DESCENDING CONTROL IN SENSITIZATION OF REFLEXES IN THE RAT

KATHARINE L. DOBSON, BSc

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ABSTRACT

Electrical stimulation of the heel or toes evokes short latency polysynaptic reflexes in muscles of the ankle extensor medial gastrocnemius (MG), the ankle flexor tibialis anterior (TA) and the knee flexor biceps femoris (BF), the co-ordinated actions of which form an organized protective withdrawal response. Previous studies in the rabbit have shown that such reflexes are enhanced (sensitized) or inhibited by application of the chemogenic agent mustard oil (MO) to various areas of the body surface in a manner that reinforces the protective function of these responses. The organization of these 'sensitization fields' was strictly controlled by supraspinal pathways from the brain. The aim of the present experiments was therefore to extend these studies of the spatial organization of sensitization of withdrawal reflexes into the rat, the species most commonly used in pain research.

Patterns of facilitation and inhibition of spinal reflexes were obtained and compared in decerebrate spinalized, decerebrate non-spinal, and Alfaxan- anaesthetized rats by applying mustard oil to sixteen different body locations including sites on the ipsilateral and contralateral hindlimbs as well as other off limb areas such as the snout and tail. It was found that in decerebrate spinalized animals, MO application to ipsilateral hindlimb sites enhanced but never inhibited reflex responses in the limb, whilst MO treatment to off limb sites was without effect. In contrast in anaesthetized animals the prevalent effect of MO was inhibition from treatment sites distributed across the entire animal. Reflexes in animals with an intact spinal cord (decerebrate or anaesthetized) were facilitated or inhibited by MO application to ipsilateral hindlimb sites in a way that resembled the modular organization of reflexes *per se* and previous sensitization studies in the rabbit. However clear differences were also observed in the effects of MO between the two species, including modulation of the heel-MG extensor response in spinalized animals, which in rabbit was inhibited by MO application to the ipsilateral toes whereas in the rat no inhibition by MO was found in spinalized animals. Sensitization of hindlimb reflexes by MO in the rat therefore seems to be influenced by descending inhibitory and facilitatory pathways. These influences were further investigated in subsequent studies.

Whilst the predominant effect of spinalization was a loss of inhibition and an expansion of sensitization fields, in the toes-evoked TA reflex the reverse was noted with regard to MO

treatment of distal ipsilateral sites. In this case, facilitation found in non-spinal animals did not occur in the equivalent spinalized cohort, and thereby implies that a descending facilitatory pathway is also implicated in the control of spinal reflex excitability in this model.

In decerebrate rats, the noradrenergic α_2-adrenoceptor antagonist RX 821002 or the serotonergic 5-HT_3 receptor antagonist ondansetron were administered directly to the spinal cord (intrathecally, i.t.) either alone (dose-response studies) or as a single dose between two successive MO applications to one of three ipsilateral skin sites on the hindlimb (heel, metatarsophalangeal joints or flexion of the ankle). Cumulative i.t. doses of RX 821002 revealed the presence of tonic descending inhibition of all reflex responses as well as preventing MO-evoked inhibition (and possibly facilitation) of reflex responses suggesting the involvement of the α_2-adrenoceptor subtype in mediating these effects in this model. On the other hand, cumulative i.t. ondansetron administration resulted in a decrease in the magnitude of reflex responses, thus indicating that 5-HT_3 receptors are indeed implicated in tonic descending facilitation of spinal reflexes. In addition i.t. ondansetron revealed that potentiation (and possibly inhibition) of reflexes following an acute chemogenic insult appears to involve the actions of serotonin at 5-HT_3 receptors in the spinal cord.

These studies therefore show that the organization of sensitization of hindlimb reflexes in the rat are modulated by supraspinal influences that exist as a balance of descending facilitatory and inhibitory pathways, mediated at least in part by serotonergic 5-HT_3 receptors and noradrenergic α_2-adrenoceptors.

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Papers

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1. LITERATURE REVIEW

Introduction

Noxious or potentially noxious stimulation of the hindlimb evokes short latency polysynaptic reflexes in the limb to withdraw it from the stimulus. Therefore rather than being a stereotypical response, limb withdrawal is comprised of a number of reflex contractions/relaxations within individual muscles of the limb, the actual movement being generated by activation of the most appropriate muscles to move the limb away from the stimulation site. Thus stimulation of the heel evokes responses in the ankle extensor muscle medial gastrocnemius (MG) which would lift the heel shifting weight to the toes, and toes stimulation evokes responses in the ankle flexor muscle tibialis anterior (TA) which raises the toes shifting weight to the heel. The knee flexor/hip extensor muscle biceps femoris (BF) is activated by stimulation at both the heel and toes reflecting the fact that its action is to lift the foot completely from contact with the ground. These nocifensive reflex responses therefore serve a protective function, as contraction of each muscle would cause the stimulated area to be lifted away from the source of stimulation. By studying reflexes, an investigator can therefore theoretically study a population of neurones with a known function rather than by sampling from a heterogeneous population of cells. Since these reflex pathways are organized entirely within the spinal cord, they can be used to assess the effects of physiological and pharmacological manipulations on spinal cord activity, and hence lead to a better understanding of how spinally mediated events are controlled. The present studies have therefore investigated two types of input which modify reflex function: i) noxious chemogenic stimulation of different cutaneous and deep tissue sites and ii) the influence of descending pathways from the brain.

Previous studies in this laboratory performed in decerebrate spinalized rabbits have shown that application of the noxious chemical mustard oil (MO) anywhere on the ipsilateral hindlimb is able to generate a prolonged enhancement of reflexes evoked in the knee flexor semitendinosus and the ankle flexor TA. In non-spinal animals however, only MO applied to the plantar surface of the foot caused sensitization of flexor reflexes, indicating a descending inhibitory control of reflex sensitization fields that restricts sensitization to sites which make ground contact (Harris and Clarke, 2003). In contrast, the pattern of MO-

induced facilitation of responses in the heel-MG extensor reflex was spatially not different in the spinalized preparation relative to the spinally-intact equivalent.

Intrathecal or systemic administration of the α_2-adrenoceptor antagonists yohimbine and idazoxan in decerebrate non-spinal rabbits facilitated reflex responses in the ankle extensor MG evoked by electrical stimulation of the sural nerve (Clarke et al., 1988, Harris and Clarke, 1992), suggesting that a tonically active noradrenergic pathway influences the activity of the spinal reflex arc. Reflex responses to noxious mechanical stimulation in this preparation were also enhanced by spinal administration of idazoxan (Clarke et al., 2001). Furthermore, receptors belonging to the serotonergic superfamily have been implicated as modulators of spinal reflex excitability. Antagonists for the 5-HT_{1A} and 5-HT_2 receptor subtypes applied intrathecally in decerebrated non-spinal rabbits potentiated (and reduced) the sural-MG response (Clarke et al., 1996) thus demonstrating the potential involvement of serotonergic transmission in modulating the excitability of reflexes in this model.

The aims of the present studies were therefore: i) to investigate whether a similar differential pattern of reflex sensitization by MO could be found in rats, a species more widely used in pain research; and ii) to examine the nature of the descending pathways which influence the reflex responses in the rat *per se* and the sensitization thereof.

This review is therefore divided into two main sections. The first part describes the organization of the spinal cord, concentrating on the various components that make up a spinal reflex and background to the concept of sensitization. Section II of the literature review gives a detailed account of the anatomy of noradrenergic and serotonergic descending pathways, as well as the pharmacology of their receptors and their location in the spinal cord.

I. SOMATOMOTOR INTEGRATION

I.1 Peripheral Sensory Transmission

Cutaneous receptors, which are the sensors for initiating reflex responses, are specialised to detect a variety of somatosensory modalities, including mechanical stimuli such as vibration and pressure, warm and cold thermal stimuli, as well as range of noxious inputs(McGlone and Reilly, 2010). Sensory receptors capable of transducing and encoding noxious stimuli are referred to as nociceptors (Loeser and Treede, 2008), with the definition provided by the International Association for the Study of Pain (IASP) in this case referring only to truly specialised receptors i.e. those capable of responding to noxious and non-noxious stimuli are not termed nociceptors.

