

**INTRINSIC INHIBITORY SYSTEMS AND DESCENDING
MONOAMINERGIC MODULATION OF INFLAMMATORY
NOCICEPTION IN THE RAT SPINAL CORD.**

By
MARK GREEN

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

Department of Pharmacology
University College London
Gower Street
London
WC1E 6BT

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ABSTRACT

Inflammation induced neuronal activity is generated in the periphery, and transmitted through the spinal cord. Descending monoaminergic systems that terminate in the dorsal horn of the spinal cord, and the intrinsic spinal GABAergic inhibitory system have been implicated in the modulation of pain, including the control of nociceptive transmission during inflammation. Using an *in vivo* electrophysiological approach, I investigated the role of GABA_A and GABA_B receptors, α_2 -adrenoceptors, and 5-HT_{1A}, 5-HT₃, and 5-HT₄ receptors in the dorsal horn during formalin induced peripheral inflammation.

The effects of intrathecally applied selective agonists and antagonists for these receptors on the electrically evoked A β -, A δ -, and C-fibre responses of dorsal horn neurones in normal animals were compared to the effects of pretreatment with the drugs on the formalin response, in anaesthetized rats.

My results show GABA_A and GABA_B receptor mediated inhibitions are involved in controlling the duration of the second persistent phase of the formalin response, and GABA_A receptor mediated inhibition also contributes to the manifestation of the silent interphase period and the magnitude of the second phase. The α_2 -adrenoceptor mediated noradrenergic inhibitory system, and the 5-HT₃ receptor mediated serotonergic excitatory system in the spinal cord appear to be dormant under normal conditions. However, during formalin induced inflammation these systems are activated, modulating the magnitude of the neuronal responses to formalin, and in the case of α_2 -mediated inhibition, controlling the duration of the response as well. Finally, 5-HT_{1A} and 5-HT₄ receptor mediated serotonergic systems in the spinal cord appear to have no endogenous role in the modulation of formalin induced inflammatory nociception. However, when activated, 5-HT_{1A} and 5-HT₄ receptors inhibit and facilitate respectively the neuronal responses to formalin, and may do so by selectively modulating A δ -fibre mediated afferent activity. This thesis advances knowledge of endogenous spinal mechanisms affecting inflammatory nociceptive transmission in the rat.

DEDICATION

To Rosemary and Patrick Green. Ultimately, this is all your fault !

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1. INTRODUCTION

Our perception of pain begins with the activation of peripheral nociceptors generating noxious afferent activity. Primary afferent fibres carry this information to the dorsal horn of the spinal cord where it converges with other non-noxious afferent inputs. The dorsal horn of the spinal cord is a principal site for the transmission and modulation of nociceptive information from the periphery to higher centres. Spinal mechanisms of nociceptive processing are subject to considerable plasticity, which can dramatically alter the relationship between stimulus and response. Spinal activity can be influenced by transmitters released from either intrinsic spinal systems, or the terminals of extrinsic descending neurones from the brain. Ultimately, the equilibrium between concurrent excitatory and inhibitory transmission must determine the global degree of excitability within the dorsal horn, which in turn modulates the transmission of pain information before it reaches the brain.

My study set out to investigate the intrinsic and descending inhibitory controls involved with modulation of formalin induced inflammatory nociception at the level of the spinal cord.

1.1 From the periphery to the spinal cord

1.1.1 Peripheral nociceptors

Sensory perception for sight, sound and smell occurs in highly specialised sensory organs. This is not the case for all somatovisceral perception of the body. Somatovisceral sensory receptors are widely distributed throughout the skin and in deep tissue such as muscle and joints. The receptors innervating the skin are termed cutaneous receptors and can be divided into several different categories, mechanoreceptors, thermoreceptors and nociceptors. Notably, in the context of somatovisceral sensory receptors, the term “receptor” does not refer to a cell membrane bound protein or ion channel. Rather it is used to denote a functional unit, the description of which includes the activating stimulus, transduction

mechanism and type of afferent fibre the “receptor” activates (Willis & Coggeshall, 1991; Gebhart, 1995).

Cutaneous mechanoreceptors, or low-threshold mechanoreceptors respond to mechanical changes in the skin (sense of touch). Mechanoreceptors can be activated by stimuli in the non-noxious range and are associated with A β -myelinated afferent fibres (Willis & Coggeshall, 1991; Gebhart, 1995). Thermoreceptors signal changes in skin temperature. They can be subdivided into cold and hot thermoreceptors and are associated with A δ -thinly myelinated afferent fibres and unmyelinated C-fibres. Thermoreceptors respond to heating or cooling stimuli in the non-noxious range, and become unresponsive to (noxious) extremes of heat or cold respectively (Willis & Coggeshall, 1991; Gebhart, 1995).

Nociceptors are defined as sensory receptors that signal damage has occurred, or is about to occur, to the skin. Nociceptors only respond to noxious stimuli, and have been classified into two groups, based on the stimuli to which they are most sensitive and the afferent fibre types that innervate them. A δ -mechanical nociceptors respond to potentially damaging mechanical forces such as pinch or squeeze. They may also respond to chemical or thermal stimuli if previously sensitized. C polymodal nociceptors are so named because they may be activated by either mechanical, thermal or chemical stimuli in the noxious range. A δ -mechanical nociceptors are associated with small diameter, thinly myelinated fibres, whereas C polymodal nociceptors possess small diameter, unmyelinated fibres (Willis & Coggeshall, 1991; Gebhart, 1995).

The skin and deep tissues contain an additional class of nociceptor known as silent nociceptors (or mechanically insensitive nociceptors). Silent nociceptors are associated with C-fibres and are inactive and unresponsive to high intensity mechanical stimulation under normal conditions. It is only during and after injury or inflammation has occurred that silent nociceptors become active. After inducing inflammation experimentally, silent nociceptors typically develop spontaneous activity and may subsequently respond to mechanical stimuli, even at previously non-noxious intensities (Gebhart, 1995).

1.1.2 Primary afferent fibres

Somatosensory primary afferent fibres can be distinguished by their conduction velocities, which is determined by fibre diameter and degree of myelination by insulating Schwann cells. A β -fibres are the most heavily myelinated, have the largest diameter, and consequently the fastest conduction velocity. A δ -fibres have a smaller diameter and are less myelinated than A β -fibres. C-fibres also have a small diameter, possess no myelin sheath, and consequently are the slowest conductors (see table 1) (Willis & Coggeshall, 1991; Gebhart, 1995).

Sensory fibre	Functional association	Myelination	Diameter (μm)	Conduction velocity (m/sec)
A β	Cutaneous mechanoreceptors	Heavy	6-12	25-70
A δ	Myelinated mechanical nociceptors; thermoreceptors	Light	1-5	10-30
C	Unmyelinated polymodal nociceptors; silent nociceptors; thermoreceptors	None	0.2-1.5	< 2.5

Table 1. Classification of afferent sensory nerve fibres associated with cutaneous somatosensory receptors

1.1.3 Dorsal root ganglia

The dorsal root ganglia (DRG) contain the cell bodies of primary afferent fibres. Cell body size is generally related to myelination, conduction velocity and function. Consequently, the cell bodies of different sensory fibres can be classified

on this basis. Large diameter cell bodies have myelinated axons with fast conduction velocities (A β -fibres). Small diameter cell bodies have thinly myelinated (A δ -fibres), or unmyelinated (C-fibre) axons. Synthesis of functionally important complex molecules occurs in the cell body. Receptors, enzymes, peptides and neurotransmitter precursors are synthesised and transported to either the central or peripheral terminal of the axon as required (Gebhart, 1995).

The wide range of peptides found in DRG cell bodies provides an additional criterion for subdivision of DRG cells. Peptides are found primarily in small cell bodies corresponding to A δ and C-fibre afferents. Typically, sensory neurones synthesise several putative neurotransmitters, and co-localisation of peptides is now recognised as being the norm. The excitatory amino acid transmitter glutamate is found in all DRG cell bodies. 30% of DRG cell bodies also contain calcitonin gene-related peptide, vasopressin and oxytocin, and 10-30% contain substance P. In addition, small proportions (10% or fewer) of fibres have been found to contain cholecystokinin, somatostatin, vasoactive intestinal peptide, bombesin, β -endorphin, enkephalin, galanin and corticotrophin releasing factor. Thus, in the transmission of nociceptive information from the periphery to the spinal cord, glutamate and peptides are released from the central terminals of primary afferent fibres where they function as neurotransmitters or modulate the action of other released substances (DeBiasi & Rustioni, 1988; Miller *et al.*, 1988; Battaglia & Rustioni, 1988; Miller *et al.*, 1993; Gebhart, 1995).

1.1.4 Activation of peripheral nociceptors

The classification of cutaneous nociceptors includes a description of which noxious stimulus the nociceptor will respond to. Once an injury has been sustained, nociceptor activity (and consequently, noxious afferent input to the spinal cord) is subject to activation by a spectrum of chemical mediators. These mediators are either released by the afferent fibre itself, or by non-neuronal cells as a part of the innate response of the immune system to any tissue damage that may have occurred (Gebhart, 1995).

Polymodal C-fibre nociceptors have the capacity to increase their response to repetitive noxious stimulation, and may continue to fire after the activating stimuli is

removed. As C-fibres are activated and conduct action potentials towards the spinal cord the antidromic flow of action potentials in adjacent branches of the nerve leads to the release of chemical mediators into the damaged area including substance P and CGRP. This is known as the axon reflex. These mediators then increase vascular permeability which leads to further release of pronociceptive and proinflammatory agents such as bradykinin. This acts to increase the area of sensitisation (Dray *et al.*, 1994; Gebhart, 1995).

The immune system acts to protect the body in two distinct ways. The adaptive response involves the production of immunologically specific antibodies. The innate response involves the release of immunologically non-specific chemical mediators from non-neuronal cells (e.g. mast cells, platelets), and the production of eicosanoids from the metabolism of arachidonic acid. These mediators promote the development of local inflammation, and either sensitise or directly activate nociceptors to maintain the flow of noxious afferent activity. They include bradykinin, serotonin, histamine, protons, substance P, ATP and adenosine. The eicosanoids are formed by the metabolism of arachidonic acid, which is formed from phospholipids found in cell membranes. The production of arachidonic acid is induced by tissue damage. The enzymes cyclooxygenase (COX) COX-1, COX-2 and lipoxygenase promote the conversion of arachidonic acid into prostaglandins, prostacyclins, leukotrienes and thromboxanes. In addition to sensitising nociceptors, prostaglandins are notable for playing an additional role in other aspects of inflammation, such as blood vessel dilation to produce oedema and thereby promote increased levels of serotonin and bradykinin at the site of the injury (Dray *et al.*, 1994; Gebhart, 1995).

Once injury has been sustained the surrounding area of skin typically becomes more sensitive. In some cases pain can be evoked by a previously innocuous stimulus, a phenomenon called allodynia. In addition, responses to noxious stimuli may increase in magnitude and duration, this phenomenon is termed hyperalgesia and is subdivided into primary and secondary. Secondary hyperalgesia describes increased sensitivity of the undamaged skin surrounding the site of injury, and is produced by increased excitability (plasticity) of CNS neurones. Primary hyperalgesia describes the increased sensitivity of the damaged tissue at the site of injury, and is a sensory consequence of the peripheral response to the injury (Raja *et al.*, 1984; Dickenson, 1995a; Dickenson, 1995b; Gebhart, 1995).

1.2 Anatomy and physiology of spinal nociceptive pathways

1.2.1 Segmental classification of the spinal cord

The spinal cord is organised into 31 continuous segments in man. The cervical segments (C1 - C8) innervate the arms and neck region. The thoracic segments (T1 - T12) serve the trunk and sympathetic ganglia. The lumbar segments (L1 - L5) supply the legs. The sacral segments (S1 - S5) and the single coccygeal segment innervate the saddle region, buttocks and pelvic organs. Each segment is defined by the dorsal and ventral roots that enter and leave the cord respectively. A pair of dorsal root ganglia are associated with each segment (Murray, 1995). Somatosensory afferent fibres have almost exclusively ipsilateral central projections. Furthermore, the various categories of primary afferent fibre have anatomically distinct termination sites within the dorsal horn (Sorkin & Carlton, 1997).

1.2.2 The organisation of spinal afferent processing

The central axons of primary afferent fibres enter the spinal cord through the dorsal root, divide into a series of rootlets, and synapse with spinal neurones. The spinal neurones then give rise to sensory pathways that ascend to the brain (Willis & Coggeshall, 1991; Gebhart, 1995). Interestingly, there are more axons in each dorsal root than there are dorsal root ganglion cell bodies, supporting the hypothesis that primary afferent fibres branch proximal to the ganglia (Langford & Coggeshall, 1979; Chung & Coggeshall, 1984). The anatomical components of somatovisceral sensation can be classified into several “sensory channels”. The description of a sensory channel includes the peripheral sensory receptors, the terminations of their central axons in the spinal cord, the ascending spinal pathways, and their target areas of the brain. Various sensory channels have thus been defined for mechanoreception, proprioception, thermoreception and nociception (see Gebhart, 1995).

It is significant that there is a marked segregation in spinal termination between small unmyelinated and large myelinated fibres. Generally speaking, small afferents terminate mainly in the dorsal-most region of the dorsal horn whereas large

afferents terminate more deeply (Sorkin & Carlton, 1997; see below). Electrophysiological studies have shown the principal effect at the primary afferent synapse is excitation (Hongo *et al.*, 1968; Light, 1992). Thus, as stimulus from the environment produces a pattern of afferent activity, the spinal cord is structured in such a way as to interpret and encode the spatial and temporal nature of this labelled sensory input. The net result of spinal encoding of afferent input is then communicated to higher centres by several long ascending tracts (Sorkin & Carlton, 1997) (see section 1.2.7).

1.2.3 Primary afferent transmission

Primary afferent terminals in the dorsal horn may contain and release multiple transmitters, the most prevalent example of which is the co-release of an excitatory amino acid (glutamate) and a peptide (substance P) (DeBiasi & Rustioni, 1988; Battaglia & Rustioni, 1988; Hokfelt, 1991). The postsynaptic action of glutamate is to evoke a rapid short-lasting depolarization, whereas SP produces a delayed long-lasting depolarization. Release of primary afferent transmitters is likely to be sensitive to the state of depolarization of the primary afferent terminal, and affected by the frequency of terminal depolarization. Consequently, transmitter release and postsynaptic excitation induced by a given stimulus can be significantly altered by the state and/or frequency of terminal depolarization at that particular point in time (Sorkin & Carlton, 1997).

Further complexity in primary afferent transmission occurs as a result of “volume transmission” effects. Studies conducting analysis of spinal superfusates (Go & Yaksh, 1987), using antibody-coated microelectrode probes (Duggan *et al.*, 1992), and examining receptor internalization (Mantyh *et al.*, 1995) have shown that substance P can be detected at considerable distances from the presumed point of release. It has been assumed the kinetics of substance P volume transmission in the dorsal horn are at least first order (Sorkin & Carlton, 1997). This simply means the greater the intensity and duration of the stimulus (noxious afferent input) the further the released transmitter will diffuse. Indeed, large conditioning stimuli have been shown to affect the properties of convergent neurones deep within the dorsal horn (Svendsen *et al.*, 1997, 1998).

Finally, on a “systems” level, primary afferent transmission is subject to modulation by intrinsic spinal systems and descending bulbospinal systems. There is substantial functional evidence that intrinsic and descending systems can reduce C-fibre input, enhance C-fibre evoked output, reduce A-fibre evoked output, and affect the size of the peripheral receptive field (Sorkin & Carlton, 1997).

1.2.4 The organisation of spinal neurones

The spinal cord consists of a butterfly shaped core of grey matter containing cell bodies and their processes. The grey matter is surrounded by a sheath of white matter composed primarily of myelinated axons. Grey matter in the spinal cord is either classified as sensory and assigned to the dorsal horn, or motor and assigned to the ventral horn (Murray, 1995; Sorkin & Carlton, 1997, see figure 1). On the next level of classification grey matter may be subdivided into nuclei or laminae depending on whether a longitudinal or cross-sectional view is being taken respectively. In this context the term nucleus refers to functionally related clusters of neurones forming columns which can extend the entire length of the spinal cord. Each nuclei can be distinguished histologically as well as functionally (Murray, 1995). However, in the 1950's Rexed examined the cord in cross-section and showed spinal grey matter (particularly the dorsal horn) could be organised as a laminated distribution of functionally related cells extending from the sacral to the cervical cord (Rexed, 1952, 1954). These two classification schemes are compared in table 2.

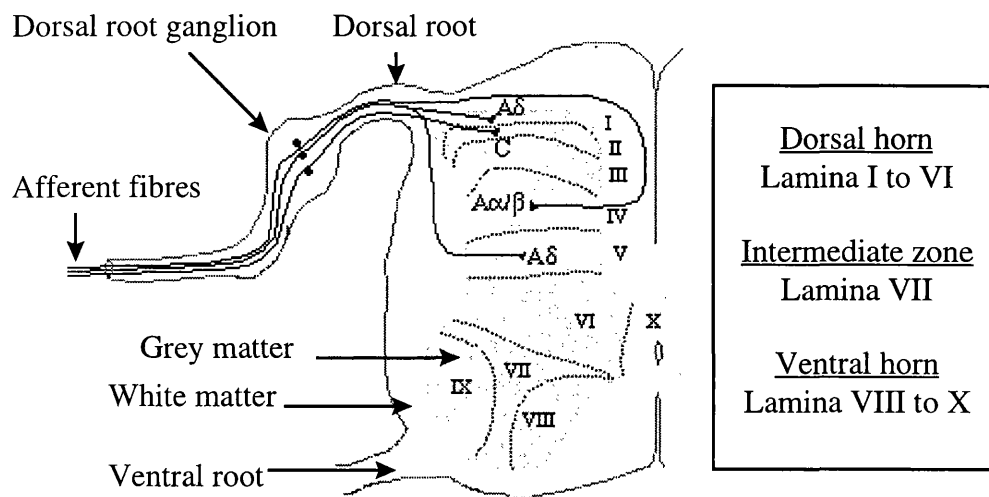


Figure 1. Cross section of the lumbar spinal cord illustrating the termination sites of afferent fibres in the dorsal horn, and the organisation of grey matter into laminae I to X

Subdivision of gray matter as sensory or motor areas	Cross-sectional division into Lamina	Corresponding longitudinal nuclei
Dorsal horn (Sensory function)	Lamina I	Marginal nucleus
	Lamina II	Substantia gelatinosa
	Lamina III, IV	Nucleus proprius
	Lamina V	Reticular nucleus
	Lamina VI	Commisural nuclei
Intermediate Zone	Lamina VII	Clarke's intermediolateral nucleus
Ventral horn (motor function)	Lamina VIII	Medial motor nuclei
	Lamina IX	Lateral motor nuclei
	Lamina X	Central Gray

Table 2. The classification of neurones in spinal grey matter. Laminae I to X, and the nuclei they include.

1.2.5 Termination of primary afferent fibres in the dorsal horn

Intraaxonal injections of the tracer horseradish peroxidase, combined with either a component of cholera toxin or with wheat germ agglutinin, are preferentially transported by large and small diameter primary afferents respectively and can be visualised using a polarized darkfield micrograph technique (Sorkin & Carlton, 1997). Studies using this method have clearly demonstrated the segregation between different classes of primary afferent fibre in the dorsal horn (see Fitzgerald, 1989 for review, see figure 1).

Generally, the terminals of large-diameter afferents are found only in the deeper laminae of the dorsal horn. A β fibres associated with hair follicle receptors terminate in laminae III and IV, whereas those associated with mechanoreceptors terminate across laminae III to V (Sorkin & Carlton, 1997). The terminals of A δ -fibres arising from D-hair receptors that carry innocuous information are found primarily in lamina III, with a small population in lamina Iii (Light & Perl, 1979). A δ -fibres associated with nociceptive high threshold mechanoreceptors distribute to laminae I, Iio and V (Sorkin & Carlton, 1997). Nociceptive C-fibre afferents terminate in the superficial horn. There is some debate as to whether C-fibre terminals are restricted to lamina II alone, or whether they include both laminae I and II. Either way, it is safe to assume lamina II is the main site of termination for nociceptive cutaneous C-fibres (Sorkin & Carlton, 1997).

1.2.6 Classification of dorsal horn neurones

There are several different systems of classification that may be used to describe neurones in the dorsal horn. In simple terms, dorsal horn sensory neurones that receive input from primary afferent fibres can be described in three categories, interneurones, propriospinal neurones, and projection neurones (Sorkin & Carlton, 1997). Interneurones have small cell bodies with axons terminating close by. They act as local relays in spinal processing and are often subdivided as excitatory or inhibitory. Propriospinal neurones have axons that extend across segments and hence are involved with information transfer across segments and heterosegmental

reflex responses. Projection neurones send axons to supraspinal centres and are responsible for the transfer of somatosensory data, including nociceptive information, from the spinal cord to the brain (Sorkin & Carlton, 1997).

Alternatively, dorsal horn neurones may be classified by their response to peripheral stimuli, laminar location of the cell body, projection site or neurochemical content (see Willis & Coggeshall, 1991 for review). As a prequel to the present study I will summarise the “intensity-dependent” classification (see Sorkin & Carlton, 1997), which includes information about peripheral input, followed by a general description of the individual lamina of the dorsal horn.

The intensity-dependent classification of dorsal horn cells has four categories, low-threshold cells (class 1), multireceptive cells (class 2), nociceptive-specific cells (class 3), and deep cells (class 4) (Sorkin & Carlton, 1997). Low threshold (LT) cells respond maximally to innocuous stimuli such as touch or hair movement and show no increase in firing after more severe noxious stimulation (Mendell, 1966). LT cells receive peripheral input from A β fibres and are found primarily in laminae III and IV (Sorkin & Carlton, 1997).

Multireceptive cells, commonly referred to as wide dynamic range (WDR) or convergent neurones, are so named because they receive afferent input from A β , A δ , and C-fibres (Mendell, 1966). Significantly, the output from WDR neurones differentially encodes the intensity of the afferent input (Mendell, 1966). This means the greater the intensity of afferent input, the higher the frequency of WDR cell firing, a phenomenon observed for both innocuous and noxious input. WDR neurones are localised in lamina V with a smaller population present in lamina I (Sorkin & Carlton, 1997). Because WDR neurones fire in response to both innocuous and noxious afferent input the question arises, how do WDR cells distinguish between noxious and innocuous stimuli and selectively signal pain? It is currently thought interpretation of the output from a single WDR neurone is unlikely to distinguish between innocuous and noxious events. Interpretation of the response of a population of these cells is thought to be required to initiate the perception of pain (Sorkin & Carlton, 1997).

Class 3 nociceptive-specific cells, or high-threshold (HT) cells, respond exclusively to noxious stimuli (Cervero *et al.*, 1976). HT cells have been subdivided into class 3A and 3B based on whether they receive afferent input from

A δ -fibre nociceptors or from both A δ and C-fibre nociceptors respectively (Cervero *et al.*, 1976). The highest concentration of HT cells is found in lamina I, with a smaller population in lamina V (Sorkin & Carlton, 1997). Class 4 deep cells respond to stimulation or manipulation of joints and muscles (Sorkin & Carlton, 1997).

Lamina I

Lamina I, sometimes referred to as the marginal layer, is a thin band capping the gray matter. It contains large marginal cells of Waldeyer and several subdivisions of smaller neurones (Bennett *et al.*, 1981; Sorkin & Carlton, 1997). The majority of lamina I cells have dendritic trees confined to lamina I itself (Light *et al.*, 1979; Lima *et al.*, 1986; Woolf & Fitzgerald, 1983). Retrograde axonal transport studies report a significant proportion of lamina I marginal cells are projection neurones. Axons from lamina I neurones contribute to the spinocervical, spinoreticular and spinothalamic tracts, as well as projecting to the periaqueductal gray and parabrachial nucleus (see refs in section 1.2.7 below). Finally, there is also evidence for lamina I cells with dense axonal structures confined to lamina I (Bennett *et al.*, 1981).

Lamina II

Lamina II, or substantia gelatinosa (SG), is composed of densely packed small neurones and is often divided into two separate layers, lamina II outer (IIo) and lamina II inner (IIi) (Sorkin & Carlton, 1997). Neurones in lamina II can be divided into Stalk cells and Islet cells. Stalk cells are localized in IIo and have dendrites that extend ventrally into lamina IV, whereas their axons project to lamina I (Gobel, 1978; Brown, 1981; Bennett, 1981). Islet cells are found predominantly in IIi and their dendrites and axons do not extend beyond lamina II (Gobel, 1978; Brown, 1981). It was suggested 20 years ago that Islet cells correspond to inhibitory interneurones (Gobel, 1978), and subsequently Islet cells have been shown to contain GABA (Todd & McKenzie, 1989). Stalk cells on the other hand are thought to be excitatory.

Interestingly, the majority of cells in the SG have different response profiles to other dorsal horn neurones. Indeed, generally speaking cells in lamina II will respond in the opposite manner to their counterparts in other laminae. Consequently, SG neurones are often described as “inverse classes” 1 to 3, and are inhibited by input that would be excitatory on dorsal horn cells in other laminae (Sorkin & Carlton, 1997). It is notable that SG cells do not appear to be subject to descending modulation by supraspinal structures (Cervero *et al.*, 1979a) but appear to be involved with tonic inhibition of dorsal horn neurones in other laminae, i.e. function as inhibitory interneurones (Cervero *et al.*, 1979b).

Lamina III

The cells in lamina III are reported to have small cell bodies (Rexed, 1952) with patterns of dendritic growth similar to cells in lamina II (Sorkin & Carlton, 1997). Lamina III also contains some projection cells which contribute to the spinocervical tract and the postsynaptic dorsal column (Brown, 1981; Bennett *et al.*, 1984). Interestingly, there is evidence that postsynaptic dorsal column projection neurones have dendrites extending to the superficial dorsal horn, and so may receive input from primary afferent C-fibres, whereas cells contributing to the SCT have dendritic trees confined to lamina III (Sorkin & Carlton, 1997).

Other (non-projection) types of cell in lamina III have extensive dendritic trees that project both dorsally and ventrally into laminae I, II, IV and V (Brown, 1981). Such comprehensive dendritic structure suggests these cells could receive input from all types of primary afferent fibre (Sorkin & Carlton, 1997). There are populations of lamina III cells with axonal projections to laminae IV, V and VI (Matsushita, 1969) and others with axons confined within the laminar boundary (Matsushita, 1969; Schoenen, 1982; Maxwell, 1985).

Laminae IV, V and VI

Laminae IV, V and VI constitute the remainder of the dorsal horn. Three types of lamina IV neurones can be distinguished by their differing patterns of dendritic growth (Sorkin & Carlton, 1997). The complex organisation of lamina IV cell dendrites suggests they play an important role integrating the different

modalities of afferent input (Sorkin & Carlton, 1997). Lamina IV cells are of uniform size and more sparse than cells in lamina III due to the greater number of nerve fibres that travel through this region (Rexed, 1952). Lamina IV also contains projection neurones that convey nociceptive information to the brain via the spinothalamic tract, postsynaptic dorsal column, and the spinothalamic tract (see refs in section 1.2.7 below). Finally, an additional category of lamina IV neurone is reported, similar in dendritic structure to projection neurones, but with locally terminating axons (Brown, 1981).

Lamina V contains an even greater variety of cell types than lamina IV. However, the dendritic pattern of lamina V cells extends upwards towards more superficial areas (Sorkin & Carlton, 1997). Lamina V also contains projection neurones that contribute to the spinothalamic tract and postsynaptic dorsal column, however the most common lamina V projection cells supply the spinothalamic tract (see refs in section 1.2.7 below). Cells in lamina V that send axons locally to laminae III, IV and V have also been reported (Matsushita, 1970).

Lamina VI is only found in the cervical and lumbar regions of the spinal cord (Sorkin & Carlton, 1997). Few studies have specifically addressed Lamina VI neurones, however they appear to be fundamentally similar those found in lamina V (Sorkin & Carlton, 1997). Labelling studies have shown there are projection cells in lamina VI, however it is thought most of the neurones in this region are propriospinal (Sorkin & Carlton, 1997).

1.2.7 Ascending pathways to higher centres

Projection neurones in the dorsal horn give rise to axons that form ascending sensory pathways to higher centres. The projecting axons are found in the spinal white matter, which is subdivided into the dorsal, lateral and ventral funiculi (Gebhart, 1995). The major ascending tracts are the spinothalamic tract (STT), the spinomesencephalic tract (SMT), the spinoreticular tract (SRT), the spinothalamic tract (SCT), the spinothalamic tract (SHT), and the postsynaptic dorsal column (PSDC) pathway (Gebhart, 1995; Murray, 1995; Sorkin & Carlton, 1997).

Ascending pathways are located in both the ventral and dorsal funiculi and most respond differentially or exclusively to noxious input. Generally speaking,

axons of projection cells in the superficial laminae (predominantly lamina I) ascend via the dorsolateral funiculus, although many join the axons of projection cells in the deeper laminae and ascend via the anterolateral funiculus. Axons from cells contributing to the STT, SRT and SMT cross to the contralateral side of the spinal cord before projecting up to the brain (Gebhart, 1995, Sorkin & Carlton, 1997 - see figure 2).

Retrograde axonal transport studies have shown low threshold cells are prevalent in the SCT and PSDC, and less common in the STT. In contrast, wide dynamic range neurones are most common in the STT, but also found in the SMT, SRT, SCT, and SHT. High-threshold nociceptive specific cells only appear to be present in the STT, SMT, and SHT ((SCT) = Brown & Franz, 1969; Brown, 1971, 1973; (PSDC) = Giesler & Cliffer, 1985; (STT) = Willis *et al.*, 1974; Owens *et al.*, 1992; (SMT) = Yeziarski & Schwartz, 1986; Hylden *et al.*, 1986; Yeziarski *et al.*, 1987; (SRT) = Brown & Franz, 1969; Brown, 1973; (SHT) = Burstein *et al.*, 1991).

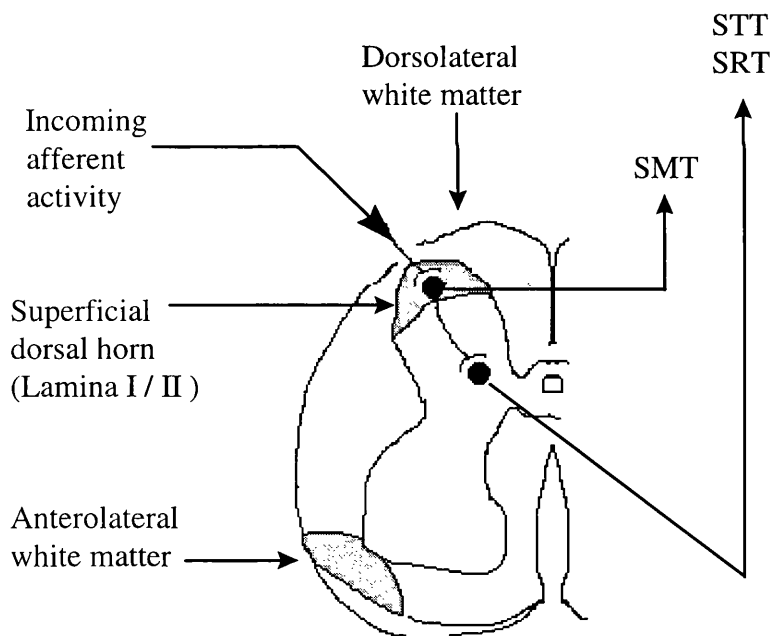


Figure 2. Cross section of the lumbar spinal cord illustrating the major dorsal and ventral ascending pathways. STT = spinothalamic tract, SMT = spinomesencephalic tract, SRT = spinoreticular tract.

1.3 Descending control from higher centres

1.3.1 A feedback loop in nociceptive transmission

Neuronal activity in the spinal cord can be influenced by transmitters released from either intrinsic spinal systems or the terminals of extrinsic descending neurones from the brain. Descending pathways that influence nociceptive transmission in the dorsal horn originate from a wide variety of midbrain and brainstem sites and use a range of neurotransmitters including NA, 5-HT, GABA and the endogenous opiates (for reviews see Basbaum *et al.*, 1983; Basbaum & Fields, 1984; Advokat, 1988; Proudfit, 1988; Reichling *et al.*, 1988; Fields *et al.*, 1991; Stamford, 1995; Millan, 1997).

The fundamental circuitry governing the descending control of nociceptive transmission is focused around several midbrain and brainstem areas. The midbrain periaqueductal grey (PAG), the brainstem rostroventromedial medulla (RVM), and the spinally projecting brainstem noradrenergic cell groups, most notably the locus coeruleus (LC), subcoeruleus (SC), A5 and A7 (see figure 3). The areas within the RVM of greatest significance in the descending modulation of nociception include the nucleus raphe magnus (NRM) and the adjacent nuclei of the reticular formation, which include the nucleus reticularis paragigantocellularis (PGi) and the nucleus reticularis gigantocellularis (Gi) (see previous reference for list of reviews). Accordingly, there have been numerous studies demonstrating spinal modulation of nociceptive transmission as a result of electrical and/or pharmacological stimulation of these and other brain regions ((LC/SC) Clark *et al.*, 1991; Kwiat & Basbaum, 1992; Proudfit, 1992; (PAG) Satoh *et al.*, 1982; Barbaro *et al.*, 1985; Jensen & Yaksh, 1986; Aimone *et al.*, 1987; (RVM/NRM) Sagen *et al.*, 1983; Jensen & Yaksh, 1984; Hammond & Yaksh, 1984; Hammond *et al.*, 1985; Barbaro *et al.*, 1985; Aimone *et al.*, 1987; Bowker & Abhold, 1990; Gebhart & Randich, 1990; Iwamoto & Marion, 1993).

The midbrain PAG constitutes a major afferent input to the RVM (Mantyh & Peschanski, 1982; Beitz, 1982a,b; Marchand & Hagino, 1983). There is substantial evidence indicating the RVM serves as a relay for midbrain modulation of spinal

nociceptive transmission (see Fields *et al.*, 1991 for review). Consequently, inhibition of nociceptive transmission evoked by electrical or chemical stimulation of the PAG can be prevented by lesion in (or a local anaesthetic injection into) the RVM (see Fields *et al.*, 1991). Other inputs to the RVM and the PAG proceed from higher centres involved with sensory, associative and perceptive function. Specifically, diencephalic and telencephalic structures including the hypothalamus, frontal cortex, amygdala, and the bed nucleus of the stria terminalis (Holstege, 1987). From within the RVM, the NRM, PGi and Gi all project primarily to the dorsal horn via the dorsolateral funiculus (Kwiat & Basbaum, 1992).

Significantly, physiological and anatomical studies have shown that these higher centres receive afferent input from the dorsal horn of the spinal cord, particularly from lamina I (which consists exclusively of nociceptive neurones) and from wide dynamic range neurones in laminae IV and V (Besson & Chaouch, 1987). The spinothalamic tract (STT) projects from both the superficial and deep laminae of the dorsal horn. The spinoreticular tract (SRT) provides a direct link between the dorsal horn and brainstem reticular structures, whereas the spinomesencephalic tract (SMT) projects from lamina I to the PAG (see refs in Sorkin & Carlton, 1997). It appears therefore that a feedback mechanism can exist within the spinal cord, driven by the transmission of nociceptive information from the dorsal horn to the brainstem.

1.3.2 A role for monoamine transmitters

There is considerable evidence supporting a major role for monoamines in the descending modulation of nociceptive transmission. Spinal projections from the RVM contain 5-HT, whereas those from the LC, SC, A5 and A7 cell groups contain NA (Azmitia & Gannon, 1986; Coote & Lewis, 1995). The antinociception induced by electrical stimulation or opiate microinjection into brainstem regions such as the PAG or the RVM reticular formation is reported to be antagonised by intrathecal administration of serotonergic and/or noradrenergic antagonists (Hammond & Yaksh, 1984; Camarata & Yaksh, 1985; Jensen & Yaksh, 1986). Furthermore, there is evidence to suggest modulation of nociceptive thresholds by focal stimulation of the PAG or RVM is associated with increased release of NA and 5-HT in the spinal cord

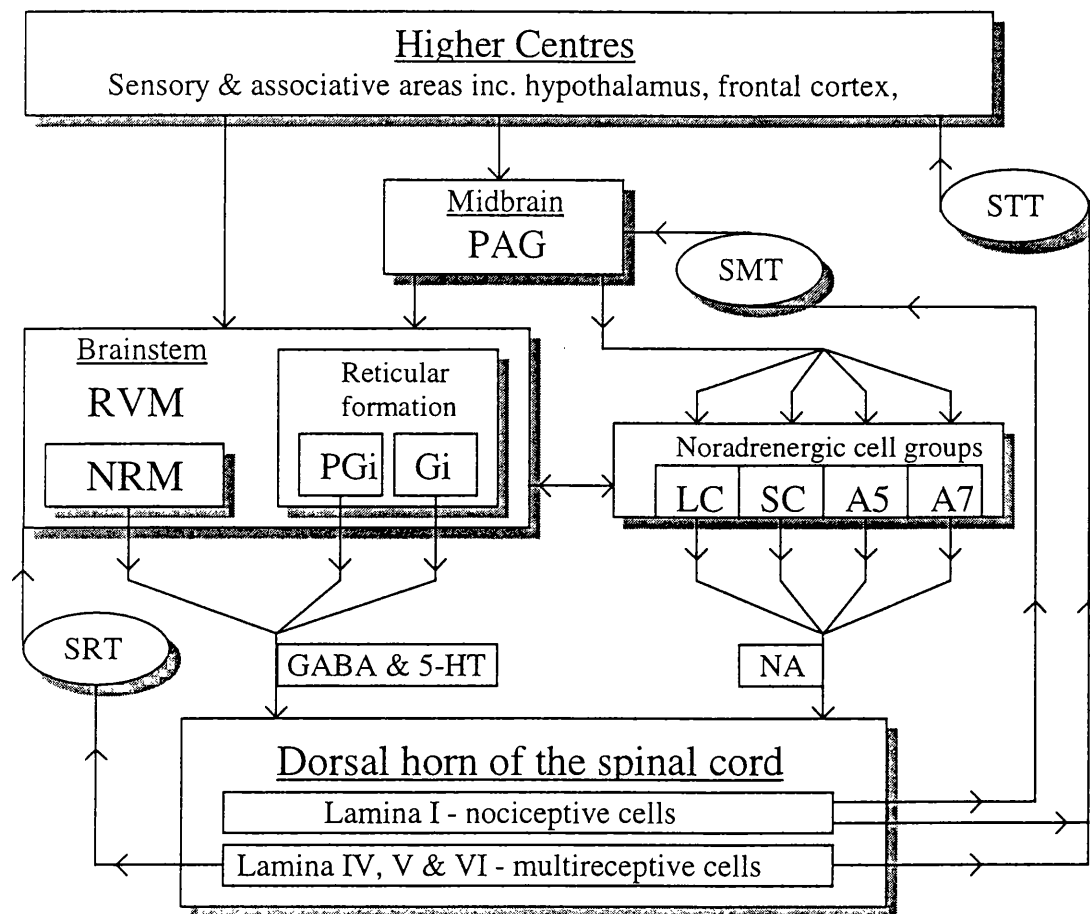


Figure 3. Basic circuitry of the putative feedback loop in nociceptive transmission between the brainstem and the dorsal horn of the spinal cord. STT = spinothalamic tract, SMT = spinomesencephalic tract, SRT = spinoreticular tract, PAG = periaqueductal gray, RVM = rostroventromedial medulla, LC = locus coeruleus, SC = subcoeruleus, PGi = nucleus reticularis paragigantocellularis, Gi = nucleus reticularis gigantocellularis.

