and metabotropic glutamate receptors (mGluR), have received much attention and have been recognized as possible targets for analgesia. To date, several selective kainate antagonists, such as LY-294486 and LY-382884 (both Eli Lilly & Co), have been developed, and these compounds have been reported to be efficacious in chronic pain models. Kainate receptors are predominantly located on nociceptive primary afferents and antagonists at this receptor can block glutamate release, thus resulting in analgesia.

There is substantial evidence to suggest that metabotropic glutamate receptors may also play a role in pain transmission. Group I receptors (mGluR1 and mGluR5) are involved in the excitatory transmission of nociceptive information and there has been recent progress in the development of novel antagonists at this site (LY-393675; Eli Lilly and NPS-2390; NPS Pharmaceuticals Inc). The activation of Group II receptors (mGluR2 and mGluR3), on the other hand, produces a presynaptic inhibition of neurotransmitter release and selective mGluR2/3 agonists have been introduced (LY-379268; Eli Lilly). These agents may provide a novel target for pain relief. However, the data available on these compounds at present is still preliminary and further research is clearly needed to fully investigate their efficacy and side effect profiles before they can be taken further into the clinic.

Cannabinoids

The CB₁ receptor antagonist, SR-141716A (Sanofi-Synthelabo), the CB₂ receptor antagonist SR-144528 (Sanofi Recherche SA), and the non-selective CB receptor agonist,

HU-210 (Hebrew University of Jerusalem), were described, in addition to the endogenous cannabinoid, anandamide. These endogenous and synthetic tools will allow the role of this novel receptor system in the modulation of pain to be fully characterized in animals.

Capsaicin analogs

Capsaicin, the 'hot' component of chilli peppers is widely used in pain research as a tool to identify nociceptive C-fibers. The activation of the vanilloid receptor by capsaicin results in both the depolarization and desensitization of nociceptive fibers, the latter action providing a basis for its analgesic effects. Synthetic analogs which avoid the initial activation and target and inactivate C-fibers may have potential as analgesics and should lack the problematic burning pain felt with capsaicin on administration. Data was presented on two compounds with this profile (EC-665 and SDZ-249665; Novartis AG), which were antihyperalgesic both *in vivo* and *in vitro*.

Novel opioids

Development of opioid analgesics lacking the side effects of μ receptor-selective agonists, such as morphine, is limited by the peptide nature of most receptor ligands and their poor selectivity between the μ , δ and κ receptors. Evidence was presented that peripheral administration of κ receptor-selective compounds, ADL-10-0101 (Adolor Corp), TRK-820 (Toray Industries Inc), and EMD-61753 (Merck KGaA), had moderate antihyperalgesic effects in behavioral studies and very limited or no antinociceptive effects in normal animals. ADL-10-0101 cannot penetrate the blood-brain barrier and as such is peripherally limited; a potentially useful method to avoid central side effects. Novel δ receptor-selective compounds were also discussed with better selectivity than compounds such as the agonist SNC-80 (National Institutes of Health) which has 300-fold and 1500-fold selectivity for δ over μ and κ , respectively and limited analgesic effects in primate behavioral studies. A δ receptor antagonist, AR-M100150, has improved selectivity for the δ receptor (100-fold) over the previously most selective δ receptor antagonist, naltrindole (20 to 30-fold).

The newest member of the opioid family is nociceptin (F Hoffmann-La Roche Ltd) - the endogenous ligand for the opioid receptor-like (ORL)-1 receptor. It has attracted much recent attention, as it appears to have antinociceptive effects at the spinal level and is likely to be involved in changes occurring during persistent pain states (inflammation and neuropathy). The central effects of nociceptin indicate a low abuse potential unlike morphine, and clear potential for the development of analgesic alternatives to morphine based on this system. However, sufficiently selective tools for the receptor are lacking; the peptide itself is the only agonist as yet, and the putative antagonist, ([Phe¹ψ(CH₂-NH)Gly²]nociceptin-(1-13)-NH₂), appears to be at best a partial agonist. The apparently paradoxical site-dependent antinociceptive/hyperalgesic effects of this peptide are yet to be resolved.

COX inhibitors

Although a series of selective COX-2 inhibitors are on the market or about to be launched, a key issue is efficacy - how will these agents compare to existing NSAIDs? Thus,

although these agents will have reduced side effects as a result of actions on this form of the enzyme that is induced in damaged tissue and constitutively expressed in the central nervous system, the degree of analgesia they produce is yet unclear. At the meeting, celecoxib (GD Searle & Co), parecoxib (Monsanto Co), and nimesulide (3M Pharmaceutical Division) were reported to be effective in studies on patients with osteoarthritis, back and dental pain and were shown to generally be as effective as ibuprofen, naproxen and Toradol (Roche Bioscience), while showing reduced gastrointestinal side effects.

Substance P

There are discrepancies between the optimistic preclinical data on antagonists of the neurokinin-1 receptor (drugs active at this site have been shown to be effective in a number of models of inflammatory pain), while a number of clinical studies have been negative. Post-operative pain patients have been studied in a number of contexts yet only a trial in dental pain showed efficacy (using the antagonist CP-99994; Pfizer). Reasons are likely to include the poor ability of many of the antagonists to penetrate the CNS, compensatory mechanisms within the tachykinin system, and sufficient residual activity in co-transmitter systems (eg, glutamate and calcitonin gene-related peptide (CGRP)).

Summary

The majority of new drugs on which data were presented at this meeting were improved tools to study transmitter systems with established roles in nociception.

There is considerable interest in a number of new areas where there are few/no drugs as yet to be used as experimental tools. Mention can be made of cytokines and nerve growth factors where using the endogenous mediators and antisera, good evidence has accumulated for roles in both inflammatory and neuropathic pains.

Molecular biology is identifying subtle differences in molecules (receptors, channels, etc) in different pain models in animals and there is much data on the regulation of gene expression. However, this is an area where chemistry and pharmacology are still way behind in being able to provide drugs that target these novel sites precisely. Not surprisingly, much emphasis was put on transgenic techniques, with both knockin and knockout animals being used in behavioral and electrophysiological studies. However, caution was stressed with regard to compensatory mechanisms and very different basal pain responsiveness and analgesic effectiveness was seen in different strains of mice.

Finally, the basic mechanistic studies provide evidence that different pain states and symptoms may have different underlying mechanisms. Although difficult in practice, it should be fruitful for clinical studies and trials to attempt to separate out and quantify different pain symptoms in human studies. This may prevent drugs with particular mechanisms being discarded if their specific actions on selected symptoms were masked in studies where only global estimates of pain are monitored.



Unaltered peripheral excitatory actions of nociceptin contrast with enhanced spinal inhibitory effects after carrageenan inflammation: an electrophysiological study in the rat

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Abstract

Nociceptin (orphanin FQ) is the endogenous agonist of the opioid receptor-like (ORL-1) receptor. The actions of this peptide have been studied extensively at a number of sites with diverse actions being reported. Here, in a rat model of peripheral inflammation, we examine the effects of nociceptin on the responses of dorsal horn neurones when applied directly to the spinal cord and, in separate studies, into the peripheral receptive fields in the hindpaw of the halothane anaesthetized rat. As changes in the receptor density and expression of the message for nociceptin have been reported after inflammation we have compared these actions to previously reported effects in normal animals. The dose-dependent inhibitory actions of nociceptin on C-fibre evoked responses and input (measures of presumed pre-synaptic excitability) are increased 3–4 h after inflammation whereas its inhibitory effects on post-synaptic mechanisms (wind-up) remain unchanged. These inhibitory effects were partly reversible by high doses of naloxone. This increased potency of nociceptin after inflammation is consistent with an increased receptor density in the superficial spinal cord. In contrast, the peripheral administration of nociceptin produced dose-dependent excitations of dorsal horn neurones and a degree of sensitization to mechanical stimuli. This peripheral action was unchanged after inflammation. These diverse site-dependent actions of nociceptin further emphasize the complexities of this novel opioid system. © 2000 International Association for the Study of Pain. Published by Elsevier Science B.V. All rights reserved.

Keywords: Nociceptin/orphanin FQ; Inflammation; ORL-1 receptor; Peripheral nerve; Spinal cord

1. Introduction

With the application of molecular biology techniques to the characterization of receptors, many new receptor genes were identified through homology screening of related and previously cloned receptors. The pharmacological characterization of these novel receptors often lags behind the structural characterization and in many cases the endogenous ligands for the receptors remain unidentified. One such orphan receptor was the opioid receptor-like (ORL-1) receptor (Wick et al., 1994), identified following the cloning of the opioid receptors (μ , δ and κ). Like the opioid receptors, the ORL-1 receptor is a seven trans-membrane spanning receptor, coupled to inhibitory G-proteins (G_i) to activate inwardly rectifying potassium channels and inhibit voltage-gated calcium channels (see Meunier, 1997 for review). Despite a marked structural homology between the ORL-1 receptor and opioid receptors, pharmacologi-

cally, they are quite distinct. The ORL-1 receptor exhibits low affinity for the endogenous opioids, opioid receptor agonists such as morphine, and for opioid receptor antagonists such as naloxone (Wang et al., 1994; Wick et al., 1994). This suggested the existence of a previously unknown endogenous opioid. Nociceptin (orphanin FQ), a heptadecapeptide (FGGFTGARKSARKLANQ), exhibits a high affinity for the ORL-1 receptor and is widely accepted to be the endogenous ligand for this receptor (Meunier et al., 1995; Reinscheid et al., 1995). Functionally, nociceptin has been implicated in many biological processes including pain. Whilst the ORL-1 receptor seems to couple to similar effector mechanisms as the more extensively studied classical opioid receptors, functionally nociceptin has effects that differ from and even oppose those of opioids. In initial behavioural studies, the intracerebroventricular (i.c.v.) injection of nociceptin was found to induce hyperalgesia – an increased sensitivity to noxious stimulation – in mice, using the hot-plate (Meunier et al., 1995) and tail-flick (Reinscheid et al., 1995) tests. However, on closer inspection, the i.c.v. injection of nociceptin was found to induce a

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biphasic response, comprising a brief period of hyperalgesia followed by a prolonged period of naloxone-sensitive analgesia (Rossi et al., 1996). This has been attributed to the fact that the i.c.v. injection route of administration may be stressful enough to induce an opioid-mediated analgesia which outlasts the transient reversal and hyperalgesia caused by nociceptin (Grisel et al., 1996; Mogil et al., 1996). This reversal of opioid-mediated analgesia by nociceptin may occur at the site of the periaqueductal grey (PAG), as terminals in the PAG have been shown to contain both the nociceptin peptide and receptors. Furthermore, Morgan et al. (1997) have demonstrated an inhibition of PAG output neurones by nociceptin – a mechanism which could explain its reversal of stress-induced opioid-mediated analgesia.

Supraspinal nociceptin does not have anti-opioid effects in motivational paradigms, although abundant in the locus coeruleus, nociceptin does not precipitate a withdrawal syndrome when injected into the lateral ventricle in morphine-dependent rats (Tian et al., 1997). In fact, i.c.v. injection of nociceptin was found to be motivationally neutral in the rat place preference/aversion test (Devine et al., 1996) indicating that the peptide lacks abuse liability.

At the level of the spinal cord, nociceptin behaves as an analgesic. Previous electrophysiological studies have shown that spinal administration of nociceptin inhibits the C-fibre-dependent wind-up and post discharge of dorsal horn neurones, whilst sparing the A-fibre evoked responses (Stanfa et al., 1996). Other studies have supported this with evidence that C-fibre evoked responses are inhibited in preference to A-fibre-evoked responses (Faber et al., 1996), demonstrating a tendency towards selective effects on noxious evoked activity and hyperalgesia (Hao et al., 1998).

Acute pain rarely presents as a clinical problem; clinically relevant pain states include more persistent inflammatory- and neuropathic-related pain. Therefore in order to conduct clinically relevant studies, it is necessary to employ models of these more persistent pain states. Recent reports have suggested that there is plasticity within the nociceptin system following peripheral inflammation. Both increased levels of the nociceptin gene transcript in the dorsal root ganglia (DRG) (Andoh et al., 1997) and increased nociceptin binding in the superficial laminae of the spinal cord (Jia et al., 1998) have been found.

The present study investigates the effect of intrathecal (i.t.) nociceptin on spinal neuronal responses in the anaesthetized rat after induction of carrageenan inflammation, and compares these effects to those of nociceptin in normal animals using the same experimental set-up (Stanfa et al., 1996). This should reveal any plasticity in the nociceptin system in a model of clinically relevant pain, and go some way towards developing alternatives to the existing opioid analgesics.

The ORL-1 receptor is detected in the DRG (Wick et al., 1994) so is presumably synthesized in the cell body of

primary afferent fibres from where receptors can migrate not only to central terminals in the spinal cord, but also to peripheral terminals. To determine whether nociceptin receptors are present and functional on peripheral terminals of primary afferent nerve fibres, nociceptin was administered into the peripheral receptive field of dorsal horn neurones in both normal and in carrageenan-injected animals.

2. Methods

2.1. Surgical procedures

Male Sprague–Dawley rats (200–225 g) were anaesthetized with 3% halothane in 66% N₂O:33% O₂, and subsequently maintained at 1.5–1.8% halothane. The animals were secured in a stereotaxic frame, the vertebrae rostral and caudal to vertebrae L1–L3 were clamped and a laminectomy performed to expose the spinal cord. A parylene-coated tungsten electrode was then descended into the cord and recordings made from neurones in segments L4–L5, the depth of the recording site was noted from the microdrive readings.