I.1.1. Primary Afferent Fibre Specialisation

The sensory neurones innervating cutaneous receptors can be divided according to their action potential conduction velocity (CV), myelination, and fibre size into three main categories, given that CV is directly proportional to diameter and myelination state (Hursh, 1939, Gasser, 1941). The values quoted below for diameter and CV are specific to rat, though much of the preliminary research into the relationship between the structural and functional properties of neurones was conducted in cats (for a review of interspecies variations of these properties see Djouhri and Lawson, 2004).

Aαβ primary afferent neurones are large myelinated fibres (5 to 14 μm diameter, Sanders and Zimmerman, 1986) with CVs in the range of 12 to 55 m/s (Harper and Lawson, 1985b, Lawson and Waddell, 1991)and are frequently classed as mechanoreceptors that have a primary role in detecting tactile stimuli, though there has been some evidence provided for a role in nociception in several species including cat, rat, and guinea pig (Lawson, 2002).

Aδ fibres are smaller myelinated neurones (a diameter of 2-5 μm, Erdine et al. , 2009) and, as might be predicted from the relationship between fibre diameter and conduction, have a lower CV ranging approximately from2 to 14 m/s (Ritter and Mendell, 1992), and transduce cold thermal stimuli (Simone and Kajander, 1997)and early-phase nociceptive input (a sharp, well localised pain). In human volunteers undergoing electrical cutaneous

stimulation, amplitudes greater than the threshold for Aδ fibres were perceived as a sharp or pricking sensation (McAllister et al., 1995), characteristic of early-phase pain. There is some discrepancy between the reported CVs for Aβ and Aδ fibre types, with mid-range conduction velocities either arbitrarily assigned as one type or the other, or differentiated on the basis of a range of properties such as stimulus modality responded to, compound action potential waveform, or a statistical analysis of CV clustering (Burgess and Perl, 1967, Villiere and McLachlan, 1996, Fang et al., 2002). As CVs are dependent on factors such as age of the animal, temperature at which the recording was performed, and distance from the cell body (Birren and Wall, 1956, Hopkins and Lambert, 1973, Waxman, 1980, Harper and Lawson, 1985a, Sato et al., 1985), it is not appropriate to apply a generalised figure to the two primary afferent subtypes. The ranges quoted above are therefore supplied for comparison only.

The fibres generally classified as nociceptors are known as C-type fibres, which are non-myelinated and therefore have a CV in the rat of less than 2 m/s (Hopkins and Lambert, 1973, Fitzgerald and Woolf, 1981, Grudt and Perl, 2002). These fibres are considered the primary nociceptors of the peripheral nervous system, with histological analyses showing that they constitute between 75 and 80%of the axons in the rat saphenous nerve and the human sural nerve (Ochoa and Mair, 1969, Scadding, 1980, Carter and Lisney, 1987), and are responsible for conveyance of the second phase of pain (a dull aching sensation) as well as warm/hot thermal stimuli (Ochoa and Torebjörk, 1989, Yarnitsky et al., 1992).
C-fibres may be described as either: i) polymodal i.e. responding to several different modalities of mechanical, thermal, and chemical stimuli; ii) selective and respond to only one of the previously mentioned stimuli; or iii) silent, in that they are not activated under normal circumstances but respond readily following tissue damage or sensitization (Handwerker et al., 1991, Schmidt et al., 1995).

I.1.2. Cutaneous Receptor Specializations

The peripheral cutaneous termini of primary afferents may be sub-divided on the basis of the nature of the stimulus to which they are specifically adapted to respond to, including differing forms of mechanical input, thermal stimuli, and also nociceptive inputs.

The physiologically simplest afferent fibre ending is the free ending which has no specialised somatosensory receptor unit. This is typical of nociceptive neurones, and allows the fibre direct access to extracellular secretory molecules as well as chemicals that may be absorbed into the skin. This serves to preserve the functionality of these afferents as the unprotected neuronal ending is unrestricted in terms of contact with potentially excitatory agents and also enables rapid detection of thermal variations. More complex morphology is found at mechanoreceptive endings which are classed as either slowly- or rapidly-adapting, and superficial or deep. Merkel's disks are slowly adapting endothelial cells residing at peripheral nerve terminals that respond primarily to sensations of pressure and vibration (Iggo and Muir, 1969), and are located relatively superficially in the basal layer of both glabrous and hairy skin. Also relatively superficial, Meissner's corpuscles are rapidly adapting mechanoreceptors innervated by both myelinated and non-myelinated afferents, that in addition to detecting low-threshold mechanical stimuli such as light pressure, may also have a nociceptive functionality (Cauna, 1956, Paré et al., 2001). Deeper located but also pressure-sensitive are the Pacinian corpuscles, which in addition to pressure respond to high frequency vibrational stimuli (William et al., 1968). These endings are multi-lamellar structures composed of a mass of connective tissue that encapsulates the free end of Aβ-fibres in the dermal layer (Bell et al., 1994) and are rapidly adapting i.e. respond quickly at the initial detection of a stimulus and then cease firing with ongoing unchanged input. The fourth type of mechanoreceptor is a deeply situated slowly adapting corpuscle referred to as a Ruffini ending which functions as a stretch receptor during grasping tasks (Ruffini, 1898).

I.2 Central Nervous System Organization of Sensory Inputs

The perikarya of primary afferent fibres reside in the dorsal root ganglia (DRG) as pseudounipolar neurones which project their central terminals into the dorsal horn of the spinal cord. The central axons of sensory neurones bifurcate prior to entering the dorsal horn proper and project both rostrally and caudally into the dorsolateral tract of Lissauer (Earle, 1952, Traub et al., 1986). Collateral fibres divide further from these branches which then enter the dorsal horn into the laminae described below. The structure of the spinal cord is divided by cytological architecture into ten distinct laminae, as originally described in the cat by Rexed in the 1950s (Rexed, 1952) (figure 1.1). A similar

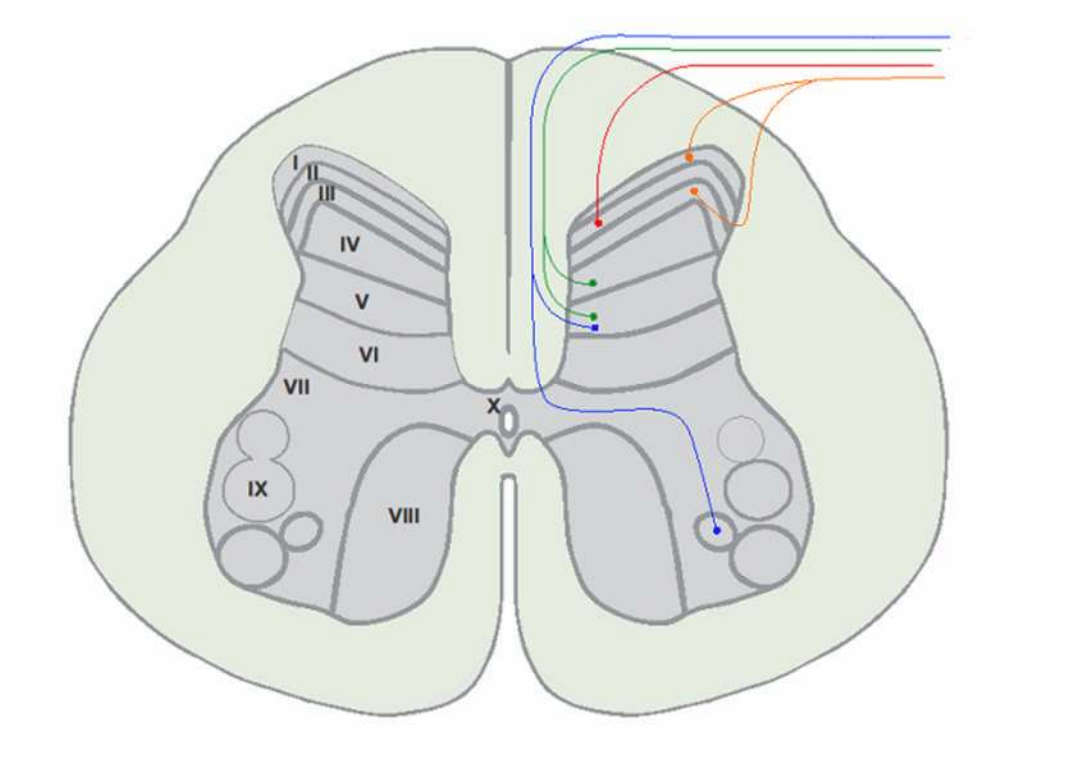


Figure 1.1: Schematic drawing of the Rexed laminar divisions of the spinal cord. The dorsal horn may be divided into superficial (laminae I-III) and deep regions (IV-VI). The ventral horn comprises laminae VII-IX with lamina X bordering the central canal.
Also shown are the laminar terminations of primary afferent fibres (Aα = blue, Aβ = green, Aδ = orange, C = red).
Figure adapted from Grottel et al. (1999).

arrangement has been demonstrated in the rat lower thoracic and lumbosacral spinal column (Molander et al., 1984, Molander et al., 1989) with subtle interspecies variation in the exact lamina position and size. In broad terms, the dorsal horn is responsible for the initial processing of somatosensory inputs, and the ventral horn co-ordinates the motor response to those inputs.