(Hammond *et al.*, 1985; Pilowsky *et al.*, 1986). Another study demonstrates release of NA and 5-HT in the spinal cord in response to high but not low intensity afferent stimulation (Tyce & Yaksh, 1981). Of direct relevance to this thesis are several studies addressing the effects of intrathecal administration (or iontophoretic application to the spinal cord) of GABAergic, serotonergic and/or noradrenergic agonists and antagonists in various models of inflammatory pain. This work will be discussed in detail in the appropriate results chapters.

1.3.3 The brainstem noradrenergic cell groups

By the end of the 1980's there was evidence to implicate all of the spinally projecting brainstem NA cell groups (LC, SC, A5, A7) in the descending control of nociception. Noradrenergic neurones of the LC, SC, A5 and A7 had all been shown to project to the spinal cord (Westlund & Coulter, 1980; Westlund *et al.*, 1983; Loewy *et al.*, 1986; Kwiat & Basbaum, 1989; Clark & Proudfit, 1991c). However, at that time it was thought the LC was the predominant source of descending noradrenergic inhibitory control (Fritschy & Grzanna, 1990). Subsequently, using localized injections of a retrograde tracer in conjunction with immunocytochemical labelling, Kwiat and Basbaum (1992) demonstrated the brainstem origin of NA innervation of the dorsal horn in the rat was widespread, including the LC, SC, A5 and A7. Significantly, Kwiat and Basbaum found no single population predominated, consistent with lesion studies that had already suggested that the non-coerulear cell groups also contributed to the noradrenergic innervation of the dorsal horn (Nygren & Olsen, 1977; Clark & Proudfit, 1991b). Therefore, NA-mediated antinociception at the spinal level produced by electrical or pharmacological stimulation of either the LC, SC, A5 or A7 is due to direct activation of a descending noradrenergic projection to the dorsal horn (Kwiat & Basbaum, 1992). However, this is not to say direct activation of these regions does not also cause indirect activation of other descending pathways (Kwiat & Basbaum, 1992).

The mechanism by which the brainstem LC, SC, A5 and A7 cell groups are activated by stimulation of the PAG or RVM is not understood. However, projections from the RVM and PAG to several brainstem noradrenergic cell groups including the LC, SC, A5 and A7 have been demonstrated (Sakai *et al.*, 1977; Cederbaum & Aghajanian, 1978; Byrum & Guyenet, 1987; Clark & Proudfit, 1991a).

1.3.4 *The rostroventromedial medulla*

The descending pathways from the RVM to the spinal cord have also been the subject of extensive investigation. Multiple lines of evidence indicate projections to the dorsal horn from within the 5-HT populations of the RVM ((anterograde tracing) Basbaum *et al.*, 1978, 1986; (retrograde labelling) Kwiat & Basbaum, 1992; (lesion studies) Martin *et al.*, 1978; Basbaum & Fields, 1979; Skagerberg & Bjorklund, 1985). The most significant projections from the RVM to the dorsal horn originate from the NRM, PGI and Gi (Kwiat & Basbaum, 1992).

Until recently it was generally assumed that spinal inhibition of nociceptive transmission resulting from RVM stimulation was due to activation of descending pathways that release 5-HT in the dorsal horn (Dahlstrom & Fuxe, 1965; Bowker *et al.*, 1981). Considerable weight was added to this hypothesis by the number of studies reporting stimulation of the NRM or intrathecal application of 5-HT produced inhibition at the spinal level (Yaksh & Wilson, 1979; Basbaum & Fields, 1984; Besson & Chaouch, 1987; Solomon & Gebhart, 1988; Crisp *et al.*, 1991; Antal *et al.*, 1996; Bardin *et al.*, 1997b). However, there is now a growing body of evidence to suggest GABAergic and glycinergic neurones of the RVM also make a significant contribution to raphe-spinal inhibitory modulation of nociceptive neurones in the spinal cord (see Antal *et al.*, 1996). In the last ten years several groups have presented evidence suggesting the RVM is not a homogeneous population of 5-HT cells (Skagerberg & Bjorklund, 1985; Bowker & Abbott, 1990; Jones & Light, 1990; Jones *et al.*, 1991; Kwiat & Basbaum, 1992; Potrebic *et al.*, 1994). Over the same period of time other groups found evidence that multiple neurotransmitter systems are involved when raphe-spinal neurones are activated (Belin *et al.*, 1983; Edwards *et al.*, 1987; Milhorn *et al.*, 1987; Sorkin *et al.*, 1993). Consequently, Sorkin *et al.* (1993) and McGowan & Hammond (1993a,b) conducted physiological and pharmacological studies that specifically pointed to a non-5-HT dependent raphe-spinal pathway, activation of which resulted in GABA and/or glycine release in the dorsal horn. They showed stimulation of raphe-spinal fibres produced a potent inhibitory effect on dorsal horn nociceptive neurones without any detectable 5-HT release, but with significant release of GABA and/or glycine (Sorkin *et al.*, 1993; McGowan & Hammond, 1993a,b). Finally, additional

anatomical evidence has been presented by Antal *et al* (1996) who traced the descending pathways from the NRM to the dorsal horn. They report raphe-spinal pathways from the NRM to lamina I, II, IV and V in the dorsal horn, a high proportion of which contained GABA, and some of which were also immunoreactive for glycine (Antal *et al.*, 1996).

1.3.5 *The On / Off cell hypothesis*

The On / Off cell hypothesis concerns three physiologically distinct classes of neurones identified in the RVM. These neurones are intermixed and distributed throughout the RVM (Fields *et al.*, 1991) and are classified as “On-cells”, “Off-Cells” or “neutral cells” based on the temporal correlation of changes in their firing when a nocifensor reflex (e.g. a tail flick) is executed. Just before the reflex occurs, On-cells increase their firing rate, Off-cells abruptly stop firing, and neutral cells display no change in firing (Fields *et al.*, 1988).

The hypothesis proposes On-cells are likely to facilitate nociceptive transmission whereas Off-cells are likely to inhibit. Certainly the fact that On-cells are active just prior to, and during, a tail flick suggests they have no potent inhibitory effect on nociceptive transmission. Furthermore, On-cells themselves are inhibited by morphine (Barbaro *et al.*, 1986; Cheng *et al.*, 1986). Conversely, Off-cells have been shown to be the only neurones in the RVM that are continuously activated by morphine when administered either systemically (Fields *et al.*, 1983) or by microinjection into the PAG (Cheng *et al.*, 1986). Therefore, since morphine selectively activates Off-cells, and since inhibition of a nocifensor reflex requires activation of an RVM output neurone (Fields *et al.*, 1991), the Off-cell must have a net inhibitory effect on such nocifensor reflexes. Additional evidence to suggest On- and Off-cells are involved in descending nociceptive modulation comes from the fact that a significant proportion of each class of neurone can be antidromically activated from the spinal cord (Vanegas *et al.*, 1984a). Also, both On- and Off-cells are reported to be excited by electrical stimulation in the PAG at intensities sufficient to inhibit the tail-flick response (Vanegas *et al.*, 1984b). However, these two populations of neurones are reported to fire reciprocally under most conditions (Fields *et al.*, 1991).

In his 1991 review Fields outlines additional evidence for the involvement of noradrenergic modulation of On-cell activity, serotonergic transmission in Off-cell activity, and GABAergic mediation of the Off-cell pause. However, the evidence described above supporting a role for GABA and questioning whether 5-HT is the predominant inhibitory transmitter released in the spinal cord following activation of descending pathways from the RVM also has implications for the On-/Off-cell hypothesis. Indeed, a recent study by Potrebic *et al.* (1994) examined 25 On-, Off-, and neutral cells in the RVM. None of the 9 Off- and 8 On-cells described in the study showed immunoreactivity for 5-HT. Of the 8 neutral cells in the study, only 4 demonstrated 5-HT immunoreactivity.

1.3.6 *From neurones to pain*

The International Association for the Study of Pain (IASP) has defined pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. Two distinct components of painful sensory experiences have been classically defined and divided into the motivational-affective component and sensory-discriminative component (Gebhart, 1995).

The motivational-affective component of pain concerns the emotional responses that make an individual’s experience of pain unique. The anatomical foundation of the motivational-affective component is not clear, but is thought to involve the spinoreticular and spinomesencephalic projections to the brainstem. Only the sensory-discriminative component of pain is directly linked to the stimulus environment, and has been associated with a defined sensory channel that includes the projections to the thalamus and somatosensory cortex via the spinothalamic tract (Gebhart, 1995; Sorkin & Carlton, 1997). In addition, the human experience of pain is thought to be influenced by learned cultural and cognitive factors such as attention, anxiety, anticipation and past experiences (Gebhart, 1995).

The experimental study of pain is limited to the sensory-discriminative component as it is not possible to assess the emotional state of an experimental animal. However, it is clear that transmitters such as serotonin and noradrenaline, which are intimately involved with the function of higher centres concerning

perceptual and emotional states, can have profound effects on putative sensory-discriminative nociceptive circuitry at the spinal level. The organisation of ascending and descending systems between the dorsal horn and brainstem is so complex that any distinction between motivational-affective and sensory-discriminative systems, at least in terms of their spinal pharmacology, would appear to be inappropriate.

1.4 An overview of the spinal cord pharmacology of pain

1.4.1 Neurotransmitter mechanisms in nociceptive processing

It is now widely accepted that mechanisms of spinal nociceptive processing are subject to considerable plasticity, which can dramatically alter the relationship between stimulus and response (for reviews see McQuay & Dickenson, 1990; Dubner & Ruda, 1992; Dray *et al.*, 1994; Dickenson, 1995a,b; Dickenson *et al.*, 1997a,b). Plasticity in spinal nociceptive transmission, combined with the profound complexity of neuronal organisation within the dorsal horn, suggests that in order to examine the effects of a given receptor system on nociceptive transmission it is appropriate to consider the dorsal horn in terms of a dynamic equilibrium between intrinsic excitatory and inhibitory transmission, subject to ongoing modulation by continuing peripheral input and descending control.

The most significant excitatory contributions to this dynamic equilibrium are AMPA and NMDA receptor mediated glutamate transmission, and the activity of excitatory peptides such as substance P which coexists with glutamate in nociceptive fibres (Battaglia & Rustioni, 1988). Inhibitory events in the spinal cord mainly arise from either interneuronal systems utilising GABA and/or the endogenous opioids, or descending inhibitory controls which encompass a range of transmitters including monoamine, opioid and GABA mediated inhibitory systems (Dray *et al.*, 1994; Dickenson, 1995a; Dickenson *et al.*, 1997a,b). Additionally however almost every neurotransmitter and receptor found in the CNS is also found in the spinal cord (Dickenson, 1995a). The majority of these transmitters and receptors are found in the substantia gelatinosa, one of the most dense neuronal areas in the CNS (Dickenson, 1995a; Dickenson *et al.*, 1997b).

In general terms of receptor localization, neurotransmitter mechanisms within the dorsal horn are commonly subdivided into presynaptic or postsynaptic to the primary afferent terminals. Presynaptic mechanisms can modulate postsynaptic activity by altering the release of primary afferent transmitters. Postsynaptic mechanisms ultimately control the output of interneurons and projection neurons to the brain.

1.4.2 Excitatory transmission

Activation of nociceptive primary afferent fibres results in transmitter release from the primary afferent terminals. The excitatory amino acids glutamate and aspartate are found in the central terminals of both large and small diameter primary afferent fibres (DeBiasi & Rustioni, 1988; Hokfelt, 1991). Release of glutamate and aspartate in the dorsal horn has been demonstrated following low and high intensity stimulation (Skilling *et al.*, 1988; Sorkin *et al.*, 1992). Spinal nociceptive transmission is thought to involve activation of both excitatory amino acid and peptide receptors. The three main receptors for glutamate in the dorsal horn are the AMPA, metabotropic, and NMDA receptors (see Monaghan *et al.*, 1989; Hollmann & Heinemann, 1994). AMPA receptor mediated events participate in the transmission of both innocuous and noxious information. In the case of noxious transmission, AMPA receptors are activated by both acute and tonic noxious stimuli, and appear to establish the baseline level of nociception (Davies & Watkins, 1983; Dickenson & Sullivan, 1990,1991; Dickenson & Aydar, 1991; Hunter & Singh, 1994). NMDA receptor activation occurs in response to repetitive noxious inputs, and serves to amplify the response of spinal neurones (Dickenson *et al.*, 1997a,b,c; see below). The role of the metabotropic glutamate receptors is not well understood, however it may serve to enhance both AMPA and NMDA mediated effects (Watkins & Collingridge, 1994).

Substance P and neurokinin A are released from the central terminals of small diameter primary afferent fibres in response to high intensity (but not low intensity) afferent input (Yaksh *et al.*, 1980; Go & Yaksh, 1987; Duggan *et al.*, 1988). There are several types of postsynaptic tachykinin receptor localized in laminae I, II and X of the dorsal horn. Substance P, neurokinin A, and neurokinin B are the preferred ligands at the neurokinin-1, neurokinin-2, and neurokinin-3 receptors respectively (see Birch, 1997). Calcitonin gene-related peptide (CGRP) is an excitatory peptide that is known to be released from primary afferent terminals in response to noxious stimuli (Miller *et al.*, 1993). CGRP has been reported to cause intraspinal diffusion of substance P (Schaible *et al.*, 1992), however because there are still no useful antagonists for the CGRP receptor the precise role of this peptide remains elusive.

Activation of the NMDA receptor requires glycine binding in addition to glutamate. Furthermore, under normal physiological circumstances in the dorsal horn, the NMDA receptor ion channel is blocked by magnesium. Acute noxious transmission in the spinal cord is carried by the AMPA receptor. If the noxious afferent input persists it is thought that peptides from primary afferent terminals in the dorsal horn start to accumulate, and peptide receptor mediated depolarization eventually results in the removal of the magnesium from the NMDA receptor channel. Once the NMDA receptor is activated calcium pours into the neurone and huge depolarizations occur, causing a significant increase in excitability (Dickenson, 1994a, 1995a; Dray *et al.*, 1994; Dickenson *et al.*, 1997a,b,c). This jump from a low baseline of nociceptive transmission to a much higher level of activity has been termed “wind-up” (Mendell, 1966) and is considered to play a central role in many prolonged pain states (Dickenson, 1994a, 1995a; Dray *et al.*, 1994; Dickenson *et al.*, 1997a,b,c).

The NMDA receptor therefore plays a key role in excitatory processes in the dorsal horn, including states of central hypersensitivity. Indeed, several behavioural and electrophysiological studies have shown that intrathecally administered NMDA receptor antagonists attenuate the second prolonged phase of the formalin response but not the first acute phase (Haley *et al.*, 1990; Dickenson & Aydar, 1991;Coderre & Melzack, 1992; Hunter & Singh, 1994; Millan & Seguin, 1994). This illustrates how prolonged inflammatory pain can be distinguished from acute pain by sensitivity to NMDA receptor antagonism. In addition, intrathecal NMDA receptor antagonists have been shown to return excessive excitatory transmission to approximately baseline levels in several other models of prolonged pain including the inflamed knee joint (Neugebauer *et al.*, 1993a,b), carrageenan induced inflammation of the paw (Ren *et al.*, 1992; Eisenberg *et al.*, 1994), behavioural models of neuropathic pain (Mao *et al.*, 1993; Bennett, 1994), models of allodynia (Yaksh, 1989; Bennett, 1994), and ischemic pain (Sher & Mitchell, 1990). Significantly, NMDA receptor antagonists do not totally abolish activity when applied in these cases, consistent with the hypothesis that NMDA receptors serve as the amplification system for established ongoing noxious transmission (see refs in Dickenson *et al.*, 1997a,b,c).

Spinal NMDA receptors are clearly involved in the induction and maintenance of many ongoing pain states and a number of recent human studies

have confirmed the clinical usefulness of NMDA receptor antagonists (Bennett, 1994; Eide *et al.*, 1994; Price *et al.*, 1994). However, it is significant that NMDA receptor activation alone is not enough to produce persistent pain, a continuing level of noxious afferent activity is also required (Dickenson & Sullivan, 1987a; Taylor *et al.*, 1995). In this respect states of spinal hypersensitivity involving NMDA receptor transmission differ from hippocampal long term potentiation which, once induced, will continue without further input (Bashir & Collingridge, 1992).

There are several other putative excitatory mechanisms in the dorsal horn. It has been proposed that the increase in intracellular calcium associated with NMDA receptor activation leads to the generation of the putative excitatory transmitter nitric oxide (NO) by activation of the enzyme nitric oxide synthase (NOS) (for review see Meller & Gebhart, 1993). There is evidence NO is involved in positive feedback, acting at presynaptic sites to further increase excitatory transmitter release (Sorkin, 1993). Furthermore, excitatory transmission may also be enhanced by prostanoids synthesised in spinal neurones in response to substance P and NMDA receptor induced neuronal activation (Malmberg & Yaksh, 1992).

1.4.3 Inhibitory transmission

There are several inhibitory systems in the dorsal horn that are able to potently suppress excitatory nociceptive transmission. Inhibitory systems are found both pre- and post-synaptically and are able to diminish activity in small-diameter afferent fibres with impressive selectivity. The opiate system is certainly the most widely studied and well understood. However, knowledge of the monoaminergic and GABAergic systems has increased in recent years with the discovery of pharmacologically distinct receptor subtypes and the development of selective drugs. Spinal inhibition can also be mediated by glycine receptors, and receptors for the inhibitory peptides neuropeptide Y, somatostatin and galanin (Dray *et al.*, 1994; Dickenson, 1995a, 1997a,b).

Opiate drugs have been employed for pain relief throughout history, and consequently have been the focus of continuing pharmacological investigation. Three subtypes of opioid receptor have been extensively investigated, μ -, δ -, and κ -receptors. The highest concentrations of opioid receptors in the dorsal horn are

around the C-fibre terminal zones in laminae I and II, with smaller populations present in deeper layers. Approximately 70% of these are μ -receptors, 24% δ -receptors, and the remaining 6% κ -receptors. The proportions of pre- and postsynaptic sites varies for the different subtypes, however dorsal rhizotomy studies have found that over 70% of μ -opioid receptors are localized presynaptically on the primary afferent terminals, illustrating the predominance of presynaptic inhibition of primary afferent transmitter release in opioid analgesia (Duggan, 1992; Dray *et al.*, 1994; Dickenson, 1994b, 1995a; Dickenson *et al.*, 1997b).

The selectivity of opioid mediated antinociception is due to the proliferation of receptors on C-fibre terminals and their absence on large A β -fibres. The mechanism of action of postsynaptic opioid receptors is harder to ascertain as direct hyperpolarisation of postsynaptic convergent neurones would inhibit both noxious and innocuous transmission. However, it is thought that the majority of postsynaptic opioid receptors are localized on interneurons or the dendrites of deep cells penetrating into the C-fibre terminal zone, allowing for selective antinociceptive inhibitory control (Dickenson, 1994b, 1995a; Dickenson *et al.*, 1997b). In addition, an orphan opioid receptor has been identified and termed ORL-1, Opiate-Like Receptor-1. The description "opiate-like" proceeds from reports that the opiate antagonist naloxone has only a weak affinity for this site. An endogenous ligand for the ORL-1 receptor has recently been described and termed nociceptin (Meunier *et al.*, 1995; Reinschild *et al.*, 1995; Carpenter & Dickenson, 1998).

Morphine, first crystallised from opium almost 200 years ago, is perhaps the most significant of the opioid analgesics. Morphine binds preferentially to the μ -receptor, and is now known to exert its analgesic effects at receptors located in the periphery and supraspinal sites as well as the dorsal horn of the spinal cord. Indeed, regardless of the modality of the test, morphine has been found to inhibit nociceptive transmission across the broad range of currently available models of nociception, with the interesting exception of those creating neuropathic pain states. It is significant that the opioid system is subject to plasticity and consequently the effectiveness of morphine and other opioid agonists can vary with different pain states. Briefly, some of the consequences of neuropathic pain may be due to a failure of spinal inhibitory systems, particularly opioid inhibitions. The converse is seen in inflammation however where alterations in the equilibrium governing

nociceptive transmission tip in favour of inhibitory systems and the effectiveness of analgesic drugs may be enhanced. In addition, this plasticity may be affected by other spinal neurotransmitter systems. Cholecystokinin, for example, acting at the CCK-B receptor will reduce the effectiveness of morphine, whereas a powerful synergistic inhibition of nociceptive transmission occurs between NA acting at the inhibitory α_2 -receptor and the effects of morphine mediated via the μ -receptor (Duggan, 1992; Dray *et al.*, 1994; Dickenson, 1994b, 1995a; Dickenson *et al.*, 1997b).

The amino acid GABA has been firmly established as the major inhibitory neurotransmitter in the central nervous system. The extensive distribution and influence of GABAergic terminals suggests the nervous system operates under considerable restraint, with GABA acting as a tonic controller-of excitation at either the GABA_A, GABA_B or GABA_C receptor. In the spinal cord, GABA is concentrated in interneurons (islet cells) of the superficial dorsal horn. Between 24-33% of neurones in laminae I-III are reported to contain GABA (Magoul *et al.*, 1987; Todd & McKenzie, 1989; Todd, 1990; Todd & Sullivan, 1990; Powell & Todd, 1992; Spike & Todd, 1992; Todd *et al.*, 1996). There are numerous reports providing evidence for the coexistence of GABA with the inhibitory amino acid glycine in the spinal cord (Aprison & Werman, 1965; Magoul *et al.*, 1987; Todd & McKenzie, 1989; Todd, 1990; Todd & Sullivan, 1990; Powell & Todd, 1992; Spike & Todd, 1992; Todd *et al.*, 1996). In addition, there is evidence GABA can coexist with other putative inhibitory transmitters in separate neuronal populations; galanin (Simmons *et al.*, 1995), met-enkephalin (Todd & Spike, 1992), and neuropeptide Y (Rowan *et al.*, 1993). The superficial dorsal horn is also the main site of termination of A δ - and C-fibre afferents, consequently GABA has been implicated in the modulation of nociceptive input.

GABAergic control of afferent input may occur presynaptically via axo-axonal synapses with primary afferent terminals (Barber *et al.*, 1978; Hunt *et al.*, 1981; Magoul *et al.*, 1987; Alvarez *et al.*, 1992; Spike & Todd, 1992; Bernardi *et al.*, 1995), or postsynaptically via axo-dendritic synapses within the dorsal horn (Magoul *et al.*, 1987; Alvarez *et al.*, 1992; Powell & Todd, 1992; Baba *et al.*, 1994). Both GABA_A and GABA_B receptors are found presynaptically on A δ - and C-fibre afferents as well as on postsynaptic sites and are discussed in detail in the following

section 1.5 (Desarmenian *et al.*, 1984; Bowery *et al.*, 1987; Price *et al.*, 1987). In spite of this substantial body of literature, until now there have been relatively few functional investigations into potentially beneficial manipulation of the GABAergic system for the treatment of pain compared to opioid mechanisms.

1.5 GABAergic, noradrenergic and serotonergic receptor subtypes

1.5.1 GABA receptors

Three pharmacologically distinct classes of GABA receptor have been described, GABA_A, GABA_B and GABA_C receptors. The GABA_A receptor is a multisubunit ligand-gated chloride (Cl⁻) channel, activation of which produces an increase in Cl⁻ channel conductance resulting in membrane hyperpolarization and reduced neuronal excitability (Schofield, 1989; Sieghart, 1992; Malcangio & Bowery, 1996). Significantly, GABA_A receptor Cl⁻ channel properties are subject to modulation by a number of allosteric binding sites on the receptor-channel complex. These allosteric sites are the targets of a diverse range of pharmacologically and clinically important drugs, most notably benzodiazepines and barbiturates (Schofield, 1989; Sieghart, 1992). Benzodiazepines enhance the action of GABA at the GABA_A receptor by increasing the frequency of the Cl⁻ channel opening, whereas barbiturates enhance the action of GABA by increasing the mean channel opening time (Schofield, 1989; Sieghart, 1992).

The GABA_C receptor is also a multisubunit ligand-gated Cl⁻ channel, however it is insensitive to the classical GABA_A receptor antagonist bicuculline (Bormann & Feigenspan, 1995; Malcangio & Bowery, 1996). In contrast, the GABA_B receptor is a GTP-binding protein coupled receptor linked to membrane Ca²⁺ and K⁺ channels. Recently, several pharmacologically distinct subtypes of the GABA_B receptor have been proposed based on their differing sensitivity to the agonists L-baclofen and 3-APPA, and the antagonists phaclofen and CGP35348 (Bonnanno & Raiteri, 1993; Malcangio & Bowery, 1996). Whereas GABA_A and GABA_C receptor mediated inhibition is associated with hyperpolarisation of the neurone, GABA_B receptor mediated neuronal inhibition is thought to be due to reduced Ca²⁺ influx into the terminals, resulting in reduced transmitter release (Malcangio & Bowery, 1996).

In the dorsal horn, inhibitory GABAergic interneurons have been found to correspond to the islet cells of the substantia gelatinosa, whereas stalked cells are thought to be excitatory interneurons (Sorkin & Carlton, 1997). GABAergic control of afferent input may occur presynaptically via axo-axonal

synapses with primary afferent terminals (Barber *et al.*, 1978; Hunt *et al.*, 1981; Magoul *et al.*, 1987; Alvarez *et al.*, 1992; Spike & Todd, 1992; Bernardi *et al.*, 1995), or postsynaptically via axo-dendritic synapses within the dorsal horn (Magoul *et al.*, 1987; Alvarez *et al.*, 1992; Powell & Todd, 1992; Baba *et al.*, 1994). Both GABA_A and GABA_B receptors are present in the dorsal horn. GABA_A receptors appear to be evenly distributed throughout the dorsal horn whereas GABA_B receptors are concentrated in laminae I-III (Desarmenian *et al.*, 1984; Bowery *et al.*, 1987; Price *et al.*, 1987; Malcangio & Bowery, 1996). Interestingly, there is now functional evidence suggesting GABA_C receptors are present in the dorsal horn, located postsynaptically on inhibitory interneurons (Stanfa, personal communication).

1.5.2 Adrenoceptors

Multiple types of α_1 -, α_2 -, and β -adrenoceptors have been cloned (see Table 3 for summary; see Millan, 1997 for review). Of these the α_2 -adrenoceptor is significantly involved with nociceptive transmission in the dorsal horn. There is no functional evidence as yet suggesting β -adrenoceptors are involved in nociception, and only low densities of α_1 -adrenoceptors are present in the dorsal horn (Yaksh, 1985; Hayes *et al.*, 1986; Jones, 1992;).

The potent antinociceptive effects of spinally and systemically administered α_2 -adrenoceptor agonists are well established and have been demonstrated both behaviourally and electrophysiologically in several different models of nociception (electrophysiology = Satoh *et al.*, 1979; Fleetwood-Walker *et al.*, 1985; Zhao & Duggan, 1987; Sullivan *et al.*, 1987, 1992b; Fleetwood-Walker, 1992; Kalso *et al.*, 1993; behavioural = Virtanen, 1986; Pertovaara *et al.*, 1990; Fisher *et al.*, 1991; Kalso *et al.*, 1991; Takano & Yaksh, 1991). Several subtypes of α_2 -adrenoceptor are now known to exist (see Table 3). Of these, the predominant α_2 -subtype (80-90% of total α_2 -adrenoceptors) in the spinal cord has been shown to be the $\alpha_{2A/D}$ -subtype (Uhlen *et al.*, 1991; Lawhead *et al.*, 1992; Renouard *et al.*, 1994, Hunter *et al.*, 1997). Significantly, recent studies of α_{2A} -adrenoceptor knockout transgenic mice

Receptor subtype	Transduction mechanism	Present in dorsal horn
α_{1A}	$\uparrow gCa^{2+}$	(++)
α_{1B}	$\uparrow PLC$	+
α_{1D}	$\uparrow gCa^{2+}$	0
human $\alpha_{2A/D}$	$\uparrow gK^+ / \downarrow gCa^{2+}$	+++
human α_{2B}	?	++
human α_{2C}	?	+
rat $\alpha_{2A/D}$	$\downarrow AC$	+++
rat α_{2B}	$\downarrow AC$	0
rat α_{2C}	$\downarrow AC$	+
β_1	$\uparrow AC$	+
β_2	$\uparrow AC$	0
β_3	$\uparrow AC$?

Table 3. Adrenoceptors in the dorsal horn of the spinal cord. Only the major transduction mechanism is given, other transduction mechanisms should not be discounted. AC, adenylyl cyclase; PLC, phospholipase C. Receptor density in the dorsal horn is given on a scale of "+" to "+++", or "0" where not present. The localization of α_{1A} -adrenoceptors is extrapolated from functional studies, not neuroanatomical investigation, and still requires direct demonstration. Adapted from Millan, 1997.

report the α_{2A} -adrenoceptor is also the primary subtype involved in mediating spinal adrenergic analgesia (Hunter *et al.*, 1997; Stone *et al.*, 1997).

Autoradiography and in situ hybridization studies show α_2 -adrenoceptors are present in high density in the superficial laminae and substantia gelatinosa of the dorsal horn, and are present in lower densities in the deeper laminae (Unnerstall *et al.*, 1984; Dashwood *et al.*, 1985; Seybold, 1986; Simmons & Jones, 1988; Nicholas *et al.*, 1993; Berkowitz *et al.*, 1994). It has been suggested spinal α_2 -adrenoceptors are located postsynaptic to the terminals of descending noradrenergic fibres because spinalisation and 6-hydroxydopamine treatment does not affect receptor numbers

(Yaksh, 1985). Furthermore, neonatal capsaicin treatment to destroy C-fibres is reported to have no effect on the number of clonidine binding sites (Wikberg & Hajos, 1987) and an immunohistochemical study reports no synaptic contacts between descending noradrenergic fibres and primary afferent terminals (Hagihira *et al.*, 1990). This would suggest α_2 -adrenoceptors are not present on primary afferent terminals. However, a recent study using subtype selective antisera to investigate the relative distributions of α_{2A} - and α_{2C} -adrenoceptors in the rat spinal cord found the primary localization of α_{2A} -adrenoceptor immunoreactivity was on the terminals of capsaicin-sensitive, substance P containing primary afferent fibres. In contrast, the majority of α_{2C} -adrenoceptor immunoreactivity was not of primary afferent origin, not strongly colocalized with substance P, and not sensitive to neonatal capsaicin treatment (Stone *et al.*, 1998).

All α_2 -adrenoceptors are linked to inhibitory $G_{i/o}$ type G-proteins. Activation of α_2 -adrenoceptors leads to inhibition of adenylyl cyclase and consequently a decrease in cellular levels of cAMP. α_{2A} -adrenoceptor activation can also lead to hyperpolarization of the cell membrane by regulation of receptor operated K^+ channels and inhibition of voltage sensitive Ca^{2+} channels (for review see Aantaa *et al.*, 1995; Schwartz & Kandel, 1995). Interestingly, a recent study reports multiple α_2 -adrenoceptor subtypes may be present in the same neurones in the rat spinal cord (Gold *et al.*, 1997).

1.5.3 Serotonin receptors

There are currently 14 recognised functional 5-HT receptors (see table 4). Their contrasting modes of coupling support Millans' hypothesis "that various 5-HT receptor types, where colocalized on individual neurones - or on separate neurones of a homogenous functional unit - differentially modify neuronal activity and correspondingly fulfil contrasting roles, for example in the control of mood" (Millan *et al.*, 1992; Millan, 1995). Millan goes on to suggest "a theoretical framework whereby individual 5-HT receptor types play differential roles in the control of nociception at the level of the dorsal horn" (Millan, 1995). This appears to be the case. The recent availability of subtype selective agonists and antagonists has enabled researchers to begin clarifying the large body of confusing and often

contradictory reports of 5-HT receptor mediated antinociception and nociception (see Millan, 1995, 1997 for review; see chapters 5, 6 & 7 for transduction mechanisms and localization of relevant subtypes).

Receptor subtype	Transduction mechanism	Present in dorsal horn
5-HT _{1A}	↓AC / ↑gK ⁺ / ↓gCa ²⁺	+++
5-HT _{1B}	↓AC	+++
5-HT _{1D}	↓AC	+++
5-HT _{1E}	↓AC	?
5-HT _{1F}	↓AC	?
5-HT _{2A}	↑PLC / ↓gK ⁺	+
5-HT _{2B}	↑PLC / ↓gK ⁺	0 / +
5-HT _{2C}	↑PLC / ↓gK ⁺	+
5-HT ₃	↑gCa ²⁺ / gNa ⁺ PLC	+++
5-HT ₄	↑AC	?
5-HT _{5A}	↓AC ?	?
5-HT _{5B}	?	?
5-HT ₆	↑AC	+ / ?
5-HT ₇	↑AC	+ / ?

Table 4. Serotonin receptors in the dorsal horn of the spinal cord. The 5-HT_{1B} (rat) and 5-HT_{1D} (human & guinea pig) are species homologues. Only the major transduction mechanism is given, other transduction mechanisms should not be discounted. AC, adenylyl cyclase; PLC, phospholipase C. Receptor density in the dorsal horn is given on a scale of “+” to “+++”, or “0” where not present. Adapted from Millan, 1997.

1.6 Characteristics of the formalin response

Activity in pathways descending from the brain to the spinal cord, descending controls, have been extensively studied using models that artificially activate their cells of origin in higher centres (see refs in section 1.3). Few studies have examined the spinal consequences of physiological activation of descending systems. Factors that influence activity in descending controls can be reflected experimentally by the parameters of the chosen noxious stimulus. The nature and duration (acute vs persistent) of the stimulus, and the extent of the induced inflammation and tissue damage must be considered. On this basis, the formalin response is appropriate since it includes both an early and a later phase that are believed to reflect activation of distinct spinal systems. Also, the formalin response has been extensively studied providing a large body of literature for comparative purposes.

As early as the 1930's researchers were injecting small volumes of hypertonic saline in human subjects to produce experimental pain (Lewis & Kellgren, 1939). Over the following decades there were several reports of the use of a peripheral injection of formalin as an inflammatory stimulus in rats (Selye, 1949; Winter, 1965) and cats (O'Keefe, 1964; Melzack & Melnickoff, 1974). Then in 1977 Dubuisson and Dennis presented the formalin test. They published both a detailed description of the behaviour, licking and flinching, indicative of nociception, induced by a peripheral subcutaneous injection of formalin, and an accompanying scheme for quantification of the assumed pain (Dubuisson & Dennis, 1977). Notably, Dubuisson & Dennis described a two phased response, the first phase lasting for approximately five minutes from the time of injection, and the second phase beginning shortly after, approximately 20 minutes post injection, and reaching a peak within an hour. Subsequently, numerous studies employed Dubuisson & Dennis' method, or adaptations of it, as a model of nociception in behavioural experiments with rodents (Dennis *et al.*, 1980; Dennis & Melzack, 1980, 1983; Abbott *et al.*, 1982; Abbott & Melzack, 1983;Coderre *et al.*, 1984). In 1987 a significant development came with the work of Dickenson & Sullivan who demonstrated a biphasic response of neurones to formalin, with the same relative magnitudes and timecourse in the dorsal horn of the spinal cord (Dickenson & Sullivan, 1987a,b). The finding of a comparable biphasic phenomenon that could be

recorded and quantified both behaviourally and electrophysiologically with such similarity reinforced the validity of the formalin test as a model of pain (see figure 4).