2.2. Electrically-evoked recordings

Extracellular recordings were made from dorsal horn neurones receiving C- and A-fibre input from the skin of the ipsilateral hindpaw, identified by their ability to respond to both noxious and innocuous stimuli (pinch and touch). Neuronal responses were elicited by transcutaneous electrical stimulation given in the centre of the receptive field of the neurone, at three times the threshold current required to elicit a C-fibre evoked response. At 10-min intervals, a train of 16 stimuli was given at 0.5 Hz and post-stimulus histograms constructed. The evoked responses were separated, according to latency, into A β -fibre evoked activity (0–20 ms); A δ -fibre (20–90 ms); C-fibre (90–300 ms) and post discharge of the neurone (300–800 ms). Responses evoked by the first stimulus of the train are referred to as 'input' and consist of the number of action potentials (90–800 ms) evoked by this stimulus. Wind-up, a measure of the enhanced neuronal response elicited by repeated stimulation, was quantified as the difference between the total number of action potentials evoked by the 16 stimuli (90–800 ms) and the input $\times$ 16.

After carrageenan inflammation had developed (3 h) a series of control responses were measured and then cumulative doses of nociceptin (5, 50, 125, 225 μ g) in 50 μ l saline, were applied to the spinal cord, and neuronal responses followed for 40–60 min per dose. After the top dose had been applied, the opioid receptor antagonist naloxone (10 and/or 50 μ g in 50 μ l saline) was applied spinally in an attempt to reverse the effect of nociceptin. All neuronal responses were expressed as a percentage of the control responses, and statistical analysis performed using the

Kruskal–Wallis (unpaired non-parametric ANOVA) test on the percentages. The effects of nociceptin on neuronal responses after induction of carrageenan inflammation were compared to the effects of nociceptin in normal animals, using the unpaired Mann–Whitney test to compare the percentages of control for each dose in each experimental group.

2.3. Peripheral administration of nociceptin

Neurons were initially characterized on the basis of their electrical-evoked activity as described above in Section 2.2, before peripheral injections of a small volume (20 μ l) of either saline or nociceptin into the receptive field of the neurone.

A 29-G insulin needle was inserted intradermally, and left for 5 min for any activity evoked by the procedure to return to baseline levels. Twenty microlitres of 0.9% saline was then administered, and the number of action potentials evoked in 60 s was recorded. Leaving the needle in place the injection was repeated after 5 min and the response counted and recorded. The needle was then removed, and after activity had returned to baseline levels, von Frey filaments (9 and 30 g) were then applied to bending force for 10 s and the evoked responses counted over 10-s periods. A needle with syringe containing 0.01 μ g nociceptin solution was then inserted as before and at 5-min intervals, two injections were made and responses recorded, followed by the von Frey filament stimuli. The saline administrations and von Frey stimuli were repeated before each successive dose of nociceptin (0.05, 0.1, 2.0 μ g) and served as a control readings. Due to the problems of multiple injections into a small area of tissue, it was not possible to test naloxone antagonism. Mean nociceptin-evoked responses (action potentials evoked in 60 s) are shown with the corresponding mean saline-evoked responses. Paired, two-tailed Student's *t*-tests were used to show any difference between the saline and nociceptin-evoked responses. Kruskal–Wallis (unpaired non-parametric ANOVA) test was used to demonstrate any overall effect of either saline or nociceptin. Values of $P < 0.05$ were taken as significant, and all data are expressed as mean $\pm$ SEM.

2.4. Carrageenan

First described by Hargreaves et al. (1988) the carrageenan model of inflammation is used to produce a state of inflammation which is stable from 3 h after subcutaneous injection in the plantar surface of the hindpaw. Here, 100 μ l of 2% carrageenan in saline solution was injected into the plantar surface of the ipsilateral hindpaw. In the case of electrical evoked responses, the injection took place after a neurone had been found: controls were taken prior to carrageenan, the evoked neuronal responses through the 3-h period were followed, at 3 h controls were again taken and the drug application commenced. In the case of peripheral administration of solutions, the injection took place as soon

as the animal was under anaesthesia, and peripheral injection of test solutions took place at least 3 h after carrageenan in all cases.

3. Results

3.1. Intrathecal nociceptin

The effect of spinally applied nociceptin (5, 50, 125 and 225 μ g) was studied on nine dorsal horn neurones (mean depth 717 ± 61 μ m from the cord surface), 3 h after the induction of carrageenan inflammation in the ipsilateral hindpaw.

The application of nociceptin caused a significant inhibition of the C-fibre evoked responses (Kruskal–Wallis, $P = 0.0268$, Fig. 1A). A maximum inhibition was achieved with 125 μ g of nociceptin ($35.4 \pm 12.5\%$ control, $n = 6$); the top dose of 225 μ g reduced neuronal responses to $39 \pm 15\%$ of control ($n = 3$). The application of 10 and 50 μ g naloxone returned the C-fibre evoked responses to $60 \pm 10\%$ control ($n = 3$) and $63.5 \pm 9\%$ control ($n = 2$) respectively, although significance was not established. Input was also significantly inhibited by nociceptin (Kruskal–Wallis, $P = 0.0272$, Fig. 1B); 225 μ g nociceptin reduced the input to $18.2 \pm 14.6\%$ of the control response. Again significance could not be established, although 10 and 50 μ g naloxone returned the input to $34 \pm 21\%$ control ($n = 3$) and $40 \pm 1.39\%$ control ($n = 2$), respectively.

Wind-up and post discharge were both inhibited by nociceptin ($P = 0.0255$ and 0.0217 respectively, Fig. 1C,D). Maximum effects were seen with 225 μ g in both cases, reducing responses to $28.3 \pm 12\%$ control and $12.1 \pm 11.5\%$ control, respectively ($n = 3$ in both cases). Top dose naloxone returned wind-up to $62 \pm 4\%$ control and post discharge to $50 \pm 36\%$ control, $n = 2$ in both cases and significance could not be established. Neither the A δ - or A β -fibre evoked responses were affected by spinal application of nociceptin; this suggests a selective action on noxious-evoked activity, agreeing with the effects of nociceptin previously seen in normal animals (Stanfa et al., 1996).

Comparing these actions of nociceptin in animals in which peripheral inflammation has been induced, to the effects of nociceptin in normal animals reveals several differences.

The inhibitory effect of nociceptin on the C-fibre evoked response is greater in carrageenan-injected rats than in normal animals, the dose-response curve for nociceptin in carrageenan-injected rats lies to the left of that obtained from normal animals (Fig. 1A, approx. 4.5-fold shift). The difference between the dose-points is significant (unpaired, two-tailed Mann–Whitney) at 5 μ g ($P = 0.0456$) and 50 μ g ($P = 0.0064$). So, whereas in normal animals 225 μ g nociceptin reduced C-fibre evoked responses to $62 \pm 15\%$ control ($n = 8$); in inflamed animals the inhibition reached with 225 μ g was much greater, reducing C-fibre evoked

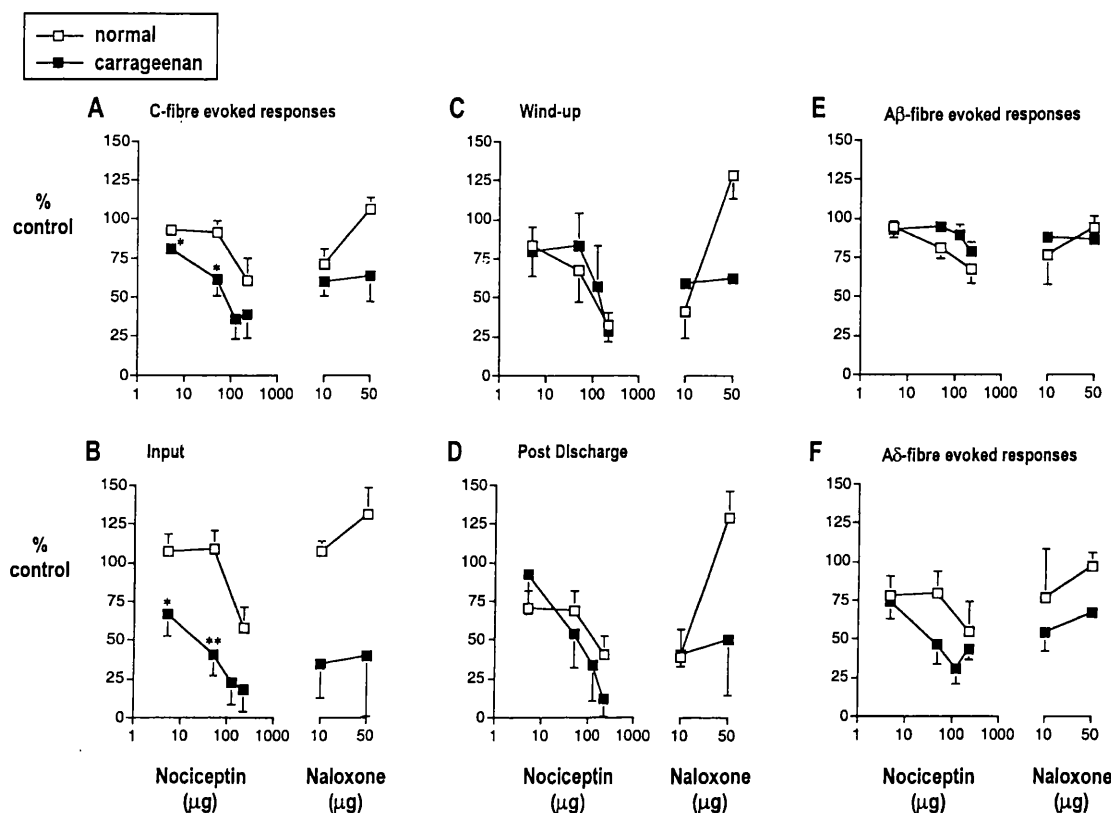


Fig. 1. Effect of spinal administration of nociceptin (5, 50, 125, 225 µg) on electrical-evoked responses of dorsal horn neurones. Carrageenan-injected animals were studied here, data for normal animals taken from previous work by Stanfa et al. (1996). Intrathecal naloxone (10 and 50 µg) partially reverses the inhibitory effects of nociceptin. All data are expressed as mean % control $\pm$ SEM. Significant difference between groups (normal vs. inflamed) is indicated (* $P \leq 0.05$; ** $P \leq 0.01$).

responses to $39 \pm 15\%$ control ($n = 3$). The difference is even more marked for inhibition of input, a parameter that was not significantly affected by nociceptin in normal animals (Fig. 1B, approx. 20-fold leftward shift). Nociceptin (225 µg) in normal animals reduced the input to $58.25 \pm 13.3\%$ control, whilst after inflammation the same dose reduces input to $18.2 \pm 14.6\%$ control.

In contrast, the inhibitory effects of nociceptin on wind-up and post discharge were not different in carrageenan-injected rats and normal animals. Fig. 1C,D shows the dose response curves correspond almost exactly. So whilst nociceptin still inhibits wind-up and post discharge, the effect is no greater in carrageenan-injected animals than in normal animals. The lack of effect on Aδ- and Aβ-fibre evoked responses is evident in both experimental groups (Fig. 1E,F).

Interestingly, the inhibitory effects of nociceptin on all parameters seem to be less sensitive to naloxone reversal after inflammation (Fig. 1).

3.2. Peripheral nociceptin

When injected into the receptive field of 16 dorsal horn

neurones (mean depth 846 ± 53 µm from cord surface) in normal animals, nociceptin evoked marked responses from 15 of these neurones. The neurone that did not respond to nociceptin was discarded from all subsequent analysis. Saline (0.9%) injected in the same manner, also caused a small amount of activity in most cases. The number of action potentials evoked within 60 s of injection was recorded, and the mean action potentials per 60 s for all cells used is shown in Fig. 2A. Saline injection caused no overall significant change in neuronal activity, whereas nociceptin (0.01, 0.05, 0.1 and 2.0 µg) caused a significant increase in mean number of action potentials evoked in 60 s (Kruskal–Wallis, $P = 0.0002$). The mean number of action potentials evoked over 60 s by each dose of nociceptin was significantly greater than the corresponding saline-evoked response (paired, two-tailed Student's *t*-tests): 0.01 µg, $P = 0.0343$, $n = 19$; 0.05 µg, $P = 0.0129$, $n = 14$; 0.1 µg, $P < 0.0001$, $n = 18$; 2.0 µg, $P < 0.0001$, $n = 9$.