I.2.1. Primary Afferent Inputs to the Dorsal Horn

The determination of primary afferent input to the various laminae of the dorsal horn may be performed by either anterograde or retrograde labelling, or by functional classification based on the stimulus modality or recruitment threshold for a particular fibre. One commonly used substance in axonal transport labelling studies has been the enzyme horseradish peroxidase (HRP) (Light and Perl, 1977), which has typically been conjugated to a macromolecule such as wheatgerm agglutinin or the B-subunit of cholera toxin (CTb) to enhance its uptake (Horikawa and Powell, 1986). Modifications of these methods have also allowed fibre types to be selectively labelled, as shown by the use of unconjugated CTb as a selective tracer for myelinated fibres (Todd et al., 2003) or of *Phaseolus vulgaris* leukoagglutinin to label only C-fibres (Sugiura et al., 1986). By using these techniques or variations thereon, the central terminations of primary afferent fibres of all classes have been established.

Rexed's lamina I (also referred to as the marginal zone) forms the dorsal-most boundary of the spinal grey matter. Small afferent fibres arising from nerves in the hindlimb of the rat terminate in lamina I (Swett and Woolf, 1985)including both Aδ and C fibres with arborizations to deeper dorsal horn laminae (Light and Perl, 1979, Sugiura et al., 1986, Todd et al., 2003). The majority of secondary neurones (i.e. potentially the first interneurones in reflex pathways) residing in this lamina are either nociceptive-specific or wide dynamic range i.e. responding preferentially to either noxious or both noxious and innocuous stimuli (Réthelyi et al., 1982, McMahon and Wall, 1983, Woolf and Fitzgerald, 1983, Cervero et al., 1988), though some studies have found a minority of cells sampled from this region that respond to innocuous cooling or light mechanical cutaneous stimulation alone (McMahon and Wall, 1983, Light and Willcockson, 1999). This range in functionality correlates with neuronal morphology, with fusiform cells characterised as

nociceptive-specific, pyramidal cells selective for innocuous cooling, and multipolar cells were polymodal or nociceptive-specific (Han et al., 1998).

Lamina II of Rexed's cytoarchitectural classification is synonymous with the substantia gelatinosa (SG) of Rolando (Cervero and Iggo, 1980), and may also be referred to as SGo and SGi (SG outer and SG inner respectively). Lamina II is the primary site for nociceptive inputs to the spinal cord, with large proportions of C-fibres terminating here (Woolf and Fitzgerald, 1983, Schouenborg, 1984, Swett and Woolf, 1985, Sugiura et al., 1986, Woodbury et al., 2000). Extracellular recordings from neurones in the superficial laminae of the dorsal horn indicate that lamina II cells are mostly of the wide-dynamic range subtype with a large sub-population falling into the nociceptive-specific class (Woolf and Fitzgerald, 1983, Cervero et al., 1988, Light and Willcockson, 1999), and chrome-silver Golgi staining revealed that cells from this lamina have long arborizations in the rostrocaudal plane (upwards of 200 μm in cat) but are sharply restricted mediolaterally (Scheibel and Scheibel, 1968, Rethelyi, 1977). Recent investigations have defined the inputs to this lamina still further, with the inner region of lamina II characterised as a site of myelinated fibre and non-peptidergic terminals, and the outer zone targeted by non-myelinated and peptidergic neurones (Woodbury et al., 2000, Neumann et al., 2008).
The functional implications of these properties are that noxious cutaneous stimulation may have a relatively restricted site of CNS integration in terms of the location of the first synapse but that projections from interneurones extending across spinal cord segments allow long-ranging transduction of that initial stimulus.

Laminae III-VI form the deep dorsal horn, of which laminae IV and V are collectively referred to as the nucleus proprius. Lamina III forms the deeper region immediately ventral to the substantia gelatinosa, and contains significantly less nociceptive primary afferent input than either of the other more superficial laminae, though some tracing studies have found primary afferent terminals in lamina III (Swett and Woolf, 1985, Molander and Grant, 1986). In contrast to laminae I and II, lamina III provides one of the major locations for Aβ fibre terminations i.e. innocuous mechanoceptors (Light and Perl, 1977, Light and Willcockson, 1999) with further Aβ inputs into laminae IV and V (Shortland and Woolf, 1993) and high-threshold mechanoceptors (Aδ fibres) arborizing to lamina V (Light and Perl, 1979). The deeper lamina are the primary projection target of myelinated fibres, with laminae III and IV showing the densest localisation when myelinated fibre types are

selectively stained (Lamotte et al., 1991, Maslany et al., 1992). However, only the simple endings of Aβ fibres are located in lamina III – complex arborizations arising from this fibre type extend both dorsally into the inner region of lamina II and ventrally into lamina IV (Shortland et al., 1989, Shortland and Woolf, 1993, Drew and MacDermott, 2009) providing greater dissemination of the initial signal.

Intermediate and ventral regions of the spinal cord (laminae VII-IX) are generally less important as a site of primary afferent input (particularly with respect to cutaneous populations) than the more dorsal laminae. Lamina VII (also known as intermediate grey) is a target for both myelinated and unmyelinated primary afferents but is generally restricted to inputs that are visceral in origin rather than cutaneous (Rivero-Melian and Grant, 1991, Wang et al., 1998). Muscle afferent input primarily terminates in the deeper dorsal horn (in particular lamina V) as well as a lesser input to laminae VI and VII of the intermediate region (Molander and Grant, 1987). One further lamina receiving peripheral information is lamina X (substantia grisea centralis), or the grey matter bordering the central canal. As with the input described to lamina VII above, fibres terminating here are mainly, though not exclusively, of visceral origin (Sugiura et al., 1989, Wall et al., 2002).

In the case of Aδ fibres terminating in lamina V, neurones that they synapse with cross the midline of the spinal cord to the lateral funiculus from which they ascend as the spinothalamic tract, or for those terminating in more superficial dorsal horn laminae may synapse with neurones forming part of the spinoparabrachial tract (Bester et al., 1995). Also, spinothalamic cells in lamina I are indirectly influenced from C-fibre terminals in the substantia gelatinosa by excitatory interneurones which link the peripheral and central components of this pathway (Lu and Perl, 2005).

I.2.2. Somatotopic Organization of the Primary Afferent Input to the Spinal Cord

The patterning of inputs to the dorsal horn provides one of the layers of organization present in withdrawal reflex pathways, as in addition to the laminar terminations dependent upon fibre type and stimulus modality, each cutaneous nerve branch has a defined three-dimensional (dorso-ventral, medio-lateral, and rostro-caudal) termination zone in the dorsal horn.

Primary afferent fibres innervating the rat hindlimb terminate in a strict topographic pattern in the superficial dorsal horn so that neighbouring skin regions are innervated by sensory receptors whose central terminals occupy slightly different yet contiguous regions (Swett and Woolf, 1985). Further transganglionic transport studies in the rat reveal that those hindlimb nerves with plantar cutaneous receptive fields (e.g. the tibial nerve) terminate in the medial dorsal horn, whereas those innervating more proximal or dorsal sites, such as the common peroneal nerve which has a receptive field focused on and around the dorsum of the foot, terminate in the lateral third of the superficial dorsal horn (Molander and Grant, 1986, Woolf and Fitzgerald, 1986, Grant, 1993). The somatotopic organization is most discernible in lamina II (as described above) although is retained through the slightly deeper laminae (laminae III/IV) and has been suggested to be absent from lamina I (Molander and Grant, 1986, Levinsson et al., 2002), though conflicting data provides a strong case to the contrary (Willis et al., 1974, Cervero et al., 1976). In addition to the mediolateral organization observed in transverse sections from thoracic, lumbar, and sacral spinal cord regions, HRP-based tracing studies also reveal a longitudinal columnar arrangement that spans several spinal segments per nerve, with the directionality and extent of arborizations dependent to some degree on the source of input. This longitudinal organization is also revealed through dermatome analysis, in which anterograde transport of a dye such as Evans Blue from individual spinal nerves may be used to visualize regions on the skin innervated by that particular neuronal bundle. As might be assumed from basic anatomy, the more caudal the spinal root examined the more caudal the skin area supplied by that nerve (Takahashi et al., 1994).