Subcutaneous injection of formalin is now an established model of inflammatory pain. It is generally accepted the first phase can last for up to 10 minutes post formalin injection before activity subsides into a silent interphase. The second prolonged tonic phase begins at about 25 min. post injection of formalin. The rate of neuronal firing and nociceptive behaviour reaches a peak and begins to subside within an hour (Dubuisson & Dennis, 1977; Dickenson & Sullivan, 1987)

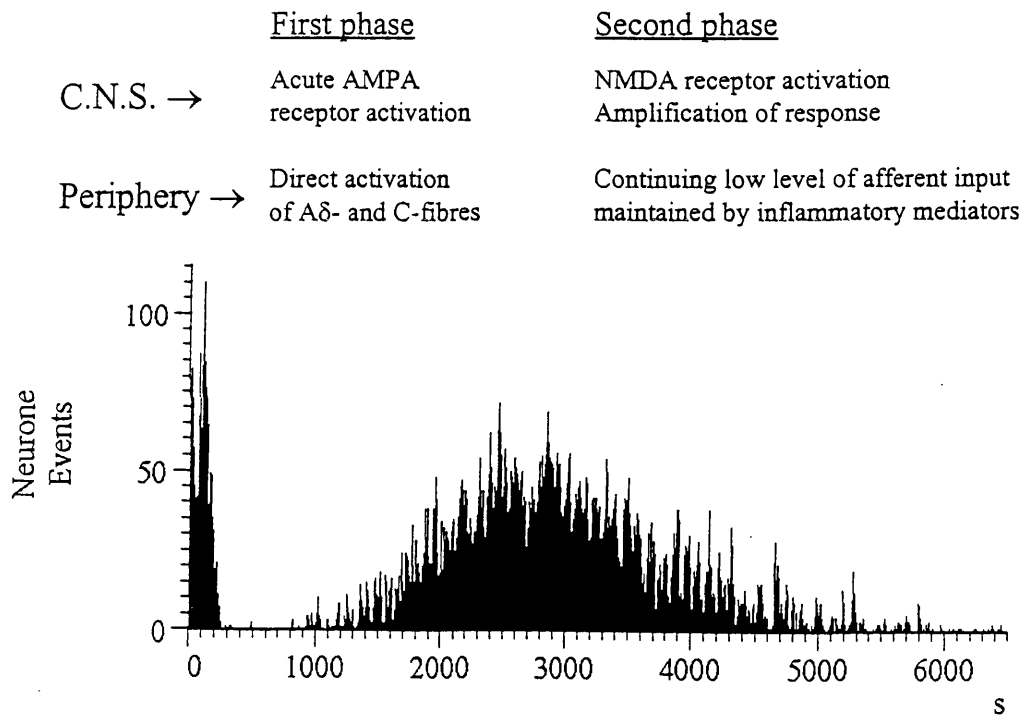


Figure 4. The response of a dorsal horn convergent neurone to a peripheral subcutaneous injection of 5% formalin solution into the receptive field. Recording presented as number of action potentials per unit time in 1 second time bins.

the second phase typically ending 30-40 minutes after it peaks (Green & Dickenson, 1997; Green *et al.*, 1998). The first phase is thought to be due to an acute afferent barrage in response to direct activation of peripheral nociceptors by the formalin. Consequently, glutamate is released from the primary afferent terminals, activating

AMPA receptors generating first phase activity in dorsal horn neurones. The tonic second phase, however, is thought to represent the consequences of continuing afferent input to the spinal cord as a result of the development of peripheral inflammation (Heapy *et al.*, 1987; Tjolsen *et al.*, 1992; Puig & Sorkin, 1995). The action of inflammatory mediators on peripheral nociceptors causes a certain level of afferent input (Heapy *et al.*, 1987; Puig & Sorkin, 1995). This continuing input to the dorsal horn has been shown to be necessary for manifestation of the second phase (Dickenson & Sullivan, 1987; Taylor *et al.*, 1995). There is evidence that bradykinin acting at peripheral B2 receptors is closely associated to second phase activity (Haley *et al.*, 1989; Shibata *et al.*, 1989), and peripheral release of serotonin and histamine may also be involved in the inflammatory response to subcutaneous formalin (Shibata *et al.*, 1989). These inputs, on arrival in the spinal cord, become amplified by mechanisms within the dorsal horn to a higher level of activity typical of the second phase. The activation of spinal NMDA receptors is known to be critical for the manifestation of second phase activity (Haley *et al.*, 1990;Coderre & Melzack, 1992; Yamamoto & Yaksh, 1992; Millan & Sequin, 1993; Vaccarino *et al.*, 1993; Chapman & Dickenson, 1995). Thus NMDA receptor antagonists reduce but do not abolish the second phase whilst having no effect on the first acute phase.

Two notable studies have addressed the question of which types of afferent fibre carry formalin evoked activity to the dorsal horn. Both report 1st phase activity in A δ fibre and nociceptive C-fibre afferents, and second phase activity in C-fibre afferents (Heapy *et al.*, 1987; Puig & Sorkin, 1995). However, Puig & Sorkin (1995) also report 1st phase activity in afferent A β fibres, and second phase activity in A δ fibres with receptive fields in hairy skin. Interestingly, there appears to be no interphase cessation in formalin evoked activity in afferent fibres, again suggesting central inhibitory mechanisms modulate formalin evoked nociception (Puig & Sorkin, 1995).

1.7 Aims of this thesis

Considerable knowledge has been accumulated on the subject of excitatory systems and the opioid inhibitory system in the spinal cord. In order to further understand different pain states, and how pharmacological intervention may be of clinical use, it would be useful to expand our knowledge of spinal inhibitory transmission. Towards this aim, my thesis will focus on the intrinsic and descending GABAergic, noradrenergic and serotonergic systems in the dorsal horn. Specifically, I investigate the endogenous inhibitory systems active during formalin induced peripheral inflammation.

The rate of neuronal firing and nociceptive behaviour in the second phase of the formalin response reaches a peak and begins to subside within an hour (Dubuisson & Dennis, 1977; Dickenson & Sullivan 1987), the second phase typically ending 30-40 minutes after it peaks (Green & Dickenson, 1997; Green *et al.*, 1998). However, the formalin induced inflammation and peripheral oedema persists for many hours after both neuronal and behavioural activity ceases. It is reported that formalin induced oedema does not peak until 4 hours post injection, by which time paw volume has increased by approximately 40% (Wheeler-Aceto & Cowan, 1993). Furthermore, studies observing formalin evoked activity in primary afferent fibres report no interphase cessation in peripheral activity (Puig & Sorkin, 1995). These observations suggest that central inhibitory mechanisms may be involved in the manifestation of the silent interphase period, the relatively abrupt decline of the second phase of the formalin response, and ultimately an ongoing suppression of formalin evoked activity in dorsal horn neurones.

2. METHODS

2.1 Anaesthesia and surgical procedure

The protocol used was based on previous studies from this laboratory (see Dickenson & Sullivan, 1986, 1987a, 1987b). Experiments were conducted on male Sprague-Dawley rats (University College London animal house) weighing 180-220g. All procedures were approved by the Home Office (Project and Personal licence) and follow IASP guidelines (International Association for the Study of Pain, see Zimmermann, 1983).

Anaesthesia was conducted using halothane in a 66% N₂O/33% O₂ mixture. Anaesthesia was induced with 3% halothane. The animal was placed in a sealed box with inflowing anaesthetic until the righting reflex was lost. Next, the animal was removed from the box, the anaesthetic was transferred to a nose cone, the trachea was cannulated and the halothane concentration reduced to 2-2.5%. Once areflexia had been confirmed, the skull was secured by ear bars in a stereotaxic frame to ensure stability during electrophysiological recording. A rectal thermometer was inserted as part of a feedback circuit with a heating blanket to regulate body temperature between 36.5-37°C. The L1-L3 lumbar vertebrae were identified and a rostral clamp was attached to the stereotaxic frame. A laminectomy was performed over this area and the dura of the exposed spinal cord removed. A caudal clamp was then secured to the frame. Halothane concentration was maintained at 2-2.5% throughout the surgery, and reduced to 1.5-2% for the duration of the experiment (levels that produced complete areflexia). The animals continued to breathe spontaneously for the duration of the experiment.

The effects of anaesthetic agents on the formalin response have been studied. O'Connor *et al.* (1995) report male Sprague-Dawley rats that received halothane, enflurane, isoflurane, desflurane or nitrous oxide during the 1st phase demonstrated a significant decrease in phase 2 activity when compared to controls. Only animals that received a combination of halothane and nitrous oxide exhibited no significant difference. The present study uses male Sprague-Dawley rats under halothane and nitrous oxide anaesthesia. Thus our anaesthetic protocol is unlikely to interfere with quantification of putative drug effects.

2.2 Electrophysiological recordings

2.2.1 *Isolating a convergent neurone*

Parylene coated tungsten electrodes were used to make single unit extracellular recordings of convergent dorsal horn neurones that received afferent input from the toes of the hindpaw. The electrode was lowered into the spinal cord in 10 μ m steps using a SCAT microdrive, which allowed measurement of the depth of the neurone relative to the surface of the dorsal horn. The receptive field was stimulated by tapping with a finger as the electrode was lowered. Cells that responded to both non-noxious (touch) and noxious (pinch) stimuli were then stimulated electrically so their identity could be confirmed. A Neurolog stimulating system was used. This consisted of period generator, digital width, pulse buffer and stimulus isolator modules. The output signal from the stimulating apparatus was applied through two electrodes (positive and negative) inserted transcutaneously into the centre of the receptive field. Criteria for identification of spinal convergent neurones was A-fibre evoked activity followed by C-fibre evoked activity in response to the electrical stimulation. The evoked responses of afferent A β -, A δ - and C-fibres were separated by latency (0-20ms, 20-90ms and 90-300ms respectively) and quantified. In addition, the post-discharge and wind-up (excess spikes) of the neurone were recorded (see below).

2.2.2 *Data capture and recording apparatus*

The signal from the isolated cell was amplified and filtered using a Neurolog system. As well as housing the spinal electrode (the A signal), the recording headstage received a second signal from a clip attached to the animal itself (the B signal), and was earthed to the stereotaxic frame. The headstage connection to the animal, or B signal, carried activity from the animal's cardiovascular, muscular and respiratory systems, and electrical interference from other nearby equipment. Initially, the Neurolog system passes the output from the recording headstage through a preamplifier. The amplified signal is then modified to minimise background activity and interference. This was achieved by subtracting the B signal

from the A signal. The differentiated signal was then amplified and filtered, and the final output sent to a loud speaker and oscilloscope (for audio and visual observation) and to a Neurolog window discriminator.

The window discriminator allows neuronal events (action potentials) above a particular amplitude to be counted. If the action potential amplitude of an isolated cell is sufficiently above background a window height may be set to ensure only the activity of that particular cell is recorded. The output from the window discriminator was sent to a CED 1401 interface (CED, Cambridge), and from the CED 1401 to a computer running complementary Spike 2 software (post-stimulus histogram and rate recording functions). The CED interface and Spike 2 process the output of the window discriminator, counting the number of action potentials recorded and separating them into A β -, A δ -, C-fibre evoked responses by latency.

2.2.3 Cell characterization

Once the signal from a convergent neurone had been identified and isolated from background activity the threshold current for C-fibre firing was established. Subsequently, transcutaneous electrical stimulation of the receptive field at three times the C-fibre threshold was applied to produce acute neuronal responses. These responses formed the basis for construction of post-stimulus histograms to a train of 16 stimuli at 0.5Hz with a 2ms pulse width. The resulting A β -, A δ - and C-fibre evoked responses were separated by latency and quantified as described above. The post-discharge of the neurone between 300-800ms was also recorded and quantified. Convergent neurones display hyperexcitability in response to repetitive stimulation. Post-discharge is a term that describes C-fibre evoked activity occurring after the 90-300ms latency band as a result of this hyperexcitability (see Dickenson & Sullivan, 1987). In addition, the number of excess action potentials, or Wind-up (the increased response of the neurone to the constant stimulus; see Mendel, 1966 and chapter 1.4.2), of the neurone was measured by recording the total C-fibre response over a latency of 90-800ms to each stimulus. This included the C-fibre evoked response over 90-300ms and post-discharge C-fibre activity. The total (90-800ms) number of C-fibre evoked action potentials fired in response to the first stimulus was multiplied by sixteen and subtracted from the total response to the stimulus train,

giving a measure of the excess action potentials over the predicted constant response. Post-stimulus histograms were constructed in this manner at ten minute intervals to ensure the response of the cell was stable (less than 10% change between tests) for three consecutive tests before continuing with drug studies (see figure 5 for example).

Number of action potentials

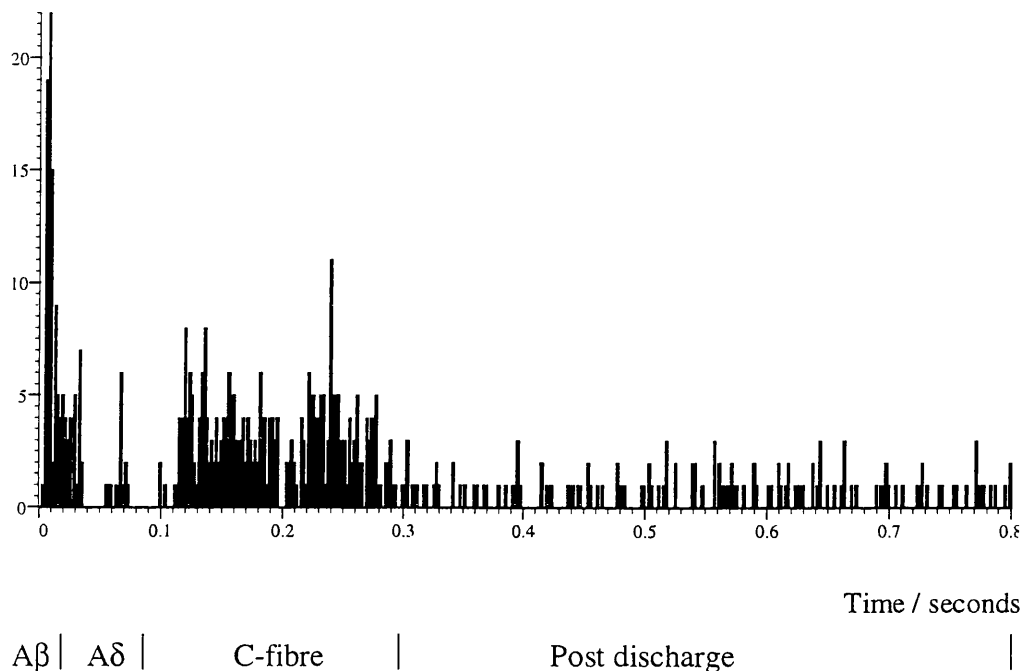


Figure 5. A typical post-stimulus histogram showing the electrically evoked A β -, A δ - and C-fibre responses, and post discharge of a convergent dorsal horn neurone in response to a train of 16 stimuli at 3 x C-fibre threshold.

2.3 Electrical studies

Experiments were conducted to assess the effect of drugs on the electrically evoked responses of convergent dorsal horn neurones. Once satisfactory control post-stimulus histograms had been acquired (see above), drugs were administered intrathecally in a volume of 50 μ l and further post stimulus histograms were constructed every 10 mins for 40-60mins following each dose. Subsequent doses were administered in a cumulative manner. These experiments were conducted to

determine the effect of drugs in normal animals with no inflammation so a comparison could be made with their effects in the formalin model.

2.4 Formalin studies

For experiments with the formalin model the neurones were characterised in the same way as the electrical studies. 50µl of 5% formalin solution was then injected subcutaneously into the centre of the receptive field (a particular toe of the hind paw) to elicit prolonged firing of the neurones. The formalin evoked activity was recorded using a rate function (Spike 2 software, C.E.D. 1401 interface). Neuronal activity during the first 10 minutes post-injection of formalin was attributed to the first phase, subsequent neuronal activity was attributed to the second phase. Control formalin responses showing the characteristic biphasic response were obtained at the start of each experiment before continuing to examine the effects of a drug on the formalin response. Doses of drug were applied directly on the spinal cord in a volume of 50µl.

2.5 Carrageenan studies

For experiments with the carrageenan model the neurones were characterised in the same way as the electrical studies. 100µl of 2% λ-carrageenan solution was then injected into the plantar region of the paw to produce inflammation. The drug effects were tested 3 hours post-carrageenan. Three post-stimulus histograms were constructed (as described above) at 10 min. intervals prior to drug administration to be used as controls. Cumulative doses of drug were applied directly on the spinal cord in a volume of 50µl. Post-stimulus histograms were constructed every 10 min. for 60 min. following the administration of each dose of drug.

2.6 Statistical analysis

The effects of the drugs on electrically evoked responses in both normal and carrageenan animals were expressed as a percentage of control \pm the standard error of the mean (s.e.m.). The effects of drugs on the formalin response were quantified by measuring the area under the curve. Further analysis of data from formalin experiments was conducted, separately comparing the number of action potentials in the first and second phases of the response to their respective controls. Control formalin responses were interspersed with the tests for drug effects within the same experimental days to control for variations in the formalin response over time.

Statistical analysis was performed only on raw data using two-way analysis of variance (ANOVA). For multiple comparisons, the Fisher's PLSD (protected least squares difference) post-hoc test was used (Statview v4.0, Abacus Concepts). Significance was set at $P < 0.05$. In formalin experiments, the area under the curve was calculated using the trapezoidal rule (PCS v2.0, Springer-Verlag).

The trapezoidal rule is given by

$$A \approx \frac{\Delta x}{2} (y_0 + 2y_1 + 2y_2 + \dots + 2y_{n-1} + y_n)$$

The area under the curve, A , is determined by this method when the x-axis is divided into an even number ($2n$) of subintervals ($y_0, y_1, y_2 \dots y_n$), each of width Δx .

The derived units for area under the curve of the formalin response calculated by this method are number of action potentials.

2.7 Drugs

(-)-Bicuculline methobromide, muscimol and 8-hydroxy-DPAT hydrobromide (8-OH-DPAT / (\pm)-8-hydroxy-2-dipropylaminotetralin) were obtained from Tocris Cookson. CGP35348 (P-(3-aminopropyl)-P-diethoxymethylphosphonic acid) was obtained from Ciba-Giegy. Atipamezole was obtained from Farnos. Idazoxan hydrochloride was obtained from RBI. Yohimbine was obtained from Sigma. Ondansetron was obtained from GlaxoWellcome. WAY100635 (N-[2-[4-(2-methoxyphenyl)-1-piperazinal]ethyl]-N-92-pyridinal)cyclohexanecarboxamide trihydrochloride) was obtained from Wyeth-Ayerst. S 18616-1 was obtained from Servier. RS67506 (1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-2-methylsulphonylamino)ethyl-4-piperidinyl]-1-propanone) and RS39604 (1-[4-Amino-5-chloro-2-(3,5-dimethoxyphenyl)methoxy]-3-[1-[2-methylsulphonylamino]ethyl]piperidin-4-yl] propan-1-one) were obtained from Tocris Cookson..

(-)-Bicuculline methobromide and ondansetron were kept in the dark at all times. All drugs were kept at 4°C except muscimol, idazoxan, RS67506, and RS39604 which were stored at room temperature. All stock solutions were made in distilled water and diluted to working concentrations in saline

3. GABA_A AND GABA_B RECEPTORS

3.1 Introduction

There is evidence of GABAergic modulation of nociceptive transmission in inflammatory pain states. Carrageenan induced unilateral inflammation has been shown to increase the number of GABA-immunoreactive cells in the ipsilateral dorsal horn, reaching a peak of 23% over the contralateral side 4 days post injection of carrageenan (Castro-Lopes *et al.*, 1994). This increase in GABA immunoreactivity could be prevented by sciatic neurectomy or neonatal capsaicin treatment, suggesting that the up-regulation of GABA in the dorsal horn is a consequence of noxious stimulation.

Benzodiazepines (BZ) are allosteric modulators of the GABA_A receptor, enhancing the action of GABA and increasing inhibitory transmission (Sieghart, 1992). Consequently, it seems reasonable to assume BZ's might have analgesic potential. Decades of clinical use of these drugs however has shown this is not the case. Indeed, an *in vivo* electrophysiological study recording, in normal animals, nociceptive spinal dorsal horn neurones demonstrated BZ's had no powerful antinociceptive properties but produced a marked depressive effect on A δ -fibre evoked responses (Clavier *et al.*, 1992).

To date several studies have investigated the role of GABAergic inhibition in the formalin model of inflammatory pain. BZ's, barbiturates and ethanol have been shown to abolish the silent interphase period but have no effect on the magnitude of either the first phase or the second phase of the response (Franklin & Abbott, 1993). GABA_A and GABA_B agonists have been reported to suppress both phases of the formalin response, and in the same study GABA_A and GABA_B antagonists were shown to have no effect (Dirig & Yaksh, 1995). However, more recently there have been brief reports that both GABA_A and GABA_B receptors can control the second phase (Ivadomi & Hammond, 1996; Kaneko & Hammond, 1996).

This diversity of results has led to some confusion in determining the contribution of GABAergic inhibitions to nociceptive processing in the spinal cord. The present study aimed to further investigate GABAergic modulation of dorsal horn neurones in inflammatory pain states. In this chapter I investigate GABA receptor mediated inhibitory control of dorsal horn neurones during formalin induced inflammation using the classical competitive GABA_A antagonist bicuculline and

CGP35348, a recently developed selective GABA_B antagonist, more potent than phaclofen and able to cross the blood-brain barrier (Bonanno & Raiteri, 1993). Antagonists were used in order to reveal the extent of endogenous activation of the receptors. The involvement of GABA inhibitions in controlling the magnitude of the formalin response was assessed by spinal administration of the antagonists simultaneously with the peripheral injection of formalin. Furthermore, any contribution of GABA controls to the duration of the response (i.e. decline of the second phase) was examined by administration of the antagonists 50 minutes post-injection of formalin, the time at which the rate of neuronal firing in the second phase typically peaks.

3.2 Methods

Post-stimulus histograms were constructed at ten minute intervals to ensure the response of the cell was stable (less than 10% change between tests). I first studied the effects of CGP35348, muscimol (a GABA_A receptor agonist) and bicuculline on the response to transcutaneous electrical stimulation in a series of animals. Control post-stimulus histograms (as described in chapter 2) were constructed at ten minute intervals until the response of the cell was stable for three consecutive tests. 50µg of CGP35348 was then administered, followed by 5µg muscimol, and finally 50µg of bicuculline to gauge the effectiveness of the antagonists at the relevant GABA receptors. Post-stimulus histograms were constructed every 10 min. for 40 min. after the administration of each drug.

A second group of animals was then used for the formalin studies; neurones were characterized in the same way as with the first series but then, once a C-fibre response of the neurones had been confirmed, 50µl of 5% formalin solution was injected subcutaneously into the center of the receptive field (a particular toe of the hind paw) to elicit prolonged firing of the neurones. The formalin evoked activity was recorded using a rate function (Spike 2 software, C.E.D. 1401 interface) and expressed as number of action potentials in ten minute time bins. Bicuculline and CGP35348 were applied in a volume of 50µl directly onto the spinal cord. The drugs were administered either simultaneously to the formalin injection, or as a post-treatment 50 minutes after formalin.

The effects of drugs on the formalin response were quantified by measuring the area under the curve. The effects of drugs on electrically evoked responses were expressed as a percentage of control \pm the standard error of the mean (s.e.m.).

3.3 Results

The results were obtained from 61 neurones, all of which displayed A β -, A δ - and C-fibre evoked responses to transcutaneous electrical stimulation (average C-fibre threshold of 1.7 ± 0.1 mA). All of the convergent neurones studied were located deep within the dorsal horn at an average depth of 819 ± 30 μ m.

3.3.1 GABA receptor antagonists and electrically evoked responses

Muscimol (the GABA_A agonist) (5 μ g) had little effect on A- β fibre evoked responses, but produced a clear inhibition of A- δ fibre and C-fibre evoked responses, post-discharge and excess spikes reducing them to $28 \pm 10\%$, $40 \pm 11\%$, $30 \pm 30\%$, and $32 \pm 21\%$ of control respectively. Bicuculline, 50 μ g, also had little effect on A- β fibre evoked responses, but partially reversed the muscimol induced inhibition of post-discharge and excess spikes, completely reversed the muscimol induced inhibition of the C-fibre evoked response, and both reversed the effects of the agonist and caused a marked facilitation of the A- δ fibre evoked response ($77 \pm 36\%$, $89 \pm 31\%$, $113 \pm 24\%$ and $204 \pm 91\%$ of control respectively) (figure 6). 50 μ g CGP35348 alone had little effect on A- β fibre, A- δ fibre and C-fibre evoked responses and excess spikes of dorsal horn neurones, however the post-discharge was increased ($183 \pm 71\%$ of control). Furthermore, CGP35348 completely failed to prevent the action of 5 μ g muscimol. 250 μ g CGP35348 has previously been shown to have little effect on C-fibre evoked responses in normal animals ($125 \pm 12\%$ of control), and to reverse the effects of the GABA_B receptor agonist baclofen using the same experimental model (Stanfa, personal communication) which confirms CGP35348 is a selective GABA_B receptor antagonist. Thus the doses of the

antagonists to be used in the formalin studies can be shown to selectively block the appropriate receptors.

3.3.2 *GABA studies with the formalin model.*

On injection of formalin the neurone, previously silent, began to fire vigorously. The characteristic biphasic response was observed in all control experiments. Initial activity subsided within ten minutes, followed by the silent interphase period. Activity recommenced approximately 25 minutes after injection and, in control responses, typically subsided within ninety minutes. Each neurone was recorded for 2 hours 20 minutes. The sum of the action potentials within the first ten minutes post-formalin was termed the first peak (mean control value of 6422 ± 1392). Subsequent activity of the neurone (10 to 140 minutes) was assigned to the second peak (mean control value of 31591 ± 6135).

3.3.3 *Bicuculline and CGP35348 administered simultaneously with formalin.*

50 μ g bicuculline administered simultaneously with formalin significantly increased the magnitude of the response (figure 7), producing an area under the curve of $253 \pm 62\%$ of control ($P = 0.0369$) for the complete response (0min to 2h 20min) (figure 8). Furthermore, the silent interphase was abolished. The level of activity that would otherwise be regarded as the peak of the first phase was sustained until the beginning of second phase activity. The timecourse of first and second phase activity was not altered, and the induction of the second phase was still marked by a further increase in the rate of firing of the neurone (figure 7). Both 50 μ g and 250 μ g CGP35348 administered simultaneous to formalin failed to significantly affect the response (figure 8). The area under the curve for the entire response was $86 \pm 40\%$ and $106 \pm 36\%$ for 50 μ g and 250 μ g respectively (figure 9). Thus GABA_A receptor mediated inhibition is involved in the manifestation of the silent interphase period (10-20 minutes) and controlling the overall magnitude of the

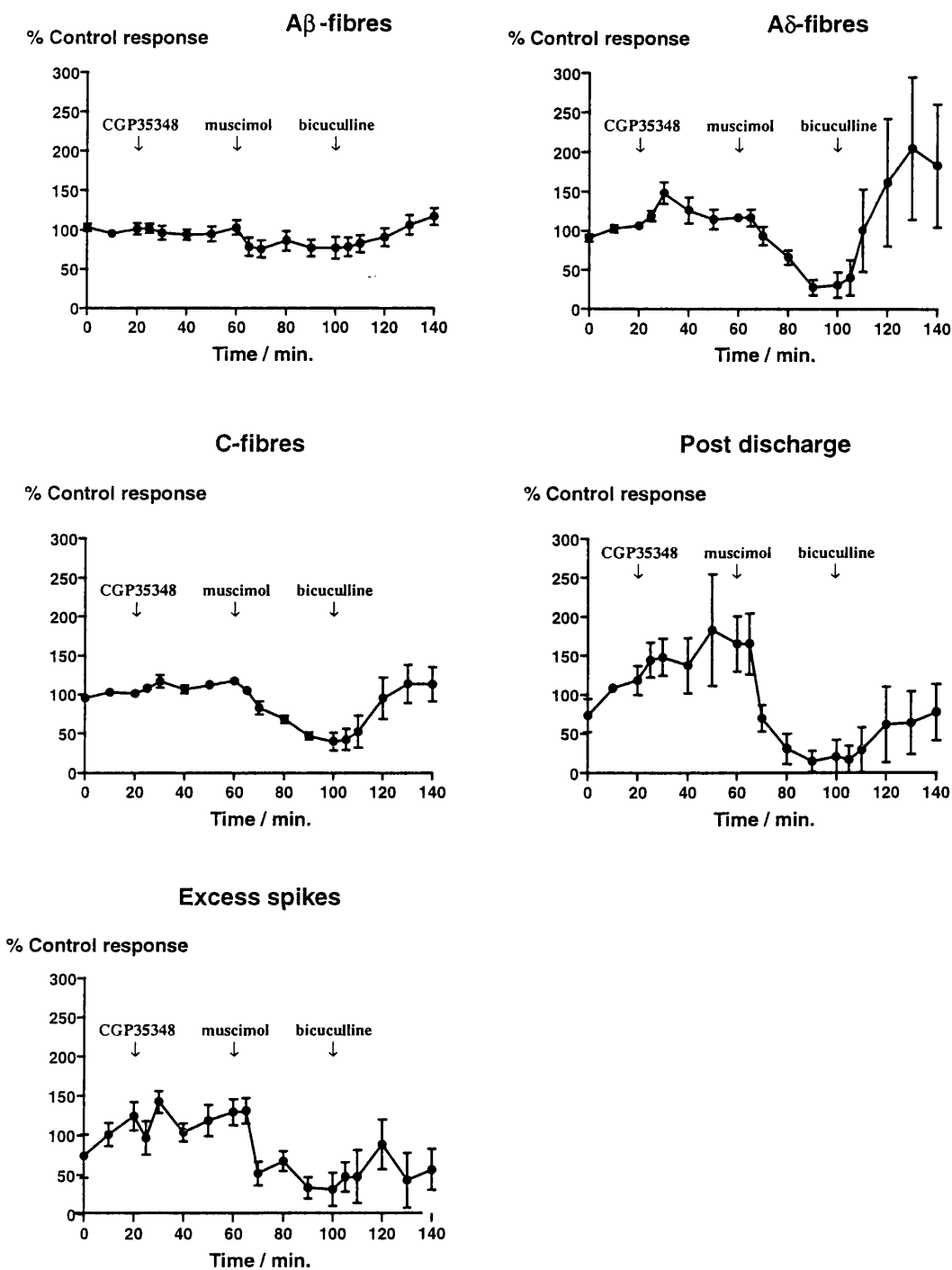


Figure 6. The effect of CGP35348 (50 μ g), muscimol (5 μ g), and bicuculline (50 μ g) on electrically evoked A β , A δ and C-fibre evoked responses, post-discharge, and excess spikes. CGP35348, muscimol and bicuculline were administered at 20 min., 60 min. and 100min. respectively (n = 5-6 neurones for all data points). All drugs were administered i.t. Results are presented as mean % of control response +/- the standard error of the mean.

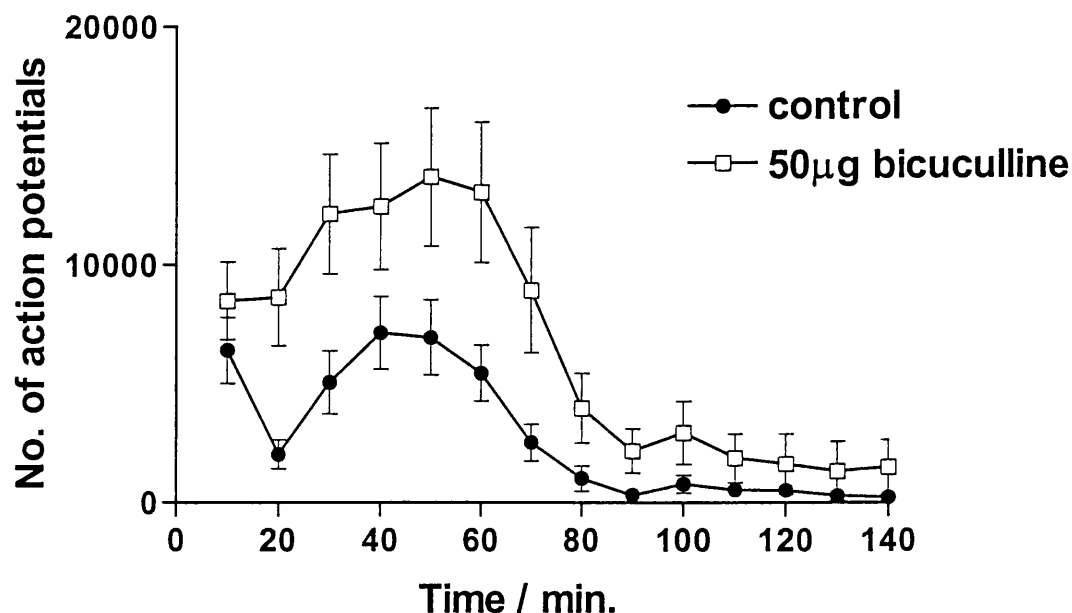


Figure 7. The effect of 50µg bicuculline (i.t.) on the formalin response ($n = 10$ and 6 neurones for control and bicuculline experiments respectively). Bicuculline was administered simultaneously with formalin. Results are presented as mean number of action potentials in 10 min. time bins +/- the standard error of the mean.

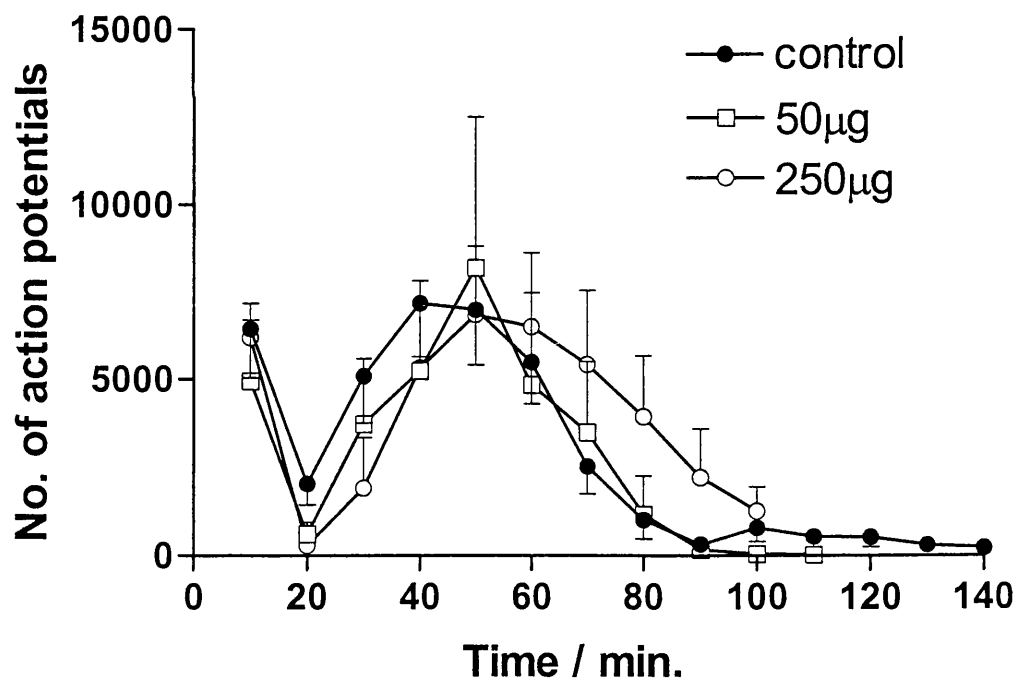


Figure 8. The effect of CGP35348 (50µg or 250µg i.t.) on the formalin response ($n = 10$ and 6 neurones for control and CGP35348 experiments respectively). CGP35348 was administered simultaneously with formalin. Results are presented as mean number of action potentials in 10 min. time bins +/- the standard error of the mean.

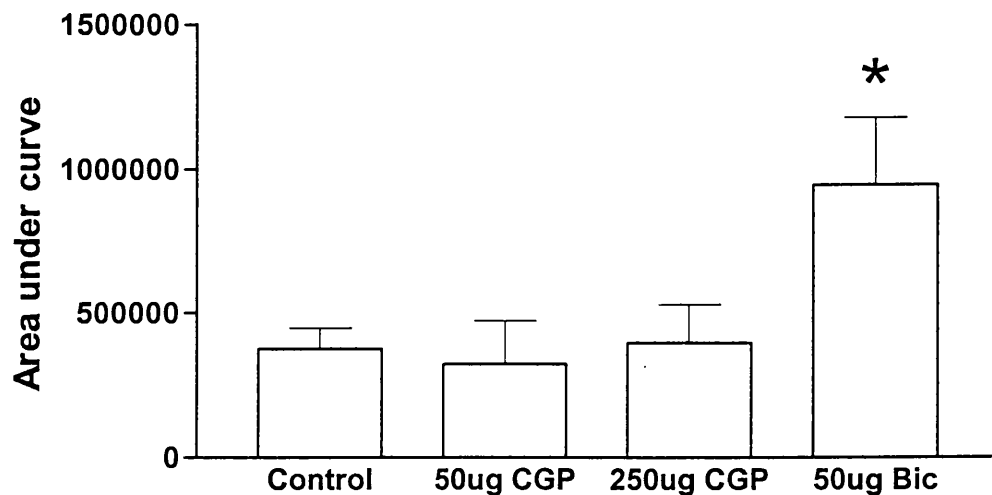


Figure 9. The effects of bicuculline and CGP35348 (50 μ g or 250 μ g i.t.) on the area under the curve of the complete formalin response ($n = 10$ for control and 6 neurones for bicuculline or CGP35348 experiments). All drugs were administered simultaneously with formalin. Results are presented as mean area under the curve +/- the standard error of the mean.

formalin response, whereas GABA_B receptor mediated inhibition does not appear to play a significant role when the antagonist is coadministered with formalin (but see page 64).

3.3.4 Bicuculline and CGP35348 administered 50 min. post-injection of formalin.

Bicuculline administered 50 min. post-injection of formalin, the time at which the second phase typically peaks, caused a dose-dependent increase in the duration of the second phase (figure 10). The total area under the curve from 50 min. to 140 min. was $264 \pm 77\%$, $295 \pm 43\%$, and $475 \pm 76\%$ of control for 5 μ g ($P = 0.0285$), 50 μ g ($P = 0.0110$), and 250 μ g ($P = <0.0001$) of bicuculline respectively (figure 11). Overall, bicuculline significantly increased the area under the curve of the formalin response ($F(3,21) = 9.996$, $P = 0.0003$). Individually, the area under the curve for 250 μ g was significantly different from both 5 μ g and 50 μ g ($P = 0.0165$ and 0.0371 respectively). Interestingly, the areas obtained for 5 μ g and 50 μ g were not significantly different from each other ($P = 0.7081$). Furthermore, 250 μ g of

bicuculline caused a marked change in the pattern of firing of the neurone, manifest in brief “burst-like” periods of intense activity that began minutes after administration and persisted for the duration of the experiment.