In carrageenan-injected animals, nociceptin evoked responses from all 10 neurones (mean depth 937 ± 44 µm). Each dose of nociceptin evoked a greater number of action potentials than the corresponding saline injection: 0.01 µg, $P = 0.0115$, $n = 16$; 0.05 µg, $P = 0.0002$,

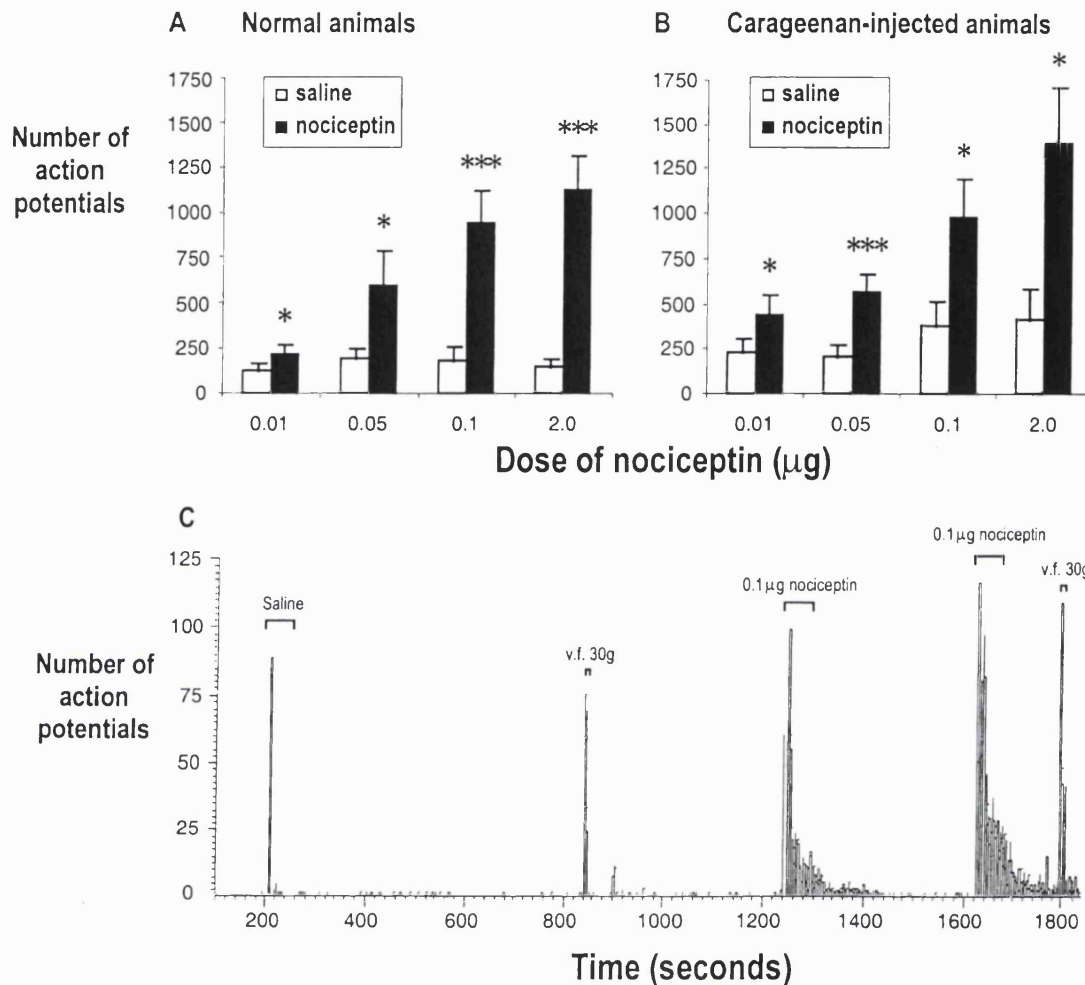


Fig. 2. Effects of peripheral administration of nociceptin (0.01, 0.05, 0.1, 2.0 μg) in normal animals (A) and after induction of inflammation with carrageenan (B). Data are expressed as mean number of action potentials evoked within 60s of injection $\pm$ SEM. Significant difference between nociceptin-evoked responses and the preceding saline control is indicated (* $P \leq 0.05$, ** $P \leq 0.01$; *** $P \leq 0.001$). Panel C is an example trace showing responses of one individual neurone to administration of saline, nociceptin and application of von Frey hairs.

$n = 17$; 0.1 μg , $P = 0.0124$, $n = 14$; 2.0 μg , $P = 0.0230$, $n = 8$. In these animals there was a significant change in the saline-evoked responses as the experiment progressed (Kruskal–Wallis, $P < 0.00001$) and the nociceptin-evoked responses also increased (Kruskal–Wallis, $P < 0.00001$). This increase in the saline controls can be seen in Fig. 2B, yet when compared to normal animals, there was no significant difference in the magnitude of either the saline- or the nociceptin-evoked responses between normal animals and carrageenan-injected animals.

3.3. von Frey-evoked responses

In normal animals, von Frey 9 g-evoked responses after 0.05 and 0.1 μg nociceptin were significantly larger than the responses evoked after saline controls (Fig. 3A) ($P = 0.0432$, $n = 3$; $P = 0.0451$, $n = 5$, respectively; paired 2-tailed

Student's t -test). No differences between the von Frey 30 g-evoked responses after saline and after nociceptin were observed (Fig. 3C). After inflammation, neither the 9 g nor 30 g von Frey stimuli-evoked responses were altered after nociceptin compared to the saline controls (Fig. 3B,D). Comparing von Frey 9 g evoked responses in normal and inflamed animals (Fig. 3A,B) there was no significant difference in the magnitude of these responses either after saline or after nociceptin – thus inflammation did not increase the neuronal responses to the stimulus in either the control situation or after nociceptin. The same is true for responses evoked by the 30 g von Frey filament. A general increase in all these mechanical-evoked responses is evident as the experiment progresses in both experimental groups, but did not reach significance in any case (Kruskal–Wallis ordinary ANOVA). Thus, overall, nociceptin sensitized the responses to low intensity mechanical stimuli in normal animals. After

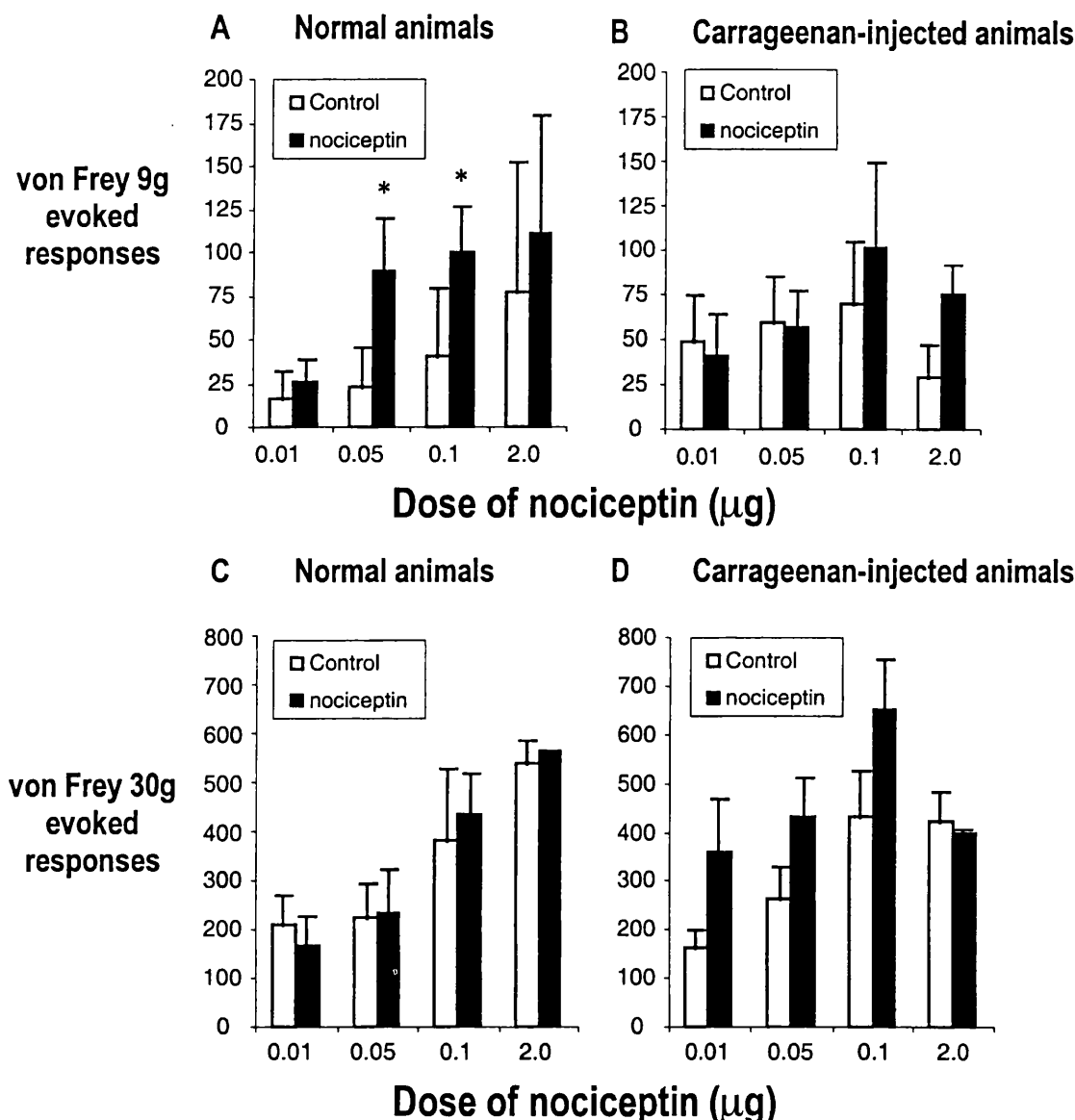


Fig. 3. Mechanical evoked responses after saline control (□) and after nociceptin (■), in normal (A,C) and inflamed animals (B,D). Both low-intensity (von Frey 9 g) and high-intensity (von Frey 30 g) mechanical stimuli were used. Data are expressed as mean number of action potentials evoked over 10 s, $\pm$ SEM. Significant difference between responses evoked after saline control and after nociceptin is indicated (* $P \leq 0.05$).

inflammation, this effect may be masked by the modestly increased baseline responses, evidenced by the augmented responses to the applied stimulus after saline alone.

4. Discussion

4.1. Spinal nociceptin

From this study it is evident that exogenous nociceptin, applied to the spinal cord, elicits antinociceptive effects and that this inhibitory effect on spinal nociceptive transmission is increased in the presence of inflammation. The most

marked action of nociceptin after inflammation was inhibition of C-fibre evoked responses and input, which were affected to a much greater extent than in normal animals (Fig. 1). This suggests an enhanced action of nociceptin at predominantly pre-synaptic sites, and this is likely as the receptor is synthesized in the DRG (Wick et al., 1994). A pre-synaptic mechanism of action is supported by evidence that nociceptin can inhibit release of sensory neuropeptides from primary afferent terminals (Helyes et al., 1997), most probably by increasing an inwardly rectifying K^+ conductance (Vaughan and Christie, 1996) and opposing transmitter release (Lai et al., 1997). Jia et al. (1998) saw increased

binding of nociceptin in superficial laminae of the spinal cord 4 days after generation of persistent inflammation with complete Freund's adjuvant (CFA) – increases in receptor number after inflammation would explain our findings of increased potency of nociceptin after inflammation. An increased pre-synaptic receptor density following inflammation would allow the same dose of nociceptin to cause a more profound inhibition of transmitter release than in normal animals.

The effects we saw were very rapid in onset (3 h), Jia et al. (1998) did not see increases in receptor number until 4 days after CFA-generated inflammation. Carrageenan inflammation develops more rapidly than that induced with CFA and Andoh et al. (1997) have described increased levels of mRNA message for the nociceptin peptide precursor 3 h after carrageenan inflammation, suggesting that changes within the system can occur rapidly.

This increased expression of prepronociceptin in the DRG after inflammation was suggested by Andoh et al. (1997) to contribute to the hyperalgesia that develops during inflammation, but the peptide more likely acts to attenuate the increased activity in nociceptive circuits. This anti-nociceptive effect of nociceptin is supported by behavioural work; i.e. administration of nociceptin induces analgesia in the rat tail-flick test and potentiates the analgesic effects of morphine (Tian et al., 1997) and exerts anti-hyperalgesic and anti-allodynic effects in the rat after nerve damage and peripheral inflammation (Hao et al., 1998). As shown here and discussed in the introduction the peptide clearly inhibits sensory transmission at the spinal level. In fact, the increased presence of the peptide in central terminals after inflammation suggests that nociceptin may begin to be released by primary afferents as a negative feedback mechanism and act at the increased number of receptor sites in the superficial dorsal horn to return spinal nociceptive transmission to baseline. The presence of nociceptin in primary afferents after inflammation is clear, but there is debate as to whether nociceptin is present in primary afferents in normal animals. Andoh et al. (1997) detected no expression in the dorsal root ganglion (DRG) in normal animals. Dorsal rhizotomy does not detectably affect nociceptin-like immunoreactivity in the spinal cord (Riedl et al., 1996) suggesting nociceptin is not expressed in primary afferents in normal animals although Saito et al. (1996) detected a putative nociceptin precursor peptide in small diameter primary afferent neurones in dorsal root ganglia of adult mice. It remains unclear therefore whether nociceptin is present in primary afferent fibres in normal animals. Nociceptin originating from intrinsic spinal neurones is a more likely source of the peptide in normal animals. Nociceptin-like immunoreactivity is detected in normal animals in the superficial layers of the spinal cord (Lai et al., 1997) and release of nociceptin-like substance has been detected from rat dorsal horn (Williams et al., 1998). This presence in and release from intrinsic spinal cord neurones adds support to the idea that nociceptin has some basal modulatory role in

sensory transmission. Without a reliable ORL-1 receptor antagonist, it will remain debatable and antagonists synthesized as yet have had mixed agonist/antagonist actions (Butour et al., 1998; Carpenter and Dickenson 1998; Xu et al., 1998) and have not helped elucidate any basal role of nociceptin.

The fact that exogenously applied nociceptin is anti-nociceptive in the spinal cord, and that the system also exhibits plasticity in inflammation as demonstrated here and by its effectiveness in behavioural studies after carrageenan inflammation (Yamamoto et al., 1997; Hao et al., 1998) and after nerve injury (Hao et al., 1998) indicates it may be a very useful target for novel analgesics. However, there is a discrepancy in the above studies with regard to whether nociceptin has increased or decreased effectiveness after carrageenan (Yamamoto et al., 1997; Hao et al., 1998); here we clearly found the former.

An increased anti-nociceptive effect of opioids has been reported after inflammation in both behavioural and electrophysiological studies (see Stanfa and Dickenson, 1995 for review). This altered potency of opioids most likely occurs not through a receptor level interaction, but via functional anti-opioid activity of the peptide CCK which is downregulated during inflammation. It is possible therefore, that the increased potency of spinal nociceptin that we see here may be mediated not only by increased receptor density but also through interaction with other spinal systems.