Somatotopic organization is also evident in the motorneurone (MN) pools of the ventral horn, which will be discussed in greater detail in section I.2.4.

I.2.3. Properties of Spinal Cord Neurones

As already alluded to above, interneurones in the dorsal horn (both in deep and superficial laminae) may be classified electrophysiologically according to the peripheral stimulus modalities capable of activating them: namely, low-threshold mechanosensitive (LTM), nociceptive-specific (NS), wide dynamic range (WDR), and proprioceptive (Kolmodin and Skoglund, 1960, Menétrey et al., 1977). LTM cells respond to innocuous cutaneous stimulation, with these responses evoked experimentally by methods such as hair

displacement, light brush strokes, pressurised air, or similar tactile stimuli and are therefore likely to receive a dominant input from Aβ fibres (Mendell, 1966, Handwerker et al., 1975). WDR cells respond to stimuli from across the intensity continuum, exhibiting high firing rates with stimuli more likely to generate tissue damage and rapidly adapting to alterations in stimulation parameters (e.g. Mendell, 1966, Woolf and Fitzgerald, 1983, Howe and Zieglgänsberger, 1987, Kawasaki et al., 2002, You et al., 2008). NS cells in the dorsal horn respond selectively to noxious stimuli only of both mechanical and thermal modalities.

Marginal zone cells have been reported to fall into either the WDR or NS subcategory in both cat and rat spinal cord (Cervero et al., 1976, Menétrey et al., 1977) of which NS neurones receiving inputs from slowly-conducting myelinated fibres may be further subcategorised as activated by noxious mechanosensation, by both noxious mechanosensation and noxious heating, and by both noxious mechanosensation and noxious heating as well as innocuous heating (Christensen and Perl, 1970). Lamina II also includes a relatively small proportion of LTM neurones in addition to the two classes that dominate lamina I (reported to range from 10 - 15%) (Price et al., 1979, Bennett et al., 1980) but is primarily populated by neurones responding to stimuli in the noxious range and therefore has a highly significant role in the integration of nociceptive inputs. Investigations into the response properties of interneurones situated in the deeper dorsal laminae have tended to focus on lamina V, given what is known about the nature of primary afferent fibres that terminate here. However, laminae III and IV in the rat and cat do contain populations of units excited by all three categories of cutaneous stimulation (Mendell, 1966, Howe and Zieglgänsberger, 1987) and receive further input from lamina II (Todd, 1989) thus enhancing the contribution of these deeper laminae of the dorsal horn in somatosensory processing. The properties of lamina V neurones responding to peripheral stimulation, either applied to cutaneous receptive fields or of deeper origin, have been extensively studied. Lamina V is a site of convergence for cutaneous and deep tissue afferents (Hoheisel and Mense, 1990) and therefore is likely to be involved in the integration of information from these sites. Cells in this region of the dorsal horn have been shown to respond to a variety of stimuli, including several modalities of thermal stimulation, innocuous and noxious mechanical stimuli (with response characteristics in the LTM, WDR and NS ranges), as well as Aδ inputs from both muscle and viscera and Aβ fibre terminations (Pomeranz et al., 1968, Willis et al., 1974, Light and Perl, 1977, Borzan et al.,

2005, You et al., 2008). Quantitative analysis of neurones situated in the neck of the dorsal horn (i.e. laminae IV-VI) classified less than 20% of cells as either LTM or NS with the vast majority therefore of the WDR type (Fitzgerald, 1982), further consolidating the theory that lamina V plays a key role in the overall sensory processing capability of the dorsal horn.

Lamina X is a further important site of nociceptive processing. Neurones in this locale have a range of response characteristics encompassing the three classes of mechanical cutaneous stimulation, as well as convergent inputs comprising visceral, proprioceptive, and thermal cutaneous stimuli (Nahin et al., 1983, Ness and Gebhart, 1987). As with the neck of the dorsal horn, lamina X is primarily composed of cells capable of transducing noxious inputs, either specifically or in addition to low-threshold responses, with over 90% falling into these two categories (Olivar et al., 2000).

It should be noted that the experimental protocol employed to investigate the differential excitability of these cell types plays a large role in the outcome of such a study i.e. anaesthetized vs. decerebrated, spinalized vs. non-spinalized, isolated skin-nerve-spinal cord preparation vs. whole animal, particularly with respect to the roles of anaesthetic agents and descending contributions to overall spinal cord excitability. For example, Menétrey et al. (1977) recorded extracellular responses in decerebrated spinalized rats, thereby negating any suppressive effects of an anaesthetic agent but also removing the tonic effects of descending inhibitory and facilitatory pathways. Opposed to this, Howe and Zieglgänsberger (1987) performed similar studies in anaesthetized spinally-intact rats and recorded a greater proportion of NS cells in those animals, as well as a small population of neurones inhibited by noxious stimuli, emphasising the impact a variation in surgical preparation can have on the outcome of such experiments and the conclusions drawn from them.

I.2.4. The Effector Component of Withdrawal Reflexes and Ventral Horn Organization

As with the sensory fibres discussed previously, MNs may be divided into categories according to their specialised structure-function relationships. In relation to the innervation of skeletal muscle, and hence relevant to the control and specificity of nocifensive withdrawal reflexes, there are two different sub-categories of MNs:- the larger more rapidly conducting α-MNs responsible for skeletal movement, and the finer more slowly

conducting γ-MNs which function as muscle spindle proprioceptors (Hunt and Kuffler, 1951). Both are myelinated fibres, with mean axon diameters in the adult rat ranging from 3.1 μm for γ fibres and 8.2 μm for α-MNs (Kaar and Fraher, 1985), and little difference from these dimensions is observed in cat when measured in the axon proximal to the ventral funiculus (Fabricius et al., 1994). These two classes of MNs may also be differentiated on the basis of their soma sizes (Kuffler et al., 1951, Hashizume et al., 1988) and the expression of transcription factor Err3 (Friese et al., 2009).

The MN cell bodies are situated within lamina IX in the ventral horn of the spinal cord, and exhibit rostro-caudal, dorso-ventral, and medio-lateral organization patterning. Clusters of MNs innervating a particular muscle are located in relatively discrete cigar-shaped longitudinal columns, termed spinal motor nuclei (Romanes, 1951), with nuclei supplying proximal hindlimb muscles showing a greater rostral extension than those supplying more distal muscles (Nicolopoulos-Stournaras and Iles, 1983). With particular reference to the muscles examined in the rat within the studies described in this thesis, tibialis anterior is innervated by a motor column extending across the third lumbar spinal cord segment (L3), medial gastrocnemius by a column which extends from the caudal-most region of L3 to mid-L5, and biceps femoris by a column that runs the length of L4 (McHanwell and Biscoe, 1981, Nicolopoulos-Stournaras and Iles, 1983, Manzano and McComas, 1988). Greater rostro-caudal discrimination in only observed when other hindlimb muscles are studied concurrently.