CGP35348, administered 50 min. post-injection of formalin, also increased the duration of the second phase (figure 12), significantly increasing the area under the curve of the formalin response between 50 min. and 2h. 20 min. ($F(2,18) = 3.768, P = 0.0430$). Mean areas under the curve were $105 \pm 79\%$ and $273 \pm 65\%$ of control for $50\mu\text{g}$ and $250\mu\text{g}$ of CGP35348 respectively (figure 13). The area under the curve for $250\mu\text{g}$ CGP35348 was also significantly different from both control and $50\mu\text{g}$ CGP35348 ($P = 0.0183$ and 0.0460 respectively). $50\mu\text{g}$ CGP35348, however, was not significantly different from control ($P = 0.9409$). Interestingly, $250\mu\text{g}$ CGP35348 did not elicit the intense “burst-like” firing pattern observed with the same dose of bicuculline. Thus both GABA_A and GABA_B receptor mediated inhibition contribute to the abrupt decline of the second phase. However GABA_A receptor mediated inhibition appears to play a greater role.

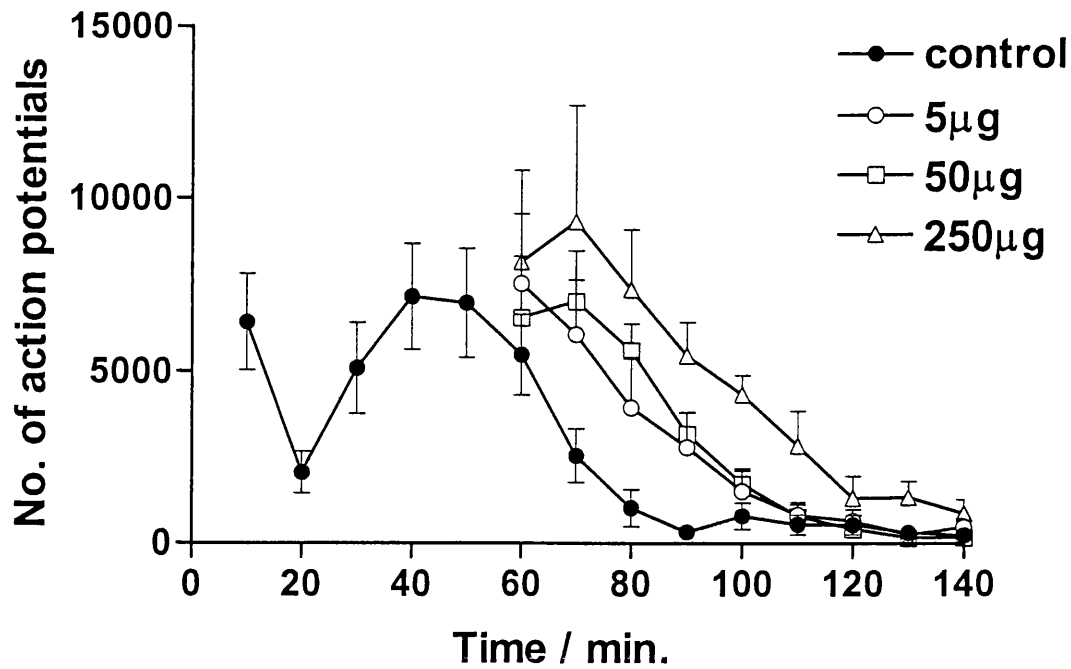


Figure 10. The effect of bicuculline (5µg, 50µg or 250µg i.t.) on the formalin response (n = 10 and 5 neurones for control and bicuculline experiments respectively). All drugs were administered 50 min. post-formalin. Results are presented as mean number of action potentials in 10 min. time bins +/- the standard error of the mean.

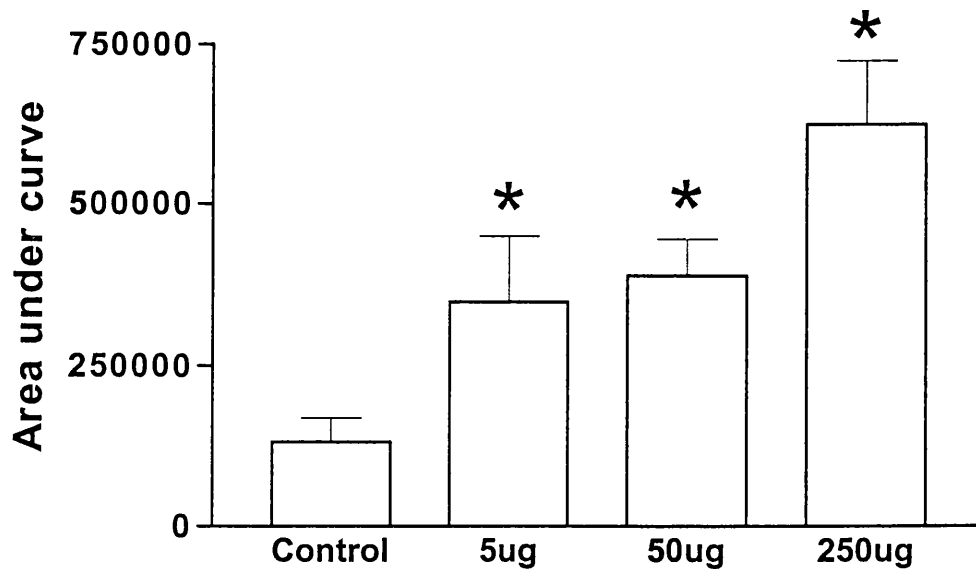


Figure 11. The effects of bicuculline (5µg, 50µg or 250µg i.t.) on the area under the curve of the formalin response (n = 10 and 5 neurones for control and bicuculline experiments respectively). All drugs were administered 50 min. post-formalin. Results are presented as mean area under the curve +/- the standard error of the mean.

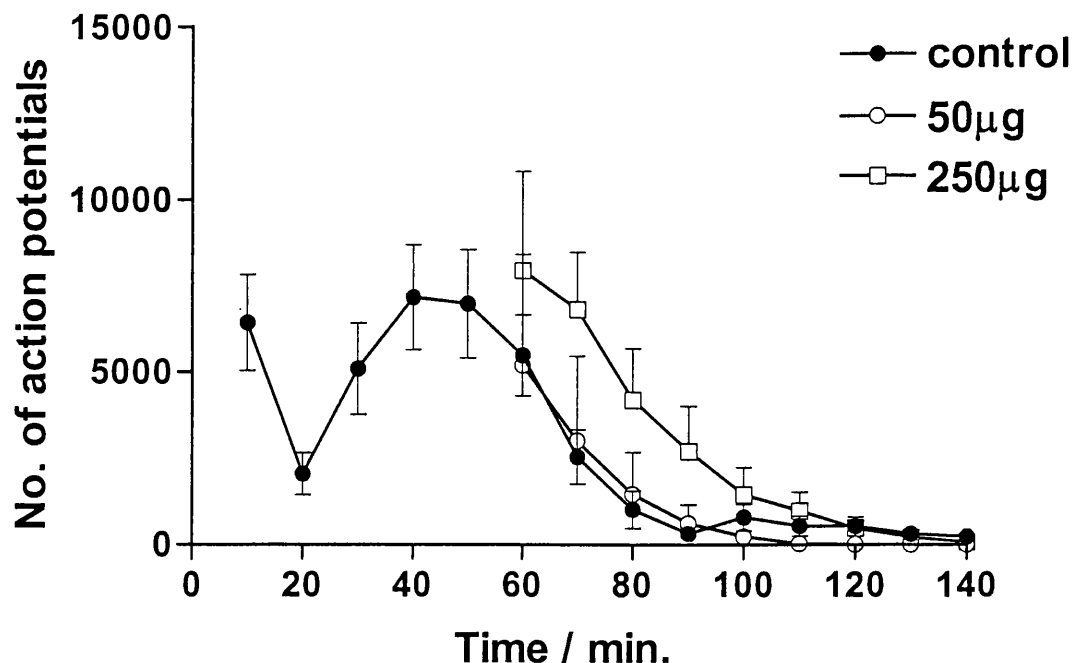


Figure 12. The effect of CGP35348 (50µg or 250µg i.t.) on the formalin response (n = 10 and 6 neurones for control and CGP35348 experiments respectively). All drugs were administered 50 min. post-formalin. Results are presented as mean number of action potentials in 10 min. time bins +/- the standard error of the mean.

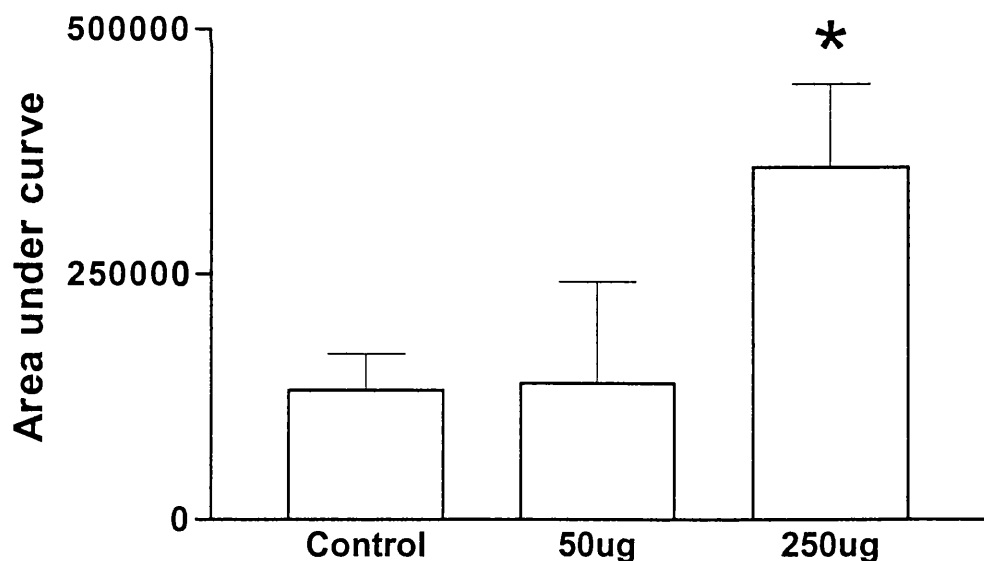


Figure 13. The effects of CGP35348 (50µg or 250µg i.t.) on the area under the curve of the formalin response (n = 10 and 6 neurones for control and CGP35348 experiments respectively). All drugs were administered 50 min. post-formalin. Results are presented as mean area under the curve +/- the standard error of the mean.

3.4 Discussion

The effects of CGP35348, muscimol, and bicuculline on the response to transcutaneous electrical stimulation were first examined. Evidence for discrimination between GABA receptor subtypes by bicuculline and CGP35348, and the selectivity of bicuculline for the GABA_A receptor is provided by the inability of CGP35348, at a dose that blocks the inhibitory effects of the GABA_B receptor agonist, baclofen, to prevent the action of muscimol, and the reversal of muscimol induced inhibition of electrically evoked neuronal responses by bicuculline.

I have already drawn attention to the fact that formalin induced inflammation and peripheral oedema persists for many hours after both neuronal and behavioural activity ceases. This suggests that central inhibitory mechanisms may be involved in the relatively abrupt decline of the second phase of the formalin response and our study strongly implicates the GABA_A receptor in these inhibitions.

A recent behavioural study monitoring the flinch response to subcutaneous injection of formalin solution (50µl of 5% solution) into the rat hindpaw reported that intrathecal GABA_A and GABA_B agonists caused a dose-dependent suppression of nociceptive behaviour in both the phase 1 and phase 2 response. However, intrathecal administration of the GABA_A and GABA_B antagonists bicuculline and CGP35348 were found to have no effect (Dirig & Yaksh, 1995), although bicuculline has been shown to increase the second phase in another behavioural study (Kaneko & Hammond, 1996)

In previous studies however, including the above, the formalin response has been typically followed for only 60min. In recording the responses of dorsal horn neurones to peripheral injection of formalin (50µl of 5% solution) for 2h 20min., administration of the same antagonists 50min. after injection of formalin produced a dose-dependent increase in the duration of the second phase (for example, neurones treated with 5µg or 50µg bicuculline maintained a rate of firing above control values for up to 2h). Bicuculline elicited a greater increase in duration of the second phase than CGP35348, suggesting predominantly GABA_A, but also GABA_B receptor mediated inhibitions are involved in the control of the duration of the formalin response, contributing to the decline of the second phase relative to the persisting formalin induced inflammation and peripheral oedema.

Another study investigated the effects of allosteric modulators of the GABA_A receptor on the pain behaviour evoked by subcutaneous injection of formalin (0.05ml of 2.5%). They found intraperitoneal BZs, barbiturates and ethanol all abolished the silent interphase period in the formalin test, but had no effect on the magnitude of either the 1st or 2nd phase of the response (Franklin & Abbott, 1993). In the present study however we found that administration of bicuculline simultaneously with formalin abolished the silent interphase, a predictable result with an antagonist of the inhibitory GABA_A receptor. Furthermore, administration of bicuculline simultaneously with formalin caused a marked increase in the area under the curve of the total response to formalin. We have therefore provided electrophysiological evidence for a GABA_A receptor mediated inhibition of the silent interphase period and also a control on the magnitude of the second phase of the formalin response. Thus, in contrast to the putative nociceptive GABA_A mediated mechanism proposed by Franklin and Abbott, our results suggest a central GABA_A receptor mediated antinociceptive system exists. Administration of CGP35348 simultaneously with formalin had no significant effect on the formalin response suggesting GABA_B receptor controls do not play a significant role in either the silent interphase or the magnitude of the second phase of the formalin response.

It has been proposed that, at most, there must be a low endogenous level of GABA inhibition associated with spinal nociceptive processing (Dirig & Yaksh, 1995). Our results however support an alternative hypothesis, namely the GABAergic inhibitory system in the cord is near maximally driven under normal physiological conditions. GABA_A potentiators such as BZ's would, therefore, have little or no effect but, as we have observed, an antagonist would block ongoing inhibitions and so produce considerable enhancement of activity. In addition to the effects of bicuculline and CGP35348 on the magnitude and duration of the formalin response, this hypothesis is further supported by the brief "burst-like" periods of intense activity revealed after administration of 250µg bicuculline 50 min. post-injection of formalin, which may represent enhanced NMDA receptor activity.

The difference in findings, however, between our electrophysiological study with GABA antagonists and behavioural studies with GABA agonists, antagonists and allosteric modulators (Franklin & Abbott, 1993; Dirig & Yaksh, 1995) may be a partial result of the sedative, myorelaxant and anxiolytic properties of the drugs.

Furthermore, the measurement of behavioural responses requires that a limit is imposed on the stimulus in order to define the threshold to produce the response. In contrast, the electrophysiological technique used in the present study examines the rate and pattern of firing of dorsal horn neurones. Subsequently our study was not subject to the limitations in quantifying the effect of a drug imposed by having to adhere to a predefined scale with a theoretical maximum. In addition, Franklin and Abbot used 2.5% formalin whereas 5% formalin solution was used in the present study. GABAergic inhibition may be related to the intensity of the noxious stimulus, and so may not be apparent when lower stimulus levels are used.

The marked facilitation of the A δ -fibre evoked response seen with electrically evoked responses after bicuculline has been previously reported (Reeve & Dickenson, 1996). Here, bicuculline alone, over the same dose-range as we show to be effective at blocking the GABA_A receptor, enhanced all noxious related neuronal responses, C-fibre and post-discharge, but had the greatest effects on the A δ -fibre evoked response. It can be noted that conversely BZ's, which act to enhance GABA_A effects, produce selective marked inhibitions of the A δ -fibre evoked responses of spinal nociceptive neurones (Clavier *et al.*, 1992). Furthermore, several reports implicate spinal GABAergic controls of incoming activity in A δ -fibres. Morphological evidence has recently been proposed for selective inhibitory gating of inputs to the superficial dorsal horn. GABAergic terminals are reported to contact more A δ -fibre terminals than non-glomerular C-fibre terminals, and form no contacts at all with glomerular C-fibre terminals (Bernardi *et al.*, 1995). Post-synaptic controls on nociceptive neurones may therefore predominate over pre-synaptic modulations. However, GABAergic control of afferent input may occur presynaptically via axo-axonal synapses with primary afferent terminals (Barber *et al.*, 1978; Hunt *et al.*, 1981; Alvarez *et al.*, 1992; Spike & Todd, 1992; Magoul *et al.*, 1987; Bernardi *et al.*, 1995), or postsynaptically via axo-dendritic synapses within the dorsal horn (Magoul *et al.*, 1987; Alvarez *et al.*, 1992; Powell & Todd, 1992; Baba *et al.*, 1994). Both GABA_A and GABA_B receptors are found presynaptically on A δ - and C-fibre afferents as well as on postsynaptic sites (Desarmenian *et al.*, 1984; Bowery *et al.*, 1987; Price *et al.*, 1987). Furthermore, a recent study examining formalin-evoked activity in primary afferent fibres observed phase 1 activity in A β - and A δ - fibres as well as C-fibres, and phase 2 activity in A δ - fibres

with receptive fields in hairy skin and in all mechanically sensitive C-fibres (Puig & Sorkin, 1995). The increase in formalin-evoked activity seen after bicuculline in the present study may, therefore, be due to enhanced activity in A δ -fibres as much as C-fibres.

In summary, I have provided electrophysiological evidence for GABAergic inhibitory control of noxious transmission in the spinal cord by predominantly GABA_A but also, GABA_B receptor mediated systems, in the formalin model of inflammatory pain. Furthermore, my results support the idea that the GABA_Aergic inhibitory system in the spinal cord is enhanced during formalin induced inflammation and acts to limit both the magnitude and duration of the neuronal responses to the peripheral noxious stimulus. This may be a physiological response to counter increased excitatory transmission under these conditions.

4. α_2 -ADRENOCEPTORS

4.1 Introduction

The potent antinociceptive effect of spinally applied α_2 -adrenoceptor agonists has been demonstrated both behaviourally and electrophysiologically (see refs. in Chapter 1.5.2). Furthermore, a synergistic relationship between α_2 -adrenoceptor agonists and mu receptor opiates, mediating inhibition in the spinal cord, has been well documented (Wilcox *et al.*, 1987; Ossipov *et al.*, 1990a,b; Sullivan *et al.*, 1992a; Kalso *et al.*, 1993). Plasticity within descending inhibitory controls has also been reported, and there are several lines of evidence implicating enhanced descending inhibition following the development of peripheral inflammation. Several studies have demonstrated increased descending inhibition during inflammatory pain by blocking or lesioning descending pathways (Cervero *et al.*, 1991; Ren & Dubner, 1996; Tsuruoka & Willis, 1996). Increased turnover of noradrenaline in the spinal cord following peripheral inflammation has been reported (Weil-Fugazza *et al.*, 1986), and more recently stimulation of peripheral C-fibres, but not A β - or A δ -fibres, has been shown to increase spinal noradrenaline release (Men & Matsui, 1994).

In keeping with the idea that α_2 -adrenoceptor controls may be activated by inflammation, a limited number of studies have demonstrated increased effects of α_2 -adrenoceptor agonists in mediating antinociception during inflammation (Hylden *et al.*, 1991; Kayser *et al.*, 1992). However, α_2 -adrenoceptor antagonists, which would reveal the degree of endogenous α_2 -adrenoceptor control, have been shown to have no effect on nociceptive thresholds or neuronal responses in normal animals (Nagasaka & Yaksh, 1990; Hylden *et al.*, 1991; Stanfa & Dickenson, 1994), but one electrophysiological study on spinal dorsal horn neurones reports the α_2 -adrenoceptor antagonist idazoxan, but not atepamizole, increased C-fibre evoked responses during carrageenan induced inflammation (Stanfa & Dickenson, 1994).

Thus the involvement of α_2 -mediated noradrenergic inhibition in the formalin response was examined using several α_2 -adrenoceptor antagonists. The selective α_2 -antagonists, atepamizole and idazoxan, were compared to the relatively non-selective yohimbine. The effects of the antagonists on the electrically evoked responses of dorsal horn neurones in normal animals were also compared to the effects of pretreatment with the antagonists on the formalin response.

In addition, I investigated the effects of a novel α_2 -adrenoceptor agonist, S 18616-1, on the electrically evoked responses of dorsal horn neurones, and on the formalin response. I then assessed the capacity of atipamezole, idazoxan and yohimbine to reverse the effects of S 18616-1 on the electrically evoked responses of dorsal horn neurones.

4.2 Methods

4.2.1 α_2 -Adrenoceptor antagonists and electrically evoked responses

I examined the effects of the α_2 -antagonists atepamizole, idazoxan and yohimbine on the response to transcutaneous electrical stimulation in a series of animals. Control post-stimulus histograms (as described above) were constructed at ten minute intervals until the response of the cell was stable for three consecutive tests. Once stable, dose-response curves to the antagonists were constructed. Doses of 1 μ g, 10 μ g and 100 μ g were applied directly on the spinal cord in a volume of 50 μ l for both idazoxan and yohimbine. Only the 10 μ g and 100 μ g doses were administered for experiments with atepamizole. Post-stimulus histograms were constructed every 10 min. for 60 min. following the administration of each dose of drug. No animal in the study received more than one of the three antagonists.

4.2.2 α_2 -Adrenoceptor antagonists and the formalin model.

A second group of animals was then used for the formalin studies; neurones were characterized in the same way as with the first series but then, once a C-fibre response of the neurones had been confirmed, 50 μ l of 5% formalin solution was injected subcutaneously into the center of the receptive field (a particular toe of the hind paw) to elicit prolonged firing of the neurones. The formalin evoked activity was recorded using a rate function (Spike 2 software, C.E.D. 1401 interface) and expressed as number of action potentials in ten minute time bins. Atepamizole, idazoxan and yohimbine were applied in a volume of 50 μ l directly onto the spinal

cord. The drugs were administered as a pre-treatment 20 minutes prior to injection of formalin.

The effects of the antagonists on electrically evoked responses were expressed as a percentage of control \pm the standard error of the mean (s.e.m.). The effects of drugs on the formalin response were quantified by measuring the area under the curve. Further analysis of data from formalin experiments was conducted, separately comparing the number of action potentials in the first and second phases of the response to their respective controls.

4.2.3 S 18616-1 and electrically evoked responses

I examined the effects of a novel selective α_{2A} -adrenoceptor agonist, S 18616-1, on the response to transcutaneous electrical stimulation in a third series of animals. Control post-stimulus histograms (as described above) were constructed at ten minute intervals until the response of the cell was stable for three consecutive tests. Once stable, dose-response curves to the agonist were constructed. Doses of 0.1 μ g, 0.3 μ g, 1 μ g and 3 μ g were applied directly on the spinal cord in a volume of 50 μ l. Post-stimulus histograms were constructed every 10 min. for 60 min. following the administration of each dose of drug. Once the dose-response curve was completed, either atipamezole (100 μ g), idazoxan (100 μ g) or yohimbine (100 μ g) was administered to determine whether each antagonist could reverse the effects of S 18616-1. Post-stimulus histograms were constructed every 10 min. for 60 min. following the administration of the antagonist. No animal in the study received more than one of the three antagonists.

4.2.4 S 18616-1 and the formalin model.

A fourth group of animals was then used for the formalin studies with S 18616-1 following the same protocol as the antagonist study. S 18616-1 was applied in a volume of 50 μ l directly onto the spinal cord as a pre-treatment 10 minutes prior to injection of formalin. The effects of S 18616-1 on electrically evoked responses

and on the formalin response were quantified and analysed in the same manner as the antagonist study.

4.3 Results

The results were obtained from 105 neurones, all of which displayed A β -, A δ - and C-fibre evoked responses to transcutaneous electrical stimulation (average C-fibre threshold of 1.4 ± 0.1 mA). All of the neurones studied were located deep within the dorsal horn at an average depth of 758 ± 22 μ m.

4.3.1 α_2 -adrenoceptor antagonists and electrically evoked responses

Overall, the α_2 -adrenoceptor antagonists had very little effect on the electrically evoked responses of spinal dorsal horn neurones. Neither atepamizole nor yohimbine had any significant effect on A β -, A δ - or C-fibre evoked responses, post discharge or excess spikes (% control after 100 μ g dose, atepamizole = A β - $99\% \pm 19\%$, A δ - $99\% \pm 26\%$, C-fibre $108\% \pm 16\%$, post discharge $190\% \pm 38\%$, excess spikes $130\% \pm 21\%$, yohimbine = A β - $96\% \pm 20\%$, A δ - $78\% \pm 18\%$, C-fibre $80\% \pm 12\%$, post discharge $179\% \pm 55\%$, excess spikes $55\% \pm 30\%$).

Overall, idazoxan also had no significant effect on any of the electrically evoked responses (% control after 100 μ g dose, A β - $96\% \pm 15\%$, A δ - $105\% \pm 15\%$, C-fibre $130\% \pm 17\%$, post discharge $198\% \pm 39\%$, excess spikes $173\% \pm 41\%$) although there was a non-dose dependent facilitation of the C-fibre evoked response ($133\% \pm 7\%$ and $130\% \pm 17\%$ of control for 10 μ g and 100 μ g respectively) that reached the limit of significance ($F(3,36) = 2.618$, $p = 0.0657$) (figure 14). Interestingly, there appeared to be a facilitation of post discharge by all three antagonists (figure 14) although this was also found to be nonsignificant.

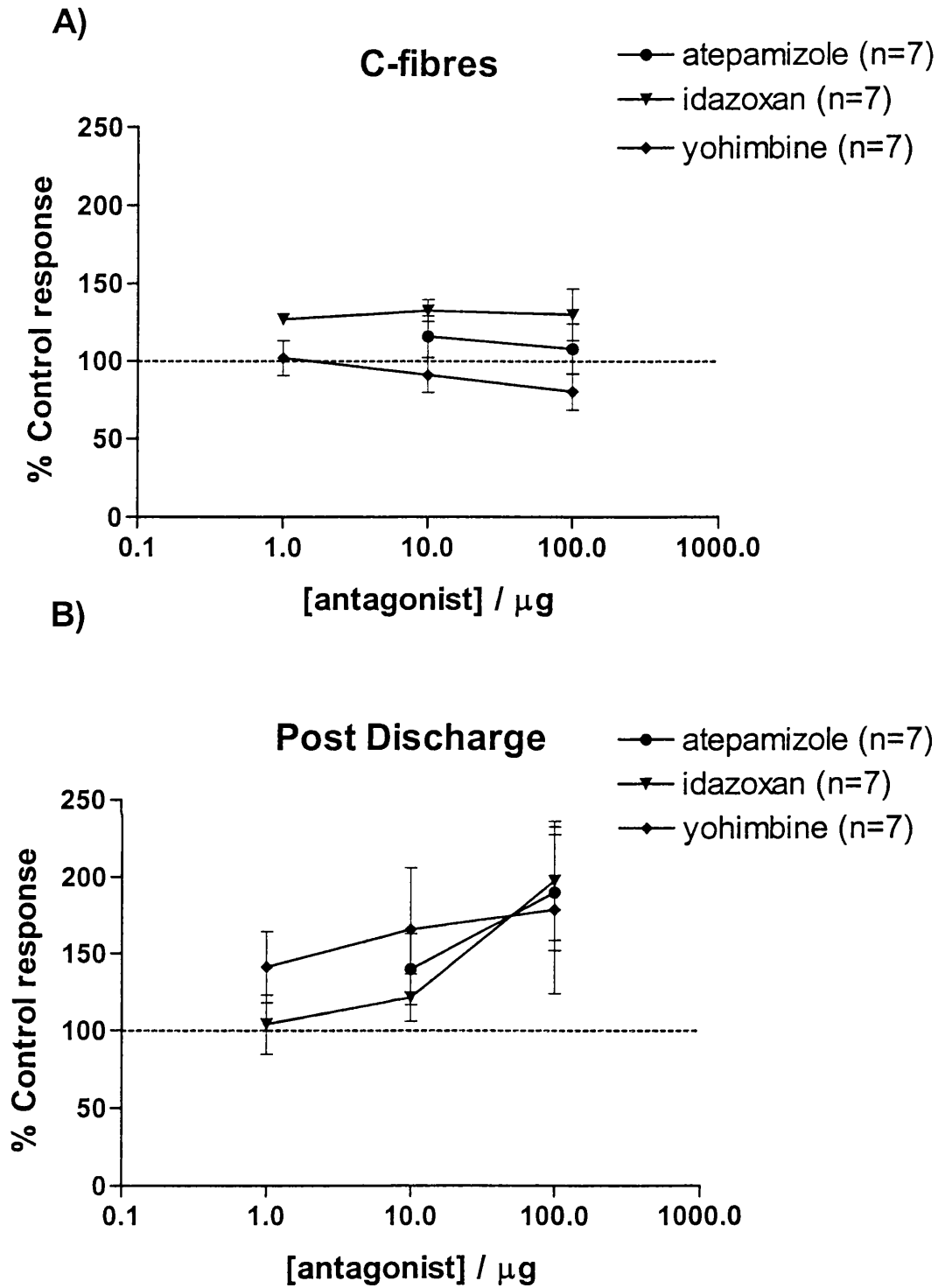


Figure 14. The effect of atepamizole, idazoxan and yohimbine on (A) electrically evoked C- fibre responses and (B) post-discharge of dorsal horn neurones. All antagonists were administered i.t. Results are presented as mean % of control response +/- the standard error of the mean.

4.3.2 α_2 -adrenoceptor antagonists and the formalin model.

On injection of formalin the neurone, previously silent, began to fire vigorously. The characteristic biphasic response was observed in all control experiments. Initial activity subsided within ten minutes, followed by the silent interphase period. Activity recommenced approximately 25 minutes after injection and, in control responses, typically subsided within ninety minutes. Each neurone was recorded for 2 hours 20 minutes. The sum of the action potentials within the first ten minutes post-formalin was termed the first phase (mean control value of 5256 ± 556). Subsequent activity of the neurone (10 to 140 minutes) was assigned to the second phase (mean control value of 24389 ± 3858).

Atepanizole, administered 20min. prior to injection of formalin, significantly increased the area under the curve of the formalin response ($F(2,26) = 10.533$, $P = 0.0004$). Mean areas under the curve were $135\% \pm 46\%$ and $357\% \pm 73\%$ of control for $10\mu\text{g}$ and $100\mu\text{g}$ of atepamizole respectively (figure 15). Post-hoc analysis revealed $10\mu\text{g}$ atepamizole had no significant effect whereas $100\mu\text{g}$ atepamizole significantly increased the response ($p = 0.0001$). Also, activity during the silent interphase period was considerably enhanced with the $100\mu\text{g}$ dose (mean spike count of 361 ± 77 and 3787 ± 1536 for control and $100\mu\text{g}$ atepamizole respectively). Interestingly, towards the offset of the second phase (from 60min onwards) neurones pretreated with $100\mu\text{g}$ atepamizole exhibited a slow, “wave-like” oscillation in their rate of firing. The timecourse of this firing pattern was similar enough between individual neurones to be apparent when mean results were plotted (figure 15), each “wave” lasting approximately 20min.

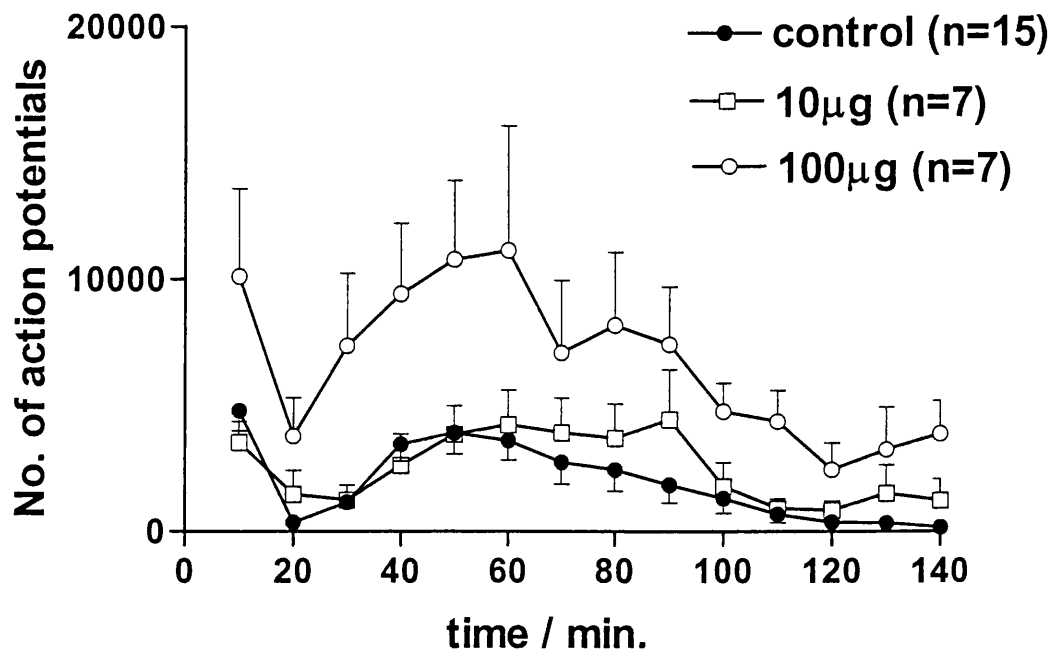
Overall, idazoxan administered 20min. prior to injection of formalin had a significant effect on the formalin response ($F(2,19) = 3.554$, $P = 0.0489$). The areas under the curve were $137\% \pm 32\%$ and $256\% \pm 72\%$ of control for $10\mu\text{g}$ and $100\mu\text{g}$ of idazoxan respectively (figure 16). Post-hoc analysis showed no significance after $10\mu\text{g}$ idazoxan, but a significant increase after $100\mu\text{g}$ ($p = 0.0163$). Activity during the silent interphase period was also enhanced with the $100\mu\text{g}$ dose (mean spike count of 813 ± 486 and 6937 ± 3082 for control and $100\mu\text{g}$ idazoxan respectively).

Furthermore, the “wave-like” oscillations in the rate of firing observed with (100µg) atepamizole treated neurones during the offset of the second phase were also discernible for neurones pretreated with 100µg idazoxan (figure 16). Although the effect was less dramatic, the timecourse was similar.

Yohimbine administered 20min. prior to injection of formalin also had a significant effect on the formalin response ($F(2,16) = 5.138, P = 0.0189$). The areas under the curve were $172\% \pm 35\%$ and $267\% \pm 44\%$ of control for 10µg and 100µg of yohimbine respectively (figure 17). Again, post-hoc analysis showed a significant increase after pretreatment with 100µg yohimbine ($p = 0.0055$) but not 10µg. Interestingly, the oscillating firing pattern observed with the 100µg doses of atepamizole and idazoxan was apparent after pretreatment with 10µg yohimbine, but not after 100µg (figure 17).

Further statistical analysis was performed separately on the first and second phases of the response (figure 18). For each antagonist, the number of action potentials in the first and second phases of the response were compared to their individual controls. Neither idazoxan nor yohimbine had any significant effect on the first phase, however atepamizole significantly increased the number of action potentials in the first phase ($F(2,26) = 3.485, P = 0.0456$). Post-hoc analysis showed no significance after pretreatment with 10µg atepamizole, however 100µg atepamizole significantly increased the first phase of the response to $210\% \pm 72\%$ of control ($p=0.0312$). Analysis of the second phase of the response revealed that all three antagonists significantly increased the second phase of the response ($F(2,26) = 11.223, P = 0.0003, F(2,19) = 3.814, P = 0.0405$ and $F(2,16) = 4.534, P = 0.0275$ for atepamizole, idazoxan and yohimbine respectively). Post-hoc analysis showed no significant effect after 10µg of either atepamizole, idazoxan or yohimbine, but significant increases after 100µg pretreatments for all three antagonists, again with atepamizole eliciting the greatest increase ($379\% \pm 73\%$ of control, $p = <0.0001$) and very similar results for idazoxan and yohimbine ($295\% \pm 86\%$ of control, $p = 0.0129$ and $288\% \pm 51\%$ of control, $p = 0.0083$ for idazoxan and yohimbine respectively).

A)

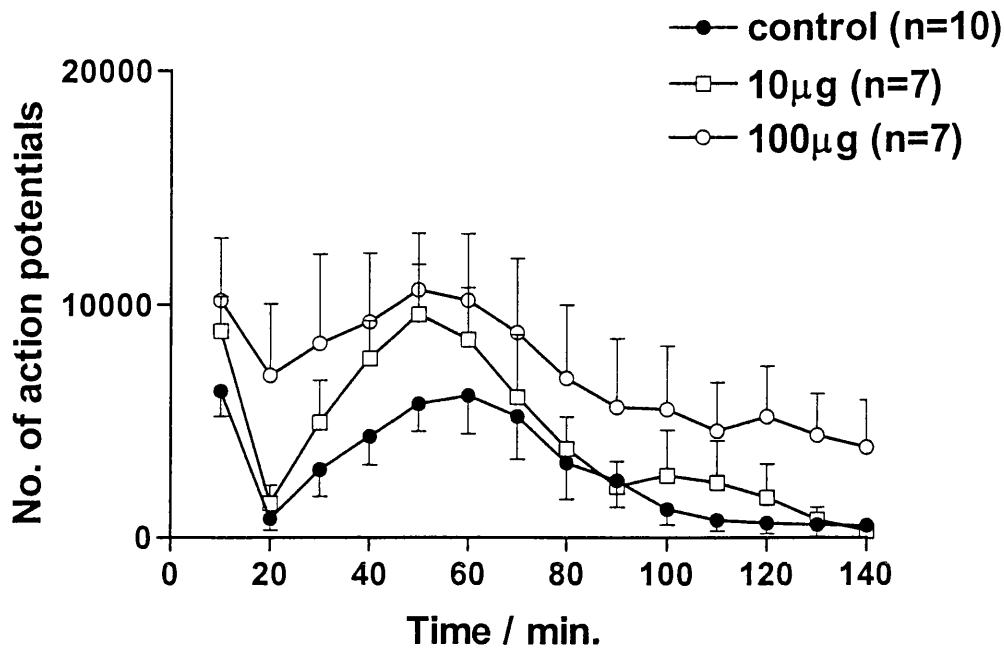


B)



Figure 15. The effect of atepamizole (i.t.) on the formalin response. Atepamizole was administered as a 20 min. pretreatment. A) Results presented as mean number of action potentials in 10 min. time bins +/- standard error of the mean. B) The effect of pretreatment with atepamizole on the area under the curve of the complete formalin response. Results are presented as mean area under the curve +/- standard error of the mean.