Interestingly, the reversibility of nociceptin-mediated inhibitions by naloxone at the spinal level was greater in the normal animals than in the group of animals studied after inflammation. This was true whether the responses were inhibited to a greater extent (C-fibre evoked responses and input) by nociceptin after inflammation or were inhibited to the same extent (post-discharge and wind-up). Thus, this differential naloxone effectiveness was not dependent on the degree of inhibition produced by nociceptin. Whether this differential reversal reflects receptor changes after inflammation remains to be determined.

4.2. *Peripheral nociceptin*

As the ORL-1 receptor is synthesized in the DRG, it follows that these bipolar primary afferent fibres may have functional receptors not only at their central terminals, but also at the terminals in the periphery. Work presented here shows that receptors are present and functional – whilst nociceptin is inhibitory in the spinal cord, administration in the periphery evoked clear excitatory responses from dorsal horn neurones. Not only did we observe neuronal firing elicited by administration of peripheral nociceptin but the peptide sensitized responses to low intensity mechanical stimuli. This is not the only study to find nociceptin eliciting a nociceptive response when administered in the periphery. Inoue et al. (1998) recently found that administration of extremely low doses of nociceptin into the hind-paw evoked nociceptive responses in awake mice and that

these flexor responses were dependent on release of substance P. The doses of nociceptin we used to evoke responses are equivalent to 5 pmol to 1 nmol – i.e. 10^3 – 10^6 times more than the amounts used by Inoue et al. (1998) to elicit behavioural responses in mice. This may be explained by the different paradigms used; behavioural tests record the threshold responses of the complete system to stimuli whereas the powerful excitatory responses we recorded from neurones may represent what would be behaviourally suprathreshold stimulation. Additionally, the difference in size between mice (as used in the original study) and rats (as we use here) may be a factor.

This excitatory action of nociceptin is unexpected as the ORL-1 receptor couples to inhibitory intracellular processes. The apparently pro-nociceptive activity of nociceptin supraspinally has been explained by a functional inhibition of analgesia – here the receptor simply inhibits neurones which subserve inhibitory (antinociceptive) functions. Direct inhibitory effects of activation of the receptor are via inhibition of adenylate cyclase and PLC, and via actions on ion channels to hyperpolarize and reduce excitability in cells expressing the receptor (see Meunier, 1997 for review). Whilst the dual effects of the peptide in the CNS can be explained by direct inhibitory actions, the peripheral excitatory actions of the peptide must be through different mechanisms. There is a precedent for bidirectional effects of opioids, in that mu opioid receptors have been reported to couple to both excitatory and inhibitory intracellular processes (Crain and Shen, 1990); opioid receptors on DRG neurones have been shown to couple via both G_s and G_i proteins (Shen and Crain, 1990) and the same could be true for the ORL-1 receptor on primary afferent fibres. However the flexor-reflex responses evoked by nociceptin in the study by Inoue et al. (1998) were blocked by pertussis toxin, showing that the receptor is still coupled to G_i – exactly how this leads to the reported substance P release is unclear. Another possibility is that the ORL-1 receptor is not a single entity and the peripheral receptor may be different from that in the CNS although nociceptin appears to be an agonist at both. Support for this premise may be provided by the observations that [Phe¹ψ(CH₂–NH)Gly²]nociceptin-(1-13)-NH₂, characterized as an ORL-1 receptor antagonist in peripheral tissues, behaves as an agonist in the spinal cord (Carpenter and Dickenson 1998; Xu et al., 1998). This may suggest the existence of different receptor sub-types – either originating from different genes or as a result of splice variants – that can couple to different intracellular mechanisms. Classical opioids, when administered in the periphery are inhibitory, but these peripheral antinociceptive actions of opioids are only revealed in inflammation and are observed before increased peripheral receptor density is evident. This effectiveness after inflammation may be due to changes around the peripheral nerve endings which facilitate access of the opioid to the receptor (see Stein, 1994 for review). Here however, the excitatory effects of nociceptin are evident

in normal animals so this large peptide appears able to reach its receptor without difficulty. That the actions are mediated via the receptor on peripheral nerves and not at other peripheral sites is quite clear from the work of Inoue et al. (1998). Antisense oligonucleotides to the receptor applied to the spinal cord for the period before testing abolished responses – if receptors on peripheral cells such as mast cells and macrophages and vascular cells were responsible for the action of nociceptin the flexor behaviour would remain after spinal antisense treatment. Release of nociceptin from these cell types however, is possible and is a potential source of the peptide in the periphery in addition to release from sensory afferent nerve endings.

That we observed no difference in the effect of nociceptin 3 h after carrageenan inflammation is not surprising – if access to the receptor is not an issue as it is with opioids then any change in peripheral activity will depend on a change in receptor density, which will not be apparent until some days after increased synthesis in the DRG. If transport of the nociceptin receptor is analogous to the opioid receptors it will take 3–4 days after inflammation for peripherally-directed axonal transport to convey these newly synthesized receptors to peripheral endings of sensory afferents (Hassan et al., 1993).

It is rapidly becoming clear that nociceptin and the ORL-1 receptor genuinely comprise a novel opioid-like transmitter system. Aspects of the function of this system resemble those of classical opioids in that spinally the consensus is that this peptide elicits antinociceptive effects. The supraspinal anti-opioid actions of nociceptin are not without precedent – there is a literature on the ability of kappa opioids to physiologically antagonize the effects of mu opioid agonists both spinally (Dickenson and Knox, 1987) and supraspinally (Pan et al., 1997). However, the peripheral pro-nociceptive actions of this peptide are surprising, novel and have not been reported with any other opioid. Indeed when considering the evidence that nociceptin appears to have low dependence liability (Devine et al., 1996) and a role in hearing (Nishi et al., 1997) manipulation of this receptor system may have important therapeutic consequences in fields other than pain control. The various and contrasting central and peripheral effects of the nociceptin peptide on pain processing beg the question as to what is the relative importance and balance of these diverse effects on nociception. Only a systemically active and stable agonist of the nociceptin receptor, able to target all sites simultaneously, will reveal the analgesic potential of drugs based on nociceptin. Along the same lines, further advances in the design of antagonists will be required to dissect out the true physiological role of this peptide.

Acknowledgements

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Neuronal inhibitory effects of methadone are predominantly opioid receptor mediated in the rat spinal cord *in vivo*

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This study aims to assess whether the antinociceptive actions of methadone are mediated solely through opioid mechanisms, or whether its reported affinity for NMDA receptors has physiological relevance *in vivo*. Methadone is a μ -opioid receptor agonist reported to relieve pain unresponsive to other opioids. It is a racemic mixture comprising *d*- and *l*-optical isomers; the *d*-isomer has a lower affinity for opioid receptors, and both also exhibit NMDA receptor binding, likely to indicate antagonist activity. *d*-Methadone is antinociceptive in behavioural studies via non-opioid mechanisms, which could include functional NMDA receptor-blocking activity. Here we investigate the ability of *d*- and *dl*-methadone to inhibit noxious and innocuous electrically-evoked responses of dorsal horn neurones in the anaesthetized rat.

Racemic methadone (5, 25, 50, 250 μ g) applied spinally, dose-relatedly inhibited the C-fibre evoked response, input and wind-up of the neurones, with a profile resembling that of morphine. *d*-Methadone (5, 25, 50, 250, 500 μ g) was also inhibitory, although less potent by a factor of between 13 and 48 depending on the neuronal measure; its profile of inhibition resembled that of the racemic mixture rather than an NMDA receptor antagonist. Both compounds had minimal effects on A β -fibre-evoked activity. The inhibitory effects of both *d*- and *dl*-methadone on noxious-evoked activity were naloxone reversible. The naloxone reversibility of *d*-methadone inhibitions is best interpreted as indicative of a purely opioid mechanism of action. However, the ability of naloxone to reverse the effects of *d*-methadone may also reflect a degree of synergy between weak NMDA antagonist and opioid agonist activity. © 2000 European Federation of Chapters of the International Association for the Study of Pain

INTRODUCTION

Methadone (a racemic mixture of *d*- and *l*-optical isomers) has long been recognized as a μ -opioid receptor agonist and is used clinically both as a replacement for heroin in withdrawal therapy and in the treatment of pain. Due to factors such as a long and unpredictable

half-life and variable pharmacokinetics, the dosage for each individual has to be carefully titrated to avoid overdose, especially in opioid naïve patients (Fainsinger *et al.*, 1993). The use of methadone in the treatment of pain is therefore usually limited to situations where the patient has become unresponsive to the opioids of choice. Methadone is atypical as an opioid, in that tolerance to its analgesic effects is slow to develop (Inturrisi *et al.*, 1990), and it has been reported to have incomplete cross-tolerance with other opioid agonists (Crews *et al.*, 1993; Thomas & Bruera, 1995). The pharmacological basis for the differences between methadone and other opioids such as

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morphine is unclear, but has been attributed in part to the affinity of both the *d*- and *l*-isomers of methadone for the non-competitive site of the NMDA receptor in rat brain membranes (Gorman *et al.*, 1997), in common with other opioids such as ketobemidone and dextro-propoxyphene and the racemic mixture (Ebert *et al.*, 1995). Ebert *et al.* (1995) not only show that the racemic mixture of methadone binds to the channel at the MK-801 site, but that it is a non-competitive antagonist of the NMDA receptor able to block responses to NMDA itself in both the cortex and spinal cord. Gorman *et al.* (1997) show that the separate isomers bind to the non-competitive site and displace MK-801—the inference is that both the isomers are non-competitive antagonists at the NMDA receptor able to block the ion channel of the receptor.

The NMDA receptor, an excitatory ionotropic receptor for glutamate, is involved in many CNS (central nervous system) functions with an important role in the spinal transmission of information in nociceptive systems (see Dickenson, 1990). In addition to its direct involvement in nociception, NMDA receptor activation can lead to a reduced sensitivity to opioids. This arises simply as a result of shifting the balance between excitatory and inhibitory pathways towards the excitatory; a higher dose of the opioid is now required to produce adequate analgesia (Dickenson, 1994). Hence, if methadone does have dual activity as an NMDA receptor antagonist and an opioid, this could explain the somewhat different functional profile of this drug compared to other opioids such as morphine. In support of this, several NMDA receptor antagonists have been shown to attenuate, but not abolish, the development of analgesic tolerance to morphine (Tiseo & Inturrisi, 1993; Elliott *et al.*, 1994). In addition, a marked synergy is observed between low doses of NMDA receptor antagonists and threshold doses of morphine (Chapman & Dickenson, 1992), the combination giving greater inhibition of neuronal responses than either drug alone. Consequently, a combination of opioid receptor activation and block of the NMDA receptor would be a desirable property of an analgesic; this alliance may exist in methadone.

As the opioid activity of methadone is predominant in the *l*-isomer whilst the *d*-isomer is weaker as an opioid by approximately 40-fold (Scott *et al.*, 1948), it would be interesting to determine whether the *d*-isomer possesses *in vivo* activity at the NMDA receptor. A recent behavioural study has shown *d*-methadone to have naloxone insensitive antinociceptive effects in the rat (Shimoyama *et al.*, 1997). Here, we have used an electrophysiological approach to compare the effects of *d*-methadone and racemic (*dl*)-methadone on evoked responses of spinal cord neurones to high intensity peripheral stimulation in the anaesthetized rat. The sensitivity of these effects to naloxone reversal was used as a criterion to distinguish opioid from NMDA receptor-mediated actions. This will reveal the potential contribution of NMDA receptor blocking actions to the well documented analgesic activity of methadone.

MATERIALS AND METHODS

The techniques used here have been described more fully in Dickenson *et al.* (1991). Male Sprague–Dawley rats (200–250 g) were anaesthetized with 2–3% halothane (in 66% N₂O and 33% O₂) and subsequently maintained at 1.8% halothane. Core temperature of the animal was monitored using a rectal thermometer probe coupled to a heating blanket. At the end of the experiment, animals were terminated with overdose of anaesthetic. A laminectomy was performed to expose the segments L4–L5 of the spinal cord, and a parylene-coated tungsten electrode was descended into the dorsal horn using an Epson Stepper device. The depth of the recording site was noted from the microdrive readings. Extracellular recordings were made from single dorsal horn neurones receiving C- and A-fibre input from the skin of the hindpaw, identified by their ability to respond to both noxious and innocuous stimuli (pinch and touch). Neuronal responses were elicited by transcutaneous electrical stimulation given in the centre of the receptive field of the neurone in the ipsilateral hindpaw, at three times the threshold current required for C-fibre evoked activity. At

10-min intervals, tests consisting of a train of 16 stimuli (2-ms-wide pulses at 0.5 Hz) were carried out and post-stimulus histograms constructed. These evoked responses were separated, according to latency, into A β -fibre evoked activity (0–20 ms post-stimulus); A δ -fibre evoked activity (20–90 ms); C-fibre evoked activity (90–300 ms) and post-discharge of the neurone (300–800 ms). The neuronal response evoked by the first stimulus of the train is referred to as 'input' and consists of the number of action potentials (90–800 ms) evoked by this stimulus. Wind-up, a measure of the enhanced neuronal response elicited by repetitive stimulation and an NMDA receptor dependent process, was quantified as the difference between the total number of action potentials produced by the 16 stimuli (90–800 ms), and the input $\times$ 16. Thus, the measure of wind-up includes both the enhanced C-fibre evoked responses and the post-discharge generated as wind-up develops.

After these evoked responses had stabilized, they were averaged to give control values. Cumulative doses of *d*-methadone (5, 25, 50, 250 and 500 μ g) or *dl*-methadone (5, 25, 50 and 250 μ g) in 50 μ l saline, were then applied to the exposed spinal cord into a 'well' formed by the laminectomy, which held the 50 μ l volume of drug. Evoked neuronal responses were followed for 40 min for each dose, after which the solution was removed and the next dose applied. Statistical analyses of drug effects as compared to control were performed using multiple paired *t*-test comparisons with the Bonferroni correction. Comparisons between the groups (*d*- vs, *dl*-methadone) were performed using unpaired *t*-tests: no correction was necessary. Values of $p \leq 0.05$ were considered significant. Data are presented as mean $\pm$ SEM.