The somatotopic organization of the ventral horn is most clearly observed in the dorso-ventral and medio-lateral axes, which in broad terms reveals that nuclei supplying muscles located proximally in the hindlimb such as sartorius or obturator internus tend to the ventral-most regions of the ventral horn, whilst nuclei supplying distal muscles such as flexor hallucis longus or peroneus brevis are located relatively dorsally within the ventral horn (Romanes, 1951, Sharrard, 1955, McHanwell and Biscoe, 1981, Nicolopoulos-Stournaras and Iles, 1983, Portal et al., 1991) (figure 1.2). The mediolateral organization of spinal motor nuclei relates more to the functional role of the muscles innervated as opposed to the physical location of the muscle within the hindlimb, though medial muscles of the thigh are innervated by MNs located more rostrally than those of the lateral thigh (Vanderhorst and Holstege, 1997). MNs supplying joint flexors are generally situated in a

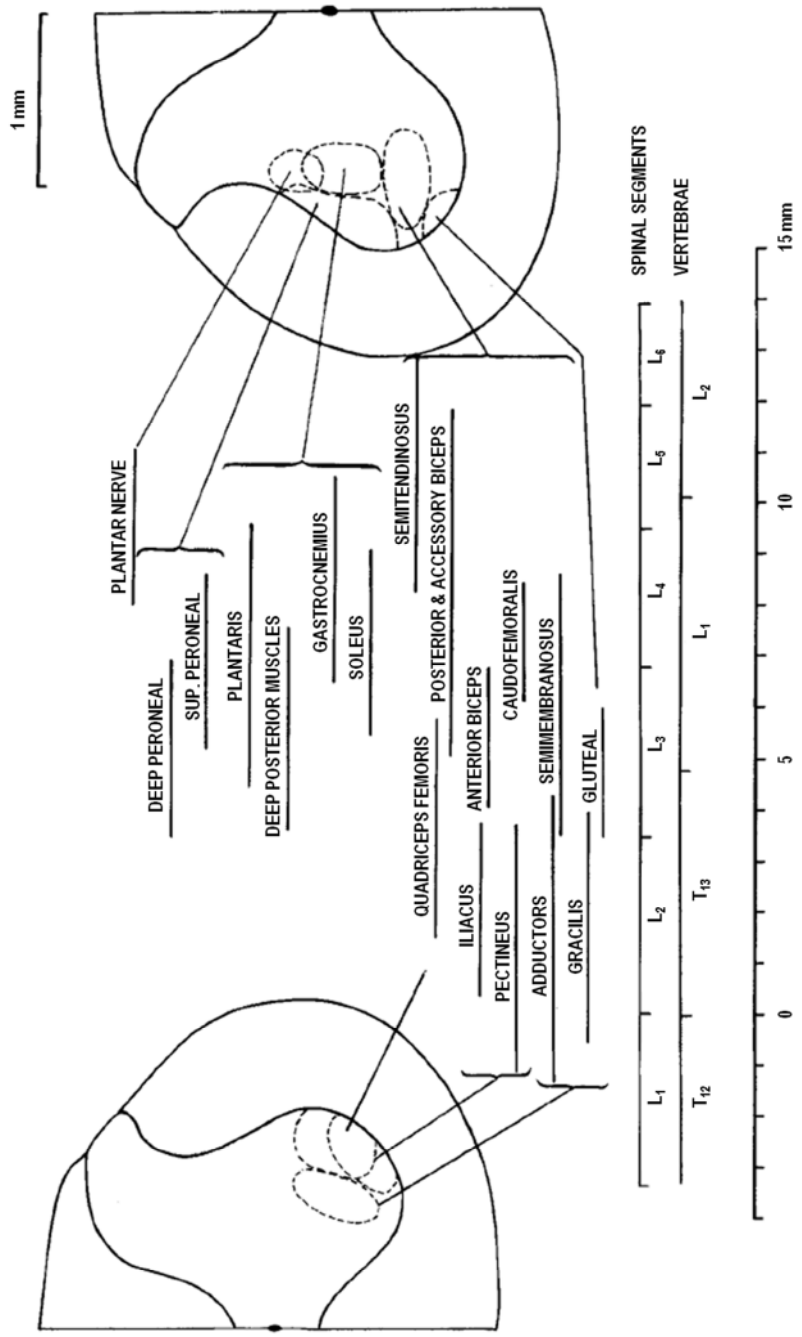


Figure 1.2: Motor neurone pools in the lumbar ventral horn of the rat delineated according to retrograde HRP labelling. Figure adapted from Nicolopoulos-Stournaras and Iles (1983).

medial position relative to those innervating the antagonistic extensor muscles, with these patterns conserved both when considering other regions of the spinal cord, such as cervical as opposed to lumbar, and across a range of species (Sharrard, 1955, Jenny and Inukai, 1983, Curfs et al., 1993, McKenna et al., 2000, Bácskai et al., 2012).

I.3 Modular Organization of Withdrawal Reflexes

I.3.1. Early Work in Animal Models

At the end of the 19th century, from collection of electromyographical data largely obtained in decerebrated cats, Charles Sherrington described an organized motor response that could be evoked by noxious stimulation of the pinna that served to move the stimulated site away from the source of noxious input (Sherrington, 1898). Based on subsequent experimentation in the same model, he proposed that withdrawal of a limb is described by a singular "flexion reflex" that serves a protective function and acts to remove the stimulated limb away from the origin of a noxious stimulus by excitation of muscles that result in flexion at the hip, knee, and ankle joints (Sherrington, 1903, 1910). Concurrent inhibition of extensor muscles antagonistic to those flexion reflex reactions were termed "reciprocal innervation" and were postulated as evidence for an integrated flexion reflex (Sherrington, 1898), wherein stimulation within a specific cutaneous receptive field (RF) or of a particular hindlimb nerve generated the same stereotyped withdrawal response, assessed primarily by qualitative analyses of evoked hindlimb movements. According to this work, the same flexion reflex response was generated from across the ipsilateral limb and was independent of stimulus location and depth. Further investigations performed by the Sherrington research group (Creed and Sherrington, 1926, Creed et al., 1932) later conceded that different input parameters (either in terms of the location of afferent stimulation or of its strength – the "local sign") were capable of evoking a motor response that was a composite of the actions of several muscles. The effects of the noxious input were also noted in the limb contralateral to the site of stimulation as an excitation of extensors with inhibition of flexors, which was termed the "crossed-extension reflex" and served to maintain the overall balance of the animal (Sherrington and Laslett, 1903, Creed et al., 1932).

Detailed cutaneous receptive field mapping to further characterise hindlimb withdrawal reflexes in terms of the actions of individual flexor and extensor muscles was performed by Hagbarth (1952). Recordings were made in decerebrate spinalized cats from α-motorneurones in response to a noxious pinch, and by recording multiple efferent responses simultaneously, the study revealed that even when a flexion response appeared "pure" i.e. no observable extensor response, that those muscles were still activated to a lesser degree and were "concealed" by the overriding action of the flexors. A complementary study on γ-motorneurones demonstrated that efferent activity was facilitated from the area overlying the muscle of interest and inhibited from elsewhere on the limb (Eldred and Hagbarth, 1954), a finding also replicated in human studies (Hagbarth, 1960). The functional implications of the organized arrangement observed was noted by Megirian (1962), who stated that "the most important factor determining the direction of reflex motor action is the locus of skin stimulation", highlighting both the protective nature of nocifensive withdrawal reflexes and their selectivity.

Later work evolved the flexion reflex theory further into an alternative modular organization theory, with strictly defined excitatory and inhibitory receptive fields for each muscle promoting or inhibiting movement accordingly as opposed to the singular action of the monolithic flexion reflex (for a detailed review of modular organization please refer to Schouenborg, 2002). By systematically measuring the myographical response to stimulation at a series of sites on the limb, the RF of that muscle may be mapped and, with the potential for recording from several muscles simultaneously, it is possible produce a detailed map of the motor response. One such study, performed in halothane-anaesthetized rats, recorded the responses in over twenty-five individual hindlimb muscles (Schouenborg and Kalliomaki, 1990). Using a focused CO_2 thermal laser stimulus, the authors found discrete RFs for muscles involved in, for example, knee and ankle flexion, specifically the ankle flexor tibialis anterior (TA) and the ankle extensor medial gastrocnemius (MG). Stimulation applied at the toe tips was found to be strongly excitatory for TA and strongly inhibitory for MG, with the reverse true when the laser was applied to the heel (see figure 1.3). The roles of these muscles in weight-bearing are to either remove pressure from the heel or the toes, with TA serving to shift weight from the toes to the heel and *vice versa* for the MG, with the excitatory RFs therefore corresponding to the spared region. Corresponding inhibitory RFs (localised to skin areas that would move towards the stimulation on contraction in the respective muscle) are also an integral part of the