A)



B)

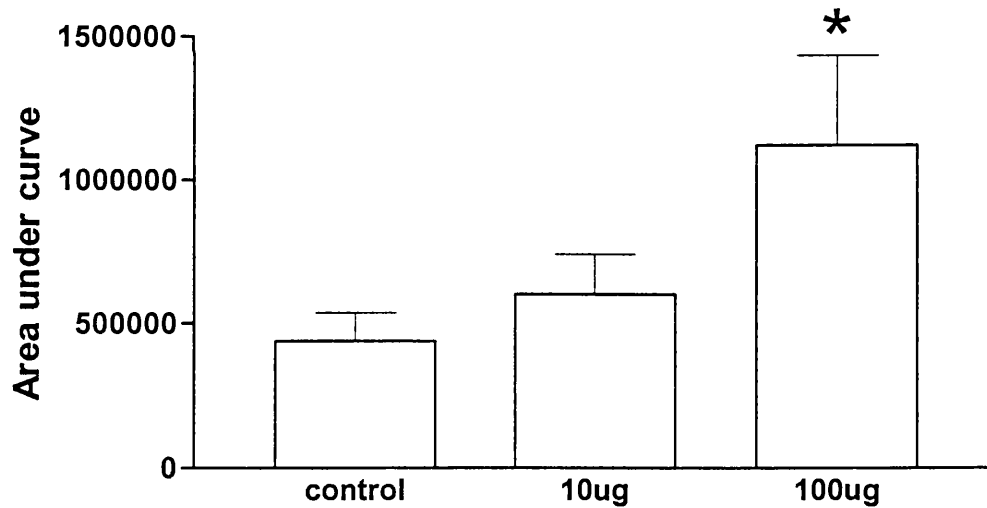
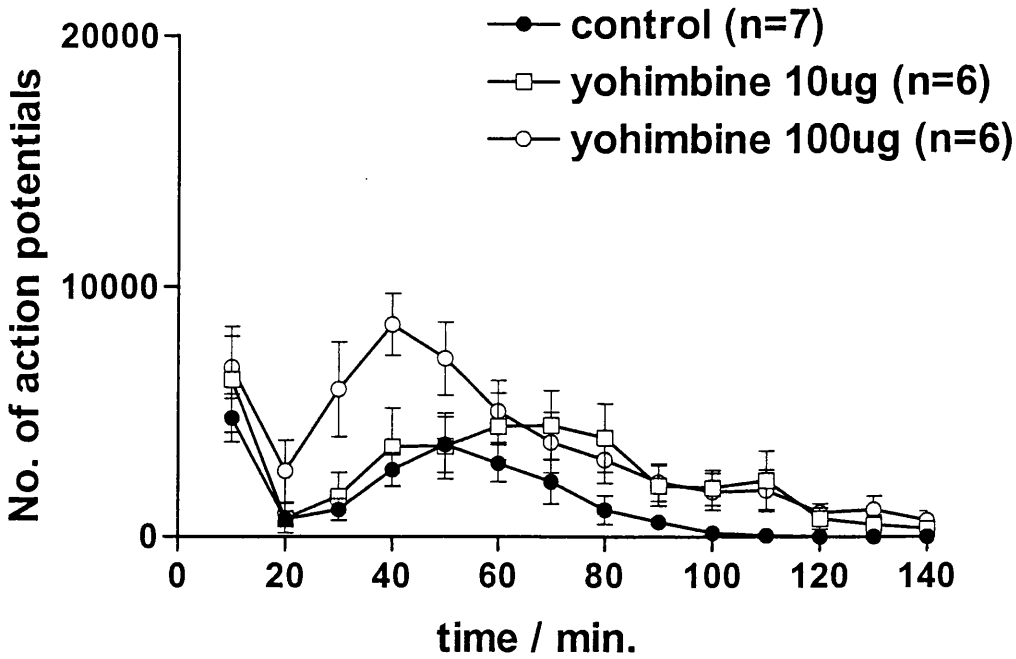


Figure 16. The effect of idazoxan (i.t.) on the formalin response. Idazoxan was administered as a 20 min. pretreatment. A) Results presented as mean number of action potentials in 10 min. time bins +/- standard error of the mean. B) The effect of pretreatment with idazoxan on the area under the curve of the complete formalin response. Results are presented as mean area under the curve +/- standard error of the mean.

A)



B)

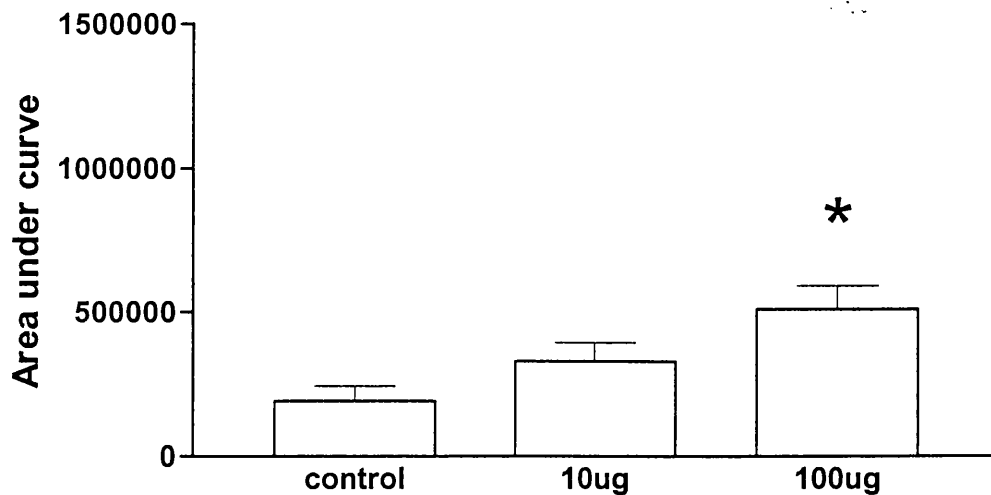
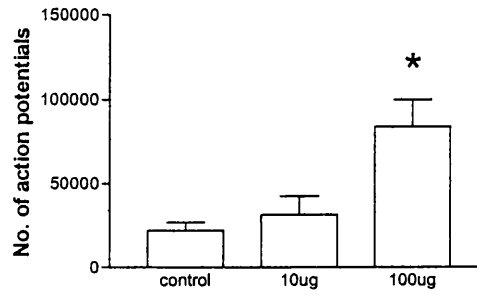
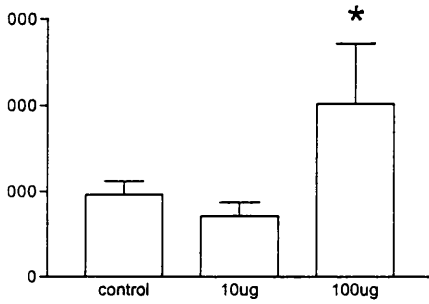


Figure 17. The effect of yohimbine (i.t.) on the formalin response. Yohimbine was administered as a 20 min. pretreatment. A) Results presented as mean number of action potentials in 10 min. time bins +/- standard error of the mean. B) The effect of pretreatment with yohimbine on the area under the curve of the complete formalin response. Results are presented as mean area under the curve +/- standard error of the mean.

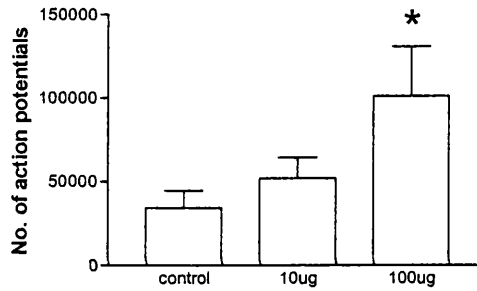
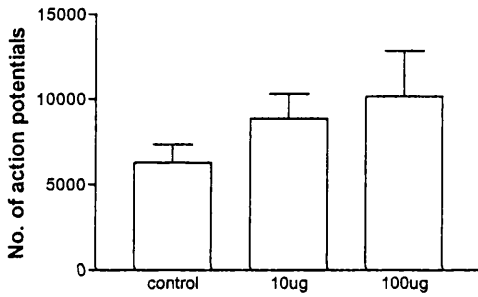
1st Phase

2nd Phase

A) Ateпамizole



B) Idazoxan



C) Yohimbine

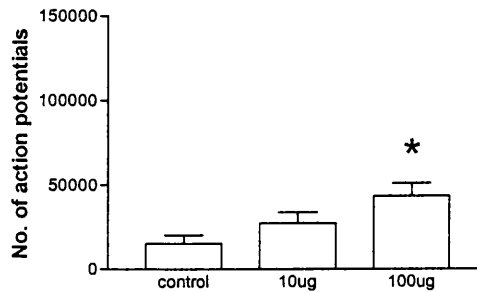
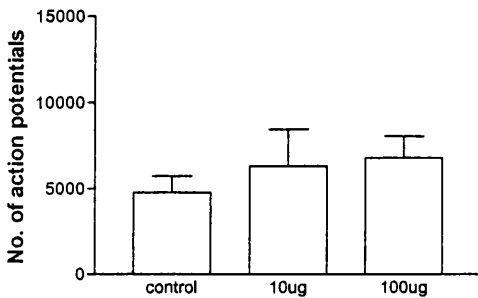


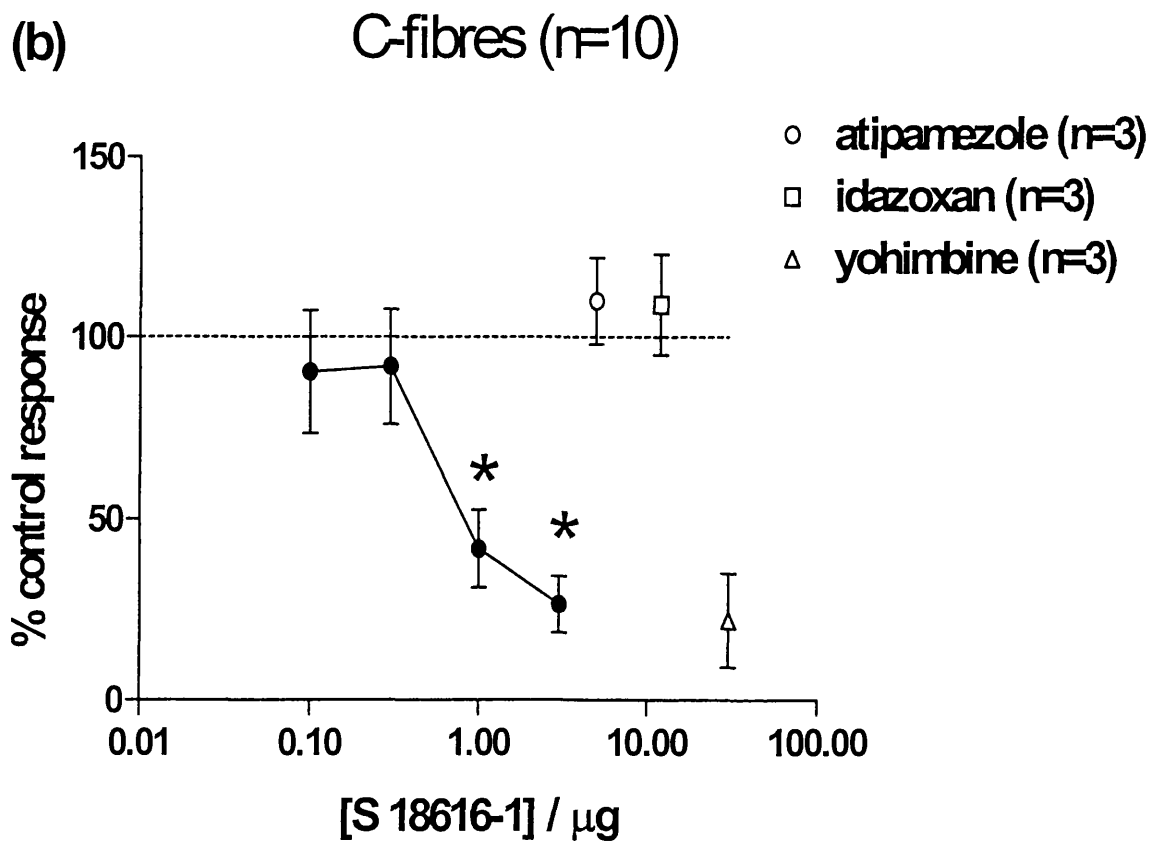
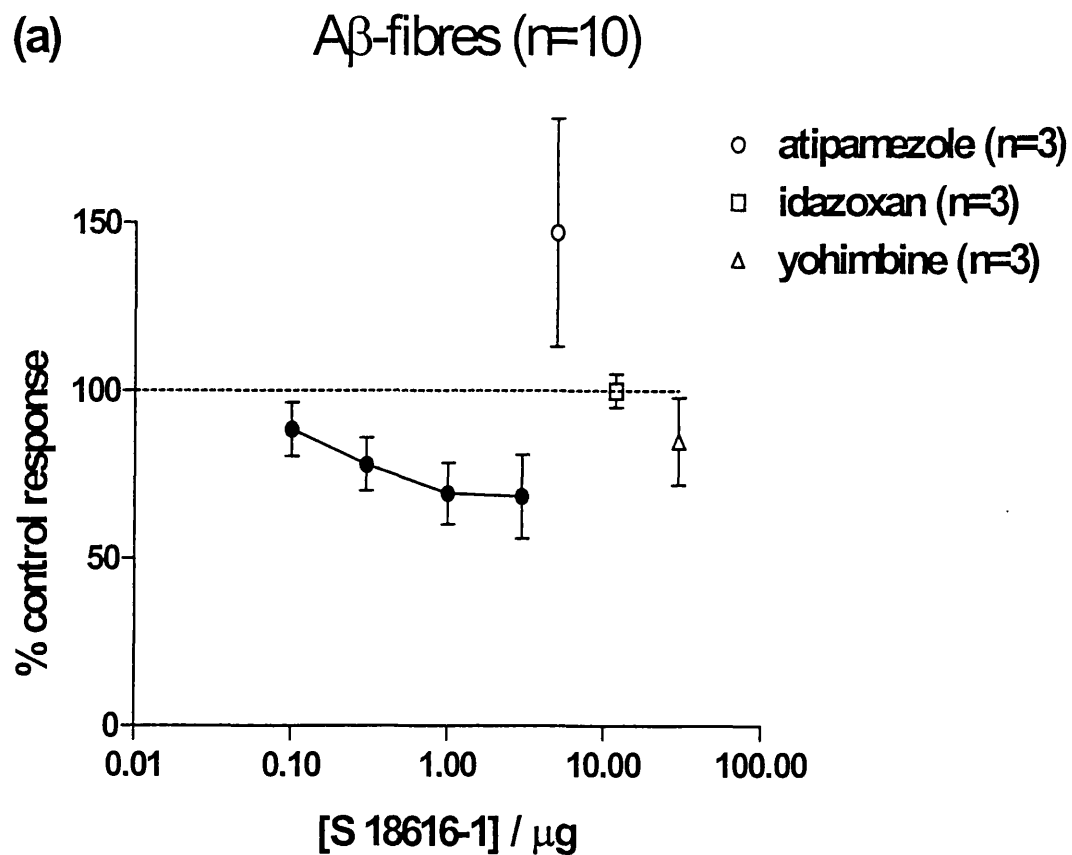
Figure 18. The effects of α_2 -adrenoceptor antagonists on the 1st phase (left column) and 2nd phase (right column) of the formalin response. Results are presented as mean number of action potentials \pm standard error of the mean for A) atepamizole, B) idazoxan and C) yohimbine.

4.3.3 *S 18616-1 and electrically evoked responses*

S 18616-1 caused a significant decrease in the electrically evoked C-fibre response and post discharge ($F(4,36) = 3.5885$, $P = 0.0146$, and $F(4,24) = 4.892$, $P = 0.005$ for C-fibre response and post discharge respectively, figure 19). % of control response was C-fibres = $90\% \pm 17\%$, $92\% \pm 16\%$, $42\% \pm 11\%$, and $27\% \pm 8\%$; post discharge = $107\% \pm 36\%$, $100\% \pm 27\%$, $7\% \pm 3\%$, and $3\% \pm 1\%$; for $0.1\mu\text{g}$, $0.3\mu\text{g}$, $1\mu\text{g}$, and $3\mu\text{g}$ S 18616-1 respectively (figure 19). Subsequent post-hoc analysis showed $0.1\mu\text{g}$ and $0.3\mu\text{g}$ S 18616-1 had no significant effect, whereas $1\mu\text{g}$ and $3\mu\text{g}$ S 18616-1 significantly inhibited both C-fibre response ($P = 0.0364$ and 0.0255 for $1\mu\text{g}$ and $3\mu\text{g}$ respectively) and post discharge ($P = 0.0052$ and 0.0089 for $1\mu\text{g}$ and $3\mu\text{g}$ respectively). Interestingly, this inhibition of C-fibre evoked response and post discharge was reversed by atipamezole and idazoxan but not by yohimbine (see figure 19). S 18616-1 had no significant effect on the electrically evoked $A\beta$ -response, $A\delta$ -response and excess spikes (not shown).

4.3.4 *S 18616-1 and the formalin model*

S 18616-1, administered 10 minutes prior to injection of formalin, significantly decreased the area under the curve of the formalin response in a dose dependent manner ($F(2,13) = 5.3924$, $P = 0.0197$) (figure 20). Mean areas under the curve were $64\% \pm 20\%$, and $20\% \pm 10\%$ of control for $0.3\mu\text{g}$ and $3\mu\text{g}$ of S 18616-1 respectively (figure 20). Post-hoc analysis revealed $3\mu\text{g}$ S 18616-1 significantly decreased the area under the curve ($P = 0.0095$), whereas $0.3\mu\text{g}$ failed to reach significance. Further statistical analysis was performed separately on the first and second phases of the response. The total number of action potentials in each phase of the response was compared to their individual controls. S 18616-1 significantly decreased the number of action potentials in the second phase of the formalin response ($F(2,13) = 4.1789$, $P = 0.0397$) (figure 21). Post-hoc analysis showed $3\mu\text{g}$ S 18616-1 produced significant inhibition of the second phase ($P = 0.0179$), whereas $0.3\mu\text{g}$ did not. S 18616-1 had no significant effect on the first phase of the formalin response.



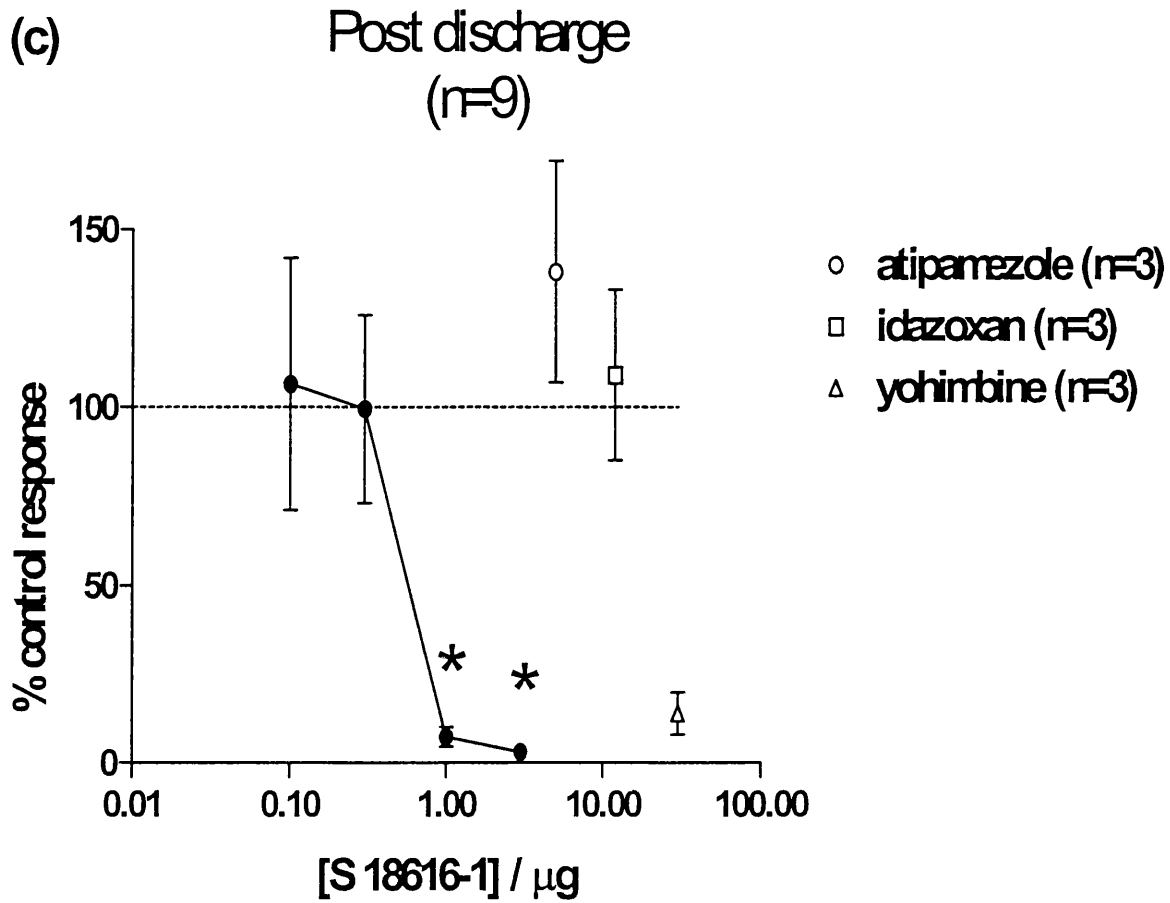


Figure 19. The effects of S-18616-1 on the electrically evoked a) $A\beta$ -fibre response, b) C-fibre response, and c) post discharge of dorsal horn neurones, and subsequent reversal using either atipamezole, idazoxan or yohimbine. All drugs were administered i.t. Results are presented as mean % of control response \pm the standard error of the mean.

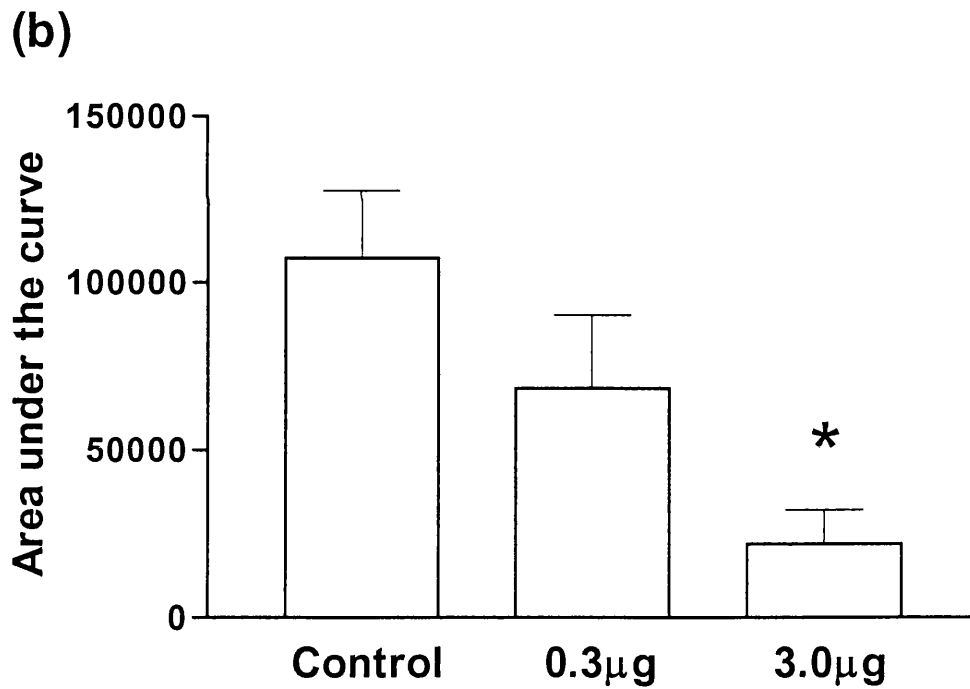
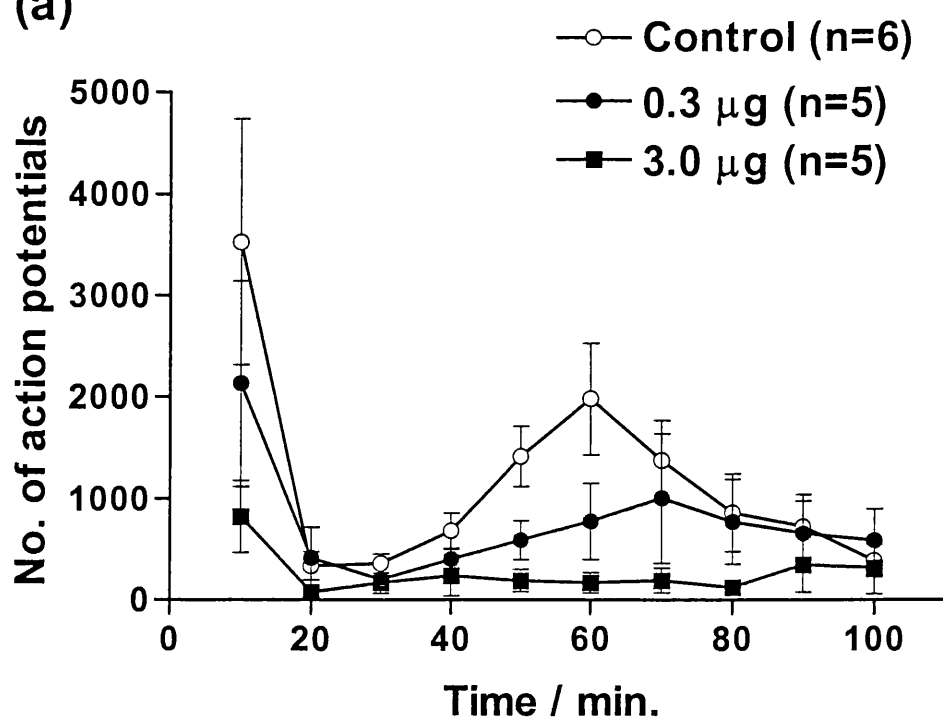


Figure 20. The effect of S 18616-1 on the formalin response. S 18616-1 was administered as a 10 min. pretreatment. a) Results presented as mean number of action potentials in 10 min. time bins +/- standard error of the mean. b) The effect of pretreatment with S 18616-1 on the area under the curve of the complete formalin response. Results are presented as mean area under the curve +/- standard error of the mean.

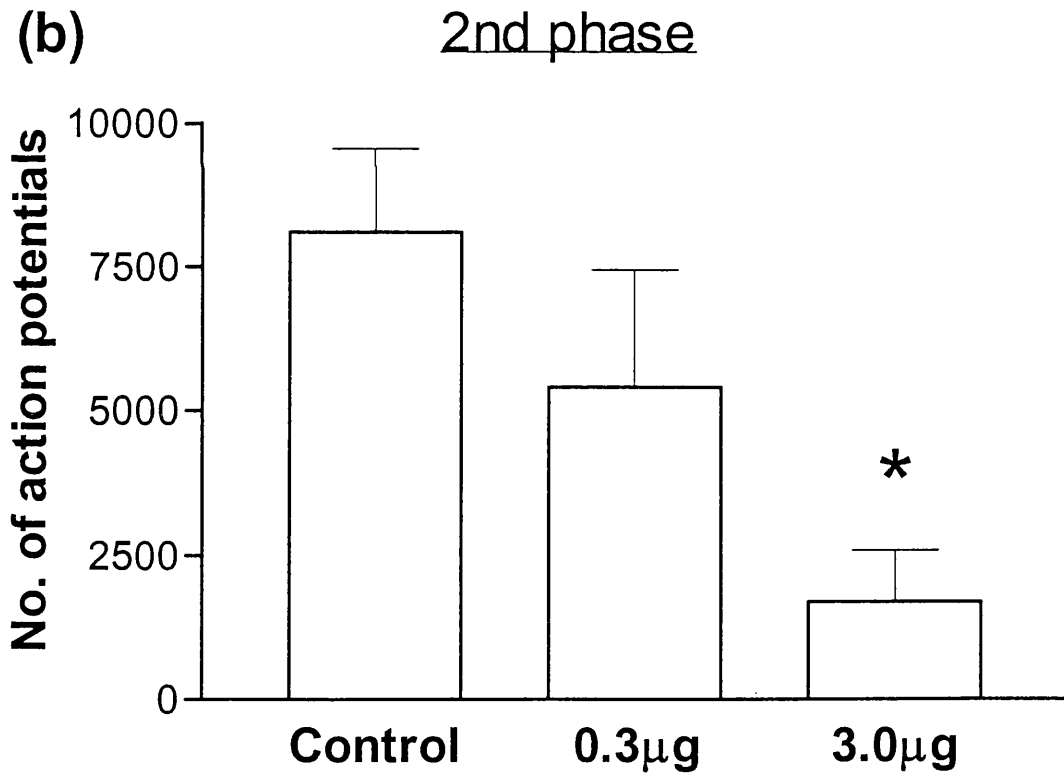
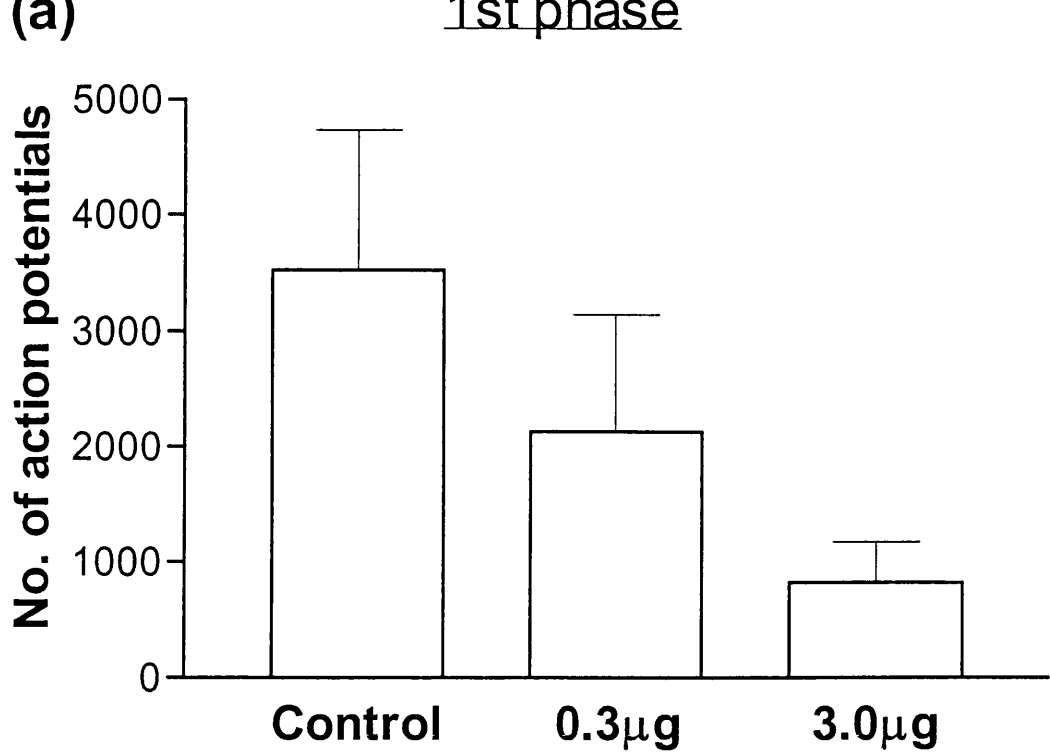


Figure 21. The effects of S 18616-1 on (a) the 1st phase and (b) the 2nd phase of the formalin response. Results are presented as mean number of action potentials \pm standard error of the mean.

4.4 Discussion

In the previous chapter I showed how GABA_A receptor mediated inhibition is involved in controlling the magnitude of the formalin response and the manifestation of the silent interphase, and how both GABA_A and GABA_B receptor mediated inhibition are involved in controlling the duration of the formalin response (Green & Dickenson, 1997, see chapter 3). In this chapter I provide electrophysiological evidence for spinal α_2 -adrenoceptor mediated noradrenergic inhibition in determining both the magnitude and the duration of the response to subcutaneous injection of formalin.

My results show that in normal animals with no inflammation, blocking the α_2 -adrenoceptor produced a slight facilitation of the C-fibre evoked response and post discharge, but overall had no significant effect on the electrically evoked responses of spinal dorsal horn neurones. This suggests that under normal conditions there is only a minor α_2 -mediated noradrenergic inhibitory control over dorsal horn neurones. During formalin induced inflammation however, atepamizole, idazoxan and yohimbine all significantly increased the magnitude of the second phase of the formalin response, suggesting an increase in α_2 -adrenoceptor mediated noradrenergic inhibition during formalin induced inflammatory pain.

The selectivity of atepamizole has already been demonstrated by our group. Sullivan *et al.* (1992b) showed complete inhibition of C-fibre evoked responses by the α_2 -agonist dexmedetomidine in spinal dorsal horn neurones that was clearly reversed by atepamizole. There are several other reports, the majority of which are behavioural studies, demonstrating atepamizole, idazoxan and yohimbine reverse dexmedetomidine induced antinociception. However, it is notable that the relatively less potent yohimbine is only able to partially reverse the effects of dexmedetomidine (see refs. in Millan, 1997).

There have been several reports suggesting an increase in descending inhibitory control following inflammation. A behavioural and electrophysiological study reports increased descending inhibitory control in rats after inflammation induced by complete Freund's adjuvant or carrageenan (Ren & Dubner, 1996). Carrageenan and kaolin induced joint inflammation in the cat has also been shown to provoke an increase in descending inhibitory control from the brainstem (Cervero *et*

al., 1991: Schaible *et al.*, 1991). There is also evidence for increased activity of noradrenergic systems in the spinal cord in response to inflammatory pain. Levels of noradrenaline in the spinal cord are reported to be increased by peripheral inflammation (Weil-Fugazza *et al.*, 1986) and peripheral C-fibre stimulation (Men & Matsui, 1994). Furthermore, an increase in the number of α_2 -adrenoceptor binding sites in the sheep spinal cord after 3 weeks of chronic pain has been reported (Brandt & Livingstone, 1990). More recently Tsuroka & Willis (1996) have shown carrageenan induced inflammation activates the noradrenergic system originating from the locus coeruleus during the initial hours following the development of the inflammation. Behavioural studies have shown atepamizole and idazoxan have no effect on nociceptive thresholds in normal animals suggesting there is little or no tonic α_2 -adrenoceptor mediated inhibitory control, in agreement with our electrophysiological study (Nagasaka & Yaksh; 1990; Hylden *et al.*, 1991). In addition, other studies examining changes in nociceptive thresholds have demonstrated the antinociceptive potency of clonidine is increased during the earlier stages of inflammation, when administered either systemically (Kayser *et al.*, 1992) or spinally (Hylden *et al.*, 1991). Again, this agrees with our study using the formalin model where α_2 -antagonists, that would block any descending noradrenergic inhibitory controls activated by the formalin-induced noxious afferent barrage, increased the magnitude and duration of the formalin response, recorded over the first few hours following the induction of inflammation.

Previous work from our group has also provided evidence for increased noradrenergic inhibitory control during inflammation. Idazoxan and atepamizole have already been shown to have no significant effect on electrically evoked responses of dorsal horn neurones in normal animals (Stanfa & Dickenson, 1994). After carrageenan induced inflammation however idazoxan, but not atepamizole, was shown to significantly increase electrically evoked C-fibre responses (Stanfa & Dickenson, 1994). The lack of effect of atepamizole after carrageenan induced inflammation compared to the marked increase in the formalin response seen after pretreatment with atepamizole in the present study may be a result of greater activation of descending systems by formalin relative to carrageenan. Subcutaneous injection of formalin elicits prolonged firing of the neurone which persists for up to 90 min. in control experiments. Subcutaneous injection of carrageenan does not

cause the neurone to fire, although responses to nociceptive stimuli are enhanced, reaching a plateau after 3 hours (Hargreaves *et al.*, 1988; Hylden *et al.*, 1991). It is plausible that the formalin model provokes a greater degree of descending inhibitory control than carrageenan due to the continuing barrage of ascending noxious information.

The differences between the results obtained with the three different antagonists used in the present study also merit discussion. It is notable that after pretreatment with 100µg doses, idazoxan and yohimbine evoked a similar increase in the total response to formalin when quantified by measuring the area under the curve. Furthermore, after analysis of the individual phases of the response, both idazoxan and yohimbine failed to have a significant effect on the first phase, but produced a similar increase in the second phase. 100µg Atepamizole however produced a greater increase in the total formalin response compared to idazoxan and yohimbine, and caused a significant increase in both the first and second phases. This is most likely due to the greater selectivity of atepamizole for the $\alpha_{2A/D}$ -subtype, which predominates in the spinal cord, relative to idazoxan and yohimbine. In addition, yohimbine probably had a lesser effect than atepamizole because of its lower potency. Unexpectedly, idazoxan caused a very similar increase to yohimbine in the formalin response. Idazoxan is more similar in potency to atepamizole than yohimbine, and is reported to have twice the potency of yohimbine to facilitate spinal reflex responses (Harris & Clarke, 1992, 1993).