The effects produced by the top dose of methadone were challenged at 40 min with 1 μ g naloxone ($n = 9$ following 500 μ g *d*-methadone and $n = 6$ following 250 μ g *dl*-methadone) and/or 5 μ g naloxone ($n = 12$ following *d*-methadone and $n = 8$ following *dl*-methadone). Both doses of naloxone were tested where possible. The term 'naloxone reversal' is used when the application of naloxone caused a significant increase in the number of action potentials from

the maximum inhibition seen, using paired, one-tailed *t*-tests with the Bonferroni correction.

RESULTS

The effects of spinal *d*-methadone were studied on 18 dorsal horn neurones (mean depth 684 ± 42 μ m from cord surface) and *d*-methadone was inhibitory, preferentially reducing the C- and A δ -fibre evoked responses, input, post-discharge and wind-up, whilst the A β -fibre evoked responses were not significantly affected (Fig. 1). C-fibre-evoked responses were significantly inhibited by 250 μ g *d*-methadone ($n = 9$, Bonferroni-corrected p -value ≤ 0.0005) and the maximum effect was seen with 500 μ g *d*-methadone ($n = 14$, Bonferroni-corrected p -value ≤ 0.001) (Fig. 1A).

The input, post-discharge and wind-up were all significantly inhibited by 500 μ g *d*-methadone ($n = 14$ and Bonferroni-corrected p -values ≤ 0.01 , ≤ 0.001 and ≤ 0.001 , respectively) (Figs 1B and 1C). The A δ -fibre evoked responses were significantly inhibited by the top dose (500 μ g) of *d*-methadone ($n = 13$, Bonferroni-corrected p -value ≤ 0.01), whilst the A β -fibre evoked responses were not changed from control values (Fig. 1D). Naloxone (1 μ g) reversed the effects of 500 μ g *d*-methadone on all modalities apart from wind-up, which was reversed by 5 μ g naloxone (Fig. 1).

An example of the effect of *d*-methadone on the wind-up of an individual neurone is shown in Figure 2A. Here, dose-related reduction of input and wind-up is seen, although low doses that reduce the input have little effect on wind-up. Only the top dose of 500 μ g *d*-methadone begins to reduce wind-up and somewhat reduces the steepness of the curve. Naloxone (5 μ g) almost completely reversed the effect of spinal *d*-methadone on this neurone.

The effects of racemic (*dl*-) methadone (5, 25, 50 and 250 μ g) were studied on 10 dorsal horn neurones (mean depth = 639 ± 74 μ m from cord surface). C-fibre, A β -fibre and A δ -fibre evoked responses and wind-up were all significantly inhibited by 25 μ g *dl*-methadone and above ($n = 8$ and Bonferroni-corrected p -values ≤ 0.01 , 0.01, 0.05 and 0.01, respectively); maximum inhibitions were reached with 250 μ g *dl*-methadone

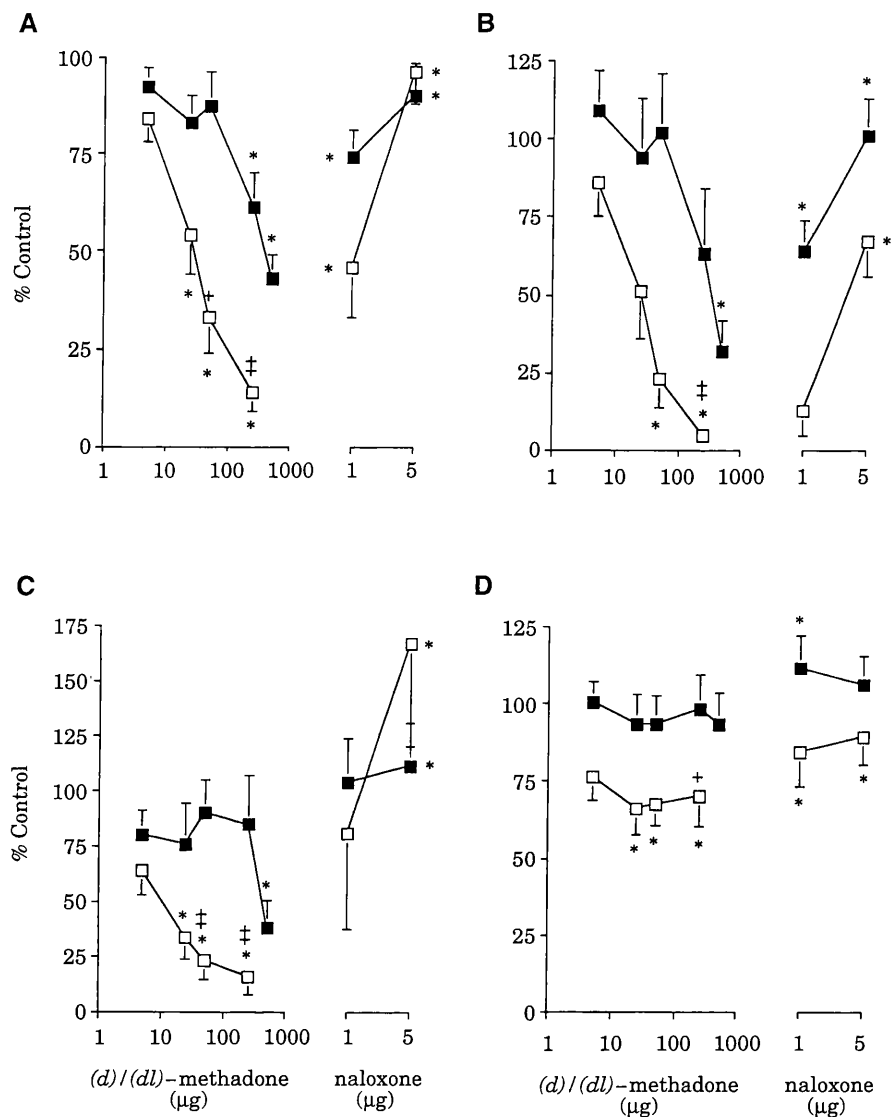


FIG. 1. Effect of spinal *d*-methadone (■) and *dl*-methadone (□) on (A) C-fibre evoked responses, (B) input, (C) wind-up and (D) Aβ-fibre evoked responses. Reversal by spinal naloxone is shown. Data are shown as mean percent control and vertical lines indicate SE mean. Significant difference as compared to control, significant reversal (* $p < 0.05$) and significant difference between groups (+ $p \leq 0.05$ and ‡ $p \leq 0.01$) are indicated.

(Figs 1A and 1D). Input was significantly inhibited by 50 µg *dl*-methadone ($n = 9$, Bonferroni-corrected p -value ≤ 0.01), and maximum inhibition was seen with 250 µg of the drug ($n = 8$, Bonferroni-corrected p -value ≤ 0.01) (Fig. 1B). Inhibition of post-discharge became significant with the top dose (250 µg) of *dl*-methadone ($n = 8$, Bonferroni-corrected p -value = 0.005). *dl*-Methadone inhibitions of C-fibre evoked responses, Aβ-fibre and Aδ-fibre evoked responses

were significantly reversed by 1 µg naloxone, whilst 5 µg naloxone was required to reverse the inhibitory effects of *dl*-methadone on input, post discharge and wind-up and to fully reverse its effects on C-fibre evoked responses back to 100% of control (Fig. 1).

An example of the effect of *dl*-methadone on the wind-up of an individual neurone is shown in Figure 2B. Irrespective of the magnitude of the neuronal response, *dl*-methadone abolishes the

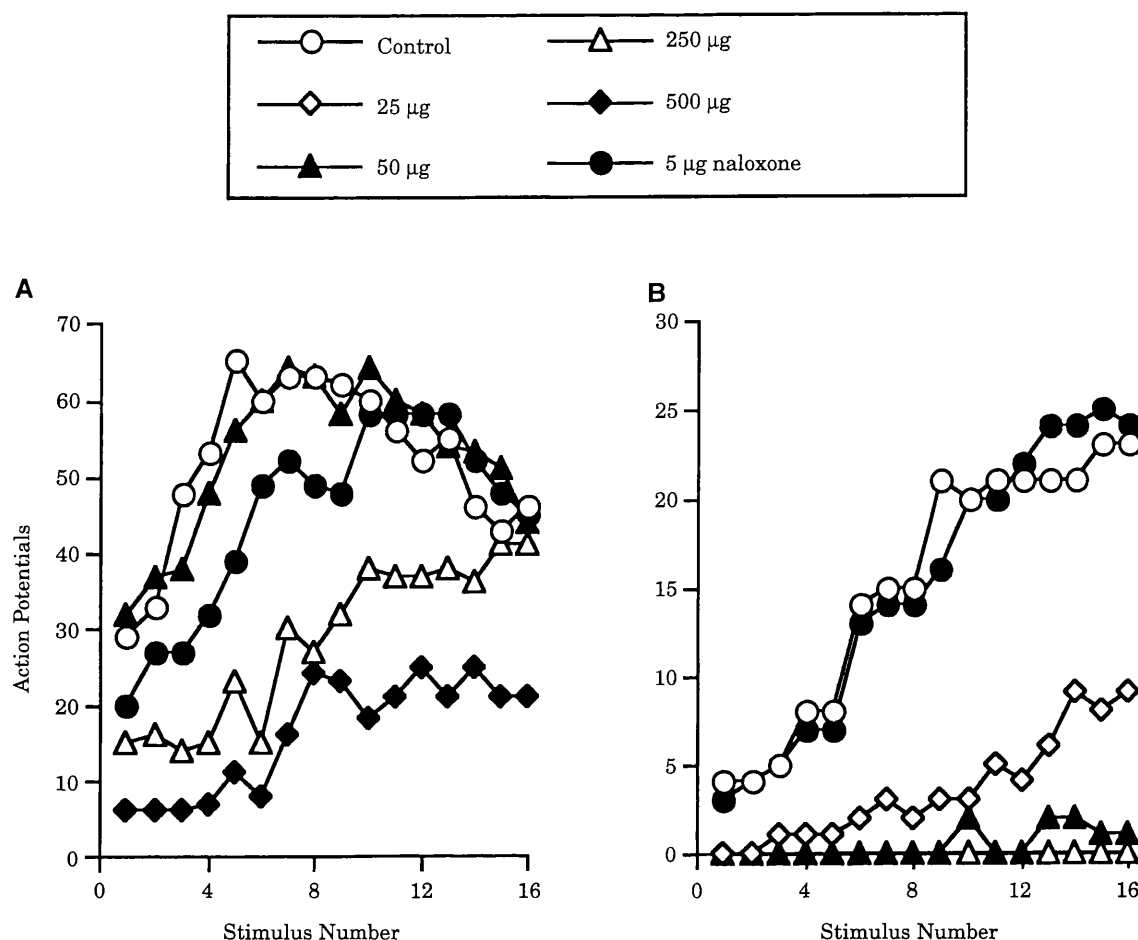


FIG. 2. Example of the inhibitory effects of spinal (A) *d*-methadone and (B) *dl*-methadone on individual neurones exhibiting wind-up. Spinal naloxone can reverse this effect.

input at 25 µg, yet some wind-up still occurs from this zero baseline. Higher doses (50 and 250 µg) abolish wind-up, and a low dose of naloxone (5 µg) completely reverses the effect of *dl*-methadone on both input and wind-up.

Before comparisons were made between the effects of *d*- and *dl*-methadone, the mean control responses for all neuronal measures were compared and found not to be different between the groups. Comparing the effects of the racemic mixture and the *d*-isomer of methadone on the different parameters shows that *dl*-methadone is more potent in all cases. At a dose of 250 µg, *dl*-methadone produced a significantly greater inhibition than the same dose of *d*-methadone on C-fibre-evoked responses, input, post-discharge and wind-up (unpaired *t*-test, $n = 9$

d-methadone, $n = 8$ *dl*-methadone, Bonferroni-corrected p -values all ≤ 0.01) (Fig. 1). The difference between the effects of the *d*-isomer and the racemic mixture was also significant at the lower dose of 50 µg for wind-up, post-discharge and C-fibre-evoked responses ($n = 14$ *d*-methadone, $n = 8$ *dl*-methadone, p -values ≤ 0.01 , ≤ 0.05 and ≤ 0.05 , respectively) and 25 µg for post-discharge ($n = 8$ *d*-methadone, $n = 7$ *dl*-methadone, $p \leq 0.05$). Graphs were re-plotted on log paper to enable the dose required to give 50% inhibition to be read accurately. *d*-Methadone is 13 times less potent than *dl*-methadone in inhibiting input and the C-fibre evoked response, 19 times less potent on post-discharge, 42 times less potent in inhibiting wind-up and 48 times less potent on Aδ-fibre evoked responses.

DISCUSSION

After spinal application in the anaesthetized rat, the highest dose of *d*-methadone (500 µg) was anti-nociceptive, showing selectivity for C-fibre and Aδ-fibre evoked neuronal responses, input, wind-up and post-discharge over Aβ-fibre-evoked responses. This broad antinociceptive action provides some clues as to the mechanism of action of this drug. NMDA receptors are predominantly located post-synaptically on spinal neurones. These receptors make a major contribution to the hyperexcitability produced by repeated C-fibre stimulation, but play a minimal role in the underlying baseline responses of the neurones. Thus, pure NMDA receptor antagonists have pronounced inhibitory effects on measures of hyperexcitability, such as wind-up and post discharge, and tend to spare the input, Aβ- and Aδ-fibre-evoked responses (Dickenson, 1990). Conversely, the inhibitory µ-opioid receptors are predominantly found on the terminals of afferent C-fibres, with a much smaller post-synaptic population. Thus, opioids have pronounced effects on C-fibre-evoked responses and input (the initial response of the neurone to the afferent volley), presumably as a result of their ability to reduce primary afferent transmitter release. At higher doses, however, opioids do cause a reduction in wind-up, but examination of wind-up responses (Fig. 2) reveal that this inhibition of wind-up is secondary to the reduced baseline input responses of the cells. NMDA receptor antagonists will block wind-up without reducing the baseline (Dickenson, 1990). Another important difference between the actions of opioids and NMDA receptor antagonists is that only the former are sensitive to naloxone (Dickenson *et al.*, 1991).