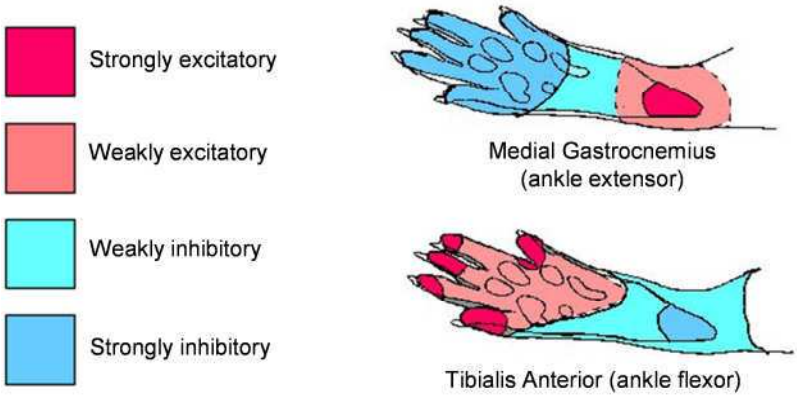


Figure 1.3: Receptive field mapping of the plantar surface of the rat hind limb. The data pictured show clear inhibitory and excitatory patterns for both flexor and extensor muscles in the anaesthetized rat. Figure adapted from Weng & Schouenborg (1996).

organization of hindlimb withdrawal reflexes (Weng and Schouenborg, 1996). Similarly selective excitatory and inhibitory RFs have also been described for other muscles of the rat hindlimb, including pronators of the hind-paw (peronei brevis and longus) and dorsiflexors of the digits (extensor digitorum longus, EDL) (Schouenborg and Kalliomaki, 1990, Weng and Schouenborg, 1996, Schouenborg, 2002). Furthermore, studies in rabbit have found a similar patterning of reflex responses. When activity in MG and the knee flexor semitendinosus (ST) muscle nerves were measured in response to noxious pinch applied at various sites across the ipsilateral hindlimb (Clarke et al., 1989) site-selective activation was observed, indicating that the modular organization recorded in rats in also present in other species. The modular organization theory was therefore lent further credibility by the publication of homologous RF patterning in both cat (Levinsson et al., 1999), mouse (Thelin and Schouenborg, 2008), and by studies in man, in which transcutaneous stimulation of nerves in the lower leg or foot evoked highly selective reflex responses (Van Wezel et al., 1997, Andersen et al., 1999).

I.3.2. Application in Humans

Investigations into the organization of withdrawal reflexes in human volunteers have elucidated a similar pattern of modular RFs as seen in animal studies. By measuring electromyographical (EMG) activity in muscles of the lower leg to electrical stimulation at various sites on the plantar surface of the foot, Kugelberg et al. (1960) and later Grimby (1963) showed clear selectivity for flexion or extension dependent on the location of the insult, with TA excitation observed in response to stimuli at proximal regions of the plantar surface such as the metatarsal joints or the medial longitudinal arch; and gastrocnemius excitation in response to stimulation of the heel. These results were confirmed in more recent and thorough studies of muscle activity in the human limb in response to noxious stimulation originating from one of sixteen possible sites on the plantar surface (Andersen et al., 1999, Sonnenborg et al., 2000). Once again the ankle extensor triceps surae muscles (MG and soleus together) responded with the greatest magnitude when regions around the heel received the electrical stimulus and the ankle flexors TA and peroneus longus when stimulus was delivered to the longitudinal arch, with corresponding inhibitory RFs. In addition to confirming the presence of a highly organized arrangement of withdrawal reflexes in the lower limb, these findings also demonstrate the variability seen between species with regard to the specific muscles activated in response to a particular stimulus

location e.g. the strong toes-TA response in rat is not as distinct in humans, though a high correlation was found in the longitudinal arch-TA response. The organization of withdrawal reflexes is therefore dependent on the normal posture and gait of the species examined, and cannot be generalised as a monolithic flexion reflex.

Activation of a lower limb withdrawal reflex in man is dependent on voluntary or ongoing activity, such as standing, knee extension, and walking (Spaich et al., 2004, Nakajima et al., 2006), and on the intensity of the stimulus applied (Andersen et al., 2001), which together with the stimulus location combine to determine the motor outcome. Electrical stimulation applied whilst the subject was standing elicited lower magnitude EMG responses (Nakajima et al., 2006), thus demonstrating a patterning in man akin to the crossed-extension reflex described by Sherrington and Laslett (1903) with the purpose of maintaining balance. Walking modulated the evoked reflexes in a more selective fashion with the outcome dependent on the phase of the gait cycle e.g. heel stimulation inhibiting the response in TA but a disinhibition was observed during the swing phase (Spaich et al., 2004). Varying the intensity of the electrical stimulation applied to the plantar surface demonstrated that not only are withdrawal reflexes selective according to the cutaneous site stimulated, but that at sufficiently high intensities the receptive field expands to encompass a greater area (Andersen et al., 2001).

Investigating functional adaptations of nocifensive withdrawal reflexes in animal models, as a means by which to deepen understanding of human conditions, is therefore a viable route of enquiry given the conserved nature of the hind- or lower-limb reflexes. As noted in the literature cited above however, direct inferences may not be possible due to the subtle intricacies of reflex patterning within each species.

I.3.3. An Alternative Theory of Reflex Organization

Whilst the modular theory of spinal reflex organization is now widely supported, the limitations of electrophysiological characterisation of the nociceptive withdrawal reflex and inferences drawn from it have been commented on detail in recent literature, and are described in review form (Schomburg, 1997a). Issues raised include the potential for depression of part of a reflex pathway to mask facilitation elsewhere, the difficulty and variability in interpretation of locomotor responses by human observers, and also the

possibility for direct recording from nerves to be confused by convergent transmission from C-type and Aδ fibres. However, the foremost concern relates to the supposition that hindlimb reflexes are organized in a modular fashion with excitatory and inhibitory cutaneous receptive fields for each muscle located at sites that would be either protected or harmed further by contraction of that muscle in response to a potentially injurious stimulus. The alternative to the modular theory of reflex organization advocated by Schomburg (1997a) is that based on the role of flexor reflex afferents (FRAs), in which it is stated that nociceptive and non-nociceptive reflex pathways belonging to the same 'system' would respond in similar ways to one another whereas nociceptive afferents outside that system cannot be considered to be part of the same reflex pathways. The integrative convergence of afferents from joint, muscle, and cutaneous origins on spinal interneurones determines the motor response produced and thus equates to a multisensory control mechanism (Eccles and Lundberg, 1959b, Lundberg, 1979). In particular, the review by Schomburg (1997a) and its subsequent discussion paper (Schomburg, 1997b) cite evidence from decerebrate spinal rats in which the EDL muscle was maximally excited from stimulation of the plantar aspect of the two lateral-most toes, though maximal inhibition was found from sites at or near the heel (Weng and Schouenborg, 1996). Strict application of the modular organization theory would predict that the dorsal surface of the toes ought to be the site capable of inhibiting that response. However, while activation of EDL does result in extension of the toes, it also generates dorsiflexion of the ankle, and in that role the site from which greatest inhibition might be predicted (under the assumption that a modular organization exists) would be sites at or near the heel. The excitatory and inhibitory fields observed therefore demonstrate a reflex organization with a preferential protective role for the plantar surface, rather than providing evidence for a shortcoming in the modular theory of reflex organization. The pattern originally described in detail in the rat (Schouenborg and Kalliomaki, 1990) has also since been recorded in other species detailed above (Levinsson et al., 1999, Sonnenborg et al., 2000, Clarke and Harris, 2004, Thelin and Schouenborg, 2008) and therefore from the evidence stated above the modular organization theory appears to be the most accurate description of the organization of withdrawal reflexes.

I.4 Hypersensitivity and the Underlying Mechanisms

Under certain circumstances the normal activation of withdrawal reflexes becomes sensitized and nociceptive sensory afferents may fire with reduced thresholds (hyperalgesia) or in the presence of previously innocuous stimuli (allodynia). Hyperalgesia, of which allodynia is a special case, is defined by IASP as any increased pain sensitivity which may be as a result of a decrease in nociceptive threshold and may include an increased suprathreshold response (Loeser and Treede, 2008).