However, in addition to their relative selectivity for the $\alpha_{2A/D}$ -subtype, the relative selectivity of the antagonists for the α_2 -adrenoceptor over the α_1 -adrenoceptor is likely to influence the results of our study. A study investigating the relative α_2/α_1 selectivity of the three antagonists in the rat Vas Deferens reports a ratio of 8526 / 27 / 40 for atepamizole, idazoxan and yohimbine respectively (Virtanen *et al.*, 1989). α_1 -adrenoceptors in the dorsal horn of the spinal cord are known to be excitatory (see refs. in Millan, 1997). Excitatory α_1 -activity would therefore oppose inhibitory α_2 -activity, decreasing the potentiation of the formalin response evoked by antagonising spinal α_2 -adrenoceptors. Thus, the far lower α_2/α_1 selectivity of idazoxan and yohimbine relative to atepamizole may well be a significant influence on our results, accounting for the similarity in effect of idazoxan and yohimbine, and the relatively greater effect of atepamizole.

Another contributory factor may be effects of idazoxan at other non-adrenergic sites in the spinal cord. It has been reported that idazoxan is also an agonist at the 5-HT_{1A} receptor (Llado *et al.*, 1996). The 5-HT_{1A} receptor is widely accepted to be inhibitory in the spinal cord, and the 5-HT_{1A} receptor agonist 8-hydroxy DPAT has been shown to dose dependently inhibit the second phase of the formalin response (see chapter 6). Thus, 5-HT_{1A} agonist activity may to some degree oppose the facilitation of the formalin response produced by α_2 -adrenoceptor antagonism. Activity at the imidazoline I₂ binding site could also affect α_2 -antagonist mediated facilitation of the formalin response. Idazoxan has high affinity for imidazoline I₂ receptors (Michel & Ernsberger, 1992). Diaz *et al.* (1997, and unpublished data) have demonstrated complete inhibition of electrically evoked C-fibre responses of dorsal horn neurones in normal animals, and after carrageenan induced inflammation, by BU224, a putative I₂ receptor agonist. Although antagonists of this site have not yet been studied, it seems plausible that the imidazoline activity of idazoxan may influence the results of the present study.

Additional evidence is revealed by the reversal experiments with the novel α_2 -adrenoceptor agonist S 18616-1. The antinociceptive effects of S 18616-1 were returned to control values by atipamezole and idazoxan, but not by yohimbine. The lack of effect of yohimbine is again likely to be a reflection of the lower potency of this drug relative to atipamezole and idazoxan as an antagonist of α_2 -adrenoceptors. However, these data also support the theory that activity at other non-adrenergic sites may have influenced the results obtained with idazoxan pretreatment on the formalin response. Idazoxan produced a similar reversal of S 18616-1 mediated inhibition of neuronal responses to atipamezole, indicating similar potency as an α_2 -antagonist, but had a reduced effect similar to yohimbine in the formalin model. Thus, it is possible that activity at non-adrenergic sites may have opposed the facilitation of the formalin response resulting from antagonism of α_2 -adrenoceptors.

As would be expected, intrathecal administration of the novel α_2 -adrenoceptor agonist S 18616-1 produced a potent antinociceptive effect on both the electrically evoked responses of dorsal horn neurones and in the formalin model. In electrical experiments S 18616-1 selectively inhibited the C-fibre evoked response and post discharge. Both the A β and A δ fibre evoked responses were also slightly decreased, but in both cases this minor inhibition failed to reach significance. The

pharmacological profile of S 18616-1 is in clear agreement with a previous study from our group examining the effects of the highly selective α_2 -adrenoceptor agonist dexmedetomidine (Sullivan *et al.*, 1992b). The estimated ED_{50} of intrathecal dexmedetomidine on the C-fibre evoked response was reported to be 2.5 μ g in our model (Sullivan *et al.*, 1992b). In the present study S 18616-1 appears to be slightly more potent, having an ED_{50} of approximately 1 μ g on the C-fibre evoked response, a comparable figure to that of morphine (Dickenson & Sullivan, 1986). The reversal of the antinociceptive effects of S 18616-1 by atipamezole confirms the involvement of α_2 -adrenoceptors. In formalin experiments, S 18616-1 produced opposing effects to the α_2 -antagonists, significantly inhibiting the second phase of the formalin response but not the first phase. These results are in agreement with other studies describing the antinociceptive effects of spinally applied α_2 -adrenoceptor agonists, and supports the hypothesis that α_2 -adrenoceptors may be localised postsynaptic to the primary afferent terminals (see refs in chapter 1.5.2).

In summary, I have provided electrophysiological evidence for α_2 -adrenoceptor mediated noradrenergic inhibitory control of formalin induced noxious transmission in the spinal cord. My results suggest this noradrenergic inhibitory system is dormant under normal conditions. However, during formalin induced inflammation this system is activated, contributing to control of both the magnitude and duration of the neuronal responses to the peripheral inflammatory stimulus.

5. 5-HT₃ RECEPTORS

5.1 Introduction

Both stimulation of the NRM and intrathecal application of 5-HT have been shown to produce inhibition at the spinal level (Yaksh & Wilson, 1979; Basbaum & Fields, 1984; Besson & Chaouch, 1987; Advokat, 1988; Solomon & Gebhart, 1988; Crisp *et al.*, 1991; Antal *et al.*, 1996; Bardin *et al.*, 1997b). Subsequently, the focus of investigation in recent years, combined with the search for novel analgesic agents, has concentrated on the inhibitory mechanisms of action of 5-HT in the spinal cord. However, the diversity of subtypes of 5-HT receptor identified to date, and their contrasting modes of coupling and functional roles, support the hypothesis that 5-HT receptors may also be involved in excitatory transmission in the dorsal horn (Todd & Millar 1983; Ren *et al.*, 1988, 1991; Fields *et al.*, 1991; Zhuo & Gebhart, 1991; Millan, 1995, 1997).

Unlike other 5-HT receptors described to date, which are 2nd messenger linked, the 5-HT₃ receptor is an excitatory ligand-gated ion channel (Derkach *et al.*, 1989). There is now substantial anatomical and functional evidence for the participation of 5-HT₃ receptors in spinal nociceptive processing. Autoradiography, in situ hybridization, and immunocytochemical studies describe a dense band of 5-HT₃ receptors in the superficial laminae of the dorsal horn (Waeber *et al.*, 1989; LaPorte *et al.*, 1992; Kia *et al.*, 1995; Morales *et al.*, 1996). Furthermore, the number of these receptors is reported to be substantially reduced after dorsal rhizotomy, indicating the majority are located presynaptically on the primary afferent terminals (LaPorte *et al.*, 1992; Kia *et al.*, 1995).

Situated on the primary afferent terminals, activation of an excitatory ion channel such as the 5-HT₃ receptor could reasonably be expected to increase transmitter release, enhancing the flow of noxious afferent information. However, there are reports of spinal 5-HT₃ receptors mediating both nociception and antinociception (Glaum *et al.*, 1988, 1990; Crisp *et al.*, 1991; Danzebrink & Gebhart, 1991; Giordano, 1991; Rodgers & Shepherd, 1992; Takeshita & Yamaguchi, 1995; Ali *et al.*, 1996; Oyama *et al.*, 1996;), and some authors suggest the 5-HT₃ receptor may not be involved in the modulation of pain transmission at the spinal level (Xu *et al.*, 1994; Bardin *et al.*, 1997a).

The involvement of 5-HT₃ receptor mediated serotonergic modulation of formalin and carrageenan induced inflammatory pain transmission was determined using the classical 5-HT₃ receptor antagonist ondansetron. The effects of ondansetron on the electrically evoked responses of dorsal horn neurones in normal animals was compared to the effects on electrically evoked responses following carrageenan induced inflammation, and the effects of pretreatment on the formalin response.

5.2 Methods

5.2.1 *Electrically evoked responses*

I examined the effects of the 5-HT₃ receptor antagonist ondansetron on the response to transcutaneous electrical stimulation in a series of animals. Control post-stimulus histograms (as described above) were constructed at ten minute intervals until the response of the cell was stable for three consecutive tests. Once stable, dose-response curves to the drugs were constructed. Doses of 1µg, 10µg and 100µg of ondansetron were applied directly on the spinal cord in a volume of 50µl. Post-stimulus histograms were constructed every 10 min. for 60 min. following the administration of each dose of drug.

5.2.2 *Formalin induced inflammation*

A second group of animals was then used for the formalin studies; neurones were characterized in the same way as with the first series but then, once a C-fibre response of the neurones had been confirmed, 50µl of 5% formalin solution was injected subcutaneously into the centre of the receptive field (a particular toe of the hind paw) to elicit prolonged firing of the neurones. The formalin evoked activity was recorded using a rate function (Spike 2 software, C.E.D. 1401 interface) and expressed as number of action potentials in ten minute time bins. Ondansetron (10µg, 50µg or 100µg) was applied in a volume of 50µl directly onto the spinal cord.

The drugs were administered as a pre-treatment 10 minutes prior to injection of formalin.

5.2.3 Carrageenan induced inflammation

A third group of animals was used for the carrageenan studies; neurones were characterized in the same way as with the first series but then, once a C-fibre response of the neurones had been confirmed, 100µl of 2% λ-carrageenan solution was injected into the plantar region of the paw to produce inflammation. The drug effects were tested 3 hours post-carrageenan. Three post-stimulus histograms were constructed (as described above) at 10 min. intervals prior to drug administration to be used as controls. Doses of 1µg, 10µg and 100µg of ondansetron were applied directly on the spinal cord in a volume of 50µl. Post-stimulus histograms were constructed every 10 min. for 60 min. following the administration of each dose of drug.

5.3 Results

The results were obtained from 83 neurones, all of which displayed Aβ-, Aδ- and C-fibre evoked responses to transcutaneous electrical stimulation (average C-fibre threshold of 1.5 ± 0.1 mA). The population of neurones studied were located deep within the dorsal horn (average depth of 813 ± 19 µm).

5.3.1 Electrically evoked responses

Ondansetron had no significant effect on the electrically evoked Aβ-, Aδ- or C-fibre responses, or post discharge, of dorsal horn neurones in normal animals with no inflammation. The values after 100µg ondansetron were Aβ-fibre 111% \pm 16%, Aδ-fibre 81% \pm 20%, C-fibre 117% \pm 18%, post discharge 87% \pm 20%.

5.3.2 Formalin induced inflammation

On injection of formalin the neurone, previously silent, began to fire vigorously. The characteristic biphasic response was observed in all control experiments. Initial activity subsided within ten minutes, followed by the silent interphase period. Activity recommenced approximately 25 minutes after injection and, in control responses, typically subsided within ninety minutes. Each neurone was recorded for 1 hour 40 minutes. The sum of the action potentials within the first ten minutes post-formalin was termed the first phase (mean control value for the population of 6511 ± 839). Subsequent activity of the neurone (10 to 100 minutes) was assigned to the second phase (mean control value of 33547 ± 6470).

Ondansetron, administered 10 minutes prior to injection of formalin, significantly decreased the area under the curve of the formalin response in a dose dependent manner ($F(3,30) = 6.325$, $P = 0.002$) (figure 22). Mean areas under the curve were $52\% \pm 13\%$, $33\% \pm 7\%$, and $6\% \pm 2\%$ of control for $10\mu\text{g}$, $50\mu\text{g}$ and $100\mu\text{g}$ of ondansetron respectively (figure 22). Post-hoc analysis revealed all three doses significantly decreased the area under the curve ($P = 0.04$, 0.006 , and 0.0004 for $10\mu\text{g}$, $50\mu\text{g}$, and $100\mu\text{g}$ respectively) (figure 23). Further statistical analysis was performed separately on the first and second phases of the response. The total number of action potentials in each phase of the response was compared to their individual controls. Ondansetron significantly decreased the number of action potentials in both the first and second phase of the formalin response ($F(3,30) = 2.99$, $P = 0.04$ and $F(3,30) = 5.167$, $P = 0.005$ for the first and second phase respectively). Post-hoc analysis showed both $50\mu\text{g}$ and $100\mu\text{g}$ ondansetron significantly decreased the number of action potentials in the second phase to $32\% \pm 7\%$, and $4\% \pm 3\%$ of control respectively ($P = 0.01$ and 0.001 for $50\mu\text{g}$ and $100\mu\text{g}$ respectively), however only $100\mu\text{g}$ ondansetron significantly attenuated the first phase ($35\% \pm 11\%$ of control, $P = 0.007$).

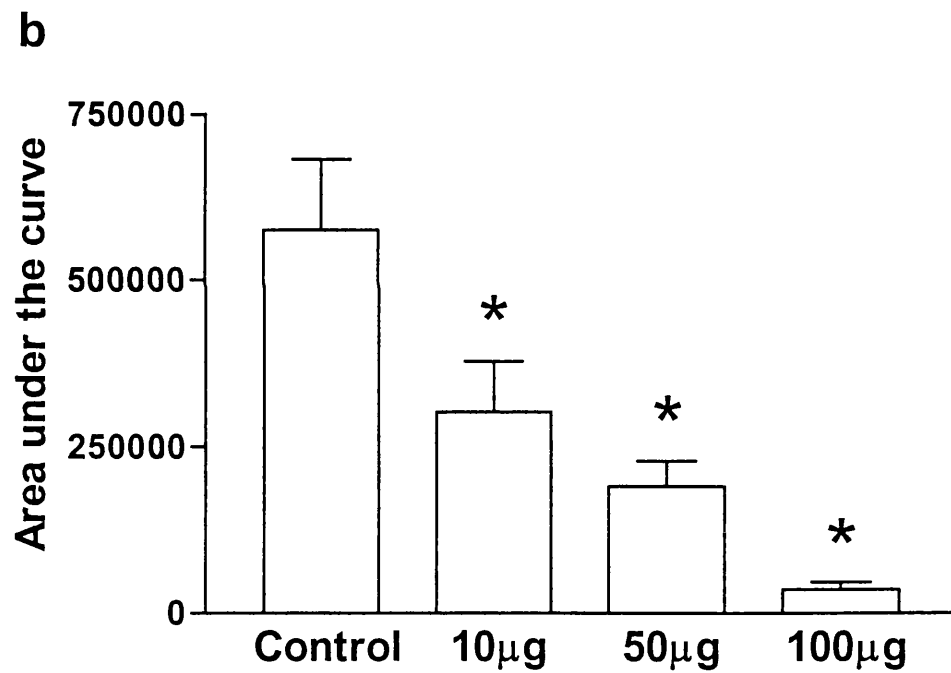
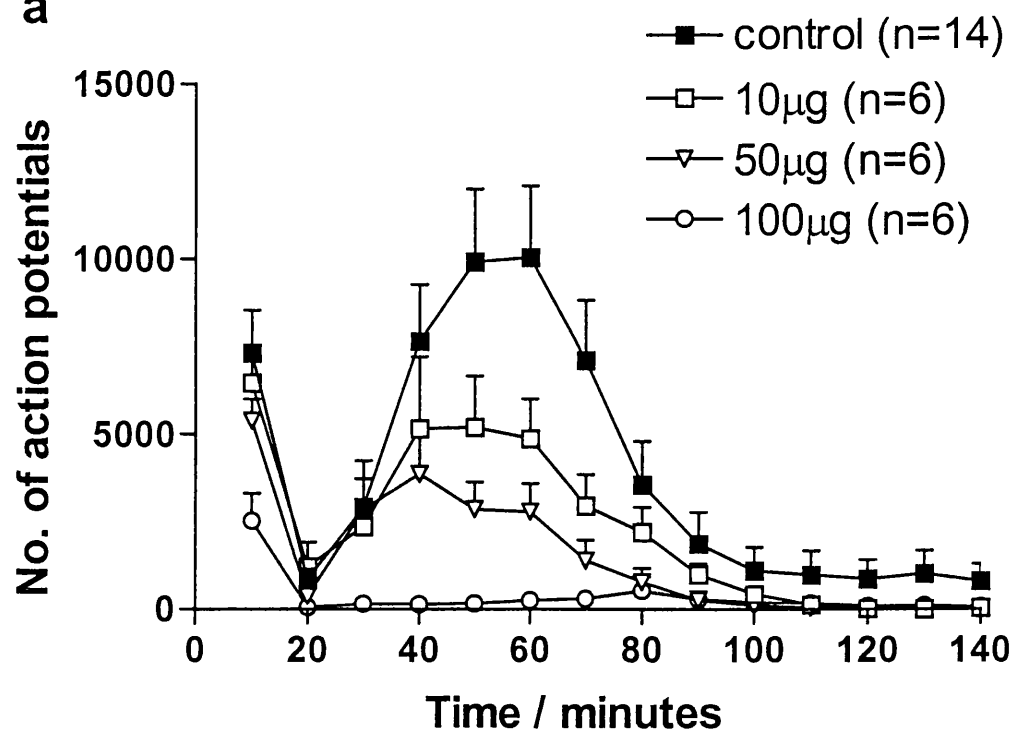


Figure 22. The effect of ondansetron (i.t.) on the formalin response. Ondansetron was administered as a 10 minute pretreatment. (a), Results presented as mean number of action potentials in 10 minute time bins +/- standard error of the mean. (b), The effect of pretreatment with ondansetron on the area under the curve of the complete formalin response. Results are presented as mean area under the curve +/- standard error of the mean.

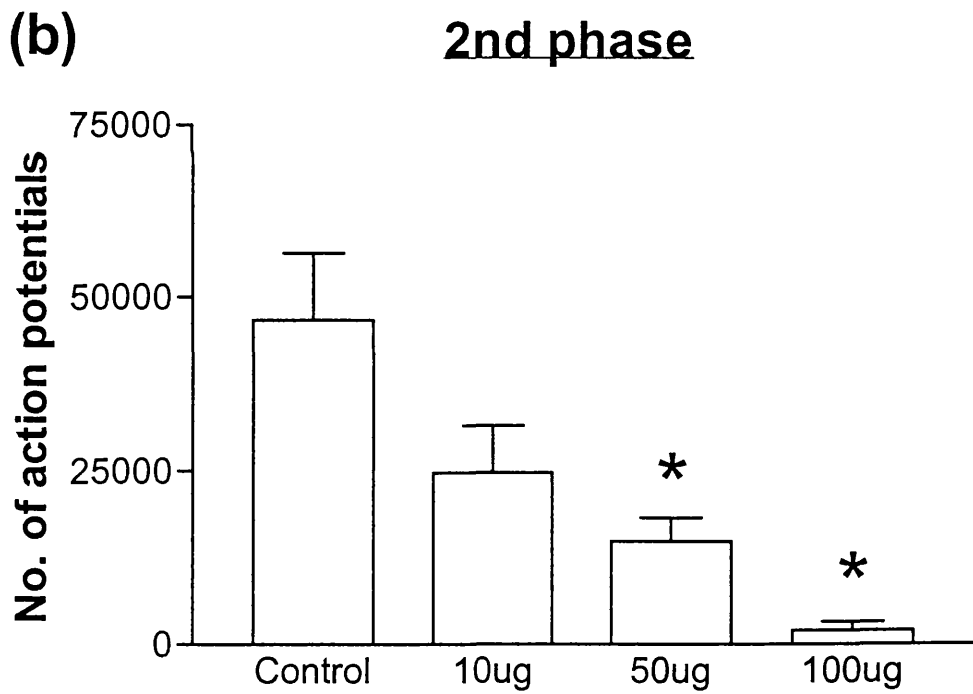
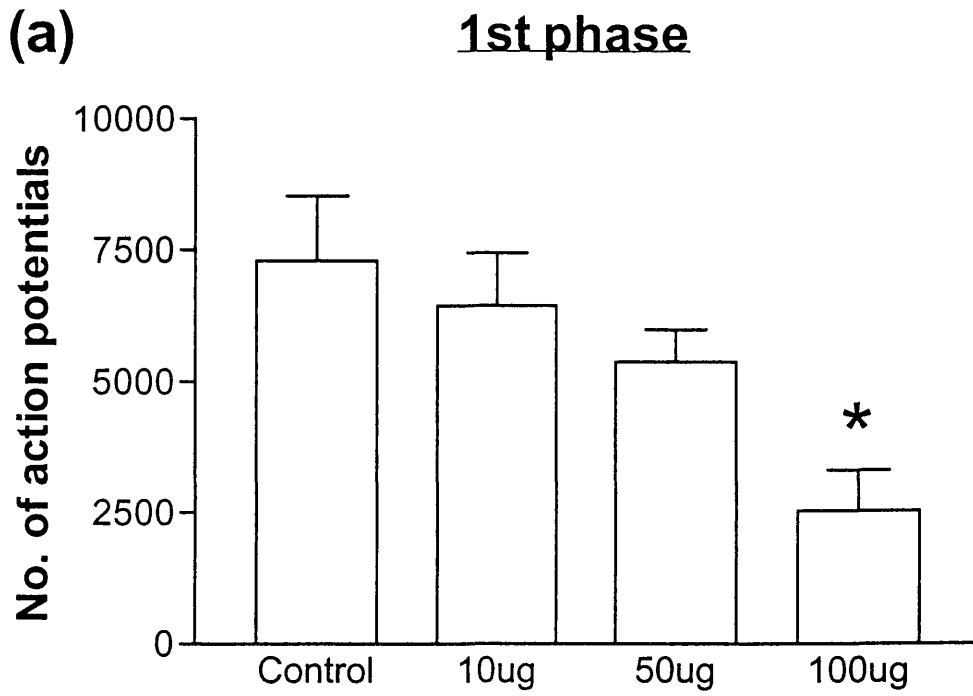


Figure 23. The effects of ondansetron on (a) the 1st phase and (b) the 2nd phase of the formalin response. Results are presented as mean number of action potentials \pm standard error of the mean.

5.3.3 Carrageenan induced inflammation

Ondansetron had no significant effect on the electrically evoked A β - and C-fibre responses, or post discharge, of dorsal horn neurones in animals with carrageenan inflammation. % control after 100 μ g ondansetron = A β -fibre 106% \pm 10%, A δ -fibre 82% \pm 17%, C-fibre 99% \pm 11%, post discharge 99% \pm 22%.

5.4 Discussion

I have already presented evidence suggesting the descending noradrenergic system is enhanced during formalin induced inflammation, acting at inhibitory α_2 -adrenoceptors in the dorsal horn of the spinal cord to limit both the magnitude and duration of the formalin response (Green *et al.*, 1998, see chapter 4). Here I provide electrophysiological evidence showing the descending serotonergic system is also enhanced during formalin induced inflammation, acting via excitatory 5-HT₃ receptors in the dorsal horn to maintain or enhance the response of nociceptive spinal neurones to formalin induced peripheral inflammation.

My results show that in normal animals with no inflammation, blocking the 5-HT₃ receptor had no significant effect on the electrically evoked responses of spinal dorsal horn neurones. This suggests that under normal conditions, or in response to acute noxious stimuli, there is little or no 5-HT₃ receptor mediated control over nociceptive neurones in the dorsal horn. During formalin induced inflammation however, ondansetron significantly decreased the magnitude of both the first and second phases of the formalin response, suggesting an increase in 5-HT₃ receptor mediated excitation during formalin induced inflammatory pain. Furthermore, when the total numbers of action potentials in each phase of the response were separately compared to their individual controls, ondansetron significantly decreased the number of action potentials in both the first and second phase of the formalin response. This is consistent with both a pre- and post-synaptic site of action, in agreement with the anatomical studies on spinal 5-HT₃ receptor localization (Waeber *et al.*, 1989; LaPorte *et al.*, 1992; Kia *et al.*, 1995; Morales *et al.*, 1996).

In contrast to subcutaneous injection of formalin, intraplantar injection of carrageenan does not produce spontaneous activity in dorsal horn neurones even though peripheral inflammation and oedema develop over several hours in a similar manner. Carrageenan induced peripheral inflammation is even more prolonged than that seen following subcutaneous injection of formalin, and can last for several days (Dickenson & Sullivan, 1987; Kayser & Guilbaud, 1987; Hargreaves *et al.*, 1988; Hylden *et al.*, 1991; Stanfa *et al.*, 1992). This is accompanied by behavioural hyperalgesia and alterations in the evoked responses of spinal neurones to peripheral noxious stimuli (Kayser & Guilbaud, 1987; Hargreaves *et al.*, 1988; Hylden *et al.*, 1991; Stanfa *et al.*, 1992). From the results of the present study an interesting comparison can be made between these two established models of inflammatory pain. Ondansetron, which produced clear, dose-dependent inhibitions of the formalin response, had no significant effect on the electrically evoked responses of dorsal horn neurones in animals with carrageenan induced inflammation. This suggests the formalin model of inflammatory pain activates the descending serotonergic system, whereas the carrageenan model does not. A likely explanation for this is that a certain degree of ongoing noxious spinal activity, such as that evoked by subcutaneous injection of formalin, is necessary for activation of the descending serotonergic system. This hypothesis is further supported by previous work from our group on the descending noradrenergic system. In two separate studies, we have shown intrathecal administration of the selective α_2 -adrenoceptor antagonist atipamezole had no significant effect on the electrically evoked responses of dorsal horn neurones following carrageenan induced inflammation (Stanfa & Dickenson, 1994) but increased in the magnitude and duration of the formalin response (Green *et al.*, 1998).

A pronociceptive role for 5-HT₃ receptors has been proposed (Danzewitz & Gebhart, 1991; Rodgers & Shepherd, 1992; Ali *et al.*, 1996; Oyama *et al.*, 1996). Presynaptic 5-HT₃ receptor mediated facilitation of glutamate release, and consequently postsynaptic NMDA receptor activation, has been demonstrated in brainstem areas receiving serotonergic input from the raphe nuclei (Wang *et al.*, 1998). Furthermore, it has been suggested that descending excitatory serotonergic influences on spinal neurones evoked by prolonged noxious stimulation could contribute to secondary hyperalgesia (Calejman *et al.*, 1998). More specifically,

there is electrophysiological (Ali *et al.*, 1996) and behavioural (Oyama *et al.*, 1996) evidence that 5-HT₃ receptors are excitatory in the spinal cord and perform a pronociceptive function. However, several behavioural studies report a paradoxical antinociceptive role for this receptor (Glaum *et al.*, 1988, 1990; Crisp *et al.*, 1991; Giordano, 1991) whilst others find no role for the 5-HT₃ receptor (Xu *et al.*, 1994; Bardin *et al.*, 1997a).

This diversity of opinion may simply reflect the complexity of spinal serotonergic mechanisms, but it may also suggest differing experimental paradigms employed by individual groups as a basis for such inconsistent results. Indeed, it has already been suggested that the contradictory results may arise from differential activation of nociceptive systems (Ali *et al.*, 1996). To clarify the situation, one recent study reports mechanical nociception as the most sensitive to intrathecal administration of 5-HT, followed by chemical nociception (5% formalin solution), and finally thermal nociception (Bardin *et al.*, 1997b).

Almost all the studies providing evidence for an antinociceptive role for the 5-HT₃ receptor used a thermal nociceptive stimulus, measuring hot plate withdrawal latency (HPL) and/or tail flick latency (TFL) (Glaum *et al.*, 1988, 1990; Crisp *et al.*, 1991). Furthermore, another TFL study suggested the 5-HT₃ receptor may not be involved in spinal nociceptive processing (Xu *et al.*, 1994). However, a recent behavioural and electrophysiological study found 5-HT₃ receptor agonists and antagonists had no effect on TFL but, in agreement with my results, report 5-HT₃ receptor agonists and antagonists respectively increased and decreased the response of dorsal horn neurones to a stronger noxious heat stimulus (Ali *et al.*, 1996). Therefore, although it appears the degree of noxious thermal stimulation necessary to initiate a withdrawal reflex does not activate the descending serotonergic system, sufficiently high temperatures may provide adequate noxious spinal activity to do so. Therefore, in addition to the nature of noxious stimuli employed, it appears the intensity of the stimulus, specifically, whether it is sufficient to activate the descending serotonergic system, may have contributed to contradictory results from individual studies.

Using subcutaneous injection of formalin as a noxious stimulus, Giordano (1991) and Takeshita *et al.* (1995) also suggested an antinociceptive role for spinal 5-HT₃ receptors. However, both of these studies used 1% formalin solution compared to 5% in the present study and it is possible 1% formalin solution does not

produce sufficient noxious spinal activity to activate the descending serotonergic system. Similar discrepancies between results obtained using 1% and 5% formalin solution have been reported elsewhere (Rosland *et al.*, 1990; Green & Dickenson, 1997). Interestingly, one study conducting orofacial formalin tests with a range of concentrations of formalin (from 0.2% to 10%) found that at least 1.5% formalin solution was required for manifestation of the second phase (Clavelou *et al.*, 1995). Another factor that must be taken into account when considering behavioural paradigms is that manipulation of the serotonergic system may alter autonomic functions and motor responses (LeBars, 1988), and consequently affect behavioural responses to noxious stimuli.

The use of agonists vis-à-vis antagonists may be another factor obscuring the role of spinal 5-HT₃ receptors in pain transmission. Many of the studies conducted to date used 5-HT and/or selective 5-HT₃ receptor agonists as pharmacological tools to study the spinal mechanisms of action of the descending serotonergic system. In several cases (Glaum *et al.*, 1988, 1990; Giordano *et al.*, 1991; Xu *et al.*, 1994) putative 5-HT₃ receptor mediated antinociception was observed using i.t. concentrations of 5-HT or selective 5-HT₃ receptor agonists significantly greater than the physiological concentrations of 5-HT found in the lumbar spinal cord (see Bardin *et al.*, 1997b). It may also be that with some of these studies, activation of 5-HT₃ receptors may result in a partial depolarization of a neuronal terminal so that subsequent transmitter release to an afferent volley is reduced. Thus, agonists at this excitatory receptor may appear to be inhibitory.

Thus, the use of spinally applied 5-HT or 5-HT₃ receptor agonists in models of pain which do not appear to activate the descending serotonergic system (2% carrageenan solution, 1% formalin solution, HPL, TFL) may only be appropriate for determining the therapeutic potential of such drugs. In terms of elucidating the mechanisms of action of the descending serotonergic system, only an antagonist will reveal the extent of endogenous serotonergic tone at 5-HT₃ receptors during different pain states and the role of the receptor in physiological processes. Indeed, in this context our results are supported by the lack of effect of 5-HT₃ receptor antagonists in studies using nociceptive tests which I propose do not activate the descending serotonergic system (Glaum *et al.*, 1990; Crisp *et al.*, 1991; Giordano *et al.*, 1991; Ali *et al.*, 1996) and in normal animals (Bardin *et al.*, 1997a).

Finally, it has been suggested GABAergic interneurons mediate the inhibitory consequences of spinal 5-HT₃ receptor activation (Alhaider et al., 1991; Giordano, 1991). In these studies putative 5-HT₃ receptor mediated antinociception was reversed by i.t. bicuculline. However, there is evidence that the GABAergic inhibitory system in the spinal cord is near maximally driven (Green & Dickenson, 1997) and bicuculline alone has been shown to cause profound excitation of nociceptive dorsal horn neurones in both normal animals and during formalin induced inflammation (Green & Dickenson, 1997; Reeve *et al.*, 1998) and also to enhance behavioural responses (Yaksh, 1989).

In summary, I have provided electrophysiological evidence for 5-HT₃ receptor mediated serotonergic control of noxious transmission in the spinal cord. My results suggest this serotonergic system is dormant under normal conditions. However, during formalin induced inflammation this system is activated, enhancing the flow of noxious information via 5-HT₃ receptors during both the first and second phase of the response.

6. 5-HT_{1A} RECEPTORS

6.1 Introduction

5-HT₁ receptors, comprising the largest subclass of 5-HT receptor, belong to the superfamily of G-protein linked receptors (Hoyer *et al.*, 1994; Gerhardt & van Heerikhuizen, 1997). Initially, 5-HT₁-like receptors were grouped together because they shared a high affinity for 5-HT and 5-CT (5-carboxyamidotryptamine) and are antagonised by methiothepin and methysergide (Bradley *et al.*, 1986). However the defining characteristic of a receptor is the primary sequence and hence it is now clear that a large number of 5-HT₁ receptor subclasses can be defined, 5-HT_{1A, B, D-F} (Gerhardt & van Heerikhuizen, 1997). 5-HT_{1A} receptors are coupled to the G_{i/o}-protein, activation of which leads to inhibition of adenylyl cyclase (Gerhardt & van Heerikhuizen, 1997). Radioligand binding, mRNA and antibody immunohistochemical studies have detected 5-HT_{1A} receptors throughout the dorsal horn of the spinal cord, with the highest concentrations found superficially in laminae I and II. In contrast 5-HT_{1A} receptors are practically undetectable in the ventral horn of the rat (Marlier *et al.*, 1991; Riad *et al.*, 1991; Thor *et al.*, 1993; Kia *et al.*, 1996).

When considering which of the numerous 5-HT receptors mediate the antinociception that has been widely reported following intrathecal administration of 5-HT (see chapter 5), the 5-HT_{1A} receptor initially appeared to be a good candidate. Indeed, several of the initial studies reported 5-HT_{1A} receptor mediated antinociception in both behavioural and electrophysiological paradigms (Fasmer *et al.*, 1986; El-Yassir *et al.*, 1988; Eide *et al.*, 1990; Mjelle *et al.*, 1992). However, many other groups found activation of spinal 5-HT_{1A} receptors to be pronociceptive (Fasmer *et al.*, 1986; Solomon & Gebhart, 1988; Crisp *et al.*, 1991; Ali *et al.*, 1994). Nonetheless, several recent studies have provided further evidence that 5-HT_{1A} receptors can specifically inhibit ascending nociceptive transmission (Xu *et al.*, 1994; Millan, 1995; Takeshita & Yamaguchi, 1995; Gjerstad *et al.*, 1996; Millan *et al.*, 1996; Oyama *et al.*, 1996). However, it is now becoming increasingly apparent that spinal 5-HT_{1A} receptor activation may result in either pro- or antinociceptive effects, depending on the algesiometric paradigm employed and the nature of the noxious stimulus (Clarke *et al.*, 1996, Millan *et al.*, 1996).

The involvement of 5-HT_{1A} receptor mediated serotonergic modulation of formalin induced inflammatory nociception was determined using the classical 5-HT_{1A} receptor agonist (±)-8-hydroxy-2-dipropylaminotetralin (8-OH-DPAT), and the potent, selective 5-HT_{1A} receptor antagonist WAY100635 (Forster *et al.*, 1995). The effects of these drugs on the electrically evoked responses of dorsal horn neurones in normal animals was compared to the effects of pretreatment on the formalin response.

6.2 Methods

6.2.1 Electrically evoked responses

We examined the effects of the 5-HT_{1A} receptor agonist 8-OH-DPAT, and the 5-HT_{1A} receptor antagonist WAY100635 on the response to transcutaneous electrical stimulation in a series of animals. Control post-stimulus histograms (as described above) were constructed at ten minute intervals until the response of the cell was stable for three consecutive tests. Once stable, dose-response curves to the drugs were constructed. Doses of 1µg, 3µg, 10µg and 30µg of 8-OH-DPAT, or 1µg, 10µg and 100µg of WAY100635 were applied directly on the spinal cord in a volume of 50µl. Post-stimulus histograms were constructed every 10 min. for 60 min. following the administration of each dose of drug. No animal in these experiments received more than one of the two drugs.

In addition, we examined the capacity of WAY100635 to reverse the effects of 8-OH-DPAT. In a third series of animals dose-response curves to 8-OH-DPAT were constructed as described above until the response of the neurone was completely abolished or a concentration of 30µg was reached. A single dose of WAY100635 (100µg) was then administered (i.t.). Post-stimulus histograms were constructed every 10 min. for 60 min. following the administration of the antagonist.

6.2.2 Formalin induced inflammation

A second group of animals was then used for the formalin studies; neurones were characterized in the same way as with the first series but then, once a C-fibre response of the neurones had been confirmed, 50 μ l of 5% formalin solution was injected subcutaneously into the centre of the receptive field (a particular toe of the hind paw) to elicit prolonged firing of the neurones. The formalin evoked activity was recorded using a rate function (Spike 2 software, C.E.D. 1401 interface) and expressed as number of action potentials in ten minute time bins. 8-OH-DPAT (1 μ g, 3 μ g, 10 μ g or 30 μ g) and WAY100635 (1 μ g, 10 μ g or 100 μ g) were applied in a volume of 50 μ l directly onto the spinal cord. The drugs were administered as a pre-treatment 10 minutes prior to injection of formalin. No animal in the study received more than one of the two drugs.

6.3 Results

The results were obtained from 104 neurones, all of which displayed A β -, A δ -, and C-fibre evoked responses to transcutaneous electrical stimulation (average C-fibre threshold of 1.4 ± 0.1 mA). The population of neurones studied were located deep within the dorsal horn (average depth of 775 ± 26 μ m).

6.3.1 Electrically evoked responses

8-OH-DPAT had no significant effect on the electrically evoked A β - and C-fibre responses, post discharge and excess spikes, of dorsal horn neurones in normal animals with no inflammation. The values as % control after 30 μ g 8-OH-DPAT = A β -fibre $103\% \pm 7\%$, C-fibre $107\% \pm 15\%$, post discharge $112\% \pm 34\%$, excess spikes $123\% \pm 27\%$. However, 8-OH-DPAT caused a significant decrease in the electrically evoked A δ -fibre response ($F(4,37) = 4.128$, $P = 0.0073$). The values as % of control response were $92\% \pm 10\%$, $84\% \pm 15\%$, $61\% \pm 10\%$, and $44\% \pm 12\%$ for 1 μ g, 3 μ g, 10 μ g, and 30 μ g 8-OH-DPAT respectively (figure 24). Subsequent post-

hoc analysis showed significance for 10 μ g and 30 μ g 8-OH-DPAT (P = 0.0091 and 0.0046 for 10 μ g and 30 μ g respectively), but not 1 μ g and 3 μ g.