The effects of *d*-methadone on neuronal responses did not resemble the profile expected of an NMDA receptor antagonist, but rather mirrored the profile of the racemic mixture, albeit with a lower potency (Figs 1 and 2). In addition, the inhibitory effects of this isomer were clearly and fully reversed by low doses of naloxone (1 and 5 µg). Overall, this could suggest that the *d*-methadone isomer has little or no

NMDA receptor antagonist activity *in vivo*. We would attribute the antinociceptive effects seen, requiring relatively high doses, to an agonist action at µ-opioid receptors, based not only on the profile of the drug on the various neuronal measures but also the full reversal by naloxone. Racemic (*dl*-) methadone was more potent than the *d*-isomer by a factor of between 10- and 50-fold, a similar order of magnitude to that previously reported for these optical isomers *in vitro* (Scott *et al.*, 1948) and in humans (Olsen *et al.*, 1976). Interestingly, the difference in potency between the two drugs is greatest for the measures of wind-up and Aδ-fibre evoked responses. If *d*-methadone had predominant NMDA-receptor blocking actions it would be expected to be more potent than *dl*-methadone on the measure of wind-up—an NMDA receptor-dependent process. This is not the case, and the inhibition of wind-up by *dl*-methadone is probably due to its profound inhibition of input.

Shimoyama *et al.* (1997) have reported on the opioid and NMDA receptor affinities of methadone and the isomers, and have studied extensively the antinociceptive actions of the *d*-isomer in behavioural studies. After spinal application, they have shown that the second phase of the formalin response is reduced by *d*-methadone and that this is not reversible by naloxone. Although this second phase is dependent on NMDA receptor mechanisms (Dickenson, 1997), it can also be inhibited by classical opioids such as morphine. In our hands, the effects of *d*-methadone, administered spinally and over a similar dose-range to that used by Shimoyama *et al.* (1997), were naloxone-sensitive. This difference may well result from the different conditions that would be present in behavioural and electrophysiological approaches. Thus, in a behavioural study where threshold responses are measured, the relative receptor profile of the drug may be different from that when suprathreshold, neuronal responses are measured. Consequently, at threshold, there may be a large NMDA receptor component to the second phase of the formalin response which is attenuated by *d*-methadone, the effects of which cannot be reversed by naloxone. The opioid

component of *d*-methadone activity may become more apparent at the suprathreshold neuronal response levels used by us, leading to either predominantly opioid receptor-mediated, or synergistic inhibition of neuronal responses which can be restored to control levels with the application of naloxone. Our model, where suprathreshold responses of sensory neurones are measured, is perhaps more relevant to the situation of moderate to severe clinical pain.

Synergy might contribute to the activity of methadone. Chapman and Dickenson (1992) showed that doses of an opioid and an NMDA receptor antagonist, which individually had little or no effect on either input (opioid-sensitive) or wind-up (NMDA receptor-dependent), showed profound synergy when applied together and abolished both input and wind-up. It follows that the inhibition of neuronal responses we saw with the highest dose of *d*-methadone could be due to synergy between weak opioid and weak anti-NMDA receptor activity. In this case, naloxone would be expected to fully reverse the effects, as the final effect is dependent on both pharmacological components. Therefore, the reversal of effects we saw with naloxone does not preclude an NMDA receptor antagonist activity for *d*-methadone.

Unlike methadone, ketobemidone, another opioid with NMDA receptor blocking effects, has a different profile in that it inhibits nociceptive responses of spinal neurones, but importantly reduces wind-up in a non-naloxone-reversible manner in the same experimental model (Andersen *et al.*, 1996). This would suggest that NMDA receptor-blocking actions of ketobemidone can be dissociated from its opioid actions. Amongst other opioids with affinity for the NMDA receptor is dextropropoxyphene (Ebert *et al.*, 1998), pointing toward a family of mixed opioid/NMDA receptor antagonist compounds, probably all with differing profiles and different effectiveness in pain conditions.

Whilst a blend of desirable pharmacological actions in a single molecule is obviously advantageous, the same goals may be achieved by the administration of combinations of individual drugs. Morphine analgesia, for example, can be

potentiated by the co-administration of NMDA receptor antagonists (Chapman & Dickenson, 1992; Hoffmann & Weisenfeld-Hallin, 1996). This synergy has also been seen in animal models of neuropathic pain, an experimental situation where the effectiveness of morphine is reduced (Yamamoto & Yaksh, 1992; Advokat & Rhein, 1995; Nichols *et al.*, 1997). This combination of opioids and NMDA receptor antagonist has been investigated in the clinic; ketamine as a licensed NMDA receptor antagonist is most often used. Yang *et al.* (1996) and Javery *et al.* (1996) found that the combination of morphine and ketamine enhanced the analgesic effect of morphine against terminal cancer pain and post-operative pain, respectively.

Combining opioid actions with NMDA receptor antagonism, either in a single molecule or by combination therapy, can provide superior pain relief with fewer side effects than could be achieved with either drug alone. However, we would conclude that the potential NMDA receptor blocking component to the actions of methadone is minor under these experimental conditions, and so may not contribute to the clinical profile of this drug (Fainsinger *et al.*, 1993). Interestingly, methadone may have other non-opioid activity apart from any NMDA-receptor antagonist actions. A recent study has pointed out that, unlike morphine, methadone has effects on 5-HT uptake, akin to tramadol (Giusti *et al.*, 1997). This is unlikely to contribute to the actions of methadone in our hands, as 5-HT-mediated controls are negligible in the spinal cord in acute pain conditions such as the present model (G.M. Green, personal communication), and all effects were naloxone reversible. However, a 5-HT component to the actions of methadone (or the *d*-isomer) may be present in models of more prolonged pain states such as the formalin test used by Shimoyama *et al.* (1997). Here the naloxone insensitive effects of *d*-methadone may reflect inhibition of 5-HT uptake and thereby increase serotonergic spinal control of nociceptive transmission in this inflammatory state.

A re-examination of potential non-opioid actions of existing opioids may be fruitful.

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Peripheral administration of low pH solutions causes activation and sensitisation of convergent dorsal horn neurones in the anaesthetised rat

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Abstract

This is the first study to examine the effects of peripheral administration of acid on the activity of dorsal horn neurones *in vivo*. Extracellular recordings from convergent neurones revealed increases in neuronal activity evoked by administration of low pH solutions into the peripheral receptive field. Threshold for activity ranged from pH 5.85 to 2.5. The magnitude of responses increased with decreasing pH; maximum effects were achieved with pH 2.5 (648 ± 181 action potentials/60 s, as compared to control-evoked activity of 86.3 ± 29 action potentials/60 s). Activity lasted for up to 60 s, likely to represent the time for which the solutions were able to surmount the buffering capacity of the intact hindpaw. Significant sensitisation of the neurones to both innocuous (von Frey filament 9 g) and noxious (30 g) mechanical punctate stimuli was also observed. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Protons; Acid sensing ion channels; Pain; Nociception; Inflammation; Tissue acidosis; Electrophysiology; Spinal cord

Protons are increasingly implicated in pain. Low pH has been recorded in inflamed tissue and in arthritic joints, skeletal and heart muscle during ischaemia and in malignant tumours as a result of both inadequate perfusion and altered local metabolism. Generation of muscle ischaemia in human volunteers causes a drop in muscle pH to 6.2 [12] and is associated with sensations of pain [9]. Direct infusion of acidic solutions (pH 5.2) into the skin of human volunteers gives rise to reports of sustained pain [13], nociceptor sensitisation to heat [8] and to mechanical punctate stimuli [13]. Single unit recordings from sensory nerve endings have established that protons can activate subsets of primary afferent nerve fibres innervating skin, cornea, cardiac muscle, and the viscera ([3] for review).

Acid-sensing ion channels (ASICs) belong to the amiloride sensitive Na^+ /degenerin family of ion channels and have been suggested as the substrates responsible for proton transduction. Several different genes and splice variants are now known to exist – heteromultimers of different subunits are probably responsible for the characteristics of native channels. When expressed as homomeric channels, all of the ASICs cloned so far can be eliminated as the channel under-

lying response of sensory neurones to acid. Recording whole cell currents in small diameter rat sensory nerves reveals biphasic currents activated at a threshold of pH 6.2. The transient component (which can be recorded in other neuronal cell types and has a threshold of pH 6.8) is Na^+ -selective whilst the sustained currents show little selectivity between Na^+ , K^+ and Cs^+ [4]. ASIC1 and ASIC β give rise to only transient currents [7,11,17]; ASIC2 (MDEG1) is only expressed in the brain and requires a pH of <4.5 for activation [10]. DRASIC (ASIC3), although it is expressed in sensory neurones and gives rise to a persistent non-selective cation current in keeping with native channels, requires a lower pH for activation (pH 4.0) than do proton-activated sensory neurones [16]. ASIC2b (MDEG2) is also present in sensory neurones, but does not form functional channels on its own [10], nor does the recently cloned ASIC4 (SPASIC) [1,10]. Expressing combinations of these channels gives rise to currents that share more similarities with currents recorded from sensory nerves, suggesting that the ASICs cloned so far represent no more than potential sub-units of functional acid-sensing channels. However, as no combination has yet mimicked all the characteristics of native proton-sensation, post-translational modifications and the presence of as-yet unidentified channel genes must be considered. The capsaicin receptor VR1, the puta-

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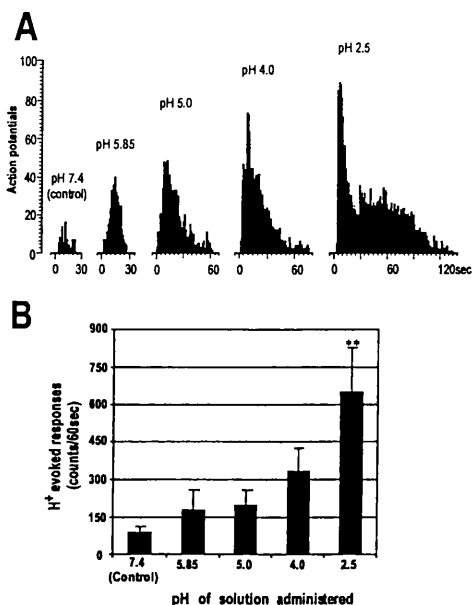


Fig. 1. Proton-evoked responses of convergent dorsal horn neurones. (A) Illustrates the response of one individual neurone to separate administrations of saline and pH 5.85–2.5. Examples of raw data are selected from a trace that was too long to show in its entirety (spanning 2 h) and therefore shows the neuronal response (action potentials/s) to just one of four separate administrations of each solution. (B) Shows the mean ($n = 21$) pH-evoked responses $\pm$ SEM (counts/60 s) following administration of solutions of decreasing pH. The increase in neuronal activity was significant ($P < 0.001$, repeated measures ANOVA) and pH-dependent (linear trend $r = 0.362$; $P < 0.001$). **Significant difference in pH-evoked activity vs. saline control is indicated ($P < 0.01$, Dunnett multiple comparisons test).

tive endogenous sensor for noxious heat, may also be modulated by protons. Under moderately acidic conditions the temperature threshold for VR1 activation is reduced such that VR1 may be activated at ambient temperatures [15].

Previous studies into ASICs have focused on the dynamics (whole cell voltage-clamp and single channel) of cloned channels in expression systems or native channels in isolated neurones, direct application of low pH onto dissected nerves in vitro or human psychophysical studies. The consequences of activation of peripheral ASICs on the activity of dorsal horn neurones in vivo is as yet unknown. The spinal cord, as the site for integration, relay and modulation of nociceptive information from primary afferents is an important area to investigate.

Here we record extracellularly the activity of convergent dorsal horn neurones in the anaesthetised whole rat. Using this approach we demonstrate that administration of low pH solutions into the peripheral receptive field of convergent dorsal horn neurones evokes graded responses from these neurones. The effect of low pH on punctate mechanical-evoked responses is also studied.

Male Sprague–Dawley rats (200–225 g) were anaesthetised with halothane (2–4% for induction, maintained at

1–1.5%) and a laminectomy performed to expose the spinal cord at the level of segments L4–L5. Extracellular recordings were made from single dorsal horn neurones in this region receiving both C- and A-fibre input from the ipsilateral hindpaw. Such neurones were identified by their ability to respond to both pinch (noxious, predominantly C-fibre mediated) and touch (innocuous, predominantly A-fibre mediated) applied to the plantar surface of the hindpaw in the receptive field of the neurone. The convergent nature of each neurone was further confirmed by electrical stimulation in the receptive field; electrical-evoked responses revealed C-fibre, A β -fibre and A δ -fibre inputs onto the neurone. Baseline C-fibre input, post discharge and wind-up were also quantified (see Ref. [5] for a full description of quantification of electrical-evoked responses). After each neurone had been characterized and allowed recovery time (10 min), injections of 0.9% saline solutions (20 μ l) titrated to a range of pH (5.85, 5.0, 4.0, 2.5) with HCl were begun. A 29G insulin syringe needle was inserted subdermally into a toe in the centre of the receptive field and remained in place while at 5-min intervals single injections of 20 μ l saline (pH 7.4) were made (four separate injections in total) and the response evoked by each one recorded. In all cases the volume of fluid administered had dissipated during the 5-min interval. The syringe was then removed and replaced with another containing saline at pH 5.85 and the process repeated. Intervals of 10 min elapsed between administration of each subsequent solution of decreasing pH. No obvious damage of the skin was observed after completing administration of the full range of pH solutions.