I.4.1. Primary Hyperalgesia and Peripheral Sensitization

Hyperalgesia within the area of injury i.e. a reduced threshold response to a noxious stimulus applied to that region is termed primary hyperalgesia (Sandkühler, 2009). A hyperalgesic response of this type may be attributed to sensitization of primary afferents. For example, the perceived level of pain caused by a thermal stimulus is significantly elevated following an injurious thermal stimulus and correlates to increased responsiveness of A-fibres (Meyer and Campbell, 1981); the increased sensitivity is also apparent as either an increased firing rate or reduced response threshold when a mechanical test stimulus is applied following a priming noxious treatment (Schaible and Schmidt, 1988, Brennan et al., 1996, Chen et al., 1999). The recruitment of these additional primary afferent fibres to the site of inflammation then has the effect of lowering the overall threshold to nociceptive input in the immediate surrounding tissues and generating primary hyperalgesia. At a molecular level, this nociceptor sensitization process is mediated by inflammatory molecules such as neuropeptides and amines (e.g. serotonin and substance P) as well as cytokines and cellular cations (Kessler et al., 1992, Sufka et al., 1992, Julius and Basbaum, 2001), and it is the diffusion of these agents which is responsible for the relatively limited zone of primary hyperalgesia around the initial stimulus. Thus an increased excitability of peripheral nociceptors (peripheral sensitization) results in primary afferent firing at a lower threshold than in the non-sensitized state, which in turn increases the likelihood of central neurones being activated and of the original stimulus being perceived as painful and potentially generating a withdrawal reflex.

I.4.2. Secondary Hyperalgesia and Central Sensitization

In contrast to the above, secondary hyperalgesia refers to increased painfulness of stimuli applied to the uninjured tissue outside the area of injury, sometimes at sites relatively distant to the location of the initial insult (Ali et al., 1996a, Brennan et al., 1996). Early work by Hardy et al. (1950) on the underlying mechanisms led to the hypothesis that secondary hyperalgesia was not produced in response to the diffusion of an inflammatory substance released from the site of injury, rather it is mediated by a central process and relies on the function of the nerve itself as local anaesthetic block eliminated hypersensitivity. Prior evidence generated by Lewis (1937) had suggested a peripheral mechanism was responsible for the hyperalgesic responses observed post-anaesthesia, though in this case ongoing tissue damage was also a factor given that the original stimulus was skin crush and is capable of generating a continued peripheral input. Secondary hyperalgesia may therefore occur as a result of the initial peripheral stimulus generating an afferent barrage that sensitizes spinal interneurones, thereby reducing the threshold required for a response to subsequent stimulation in that same receptive field. This neural plasticity is termed “central sensitization” (Woolf, 1983, Woolf and Salter, 2000), and is defined by IASP as increased responsiveness of nociceptive neurones in the central nervous system (Loeser and Treede, 2008). The increased responsiveness also describes a reduced latency of the reaction time to the noxious input and a lower firing threshold. Further evidence for central factors underlying the hyperalgesic state is demonstrated by the application of a local anaesthetic at the site of noxious stimulus which failed to reverse receptive field expansion of WDR neurones (Woolf, 1983). This is supported by data collected from studies involving human volunteers which used the chemogenic noxious stimulus of capsaicin followed by application of either dissociative or opioid anaesthetic, and showed central effects to be involved in ongoing hyperalgesia (Woolf, 1983, Park et al., 1995).

Another characteristic observed during secondary hyperalgesia is the expansion of the RF of sensitized dorsal horn neurones (Woolf and King, 1990). Research carried out in macaques demonstrated that NS and WDR interneurones differ in their response to capsaicin sensitization with the WDR sub-class displaying a significant expansion of the receptive field when analysed for reaction to noxious and innocuous stimuli whilst NS cells remained unchanged from the control state (Simone et al., 1991, Dougherty and Willis,

1992). The use of a non-physical stimulus such as capsaicin lowers the likelihood of on-going input due to tissue damage influencing spinal neuronal activity.

Expansion of receptive fields occurs following stimuli of sufficient intensity (or several that summate to generate a similar effect) to recruit neurones spatially remote from those activated by the initial stimulus, indicating an underlying heterosynaptic facilitatory mechanism i.e. facilitation in synapses outside those activated by the injurious stimulus (Kandel and Schwartz, 1982, Latremoliere and Woolf, 2009). Heterosynaptic plasticity is the mechanism by which activity at one synapse or pathways modulates the responsiveness of another i.e. outside of those activated by the injurious stimulus, whereas in homosynaptic plasticity mechanisms the alteration in responsiveness is induced by prior activity at that same synapse (Chen and Sandkühler, 2000, Woolf and Salter, 2000). Heterosynaptic sensitization also accounts for the reduction of threshold observed in secondary hyperalgesia, as lower-threshold fibres are recruited and fire concomitantly with those responding to such a stimulus in a non-sensitized state e.g. a C-fibre mediated conditioning stimulus such as an electrical pulse is capable of facilitating the responses of Aδ- and Aβ-fibres (Ziegler et al., 1999, Klein et al., 2008).This may also be described in terms of the responsiveness of spinal interneurones which are now activated by both A- and C-fibres, indicating a transition from a NS neurone to one that may now be categorised as being of the WDR type (Simone et al., 1989, Thompson et al., 1993).

A process related to central sensitization is a temporal summation wind-up phenomenon, in which a dorsal horn interneurone receives a series of action potentials from C-fibre nociceptor afferents and as a consequence responds more strongly to subsequent stimulation (Mendell and Wall, 1965, Herrero et al., 2000). Neurones experiencing wind-up return to their previous non-sensitized state within a matter of seconds, whereas in 'true' central sensitization changes are much more prolonged with a time course of hours or even longer due to transcriptional changes occurring in these neuronal populations (Price et al., 1971, Woolf, 1984, Ji et al., 2003). Wind-up may therefore be described as transcriptional-independent central sensitization *cf.* long-lasting transcriptional-dependent alterations in spinal cord neurone excitability.

Another closely related process is that of long-term potentiation (LTP) (Bliss and Lømo, 1973), of which central sensitization may be considered a subtype and *vice versa*. LTP is

also a long-lasting increase in synaptic strength as a result of alterations in levels of protein synthesis and is a process implicated in memory and learning as well as contributing to hyperalgesia (Sandkühler, 2007). While these two processes share many similarities (Ji et al., 2003), they are distinguished from one another by the region of the CNS to which they pertain – LTP generally refers to plasticity of cortical neurones and central sensitization to that of spinal cord neurones.

I.5 Pharmacology of Sensitization

I.5.1. Molecular Mechanisms of Sensitization

The cellular mechanism underlying both LTP and wind-up is homosynaptic sensitization, in which the synapses displaying an increase in responsiveness are restricted to those that were activated by the initial conditioning stimulus, with the form of plasticity generated dependent upon the frequency of the initiating stimuli (low frequency triggers wind-up with high frequency initiating LTP) (for reviews on wind-up and LTP see: Herrero et al., 2000, Ji et al., 2003). In the case of wind-up, the plasticity is a transient effect generated due to cumulative depolarisation caused by the excitatory postsynaptic potentials of sequential presynaptic action potentials (Mendell and Wall, 1965, McMahon et al., 1993). The high level of depolarisation at the post-synaptic membrane is mediated by the relatively slow recovery rate of α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptors following activation by glutamate (Trussell and Fischbach, 1989, Jones and Westbrook, 1996). This depolarisation may also be sufficient to activate N-methyl D-aspartic acid receptors (NMDAR, a post-synaptic receptor for the excitatory neurotransmitter glutamate) by relieving the covalent magnesium ion block which is present at and around resting potential, which will then subsequently allow greater influx of calcium and greater depolarisation (Mayer et al., 1984, Nowak et al., 1984, Dickenson, 1990, Dickenson et al., 1997), though this is mediated in part via NMDA-independent mechanisms (Baranauskas and Nistri, 1996). The increase in post-synaptic intracellular Ca^{2+} concentration triggers downstream signalling cascades and has the potential to alter levels of gene expression and protein synthesis. Studies in the CA1 region of the hippocampus, the part of the brain associated with learning and memory, have detailed a mechanism behind LTP in response to a high frequency or tetanic stimulation (Frey et al., 1993, Cao and Harris, 2012, Szabo et al., 2012). In this case, activation of receptor tyrosine kinases by

neurotrophic factors and of G-protein coupled-receptors such as those of the metabotropic glutamate family leads to an up-regulation of the activity of further kinases, culminating in the phosphorylation of Src, a non-receptor tyrosine kinase. Src then functions to phosphorylate tyrosine residues of the NR2 subunits of the NMDAR, resulting in increased probably of the channel remaining open (Sprengel et al., 1998, Ali and Salter, 2001, Rossi et al., 2002). The mechanism by which LTP occurs in the dorsal horn is thought to be analogous to that seen in the hippocampus, as the key components of that pathway as described above are also expressed in dorsal horn neurones and are implicated in plasticity at that site (Woolf and Salter, 2000, Brenner et al., 2004, Kawasaki et al., 2004).