WAY100635 alone had no significant effect on the electrically evoked responses of dorsal horn neurones in normal animals with no inflammation. Thus, % control after 100 μ g WAY100635 = A β -fibre 105% \pm 7%, A δ -fibre 84% \pm 8%, C-fibre 101% \pm 17%, post discharge 86% \pm 15%, excess spikes 83% \pm 24%. However, in the third group of experiments 100 μ g WAY100635 partially reversed 8-OH-DPAT mediated inhibition of the A δ -fibre evoked response to 72% \pm 12% of control (figure 24).

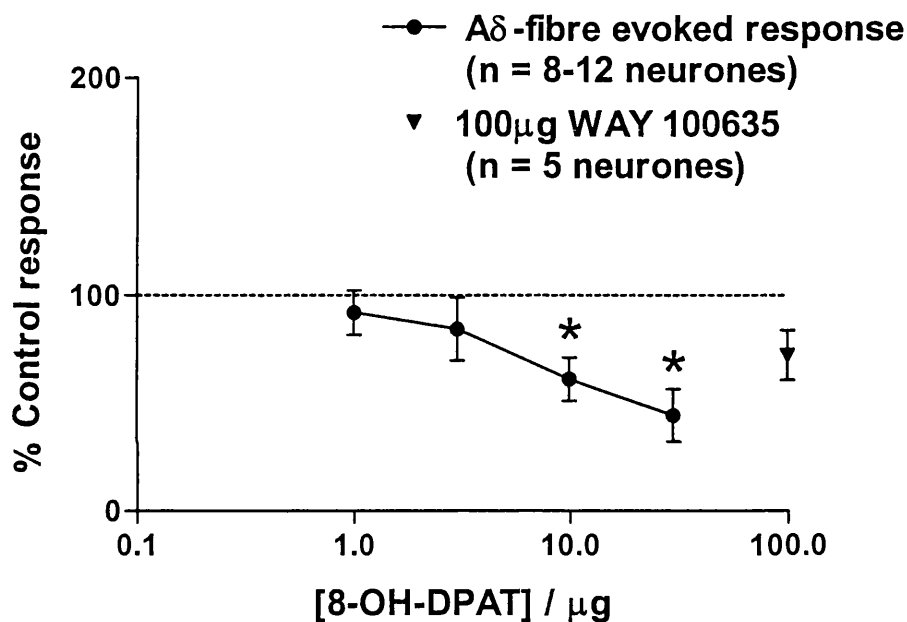


Figure 24. The effect of 8-OH-DPAT, the 5-HT_{1A} receptor agonist, on the electrically evoked A δ -fibre response of dorsal horn neurones in normal animals. 8-OH-DPAT was administered i.t. Results are presented as mean % of control response \pm the standard error of the mean.

6.3.2 Formalin induced inflammation

On injection of formalin, the neurone, previously silent, began to fire vigorously. The characteristic biphasic response was observed in all control experiments. Initial activity subsided within ten minutes, followed by the silent

interphase period. Activity recommenced approximately 25 minutes after injection and, in control responses, typically subsided within ninety minutes. Each neurone was recorded for 1 hour 40 minutes. The sum of the action potentials within the first ten minutes post-formalin was termed the first phase (mean control value for the population of 5904 ± 572). Subsequent activity of the neurone (10 to 100 minutes) was assigned to the second phase (mean control value of 33005 ± 4444).

8-OH-DPAT, administered 10 minutes prior to injection of formalin, significantly decreased the area under the curve of the formalin response in a dose dependent manner ($F(4,44) = 4.204$, $P = 0.0057$) (figure 25). Mean areas under the curve were $81\% \pm 16\%$, $42\% \pm 14\%$, $33\% \pm 11\%$, and $11\% \pm 5\%$ of control for $1\mu\text{g}$, $3\mu\text{g}$, $10\mu\text{g}$ and $30\mu\text{g}$ of 8-OH-DPAT respectively (figure 25). Post-hoc analysis revealed $3\mu\text{g}$, $10\mu\text{g}$ and $30\mu\text{g}$ 8-OH-DPAT significantly decreased the area under the curve ($P = 0.0255$, 0.0158 , and 0.0016 for $3\mu\text{g}$, $10\mu\text{g}$ and $30\mu\text{g}$ respectively), whereas $1\mu\text{g}$ failed to reach significance. Further statistical analysis was performed separately on the first and second phases of the response. The total number of action potentials in each phase of the response was compared to their individual controls. 8-OH-DPAT significantly decreased the number of action potentials in the second phase of the formalin response ($F(4,44) = 3.894$, $P = 0.0086$, figure 26). Analysis of the number of action potentials in the first phase showed no overall significance. Post-hoc analysis showed $3\mu\text{g}$, $10\mu\text{g}$ and $30\mu\text{g}$ 8-OH-DPAT significantly decreased the number of action potentials in the second phase to $40\% \pm 15\%$, $29\% \pm 11\%$, and $8\% \pm 5\%$ of control respectively ($P = 0.032$, 0.0181 and 0.0025 for $3\mu\text{g}$, $10\mu\text{g}$ and $30\mu\text{g}$ respectively), however only $30\mu\text{g}$ 8-OH-DPAT significantly attenuated the first phase ($44\% \pm 15\%$ of control, $P = 0.0238$).

Pretreatment with WAY100635 ($1\mu\text{g}$, $10\mu\text{g}$, and $100\mu\text{g}$) had no significant effect on either the area under the curve of the formalin response, or the number of action potentials in the first or second phase of the response (figure 27 and figure 28).

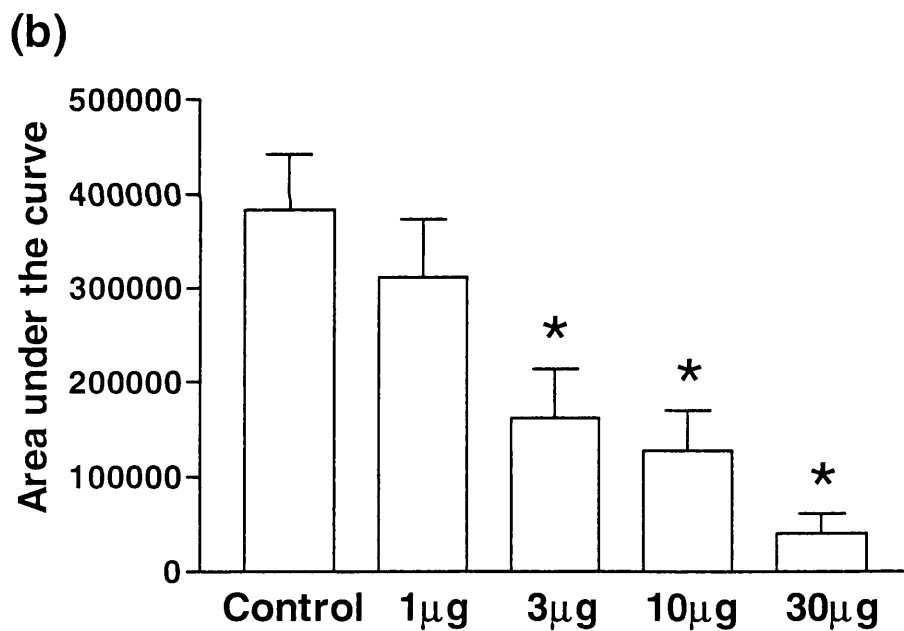
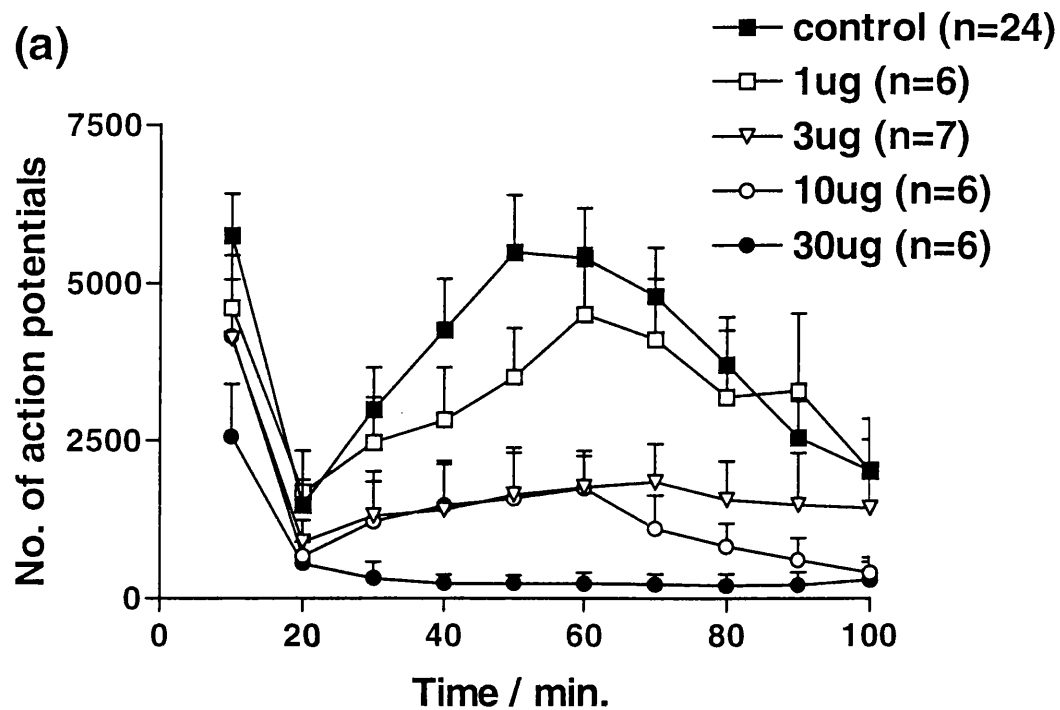


Figure 25. The effect of 8-OH-DPAT (i.t.) on the formalin response. 8-OH-DPAT was administered as a 10 minute pretreatment. (a), Results presented as mean number of action potentials in 10 minute time bins +/- standard error of the mean. (b), The effect of pretreatment with 8-OH-DPAT on the area under the curve of the complete formalin response. Results are presented as mean area under the curve +/- standard error of the mean.

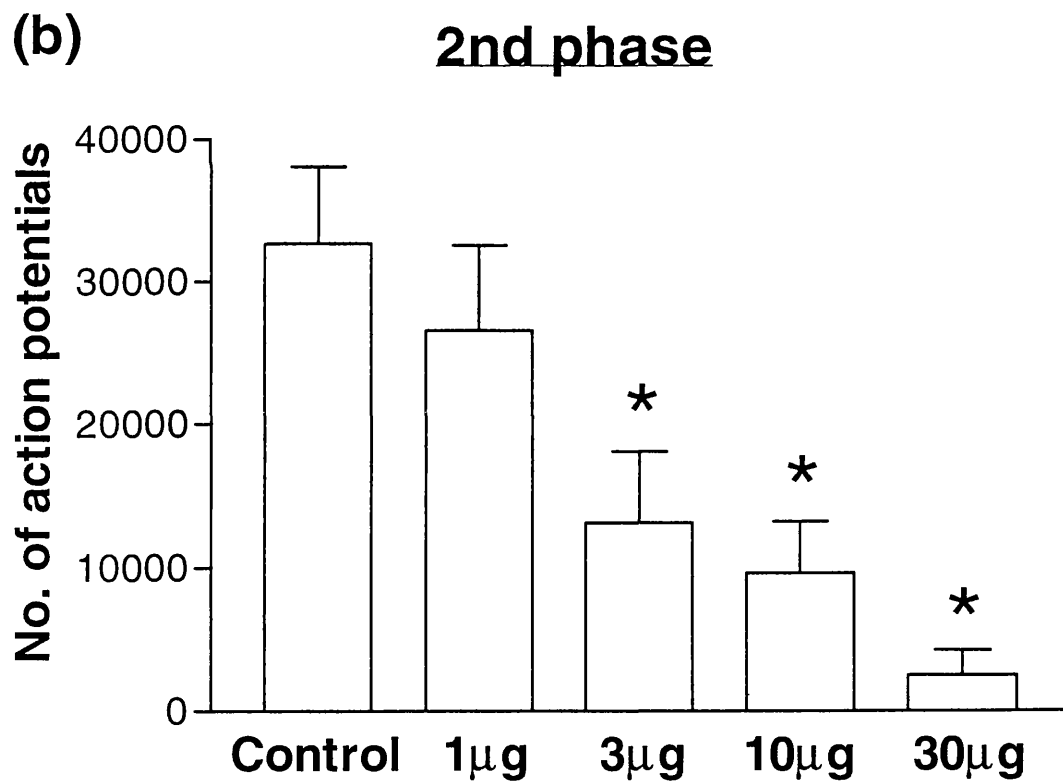
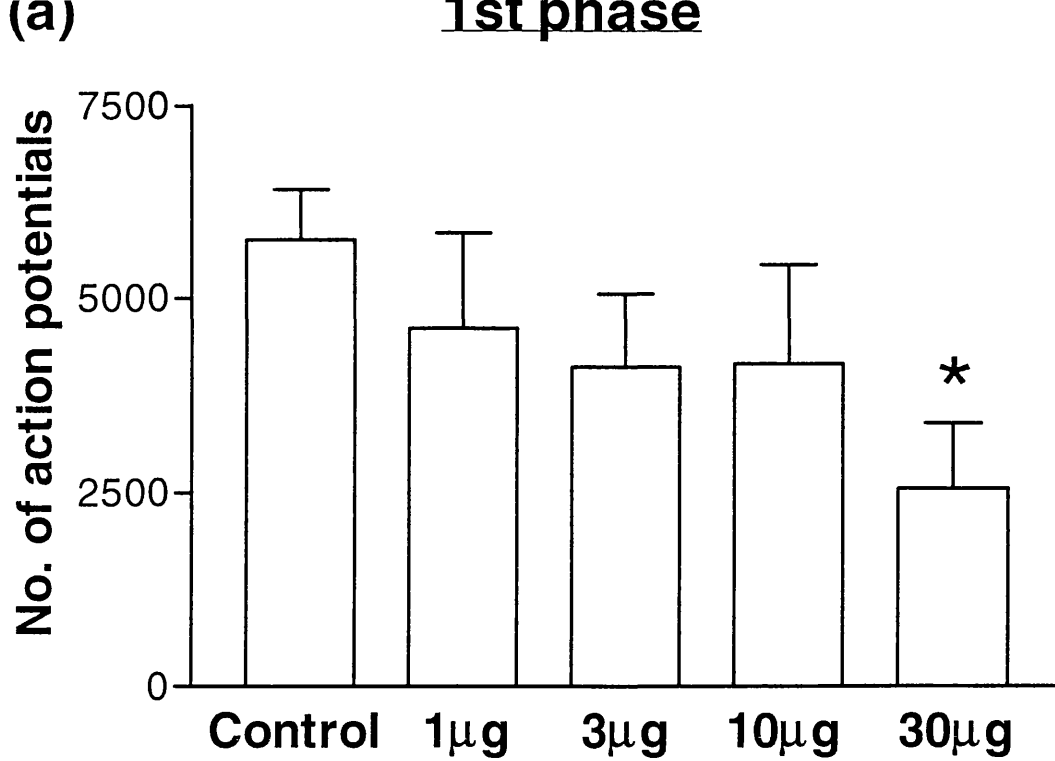


Figure 26. The effects of 8-OH-DPAT on (a) the 1st phase and (b) the 2nd phase of the formalin response. Results are presented as mean number of action potentials \pm standard error of the mean.

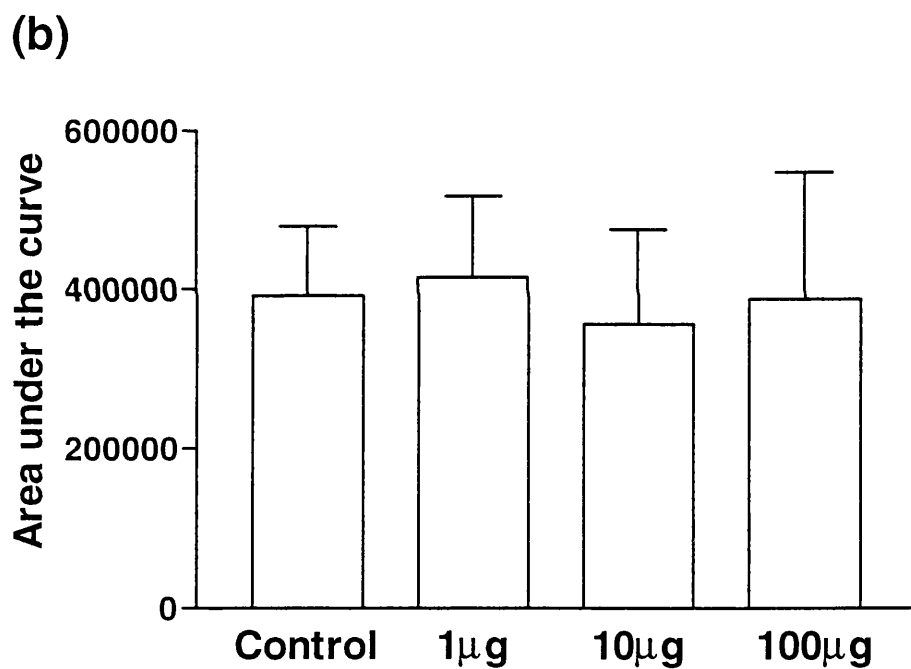
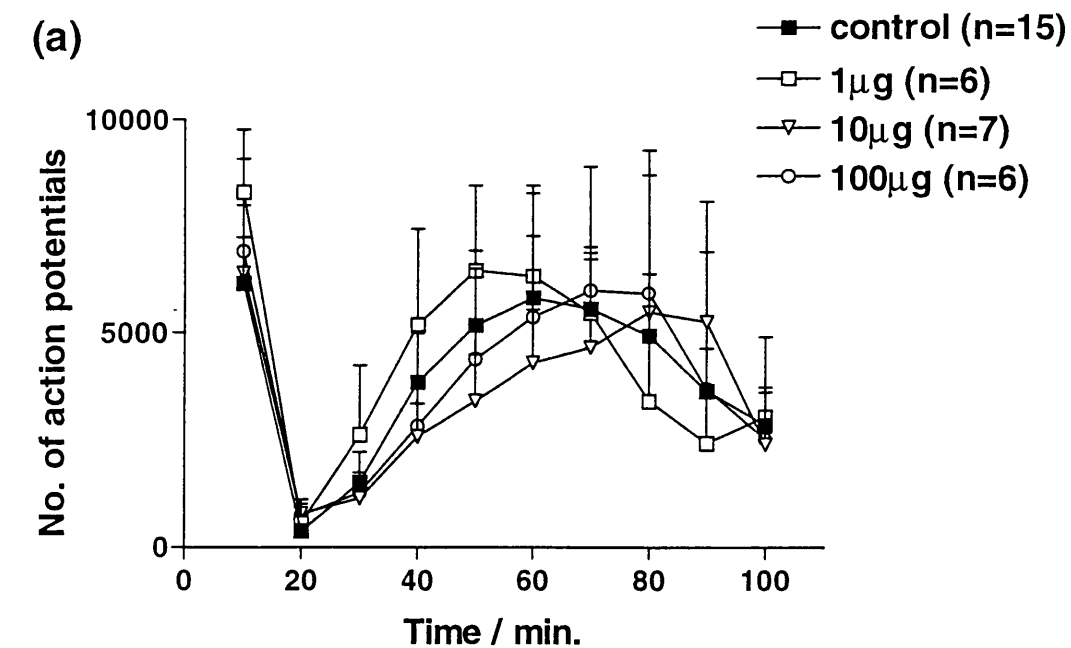
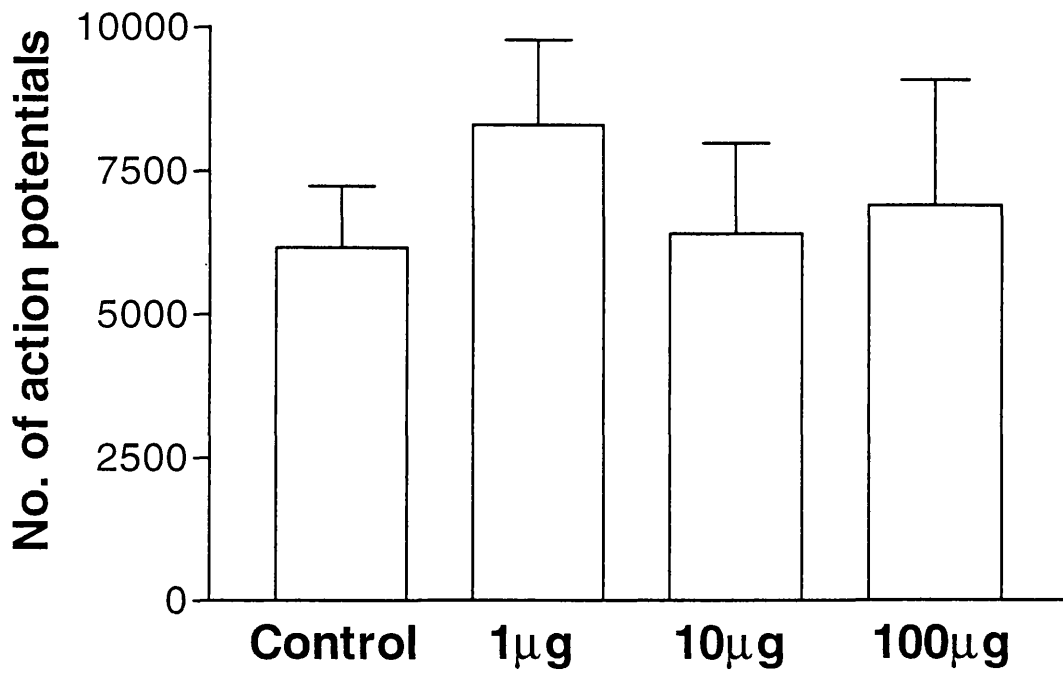


Figure 27. The effect of WAY100635 (i.t.) on the formalin response WAY100635 was administered as a 10 minute pretreatment. (a), Results presented as mean number of action potentials in 10 minute time bins +/- standard error of the mean. (b), The effect of pretreatment with WAY100635 on the area under the curve of the complete formalin response. Results are presented as mean area under the curve +/- standard error of the mean.

(a)

1st phase



(b)

2nd phase

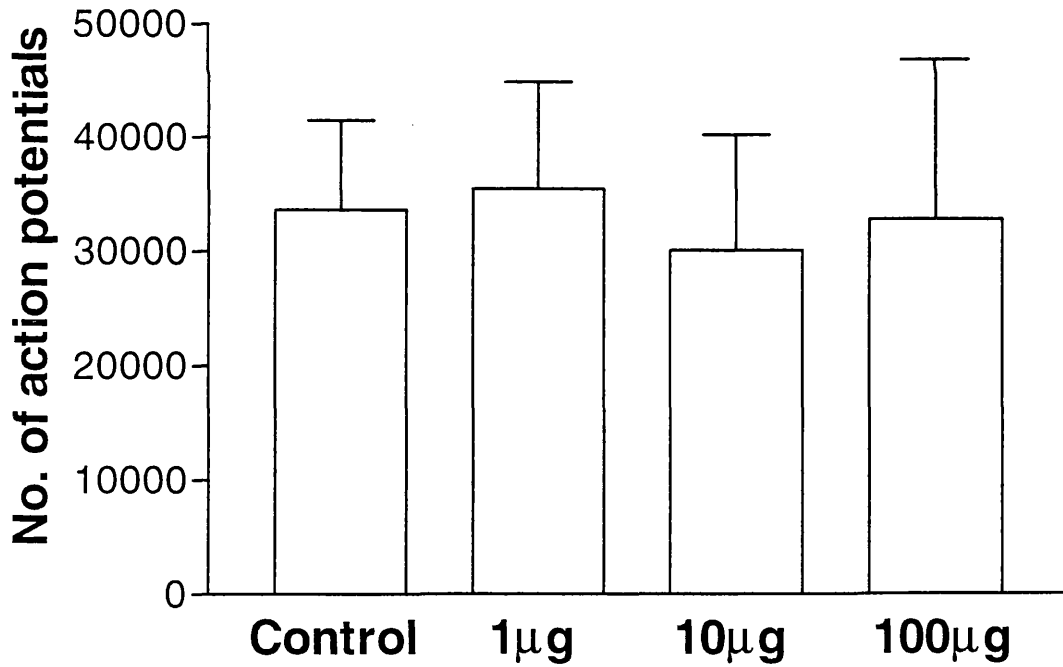


Figure 28. The effects of WAY100635 on (a) the 1st phase and (b) the 2nd phase of the formalin response. Results are presented as mean number of action potentials \pm standard error of the mean.

6.4 Discussion

My results show that in both normal animals and animals with formalin induced peripheral inflammation, blocking the 5-HT_{1A} receptor had no significant effect on the nociceptive responses of spinal dorsal horn neurones. This suggests there is little or no endogenous 5-HT_{1A} receptor mediated modulation of WDR neurones in the dorsal horn under normal circumstances or indeed, during formalin-induced inflammation. However, activation of the receptor by the 5-HT_{1A} receptor agonist 8-OH-DPAT, produced significant inhibition of both neuronal responses to peripheral formalin, and of the A δ -fibre evoked response following acute electrical stimulation. It is significant that WAY100635 only partially reversed the inhibition of A δ -fibre evoked responses implicating activity at sites other than 5-HT_{1A} receptors in the action of 8-OH-DPAT (see below).

During formalin induced inflammation, 8-OH-DPAT reduced the magnitude of the second phase of the response in a dose dependent manner, but only the highest dose (30 μ g) significantly decreased the first phase. Thus the results suggest 5-HT_{1A} receptors in the dorsal horn have a predominantly post-synaptic mechanism of action, in agreement with the anatomical studies on spinal 5-HT_{1A} receptor localization (Marlier *et al.*, 1991; Riad *et al.*, 1991; Bervoets *et al.*, 1993; Thor *et al.*, 1993; LaPorte *et al.*, 1995; Kia *et al.*, 1996). However, the significant effect on the first phase of the formalin response suggests spinal 5-HT_{1A} receptors are also found presynaptically. This is something of a controversy. Destruction of primary afferent terminals has been shown to cause a minor reduction in 5-HT_{1A} receptor density, suggesting a small population (20%) of these receptors are to be found on primary afferent terminals (LaPorte *et al.*, 1995). This is supported by one study that found 5-HT_{1A} receptor mRNA in the dorsal root ganglion (DRG) (Pompeiano *et al.*, 1992). However, this has been recently disputed by another group who also examined the DRG and failed to detect 5-HT_{1A} receptor mRNA (Pierce *et al.*, 1996). In addition, 8-OH-DPAT has been shown to have no effect on substance P release in dorsal horn slice preparations (Bourgoin *et al.*, 1993). It could be argued that the lack of effect of 8-OH-DPAT on C-fibre evoked responses and selective inhibition of A δ -fibre evoked responses observed in the present study could account for the inability of 8-OH-DPAT to alter SP release. Colocalization of SP with glutamate in the central

terminals of A δ -fibres has not been conclusively demonstrated. However, this is most likely due to the fact that anatomical studies are not yet able to distinguish A δ -fibre cell bodies in the DRG from those of C-fibres. Nonetheless, lesion studies also report the majority of 5-HT_{1A} receptors are localized postsynaptically to serotonergic neurones, and are not found on descending noradrenergic pathways (Bervoets *et al.*, 1993; LaPorte *et al.*, 1995). It has also been hypothesised postsynaptic 5-HT_{1A} receptors are present on intrinsic interneurons (LaPorte *et al.*, 1995).

In the previous chapter I reviewed the results obtained from several different models of nociception investigating the role of spinal 5-HT₃ receptors. The evidence suggested the descending serotonergic system was not significantly involved with acute nociceptive transmission, but became active in response to more severe noxious stimuli. It is also apparent that several inconsistencies in the literature may be accounted for if the descending serotonergic pathways that activate spinal 5-HT₃ receptors respond differentially to thermal, chemical and mechanical noxious input. A similar situation surrounds the role of spinal 5-HT_{1A} receptors in nociception, however a more substantial body of evidence exists.

A number of hot-plate latency (HPL) and tail-flick latency (TFL) studies have examined spinal 5-HT_{1A} receptors, reporting both pro- and antinociceptive effects (Fasmer *et al.*, 1986; Solomon & Gebhart, 1988; Eide *et al.*, 1990; Crisp *et al.*, 1991; Mjellem *et al.*, 1992; Ali *et al.*, 1994; Xu *et al.*, 1994; Takeshita & Yamaguchi, 1995). However, a recent, comprehensive behavioural study has shown the 5-HT_{1A} receptor has little ability to modulate *acute* nociception whether it was produced by thermal, mechanical or chemical stimuli. Furthermore, in the cases where 5-HT_{1A} agonists did elicit antinociceptive behaviour it was α_2 -adrenoceptors that were implicated rather than 5-HT_{1A} receptors (Millan, 1994). The author also suggests the diversity of results from previous HPL and TFL studies may be due to the fact that 5-HT_{1A} agonists have marked cardiovascular and endocrine activity, as well as possible effects on motor function, that may modify behaviour and produce apparent antinociceptive effects (see refs in Millan, 1994). But even if spinal 5-HT_{1A} receptors do not play a significant role in acute nociception, it is reasonable to suggest the case may be different for other nociceptive modalities. Indeed, there are many convincing reports supporting a role for 5-HT_{1A} receptors in spinal cord and brainstem nociceptive circuitry.

In agreement with the present study, 8-OH-DPAT has been shown to inhibit the second phase of the formalin response (Oyama *et al.*, 1996). Also an electrophysiological study recording WDR neurones found iontophoretic application of 8-OH-DPAT suppressed the response to both noxious and non-noxious mechanical stimuli (El-Yassir *et al.*, 1988). Noxious mechanical information is likely to be relayed to the dorsal horn by both C- and A δ -fibres. Interestingly, a recent study making single unit extracellular recordings, with a similar protocol to the present study, but using urethane anaesthesia and female rats, investigated the effects of intrathecal 8-OH-DPAT and WAY 100635 on the electrically evoked responses of spinal WDR neurones. In agreement with my results they report WAY100635 alone had no effect, but found 8-OH-DPAT produced a nonselective inhibition of all evoked neuronal responses (A β , A δ , C-fibre and post discharge) which could be prevented by WAY100635 (Gjerstad *et al.*, 1996).

Other groups suggest spinal serotonergic transmission involving 5-HT_{1A} receptors has multiple effects on nociception. One reports a bell shaped dose-response curve for the antinociceptive effects of 8-OH-DPAT (Xu *et al.*, 1994) whilst another group reports a dual effect of intrathecal 5-HT, inhibiting aversive responses at a low dose and facilitating responses at higher concentrations (Oyama *et al.*, 1996). Interestingly, the inhibition induced by a low dose of 5-HT was reversed by coadministration of the 5-HT_{1A} receptor antagonist NAN190 (Oyama *et al.*, 1996). 5-HT released from descending axons has also been shown to have multiple roles in the control of spinal withdrawal reflex transmission (Clarke *et al.*, 1996). The same group also reports 8-OH-DPAT inhibited spinal polysynaptic reflex responses to low intensity input but increased responses evoked by moderate to high stimulus intensities. Significantly, only the inhibitory effects of 8-OH-DPAT could be reversed by WAY 100635 (Clarke *et al.*, 1997).

Facilitation of nociceptive responses following electrical stimulation of areas in the reticular formation of the RVM is reported to be mediated by a descending serotonergic pathway that involves spinal 5-HT_{1A} receptors (Zhuo & Gebhart, 1991). Descending facilitation following stimulation of the same area of the reticular formation has also been shown to enhance the expression of c-fos in the spinal cord elicited by peripheral inflammation (Ren *et al.*, 1997). Calejesan *et al.* (1988) report descending facilitation of the formalin response that was abolished by stimulation of

structures in the reticular formation. The authors cite work on the descending noradrenergic system that demonstrates an increase in descending inhibition of dorsal horn neurones receiving input from inflamed tissue (Cervero *et al.*, 1991; Schaible *et al.*, 1991; Ren & Dubner, 1996; see chapter 4 discussion) and suggest that while descending inhibition is likely enhanced at synapses receiving sensory input from the damaged area, descending facilitation may be dominant at distal synapses (Calejasan *et al.*, 1998).

Thus it appears the descending serotonergic system mediates multiple effects via spinal 5-HT_{1A} receptors in response to a range of intensities and modalities of noxious afferent input. Indeed, this has already been proposed (Clarke & Houghton, 1994; Millan, 1995; Clarke *et al.*, 1996; Millan *et al.*, 1996; see Millan, 1997 for review). It is probable the various excitatory phenomena observed following spinal 5-HT_{1A} receptor activation are due to suppression of GABAergic transmission via 5-HT_{1A} receptors localized on interneurons (See Millan, 1997 for review).

There are several factors which may have contributed to many of the apparent inconsistencies in the literature. One recent study reports mechanical nociception as the most sensitive to intrathecal administration of 5-HT, followed by chemical nociception (5% formalin solution), and finally thermal nociception (Bardin *et al.*, 1997b). In the present study 8-OH-DPAT had no effect on C-fibre evoked responses, but selectively inhibited responses evoked in A δ -fibres which are activated physiologically by high threshold mechanoreceptors. Thus it is possible the role of spinal 5-HT_{1A} receptors may have been obscured by the results of studies employing nociceptive tests that do not generate significant noxious activity in A δ -fibre afferents.

Differential coupling of individual populations of 5-HT_{1A} receptors may also be a factor. An electrophysiological study making patch-clamp recordings from C-like DRG cells showed that two parallel signalling pathways couple 5-HT_{1A} receptors to N-type Ca²⁺ channels by a membrane delimited pathway, and to P-type Ca²⁺ channels by a pathway dependent on a cytosolic diffusible component (most likely cAMP) and the concentration of free intracellular Ca²⁺ (Cardenas *et al.*, 1997). The authors report these two transduction pathways are different enough in mechanism that modulation of neuronal activity by either pathway may be selectively suppressed or enhanced by physiological inputs, for example cross-talk

from other active 2nd messenger systems, depolarisation or hyperpolarisation by impinging afferents, rapid firing rates, or desensitisation by prolonged exposure to agonists. Thus, selective activation or inhibition of either pathway could modify neuronal activity by altering patterns of Ca²⁺ entry into the cell (Cardenas *et al.*, 1997). Significantly, different populations of 5-HT_{1A} receptors in different areas of the brain, for example the DRG and the raphe nuclei, have been shown to have different degrees of coupling to the two transduction pathways (see refs in Cardenas *et al.*, 1997). Thus, 5-HT_{1A} receptor activation may have different effects on neuronal activity at individual sites.

Finally, the selectivity of 5-HT_{1A} receptor ligands must be considered. One study reports the effects of 8-OH-DPAT were reversed by WAY100635 in a range of behavioural tests (Sanchez *et al.*, 1996), and 8-OH-DPAT induced suppression of the second phase of the formalin response is reported to be blocked by coadministration of the 5-HT_{1A} antagonist NAN190 (Oyama *et al.*, 1996). However, 8-OH-DPAT and almost all other 5-HT_{1A} ligands have been shown to bind to sites other than 5-HT_{1A} receptors. All 5-HT_{1A} antagonists other than WAY100135 and WAY100635 are reported to have either partial agonist activity or poor selectivity relative to activity at other sites (Fletcher *et al.*, 1993; Lejune *et al.*, 1993; Munday *et al.*, 1994; Routledge, 1996). There is also evidence 8-OH-DPAT has significant affinity for 5-HT₇ sites (Ogilvie & Clarke, 1996; Pierce *et al.*, 1996; Clarke *et al.*, 1997). In the present study WAY100635 only partially reversed 8-OH-DPAT induced inhibition. Other groups have also found the effects of 5-HT_{1A} receptor agonists to be resistant to 5-HT antagonists (Danzebrink & Gebhart, 1991; Millan, 1994) and further studies have implicated α_2 -adrenoceptors and D₂ receptors in 5-HT_{1A} agonist mediated antinociception (Post & Archer, 1990; Giordano, 1991; Millan & Colpaert, 1991a,b; Millan, 1994). There is also evidence to suggest 8-OH-DPAT may possess 5-HT reuptake inhibitor properties (Assie & Koek, 1996).

In summary, I have presented electrophysiological evidence suggesting spinal 5-HT_{1A} receptors are not significantly involved in descending serotonergic modulation of spinal WDR neurones under normal conditions or during formalin induced inflammation. However, my data suggests 5-HT_{1A} receptors are involved in 8-OH-DPAT mediated spinal inhibition, which selectively suppressed A δ -fibre evoked activity.

7. 5-HT₄ RECEPTORS

7.1 Introduction

The recently classified 5-HT₄ receptor is G-protein linked to adenylate cyclase (Eglen *et al.*, 1995a). In situ hybridization and radioligand binding studies have demonstrated the presence of 5-HT₄ receptors throughout brain regions belonging to the limbic system including the amygdala, hippocampus, and olfactory system (Domenech *et al.*, 1994; Waeber *et al.*, 1994; Ullmer *et al.*, 1996; Vilaro *et al.*, 1996). Thus, the central distribution of 5-HT₄ receptors suggests a role in emotional processes, and studies have been conducted exploring their participation in learning and memory, cognition and anxiety (Barnes *et al.*, 1990; Ansanay *et al.*, 1992; Hochner & Kandel, 1992; Frey *et al.*, 1993; Waeber *et al.*, 1994; Kennett *et al.*, 1997). However, a role for the 5-HT₄ receptor in central pain transmission has yet to be addressed.