Twenty-two neurones (mean depth = 895 ± 48 μ m) from 22 different animals were studied, and all but one responded to administration of solutions of decreasing pH with a short-lived period of firing (see Fig. 1A for an example). This activity tended to have subsided within 1 min (saline-evoked activity duration = 23 ± 3 s; pH 5.85 = 25 ± 4.0 s; pH 5.0 = 28 ± 5.1 s; pH 4.0 = 31 ± 5.2 s; pH 2.5 = 59 ± 11 s), therefore, responses were counted over a period of 60 s from completion of injection. Any background activity between tests was recorded over the preceding 60 s and subtracted from the solution-evoked counts. For each neurone, the activity evoked by each of the four injections of the same solution was fairly consistent, and therefore the mean of these administrations was recorded. This mean injection-evoked counts/60 s (corrected for background) for each neurone was averaged over the 21 neurones that responded, for each pH solution, and are shown $\pm$ SEM in Fig. 1B. Administration of solutions of decreasing pH caused a significant increase in neuronal activity (repeated measures analysis of variance (ANOVA), $F = 7.5481$; $P < 0.001$) that was pH-dependent (linear trend $r = 0.362$; $P < 0.001$). Mean saline-evoked counts/60 s = 86.3 ± 28.5 ; pH 5.85 = 171 ± 82.4 ; pH 5.0 = 195.5 ± 61.0 ; pH 4.0 = 332 ± 94.5 ; pH 2.5 = 648 ± 181 . Post-hoc analysis showed that the pH 2.5-evoked activity (648 ± 181 action potentials/60 s) was significantly greater than control saline-

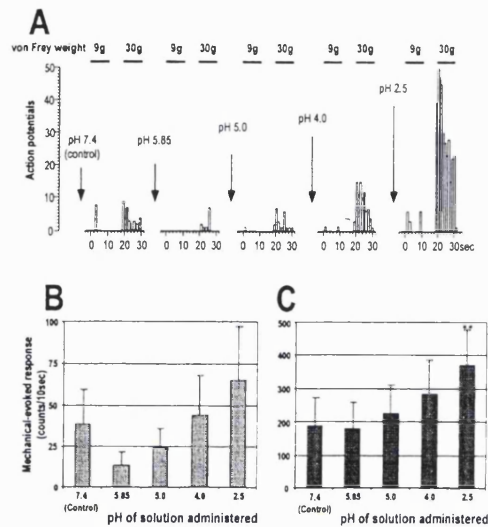


Fig. 2. Proton-induced sensitization to mechanical stimuli. (A) Illustrates the protocol adopted for application of mechanical stimuli. Raw data are shown as number of action potentials/s. The needle was removed after the administration of each pH solution and von Frey filaments (9 and 30 g) were applied to the receptive field for 10 s. Mean counts/10 s $\pm$ SEM are shown in (B) for von Frey 9 g ($n = 9$) and in (C) for von Frey 30 g ($n = 11$). Overall increase in mechanical-evoked responses was significant for both von Frey 9 and 30 g (repeated measures ANOVA, $P < 0.05$ and $P = 0.01$, respectively). **Significant difference in responses evoked with von Frey 30 g after pH 2.5 vs. saline control is indicated (Dunnett multiple comparisons test $P < 0.01$).

evoked responses (86.3 ± 28.5 action potentials/60 s; Dunnett multiple comparisons test, $P < 0.01$).

In addition to the direct effects of administration of solutions of low pH, the effect of low pH on mechanical-evoked responses of dorsal horn neurones was also studied. Of the 22 neurones studied, nine responded to application of von Frey filament of weight 9 g, and 11 neurones responded to a filament weight of 30 g. Filaments were applied to the centre of the receptive field, and once bending force had been reached pressure was sustained for 10 s. Action potentials evoked in this 10-s period were recorded and any background activity (recorded over the preceding 10 s) was subtracted. Filaments were applied after administration of neutral saline and after each successive low pH solution once the needle was removed (see Fig. 2A).

With progressive lowering of the pH administered, mechanical responses evoked by both von Frey filaments 9 and 30 g showed a significant increase from control responses evoked after saline (Fig. 2B,C, repeated measures ANOVA, $P < 0.05$ and $P < 0.01$, respectively). Maximum mechanical-evoked response was achieved after administration of pH 2.5 in both cases, significant for von Frey 30 g after pH 2.5 (Fig. 2C, Dunnett multiple comparisons test $P < 0.01$).

The only neurone that did not respond to low pH could not be differentiated from those that were sensitive to pH on the basis of the electrical threshold required for a C-fibre response, total C- or A-fibre counts, input, post discharge or wind-up (not shown). However, this non-responding neurone was not sensitive to noxious heat (application of a 5 ml gentle water jet at 45°C before beginning the pH administrations). We also recorded one neurone which did not respond to noxious heat, but had a large pH-evoked response (maximum = 1911 action potentials). Excluding both of these neurones and looking at the other 20, there is a significant correlation between the sensitivity of each neurone to noxious heat (scale 1–3; no response, moderate firing, high frequency sustained response) and the maximum pH-evoked response (action potentials/60 s) shown by that neurone (Fig. 3, Pearson correlation, $r = 0.517$; $P < 0.05$). Whilst using a more intense heat stimulus (50°C) may have revealed a more robust correlation, this could have been confounded by potential desensitization and damage to the skin.

Administration of low pH solutions into the hindpaw receptive field of convergent dorsal horn neurones evoked a series of action potentials that increased in number as the pH of the solution decreased.

Direct comparisons with in vitro experiments would indicate that a much lower pH is required to activate primary afferents in this paradigm (threshold pH required ranges from 4.0–2.5, as compared to 6.9–6.1 in Ref. [14]). However as the intact hindpaw is used, and constant infusion was not possible, the buffering capacity of the hindpaw would ensure that the proton concentration reaching the site of action would be less than if the solution were applied directly to the exposed nerve endings. Appropriately, the excitations evoked were not persistent (mean duration pH 2.5-evoked

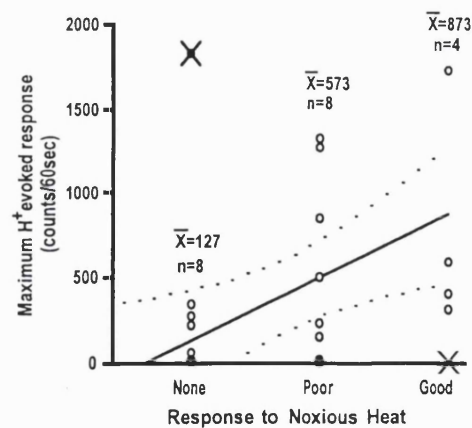


Fig. 3. Proton-evoked responses correlate with sensitivity to noxious heat. For each neurone studied, ($n = 22$) the maximum response (counts/60 s) to administration of low pH is plotted against the qualitative response of that neurone to noxious heat (water at 45°C applied to the receptive field before acid administrations were begun). Excluding the two anomalous neurones as discussed (indicated with X) the linear correlation is significant ($P < 0.05$; $r = 0.517$; $n = 20$).

responses = 59 ± 11 s), as is observed with administration directly onto exposed nerves [14] or constant infusion into the skin [13] but presumably lasted as long as the low pH solution was able to surmount the buffering capacity of the tissue.

Whilst some desensitization of the prolonged component of proton-evoked currents is observed in voltage-clamp recordings of sensory nerve [4], here the magnitude of responses was non-adapting for each pH. Large responses were recorded from the same dorsal horn neurone with repeated administration of progressively lower pH solutions over a period of several hours. This demonstrates that in a physiological setting where a group of nociceptors are present to mediate the response to protons, no desensitization occurs. This is in keeping with both human studies and in vitro work [13,14]. Also seen by Steen et al. [13,14] in both these human and rat skin-nerve experiments was a reduction in the punctate mechanical threshold required to evoke pain sensations or to activate C-fibres, respectively. We observed a significant increase in the magnitude of responses evoked by low (9 g) and high (30 g) intensity mechanical stimuli after repeated treatment with lowering pH. This demonstrates that low pH in peripheral tissues can not only directly activate nociceptive fibres but also cause sensitisation to mechanical stimuli.

The correlation between noxious heat- and pH-evoked responses of the dorsal horn neurones studied here suggests that primary afferent nociceptors sensitive to noxious heat and to protons may tend to converge on the same dorsal horn neurones. One molecular substrate thought to confer heat sensitivity is the capsaicin receptor VR1, which is also sensitive to proton activation [6,15]. Bevan et al. [2] have in fact suggested that capsaicin and protons might activate the same ion channels, as the currents evoked by these two stimulants share many biophysical properties. There are however apparent differences; proton activated channels are less permeable to Ca^{2+} than capsaicin-activated channels [18], and show much slower (or no) inactivation [4,6]. However, these properties may be altered by the presence of protons. In light of these possible differences, and the recording we have made here from several neurones sensitive to only one modality, it seems likely that heat- and proton-activated receptors are distinct entities which are expressed on similar subsets of sensory nerves, and therefore tend to converge on the same dorsal horn neurones. This pairing is not absolute.

In conclusion, despite the buffering capacity of the intact rat hindpaw, administration of low pH solutions in the receptive field caused excitation of convergent dorsal horn neurones and sensitisation of these neurones to both low and high intensity punctate mechanical stimuli. Also observed was a tendency for heat-activated and pH-sensitive primary afferents to converge on the same dorsal horn neurones. Resting half-way between the physiological nature of human studies and single-unit-recordings in skin-nerve preparations, the series of experiments described here support the findings of both.

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Amino acids are still as exciting as ever

Kate J Carpenter and Anthony H Dickenson

Glutamate is probably the most important excitatory transmitter in the vertebrate central nervous system. Its multiple functional roles in the brain and spinal cord make therapeutic manipulation of these systems fraught with difficulties. There has, however, been recent progress in pharmacological manipulations of NMDA receptor subtypes and non-NMDA receptors, and understanding of the roles of NAAG, that promise rapid advances in pain control.

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Abbreviations

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
mGluR	metabotropic glutamate receptor
NAAG	<i>N</i> -acetylaspartylglutamate
NAALADase	<i>N</i> -acetylated- α -linked-acidic dipeptidase
NMDA	<i>N</i> -methyl-D-aspartic acid

Introduction

The cell-surface receptors that mediate the effects of released glutamate are located at most excitatory synapses in the CNS. These receptors are divided into the slow G-protein-coupled metabotropic glutamate receptors (mGluRs), and the fast ligand-gated ion channels (ionotropic receptors). The latter class is again divided into α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, mediating the majority of fast excitatory neurotransmission, kainate receptors and *N*-methyl-D-aspartic acid (NMDA) receptors [1]. Of these, the NMDA receptor has been studied extensively over the past decade, as a full set of pharmacological tools has been available. This has revealed a major role in nociceptive transmission. Here we review advances made over the past few years that allow us to modulate NMDA receptor transmission more precisely, and permit characterisation of the roles of the other receptors for glutamate. Finally, we discuss the pharmacology of an endogenous neuropeptide, NAAG (*N*-acetylaspartylglutamate), which appears to interact with excitatory amino-acid-mediated events in an interesting manner.

The NMDA receptor

Activation of the NMDA receptor allows a Ca^{2+} influx powerful enough to trigger long-term changes within and around that cell. The NMDA receptor is implicated in plasticity in many systems, such as memory, motor function, vision and spinal sensory transmission. Excessive NMDA receptor activation is thought to be responsible for

some of the excitotoxic neuronal death seen after cerebral ischaemia, and is also implicated as a final mechanism of neuronal death in many neurodegenerative diseases [1].

There is evidence for involvement of the NMDA receptor in inflammatory pain, neuropathic pain, allodynia and ischaemic pain, all processes in which the receptor alters the normal relationship between stimulus and response. In these persistent pain states, the NMDA receptor is vital both in establishing the heightened pain state and in maintaining this state; parallels can be made to the phenomenon of long-term potentiation [2]. This pivotal role in the plasticity of the system makes it an attractive target for development of new analgesics.

The NMDA receptor is, of course, not restricted to spinal pain pathways and it is not surprising that NMDA receptor antagonists such as the channel blocker ketamine and competitive antagonists are associated with a range of adverse effects. One possible approach to avoid the side effects associated with global block of NMDA receptors is to target a particular receptor type by its subunit makeup. The NMDA receptor is a hetero-oligomer. The stoichiometry of the subunit composition has been in contention for some time and Laube *et al.* [3] recently presented evidence for a tetrameric structure for NMDA receptors: two NR1 and any combination of two NR2 subunits (NR2A–2D). In expression systems, combinations of the different NR2 subunits and NR1 splice variants generates a wide diversity of receptor types, which differ in single channel properties, in sensitivity to glutamate and glycine-site antagonists and, for example, sensitivity to ifenprodil antagonism (see [4]). Ifenprodil is a non-competitive NMDA receptor antagonist, selective for receptors containing the NR2B subunit [5]. It and other similar compounds with this selectivity have reduced side-effect profiles *in vivo* [6]. Therefore, if the functional significance of these various receptor types is determined, subunit-selective drugs could target the relevant systems more accurately, reducing the side effects of ubiquitous NMDA receptor block.