The involvement of 5-HT₄ receptor mediated serotonergic modulation of formalin and carrageenan induced inflammatory pain transmission was determined using the selective 5-HT₄ receptor antagonist RS39604 (Hedge *et al.*, 1995), and (as a full, selective agonist for the 5-HT₄ receptor is not yet available) the selective 5-HT₄ receptor partial agonist RS67506 (Eglen *et al.*, 1995b). The effects of these drugs on the electrically evoked responses of dorsal horn neurones in normal animals were compared to their effects on electrically evoked responses following carrageenan induced inflammation, and the effects of pretreatment on the formalin response.

7.2 Methods

7.2.1 Electrically evoked responses

We examined the effects of the 5-HT₄ receptor partial agonist RS67506, and the 5-HT₄ receptor antagonist RS39604 on the response to transcutaneous electrical stimulation in a series of animals. Control post-stimulus histograms (as described above) were constructed at ten minute intervals until the response of the cell was stable for three consecutive tests. Once stable, dose-response curves to the drugs

were constructed. Doses of 5µg, 50µg and 250µg of RS67506 were applied directly on the spinal cord in a volume of 50µl. Only a 250µg dose was administered for experiments with RS39604. Post-stimulus histograms were constructed every 10 min. for 60 min. following the administration of each dose of drug. No animal in the study received more than one of the two drugs.

7.2.2 *Formalin induced inflammation*

A second group of animals was then used for the formalin studies; neurones were characterized in the same way as with the first series but then, once a C-fibre response of the neurones had been confirmed, 50µl of 5% formalin solution was injected subcutaneously into the centre of the receptive field (a particular toe of the hind paw) to elicit prolonged firing of the neurones. The formalin evoked activity was recorded using a rate function (Spike 2 software, C.E.D. 1401 interface) and expressed as number of action potentials in ten minute time bins. RS67506 (50µg or 250µg) and RS39604 (250µg) were applied in a volume of 50µl directly onto the spinal cord. The drugs were administered as a pre-treatment 10 minutes prior to injection of formalin. No animal in the study received more than one of the two drugs.

7.2.3 *Carrageenan induced inflammation*

A third group of animals was used for the carrageenan studies; neurones were characterized in the same way as with the first series but then, once a C-fibre response of the neurones had been confirmed, 100µl of 2% λ-carrageenan solution was injected into the plantar region of the paw to produce inflammation. The drug effects were tested 3 hours post-carrageenan. Three post-stimulus histograms were constructed (as described above) at 10 min. intervals prior to drug administration to be used as controls. Doses of 5µg, 50µg and 250µg of RS67506 were applied directly on the spinal cord in a volume of 50µl. Post-stimulus histograms were constructed every 10 min. for 60 min. following the administration of each dose of drug.

7.3 Results

The results were obtained from 43 neurones, all of which displayed A β -, A δ - and C-fibre evoked responses to transcutaneous electrical stimulation (average C-fibre threshold of 1.5 ± 0.1 mA). The population of neurones studied were located deep within the dorsal horn (average depth of 801 ± 31 μ m).

7.3.1 *Electrically evoked responses*

RS67506 had no significant effect on the electrically evoked A β -, A δ - and C-fibre evoked responses, post discharge or excess spikes of dorsal horn neurones in normal animals with no inflammation. % control after 250 μ g RS67506 = A β -fibre $91\% \pm 5\%$, C-fibre $120\% \pm 6\%$, post discharge $136\% \pm 20\%$, excess spikes $96\% \pm 26\%$. RS39604 also had no significant effect on the electrically evoked A β - and C-fibre evoked responses, post discharge or excess spikes of dorsal horn neurones in normal animals. % control after 250 μ g RS39604 = A β -fibre $94\% \pm 8\%$, C-fibre $108\% \pm 12\%$, post discharge $114\% \pm 39\%$, excess spikes $136\% \pm 47\%$ (figure 29). However, 250 μ g RS39604 significantly decreased the A δ -fibre evoked response to $55\% \pm 9\%$ of control ($F(1,4) = 9.1011$, $P = 0.0393$) (figure 29).

7.3.2 *Formalin induced inflammation*

On injection of formalin the neurone, previously silent, began to fire vigorously. The characteristic biphasic response was observed in all control experiments. Initial activity subsided within ten minutes, followed by the silent interphase period. Activity recommenced approximately 25 minutes after injection and, in control responses, typically subsided within ninety minutes. Each neurone was recorded for 1 hour 40 minutes. The sum of the action potentials within the first ten minutes post-formalin was termed the first phase (mean control value for the population of 5900 ± 733). Subsequent activity of the neurone (10 to 100 minutes)

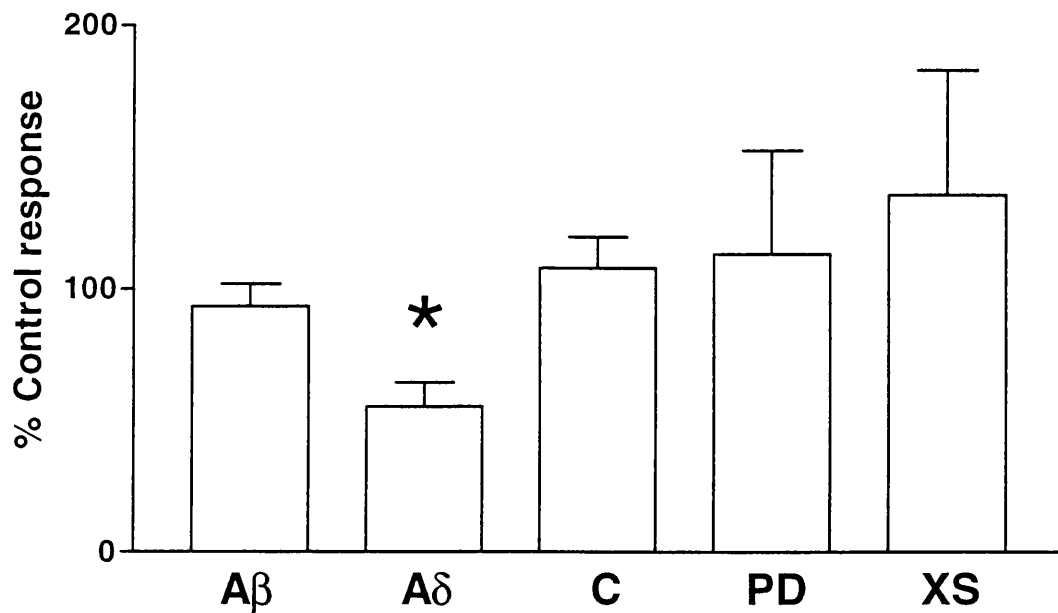


Figure 29. The effect of 250µg RS39604, the 5-HT₄ receptor antagonist, on the electrically evoked Aβ-, Aδ-, and C-fibre responses, post discharge (PD) and excess spikes (XS) of dorsal horn neurones in normal animals. RS39604 was administered i.t. Results are presented as mean % of control response +/- the standard error of the mean.

was assigned to the second phase (mean control value of 17675 ± 2547).

Pretreatment with RS67506, the 5-HT₄ receptor partial agonist, 10 minutes prior to injection of formalin, significantly increased the area under the curve of the formalin response ($F(2,18) = 4.604$, $P = 0.024$) (figure 30). Mean areas under the curve were $176\% \pm 67\%$, and $272\% \pm 49\%$ of control for 50µg and 250µg of RS67506 respectively (figure 30). Post-hoc analysis showed 250µg RS67506 significantly increased the area under the curve ($P = 0.01$) but 50µg RS67506 failed to reach significance. Further statistical analysis was performed separately on the first and second phases of the response. The total number of action potentials in each phase of the response was compared to their individual controls. RS67506 had no significant effect on the first phase of the response, but significantly increased the number of action potentials in the second phase ($F(2,18) = 4.608$, $P = 0.024$). Post-

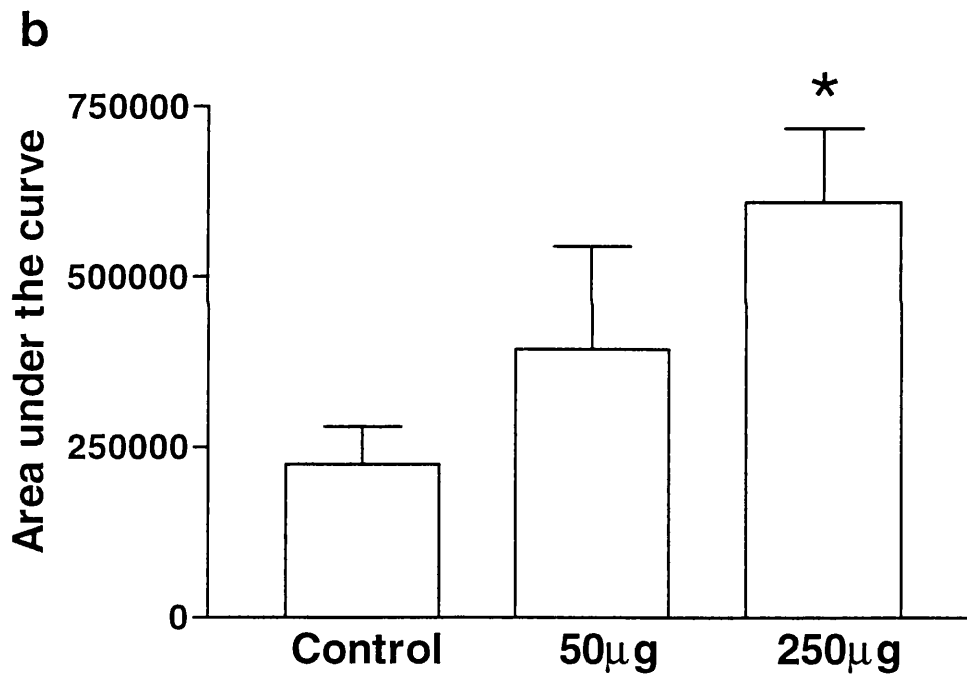
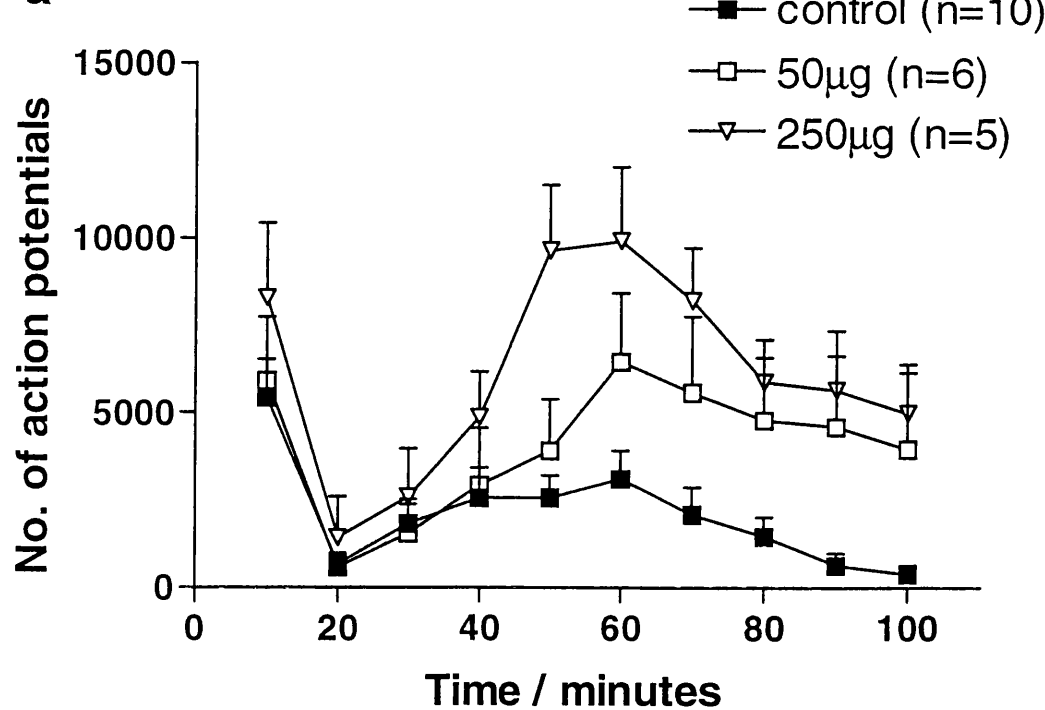


Figure 30. The effect of RS67506, the 5-HT₄ receptor partial agonist (i.t.), on the formalin response. RS67506 was administered as a 10 minute pretreatment. (a), Results presented as mean number of action potentials in 10 minute time bins +/- standard error of the mean. (b), The effect of pretreatment with RS67506 on the area under the curve of the complete formalin response. Results are presented as mean area under the curve +/- standard error of the mean.

hoc analysis showed no significance after 50µg RS67506, but a significant increase in the second phase after 250µg (385% ± 67% of control, p = 0.01).

Pretreatment with RS39604 (250µg), the 5-HT₄ receptor antagonist, had no significant effect on either the area under the curve of the formalin response, or the number of action potentials in the first or second phase of the response (figure 31).

7.3.3 Carrageenan induced inflammation

RS67506, the 5-HT₄ receptor partial agonist, had no significant effect on the electrically evoked Aβ- and C-fibre responses (% control after 250µg RS67506 = Aβ-fibre 107% ± 9%, C-fibre 99% ± 9%) but caused a significant increase in the electrically evoked post discharge of dorsal horn neurones in animals with carrageenan induced inflammation (F(3,20) = 4.644, P = 0.01) (figure 32). % of control response was 142% ± 4%, 142% ± 16%, and 170% ± 11% for 5µg, 50µg, and 250µg RS67506 respectively. Post-hoc analysis showed significance for 250µg RS67506 (P = 0.004), but not 5µg or 50µg (figure 32).

7.4 Discussion

The 5-HT₄ receptor partial agonist RS67506 had no significant effect on the electrically evoked responses of nociceptive neurones in the dorsal horn, yet produced a significant facilitation of the second phase of the formalin response, and significantly increased the electrically evoked post discharge of dorsal horn neurones in the carrageenan model. The 5-HT₄ receptor antagonist RS39604 had no significant effect on the formalin response, but significantly decreased the electrically evoked Aδ-fibre response in normal animals. To the best of my knowledge this is the first evidence for functional 5-HT₄ receptors in the dorsal horn of the rat spinal cord. My results suggest spinal 5-HT₄ receptors are involved in the maintenance or tonic enhancement of Aδ-fibre mediated acute noxious transmission, but have no significant endogenous role during the transmission of ongoing, formalin induced

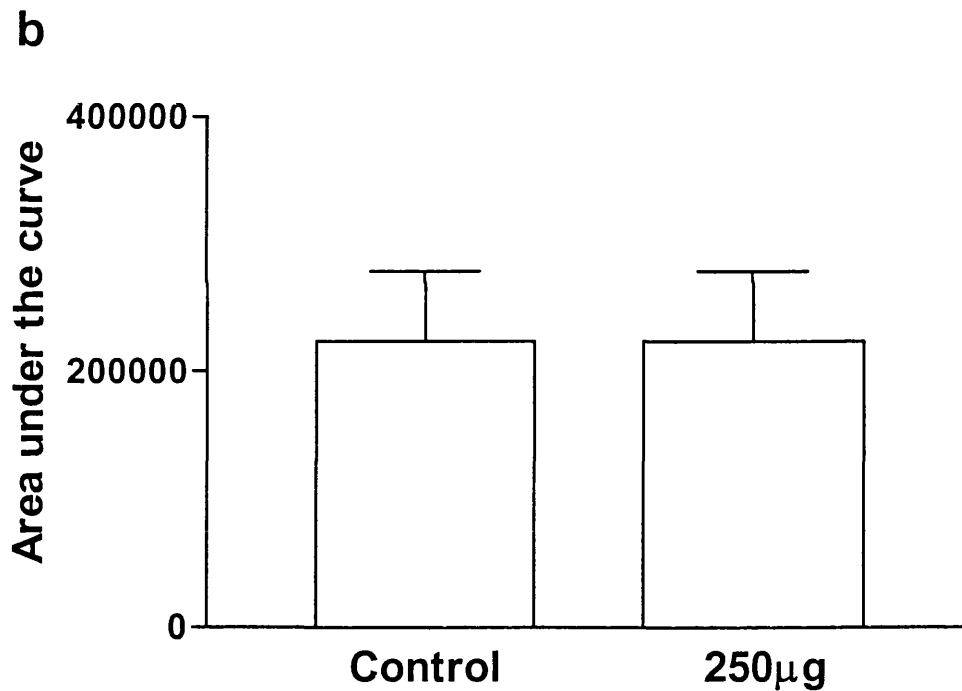
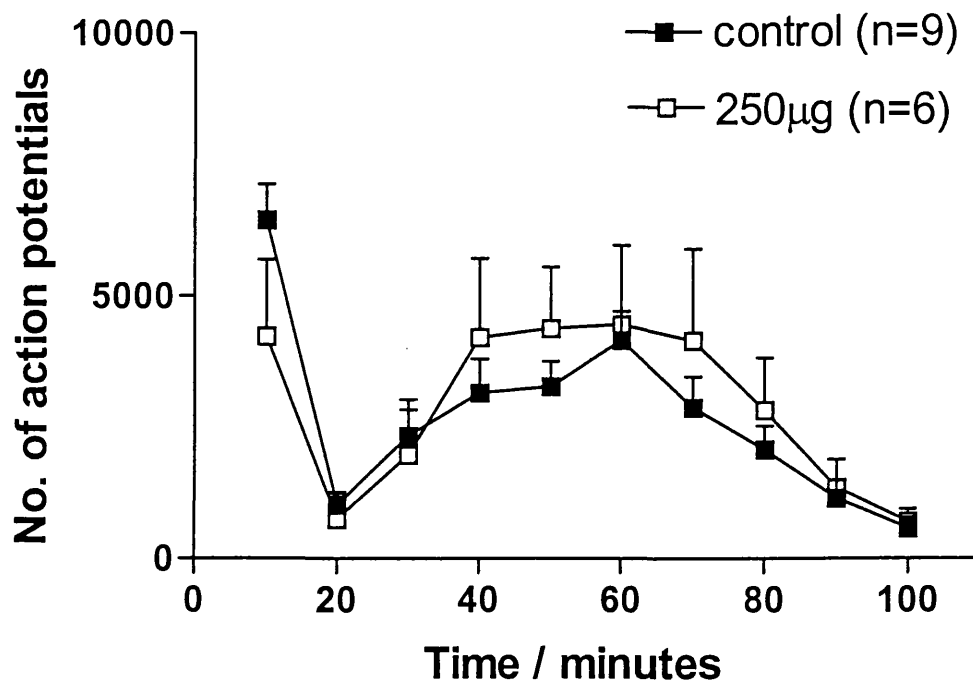


Figure 31. The effect of RS39604, the 5-HT₄ receptor antagonist (i.t.), on the formalin response. RS39604 was administered as a 10 minute pretreatment. (a), Results presented as mean number of action potentials in 10 minute time bins +/- standard error of the mean. (b), The effect of pretreatment with RS39604 on the area under the curve of the complete formalin response. Results are presented as mean area under the curve +/- standard error of the mean.

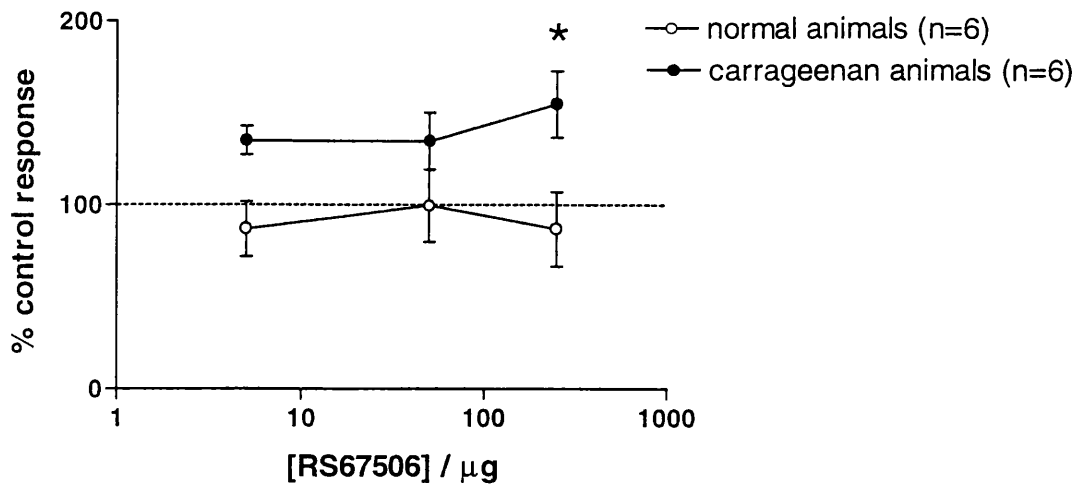


Figure 32. The effect of RS67506, the 5-HT₄ receptor partial agonist, on the electrically evoked post-discharge of dorsal horn neurones in normal animals and animals with carrageenan inflammation. RS67506 was administered i.t. Results are presented as mean % of control response +/- the standard error of the mean.

inflammatory nociception. Nonetheless, it appears a 5-HT₄ receptor mediated excitatory system exists within the dorsal horn which, when activated, may act to enhance the response of nociceptive neurones during formalin and carrageenan induced inflammation. Furthermore, our results suggest spinal 5-HT₄ receptors are located post-synaptically. RS67506 facilitated the electrically evoked post discharge, but not C-fibre or A δ -fibre response, of dorsal horn neurones following carrageenan induced inflammation, and significantly increased the second phase of the formalin response but not the first, events thought to be mediated by post-synaptic NMDA receptors (Dickenson, 1997).

There have been occasional reports of analgesia induced by an increase in central cholinergic tone involving 5-HT₄ receptors (Romanelli *et al.*, 1993, 1996; Ghelardini *et al.*, 1996). Also, one recent study demonstrates antagonism of peripheral 5-HT₄ receptors mediated antinociception in enteric viscera and, to a lesser extent, in cutaneous terminals (Espejo & Gill, 1998). In the present study, the lack of effect of RS67506 on electrically evoked C-fibre responses in the carrageenan model suggests activation of spinal 5-HT₄ receptors enhance neuronal responses during inflammation by an indirect mechanism. The 5-HT₄ receptor is G-protein linked and has been shown to facilitate an increase in intracellular cyclic

AMP (cAMP_i) via activation of adenylate cyclase (Dumuis *et al.*, 1988; Bockaert *et al.*, 1989; Monferini *et al.*, 1993). This accumulation of cAMP_i has been found to induce closure of inward K⁺ currents, providing evidence for an increase in neuronal excitability following activation of 5-HT₄ receptors, concluding with increased neurotransmitter release (Ansanay *et al.*, 1992; Fagni *et al.*, 1992; Torres *et al.*, 1995).

Clearly, more selective agents will be required for further investigation of the roles of this receptor in sensory processes. However, by way of speculation there is evidence that peripheral 5-HT₄ receptors modulate tachykinergic excitation, effecting an increase in substance P (SP) release (Briejer & Schuurkes, 1997; Goldhill *et al.*, 1997). In addition to the primary afferent terminals, release of SP in the dorsal horn has been reported from both intrinsic neurones and descending fibres (Jessel *et al.*, 1979; Yaksh *et al.*, 1988). Also, neurokinin receptors in the dorsal horn are located post-synaptically to the primary afferent terminals in laminae I, II and X (Yashpal *et al.*, 1991). One possible theory is that central 5-HT₄ receptors mediate excitation by similar means to their peripheral counterparts, increasing tachykinin release in response to a noxious inflammatory stimulus, and consequently facilitating the response of nociceptive neurones in the dorsal horn.

In summary, I have provided electrophysiological evidence for 5-HT₄ receptor mediated serotonergic modulation of noxious transmission in the spinal cord. It appears 5-HT₄ receptors may be involved in the maintenance of A δ -fibre evoked activity in the spinal cord, and that activation of 5-HT₄ receptors during both formalin and carrageenan induced inflammation may enhance the response of spinal nociceptive neurones.

8. GENERAL DISCUSSION

My study set out to investigate the intrinsic and descending inhibitory systems involved with modulation of formalin induced inflammatory nociception at the level of the spinal cord. I have provided electrophysiological evidence for GABAergic and noradrenergic inhibitory control of noxious transmission in the spinal cord by predominantly α_2 -adrenoceptor, GABA_A receptor, but also GABA_B receptor mediated systems, in the formalin model of inflammatory nociception (figure 33). My results support the idea that the GABA_Aergic inhibitory system in the spinal cord is enhanced during formalin induced inflammation and acts to limit both the magnitude and duration of the neuronal responses to the peripheral inflammatory stimulus. The α_2 -adrenoceptor mediated noradrenergic inhibitory system appears to be dormant under normal conditions. However, during formalin induced inflammation this system is activated, contributing to control of both the magnitude and duration of formalin induced neuronal responses. These inhibitory events may be a physiological response to counter increased excitatory transmission during formalin induced inflammatory nociception.

My investigation of spinal serotonergic transmission also implicates 5-HT₃ receptor mediated control of formalin induced noxious transmission in the spinal cord (figure 33). My results suggest that, like the noradrenergic system, the 5-HT₃ receptor mediated serotonergic system is dormant under normal conditions. However, during formalin induced inflammation the 5-HT₃ receptor is activated, facilitating the flow of noxious information during both the first and second phase of the response. Finally, my results suggest spinal 5-HT_{1A} receptors and 5-HT₄ receptors are not significantly involved in descending serotonergic modulation of spinal convergent neurones during formalin induced inflammation. However, my data suggests 5-HT_{1A} receptors are involved in 8-OH-DPAT mediated spinal inhibition, which selectively suppresses A δ -fibre evoked activity. Furthermore, 5-HT₄ receptors may be involved in the maintenance of A δ -fibre evoked activity in the spinal cord, and activation of 5-HT₄ receptors during both formalin and carrageenan induced inflammation may enhance the response of spinal nociceptive neurones.

Due to the design of my experiments it was not possible to stimulate in the brainstem in order to attempt antidromic activation of the spinal neurones I recorded. Consequently, although every neurone in the present study was confirmed as wide

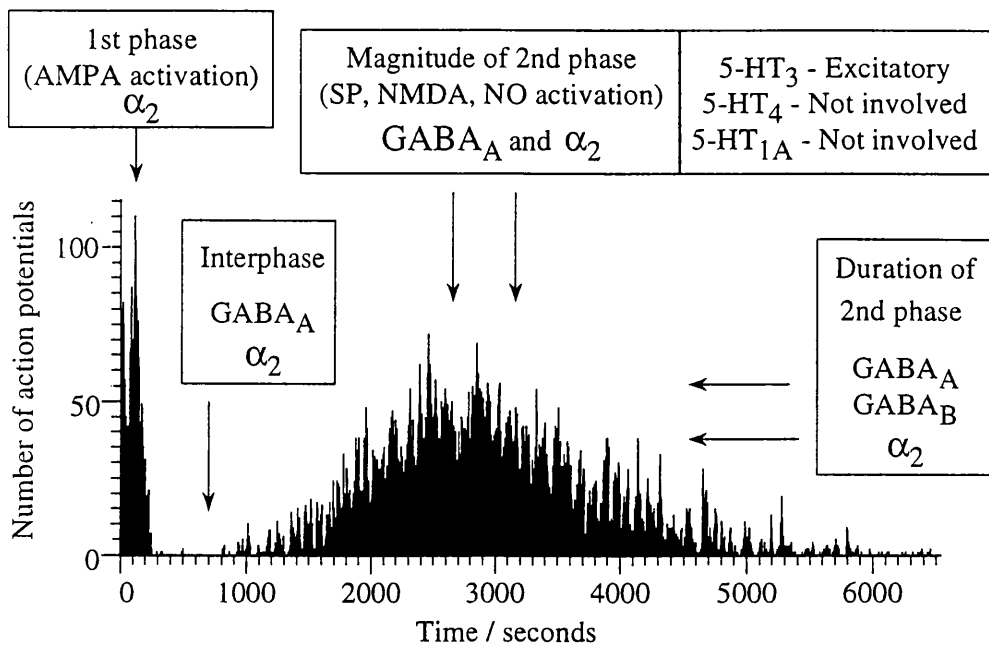


figure 33. Summary of GABAergic and monoaminergic inhibitory modulation of noxious activity in spinal dorsal horn convergent neurones induced by a peripheral subcutaneous injection of 5% formalin solution into the receptive field. Recording presented as number of action potentials per unit time in 1 second time bins.

dynamic range (class 2), whether individual neurones in the study were local or projecting to higher centres is not known. However, of the total population of 396 neurones recorded in the present study, which may include both local and projection cells, there was no evidence to suggest pharmacologically distinct subpopulations of spinal wide dynamic range neurones exist, as far as their monoamine and GABAergic pharmacology is concerned. Therefore we could presume the pharmacological effects I describe are the same for intrinsic and projection wide dynamic range neurones.

Several other interesting points are raised by the present study. Two studies have addressed the question of which types of afferent fibre carry formalin evoked activity to the dorsal horn. Heapy *et al.* (1987) report second phase activity occurs only in C-fibre afferents, whereas Puig & Sorkin (1995) report second phase activity in A δ -fibres as well. In the present study 8-OH-DPAT inhibited A δ -fibre evoked activity but did not significantly affect the C-fibre evoked response, and also produced clear inhibition of the second phase of the formalin response. Thus, my

results support the findings of Puig and Sorkin in that both A δ and C-fibres contribute to the second phase of the formalin response.

There is evidence to suggest a relationship between descending serotonergic transmission, GABAergic transmission (which may be local or descending), and modulation of A δ -fibre mediated afferent input. Electrical stimulation of the PAG has been reported to inhibit A δ -fibre and C-fibre evoked responses (Zhang *et al.*, 1991). However, recent work by Waters and Lumb has used DL-homocysteic acid, which chemically activates cell bodies and dendrites, to examine the effects of PAG activation on the responses of spinal neurones evoked by noxious mechanical stimulation (Waters & Lumb, 1996a,b, 1997). They report neuronal activation in the PAG facilitated A δ -fibre evoked responses of spinal neurones but inhibited C-fibre evoked responses (Waters & Lumb, 1996a). In a subsequent study they found the effects of chemical and electrical activation of the PAG to have differing effects on spinal neurones. Again, chemical activation of the PAG clearly facilitated A δ -fibre evoked responses, whereas electrical stimulation at identical sites inhibited A δ -fibre and C-fibre evoked responses (Waters & Lumb, 1996b). The authors suggest activation of axons of passage may have been responsible for a considerable component of the spinal inhibitory events reported following electrical stimulation of the PAG.

Thus it appears activation of a descending pathway from the PAG selectively enhances A δ -fibre mediated noxious input. The midbrain PAG constitutes a major afferent input to the RVM (Beitz, 1982a; Mantyh & Peschanski, 1982; Marchand & Hagino, 1983). There is substantial evidence indicating the RVM serves as a relay for midbrain modulation of spinal nociceptive transmission, and spinal events following PAG stimulation can be prevented by lesion in (or a local anaesthetic injection into) the RVM (see section 1.3). It also appears a feedback loop exists between these higher centres and the dorsal horn of the spinal cord. The spinoreticular tract provides a direct link between the dorsal horn and brainstem reticular structures in the RVM, whereas the spinomesencephalic tract projects from lamina I to the PAG (see refs in Sorkin & Carlton, 1997).

At the level of the spinal cord, the terminals of descending pathways from the RVM utilise GABA and 5-HT as transmitters (see section 1.3). Anatomical studies have shown GABAergic terminals contact more A δ -fibre terminals than C-fibre

terminals (Bernardi *et al.*, 1995). There is also evidence to suggest glutamate released from the central terminals of A δ -fibres activates GABAergic interneurons, which inhibit transmitter release from other A δ -fibres, or inhibit the original A δ -fibre itself (Shimizu *et al.*, 1995). Furthermore, a recent paper measuring field potentials reports long term depression (LTD) of C-fibre evoked responses following high frequency stimulation of the sciatic nerve at A δ -fibre strength. In spinalized rats however, the same protocol induced LTP rather than LTD, suggesting the original inhibitory effect is mediated by descending pathways. The authors conclude tonic descending inhibition may determine the direction of plastic changes in C-fibre mediated synaptic transmission (Liu *et al.*, 1998).

Pharmacologically, agents that alter GABAergic transmission, both agonists and antagonists, are reported to have a greater effect on A δ -fibre evoked activity than C-fibre evoked activity (Clavier *et al.*, 1992; Reeve & Dickenson, 1996). Finally, in the present study a 5-HT_{1A} receptor agonist and a 5-HT₄ receptor antagonist also selectively modulated A δ -fibre evoked responses. Thus, there may be descending pathways from the RVM that selectively modulate A δ -fibre mediated afferent input, using serotonin and GABA as neurotransmitters at the spinal level.

With regard to the role of spinal 5-HT_{1A} receptors in nociception, there is now convincing evidence the 5-HT_{1A} receptor is involved with both pro- and antinociceptive effects depending on the nature and intensity of the stimulus (see discussion chapter 6). The 5-HT_{1A} receptor agonist 8-OH-DPAT has also been shown to produce different effects when administered by different routes (Fasmer *et al.*, 1986; Millan *et al.*, 1996). Furthermore, it is possible that individual populations of 5-HT_{1A} receptors differentially modulate nociceptive activity (see discussion chapter 6). However, similar situations have been described. For example Neuropeptide FF is pronociceptive when administered supraspinally (Tang *et al.*, 1984; Kavaliers, 1990), but antinociceptive when administered intrathecally (Gouarderes *et al.*, 1993; Kontinen & Kalso, 1995). Similarly, coadministration of a benzodiazepine potentiates opiate analgesia in the spinal cord (Moreau & Pieri, 1988; Yanez *et al.*, 1990; Luger *et al.*, 1994, 1995) but reduces opiate inhibitions when the two drugs are given supraspinally (Mantegazza *et al.*, 1982; Luger *et al.*, 1994).

Regarding potential therapeutic manipulation of the descending noradrenergic system, a recent study of α_{2A} -adrenoceptor knockout transgenic mice reports that the α_{2A} -adrenoceptor is the predominant subtype involved in mediation of dexmedetomidine induced antinociception, but also of the sedative and hypothermic actions of dexmedetomidine. Consequently, the authors suggest an α_{2A} -selective analgesic will have a narrow therapeutic window (Hunter *et al.*, 1997). Another study with α_{2A} -knockout transgenic mice agrees that the α_{2A} -adrenoceptor is the primary mediator of spinal adrenergic analgesia, and further demonstrate it is the subtype necessary for synergistic effects with opiates (Stone *et al.*, 1997). Clinical exploitation of spinal α_2 -adrenoceptors may therefore be limited to coadministration with opiate analgesics although there is the hope that selective $\alpha_{2B/C}$ -adrenoceptor agonists may lack the sedative and cardiovascular effects.

Complexity in the underlying mechanisms governing interactions between monoaminergic systems is evident. For example, LaPorte *et al.* (1995) report a 42% increase in 8-OH-DPAT binding to 5-HT_{1A} receptors in the lumbar dorsal horn following the destruction of descending serotonergic pathways with 5,7-dihydroxytryptamine, whereas the levels of iodozacopride binding to 5-HT₃ receptors remained unchanged. The same study also demonstrates a 26% increase in 8-OH-DPAT binding in the lumbar spinal cord after descending noradrenergic systems had been lesioned by DSP-4, whereas iodozacopride binding to 5-HT₃ receptors again remained unchanged. This is an interesting result when considering that 5-HT_{1A} receptors are not found on descending noradrenergic pathways (Bervoets *et al.*, 1993; LaPorte *et al.*, 1995). In another example of links between noradrenaline and 5-HT neuronal systems, an electrophysiological study reports a potentially useful interaction between monoaminergic agents. Activation of 5-HT_{1A} receptors has been shown to have no effect on baseline recordings but clearly potentiates the inhibitory effects of clonidine in the locus coeruleus, lowering the ED₅₀ of clonidine by 77% (Ruiz-Ortega & Ugedo, 1997).

Therapeutic manipulation of monoaminergic and GABAergic transmission for the treatment of pain is still primitive. The complexity of these systems is such that there is a lack of specific drugs targeted at putative analgesic sites. The only exception to this would be the 5-HT_{1D} selective agonists developed for the treatment of migraine acting at receptors in the cranial vasculature (see Perry & Markham,

1998). Otherwise, nonspecific agents have been more effective in treating pain than more selectively acting drugs. However, the tricyclic antidepressant amitriptyline, commonly used to treat neuropathic pain, is still only effective in 20-30 % of neuropathic pains (see McQuay *et al.*, 1996). This could be interpreted to mean a balanced manipulation of noradrenergic and serotonergic transmission involving more than one drug will be required for successful analgesia. Combination therapy seems a more probable advance in this area of pharmacology than the discovery of novel highly selective analgesics. However, it may be that the global increase in 5-HT produced by amitriptyline activates both excitatory and inhibitory systems, in which case more selective receptor directed drugs may be more effective clinically. Clearly a far greater understanding of the relationships between inhibitory transmitter systems and their possible therapeutic manipulation is needed.

Drugs that modulate neuronal activity via GABAergic, noradrenergic or serotonergic receptors are used in the treatment of several disorders including epilepsy, anxiety, depression and insomnia. It is interesting to speculate on the relationship between these conditions and pain. Epilepsy is similar to many chronic pain syndromes in that both involve excessive central excitatory transmission. Anxiety, depression and insomnia are all themselves symptoms of many chronic pain sufferers. It has been known for some time that GABA, but more so noradrenaline and 5-HT, are intimately involved with emotional and perceptual states. Consequently, an advanced knowledge and understanding of these systems may eventually provide links between conditions such as epilepsy, anxiety, depression and pain with common therapeutic principles, and furthermore provide an insight into the relationship between such disorders and human emotional processes.

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