This approach was justified in recent study that investigated the distribution of the NR2B subunit protein in rat lumbar spinal cord and examined effects of NR2B-selective antagonists on nociception [7•]. Immunocytochemical studies showed that the NR2B subunit had a restricted distribution, with moderate labelling in the superficial dorsal horn, where nociceptive afferent fibres terminate, indicative of a possible involvement in pain transmission. In the *in vivo* studies, the NMDA/glycine antagonists (MK-801, L-687,414 and L-701,324) affected motor control at antinociceptive doses. In contrast, the NR2B-selective antagonists (+/–)-CP-101,606 and (+/–)-Ro 25-6981 caused

Figure 1

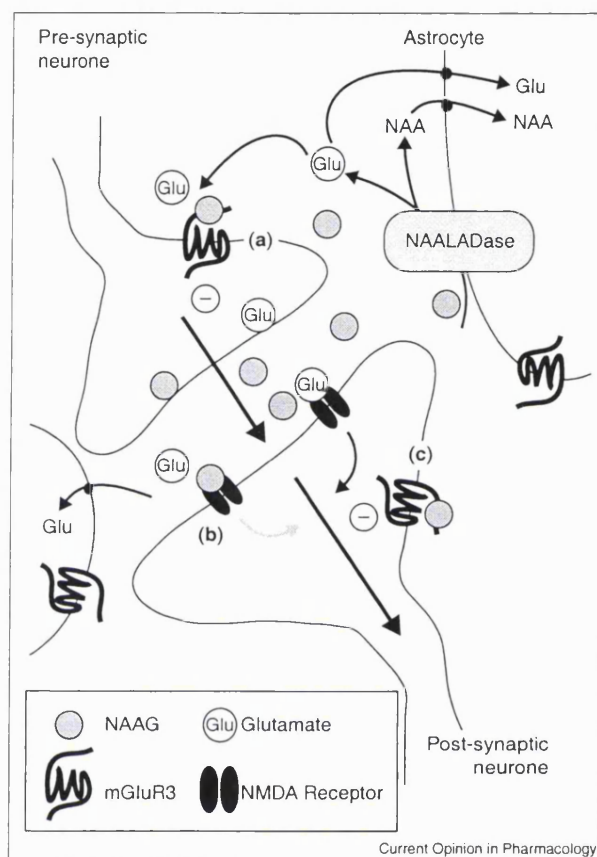


Illustration of the neurotransmitter actions of NAAG. After release, NAAG (a) activates pre-synaptic inhibitory mGluR3 to attenuate further release of neurotransmitters, including NAAG and glutamate. At the post-synaptic membrane, NAAG attenuates excitability by (b) interfering with binding of released glutamate at the NMDA receptor and (c) by the activation of post-synaptic mGluR3. Inhibition of NAALADase with 2-PMMA can reveal these effects.

no motor impairment or stimulation even at doses far in excess of those required to inhibit allodynia in neuropathic rats. These findings demonstrate that NR2B-selective antagonists might have clinical utility for the treatment of pain conditions in man with a reduced side-effect profile compared to existing NMDA receptor antagonists.

Non-NMDA receptors

Meanwhile, the roles of non-NMDA ionotropic glutamate receptors in spinal nociceptive transmission are now becoming better characterised. AMPA-preferring receptors, comprised of subunits GluR1–4 and kainate-preferring receptors, comprised of subunits GluR5–7 and KA1–2, are arranged as either homomeric or heteromeric pentamers [8]. mRNA coding for GluR1–4 subunits is found throughout the dorsal horn of the spinal cord, with strong expression of GluR2 and to a lesser extent GluR1, in the superficial laminae of the dorsal horn [9,10]. Lower levels of

mRNA coding for kainate-preferring subunits are found in the dorsal horn, with no GluR6 mRNA detected [9]. Both AMPA- and kainate-preferring subunits are found in dorsal root ganglia [11,12], with evidence for pre-synaptic AMPA and particularly kainate-preferring receptors comprised of GluR5 subunits [11,13–15].

A recent study used the competitive AMPA receptor antagonist 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione (NBQX), and the recently developed GluR5-selective antagonist LY382884 to investigate the relative contributions of the AMPA (GluR1–4) and kainate (here GluR5) preferring subtypes of non-NMDA ionotropic glutamate receptors to spinal nociceptive processing in normal animals and to follow the development of a peripheral inflammatory state [16*].

Application of LY382884 to the spinal cord *in vivo* (anaesthetised rats) revealed a small contribution of the GluR5 to spinal nociceptive transmission in normal conditions, which is in agreement with another recent report [17]. Inhibition of both the non-potentiated and wind-up enhanced noxious-evoked responses of dorsal horn neurones was seen. Excitatory GluR5 kainate receptors located on nociceptive primary afferent terminals or on post-synaptic neurones in the spinal cord may contribute to the effects seen.

In contrast, the AMPA receptor antagonist NBQX produced selective inhibitions of the non-potentiated components of the nociceptive response. Unlike kainate-preferring receptors, a significant proportion of AMPA receptors are thought to be located post-synaptically in the spinal cord, on both intrinsic and projection neurones [9,10,18]. The results of this study suggest that in normal animals, AMPA receptors contribute to only fast, faithful transmission of afferent C-fibre-evoked responses of the neurones.

Following the development of peripheral inflammation, the spinal potency of the GluR5-selective antagonist was increased, consistent with results obtained in another model of inflammatory pain, the formalin response, where the antagonist blocks the second phase of this response [19]. The enhanced potency of LY382884 in animals with inflammation may result from the increased release of glutamate within the spinal cord [20,21], leading to increased activation of putative pre-synaptic kainate receptors.

Pre-synaptic kainate GluR5 receptors are likely to represent a good analgesic target, antagonists at this receptor may be associated with less side effects, at least at the spinal level, than AMPA receptor antagonists. Furthermore, as the role of kainate receptors is greater in conditions where the release of the amino acid is enhanced, drugs targeted to the GluR5 could be particularly effective in pathological conditions such as pain and epilepsy [1].

Following inflammation, AMPA receptors make a greater contribution to previously NMDA-receptor-mediated

spinal mechanisms of central amplification and wind-up ([16*,19] also see [22]). One possible explanation for the greater involvement of AMPA receptors in wind-up following inflammation is a change in their subunit composition. Up-regulation of Ca²⁺-permeable AMPA receptor channels may occur after inflammation to facilitate excitability.

The majority of AMPA receptors are impermeable to Ca²⁺ [23]. The absence of the GluR2 subunit confers Ca²⁺ permeability on AMPA receptors (GluR1–4). Similarly, RNA editing in the same pore region controls the Ca²⁺ permeability of the kainate receptor subunits GluR5 and GluR6. Significant levels of the Ca²⁺-permeable non-NMDA receptors are present in the adult CNS [24,25].

Neurons expressing high levels of GluR1 mRNA, but lacking GluR2 are found in the superficial laminae of the spinal cord, suggesting that a subpopulation of AMPA receptors with significant Ca²⁺ permeability may play a role in pain [26]. Accordingly, functional Ca²⁺-permeable non-NMDA receptors have been demonstrated in spinal cord slices [27**]. Ca²⁺ entry through Ca²⁺-permeable AMPA receptors in the spinal cord may enhance spinal nociceptive transmission [28], whereas other studies have suggested a link between these receptors and inhibitory systems in the dorsal horn of the spinal cord [26]. A recent study demonstrated functional Ca²⁺-permeable non-NMDA receptors *in vivo* in the pathways involved in the modulation of spinal nociceptive transmission in adult rats [29**]. Spinal application of Joro Spider Toxin (JSTx), a selective blocker of Ca²⁺-permeable non-NMDA receptors [30], caused a predominant facilitation of neuronal responses. This suggests that Ca²⁺-permeable non-NMDA receptors activate, directly or indirectly, inhibitory interneurons within the spinal cord. This is consistent with anatomical data suggesting that GABAergic interneurons in the superficial dorsal horn express Ca²⁺-permeable AMPA receptors [26]. The localisation and functional role of these receptors clearly differs from the distinct roles of the other ionotropic glutamate receptors in the spinal cord: Ca²⁺-impermeable AMPA receptors mediate the fast basal excitatory response to nociceptive and non-nociceptive stimuli; NMDA receptors are responsible for the spinal amplification of nociceptive responses; and kainate receptors play a facilitatory role, possibly at pre-synaptic receptors on primary afferent fibres (see references in [31]). Thus, this diversity in the spinal receptors for glutamate, each with a distinct and defined role, allows this transmitter to play a complex role in nociceptive transmission and modulation, with much scope for plasticity in the balance between excitatory and inhibitory pathways in chronic pain states.

Metabotropic receptors

The G-protein-coupled (metabotropic) receptors for glutamate generate even more diversity in excitatory amino acid transmission. These mGluRs mediate the slower modulatory events and can be divided into three groups: group I, mGluR1 and 5; group II, mGluR2 and 3; and group III,

mGluR4, 6–8. Pharmacological tools for probing the function of these receptors are far from perfect but evidence points to a role for group I and group II receptors in the transmission and modulation, respectively, of nociceptive information in the spinal cord [32*].

The mGluR3 is inhibitory, activation reduces cAMP formation and inhibits pre-synaptic voltage-dependent Ca²⁺ channels [33]. The mGluR3 may be of particular interest in nociception. Its discrete expression in the dorsal horn is enhanced after the development of peripheral inflammation [34], where it may function as an autoregulatory control on the excessive glutamate release seen in inflammation.

N-acetylaspartylglutamate – an endogenous modulator?

While we wait for the development of drugs that can manipulate the complex and many-faceted involvement of glutamate in nociceptive transmission and modulation, could an endogenous neuropeptide already possess the ideal pharmacological profile?

NAAG is a neuropeptide localised in subsets of glutamatergic, GABA-ergic, cholinergic, noradrenergic and serotonergic neurones in the mammalian brain and spinal cord. It fulfils the criteria used to define a neurotransmitter: it is stored in synaptic vesicles at the pre-synaptic terminal [35]; exhibits Ca²⁺-dependent release [36]; is removed from the synapse both by direct uptake [37] and by enzymatic degradation [38]; and has a very interesting pharmacological profile.

NAAG is an agonist at the inhibitory mGluR3 [39] and a partial agonist/antagonist at the NMDA receptor [40,41], where the high affinity but low efficacy of NAAG interferes with normal glutamatergic transmission. This profile therefore confers a predominantly inhibitory function (Figure 1).

The functional significance of this combination of receptor effects could be interesting, and as the breakdown of NAAG by NAALADase (*N*-acetylated- α -linked-acidic dipeptidase; also known as glutamate carboxypeptidase II) liberates NAA and glutamate, the dipeptide may also function as a source of extra-synaptic glutamate. [42,43]. NAALADase is bound to the extracellular membrane of astrocytes [44], and so terminates the activity of NAAG only when it has left the synapse, and liberates glutamate into the extra-synaptic space. The enzyme NAALADase therefore regulates the balance between levels of synaptic NAAG and extra-synaptic glutamate.

Altered NAALADase activity is implicated in many disease states that involve dysregulation of glutamatergic transmission, including epilepsy, Huntington's disease and Alzheimer's disease, amyotrophic lateral sclerosis and schizophrenia [45]. NAAG is neuroprotective under pathological conditions, an effect mediated both by activation of mGluR3 and antagonism at the NMDA receptor [46,47]; however, determining the physiological function of NAAG

is limited because of its peptidergic nature. Protecting NAAG from breakdown by inhibition of NAALADase is an effective approach. The first specific inhibitor of NAALADase was described in 1996 [48] and 2-(phosphonomethyl)pentanedioic acid (2-PMPA) is now used to manipulate NAAG levels in order to study the physiological role of this dipeptide.

Inhibition of NAALADase activity with 2-PMPA increases brain levels of NAAG and protects against ischaemic injury *in vitro* [49**] and *in vivo* [50**]. Pharmacological strategies that are effective in neuroprotection paradigms often translate into effective analgesic strategies, as an underlying factor in each condition is excessive glutamate release and excess excitability. Partial agonism at the NMDA receptor, as displayed by NAAG, may be an effective strategy to circumvent ubiquitous NMDA receptor blockade, and target only areas where NMDA receptor activation is excessive. There is little literature on the role of the mGluR3 in nociception, as selective tools do not exist. The receptor is expressed strongly in laminae II–V of the dorsal horn, with very little expression in the ventral horn. Expression is enhanced after inflammation, paralleling the development of hyperalgesia [34], indicating a role in chronic pain states. Increasing NAAG levels may be an effective strategy to control nociceptive processing as NAAG-like immunoreactivity is detected in the spinal cord [51,52] and dorsal root ganglia [53,54]. Interestingly, the level of peptidase activity in the spinal cord is low in comparison to the high concentration of NAAG (64 nmol/mg protein, the highest in the CNS) [42]. This may indicate a prolonged activity of NAAG in the spinal cord. Therefore, endogenous NAAG, the enzyme responsible for its breakdown and the receptor targets of released NAAG are all present in the dorsal horn of the spinal cord, and increasing NAAG levels using 2-PMPA to protect it from breakdown could be a valid strategy for analgesia. However, the prospect of manipulating the NAAG system with a view to therapeutic function in pain, stroke, epilepsy, dementia, schizophrenia or amyotrophic lateral sclerosis must consider that this peptide is undoubtedly involved in many other physiological processes.

Conclusions

The different functional roles of the spinal receptors for glutamate, roles that are subject to plasticity, allow this transmitter to play a complex role in nociceptive transmission and modulation. There is much scope for novel therapies and recent clinical studies have indicated that targeting the NMDA receptor may be an effective strategy in the control of pain [55**]. Similar advances could soon be made through targeting non-NMDA receptors, but the issue is still going to be side effects rather than efficacy. In this context, manipulation of an endogenous transmitter may be useful and thus investigation of NAAG in nociception will be illuminating, and may also elucidate the role of the mGluR3, which is still poorly understood in this system.

References and recommended reading

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