Pharmacological factors are also implicated in central sensitization itself, in particular the role of glutamate. Recent studies have shown that application of NMDAR antagonists block the development and maintenance of central sensitization in intact rats (Woolf and Thompson, 1991, Hama et al., 2003, Qu et al., 2009, Kim et al., 2012) and rabbits (Harris et al., 2004) and either the receptor as a whole or one or more of its component subunits therefore provides a potential candidate for targeted anti-hyperalgesics (for a review of NMDAR antagonists in analgesia see McCartney et al., 2004). The role of NMDAR in the increased responsiveness of dorsal horn interneurones is related to its function as a ligand-gated non-specific cation channel, and upon activation of the receptor by NMDA, its specific agonist, undergoes a conformational change which ultimately potentiates intracellular second messenger cascades (Coderre and Melzack, 1992). One target of this system is the membrane-associated enzyme nitric oxide synthase (NOS), which in neuronal tissue exists as an isoform inducible by raised intracellular calcium levels (Wu et al., 2001, Pedersen et al., 2010). Elevated NOS levels (which may therefore lead to elevated nitric oxide levels) in the dorsal horn are associated with hypersensitivity in models of neuropathic and inflammatory pain (Lam et al., 1996, Chacur et al., 2010, Chen et al., 2010, for review see Freire et al., 2009), thus linking the activity of NMDAR with reflex excitability. Nitric oxide release, which is itself capable of functioning as a signalling molecule in the nervous system implicated in LTP (Hopper and Garthwaite, 2006, Taqatqeh et al., 2009), enhances the release of sensory neuropeptides such as substance P and other tachykinin receptor agonists (Linden et al., 1999), thereby increases the excitability of the neurones in the reflex pathway. A full account of the role of tachykinins in neuronal plasticity is beyond the scope of this thesis, therefore for a detailed review on the subject

of tachykinin functionality (including nervous system functions) please refer to Severini et al. (2002).

I.5.2. The Role of Second Messenger Systems

As discussed above, calcium plays a critical role in the intracellular signalling pathways that precede the development of central sensitization. Another molecule of high importance is cAMP (3', 5'- cyclic adenosine monophosphate), a cyclic nucleotide synthesised from ATP (adenosine triphosphate) by the membrane-bound enzyme adenyl cyclase (Sutherland et al., 1968). There is experimental evidence that up-regulation of adenyl cyclase by the exogenous activator forskolin reduces nociceptive threshold in awake rats (Taiwo and Levine, 1991, Lee et al., 2012) thus implying that increased levels of cAMP are at least in part responsible for the onset of hyperalgesia. Additional evidence that changes in the intracellular concentration of cAMP are relevant to the study of nociception and sensitization is the ability of analogues of cAMP to produce mechanical hyperalgesia (Sluka, 1997, Dolan and Nolan, 2001, Levy and Strassman, 2002, Parada et al., 2005). One of the downstream effects of elevated cAMP levels is activation of protein kinase A (PKA), which via both pharmacological and mutation studies has been shown to be necessary for the development and maintenance of thermal hyperalgesia (Malmberg et al., 1997, Aley and Levine, 1999). Other enzymes involved in include protein kinase C (PKC) and members of the mitogen-activated protein kinase (MAPK) family such as extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Sweitzer et al., 2004, Hucho and Levine, 2007). Of particular interest to the study of hyperalgesia and sensitization is the range of stimuli that are capable of activating ERK1/2, from chemogenic stimuli such as capsaicin and nerve growth factor (NGF) to electrical stimulation and nerve injury (Ji et al., 1999, Liu et al., 2001, Zhuang et al., 2004, Agthong et al., 2006).

The epsilon isoform of PKC (PKCε) is selectively activated by inflammatory mediators such as bradykinin and tumour necrosis factor-alpha (TNF-α) that are released in response to peripheral tissue injury (Cesare et al., 1999), and high levels of expression of this isozyme have been found in DRG cells in a rat model of neuropathic pain (Dina et al., 2000, Zhang et al., 2008). In addition to PKCε, calcium-dependent inducible NOS expression increases in DRG following peripheral nerve injury (Mizusawa et al., 2003, Martucci et al., 2008). Peripheral inflammatory mediators and neuropathies are therefore capable of increasing

the excitability of primary afferent somata that then project into the dorsal horn and initiate central changes with the potential to induce sensitization.

I.5.3. Pharmacological Action of Chemogenic Sensitizing Agents

Chemical agents, such as capsaicin (8-methyl-N-vanillyl-6-nonenamide), are frequently employed in studies of allodynia and hyperalgesia in pre-clinical species in addition to investigations undertaken in man, and also include allyl isothiocyanate (mustard oil) and trans-cinnamaldehyde for cutaneous sensitization or formaldehyde (also known as formalin) for deeper structures (e.g. Koltzenburg et al., 1992, Jiang and Gebhart, 1998, Fu et al., 2001).

Chemical stimuli are used in these studies because not only are they capable of generating high frequency nociceptive input they also preserve the integrity of peripheral structures and do not therefore produce ongoing nociceptor activity. These molecules act via the transient receptor potential (TRP) cation channels expressed at the peripheral terminals of C- and Aδ- primary afferent fibres (Dhaka et al., 2006). Mustard oil and cinnamaldehyde both trigger intracellular signalling cascades by interacting with the TRPA1 (member 1 of the ankyrin sub-family) receptor, specifically, by covalently binding to cysteine residues (Macpherson et al., 2007a). Formalin hyperalgesia is also initiated by interacting with the TRPA1 receptor, as activation of TRPA1 allows influx of cations (including calcium) and thus triggers downstream signalling to heighten the responsiveness of TRPA1-expressing neurones (Jordt et al., 2004, McNamara et al., 2007, Kerstein et al., 2009). Competitive inhibition studies performed in rats, in which methanol was used as a reversible blockade on the channel, determined that formalin interacts with the receptor complex in a similar way to both mustard oil and cinnamaldehyde (Macpherson et al., 2007b). Further evidence for the role of TRPA1 in formalin-induced hyperalgesia was provided by studies in knock-out mice in which animals lacking this receptor displayed marked attenuation of pain-like behavioural responses (McNamara et al., 2007).

II. DESCENDING CONTROLS

II.1 Supraspinal Control of Hindlimb Reflexes

The factors determining the outcome of a peripheral stimulus i.e. the magnitude and directionality of a withdrawal reflex, and whether or not such an action occurs, are not resolved purely at the level of the spinal cord in the dorsal and ventral horns. Tracts which ascend from the spinal cord to specific brain regions enable the experiential component of that stimulus as either tactile sensation or as pain, and corresponding descending pathways exert a modulatory control over the effect of such a sensation.

II.1.1. Ascending Pathways

Ascending pathways involved in the transmission of various peripheral stimuli may be sub-categorised into those relaying exteroceptive information and those responsible for proprioceptive information. The spinothalamic tract comprises projection fibres originating in the marginal zone (lamina I), intermediate dorsal horn (laminae III-IV), and the neck of the dorsal horn (lamina V) when quantified in the lumbar region of the rat spinal cord (Giesler et al., 1979, McMahon and Wall, 1983, Kemplay and Webster, 1986, Burstein et al., 1990, Marshall et al., 1996) with laminae I and V the main sources, and therefore in terms of peripheral sensory modalities carried is primarily responsible the transmission of nociceptive, tactile, and thermal inputs to the thalamus. The dorsal column-medial lemniscal (DCML) tract relays proprioceptive and discriminative tactile information and may be divided into the gracile fasciculus (medial; lower limbs) and the cuneate fasciculus (lateral; upper limbs, trunk, and neck) (Langley, 1886, Mott and Sherrington, 1894), again with thalamic projections. Spinothalamic neurones ascend the tract on the side contralateral to the site of input, hence demonstrating that afferent axons decussate within this branch of the somatosensory pathways, whereas DCML projections ascend ipsilateral to the original stimulus. Proprioceptive feedback is also relayed via the spinocerebellar tract (for review see Bosco and Poppele, 2001) which without direct thalamic projections does not therefore play a role in perception.

In addition to the thalamic projections already described, several spinobulbar pathways are integral in pain processing which includes such ascending columns as the spinoreticular and spinomesencephalic tracts, originating primarily in laminae VII and VII and laminae I/IV-VI

respectively (Menétrey et al., 1982, Chaouch et al., 1983). These spinobulbar tracts project ultimately to the amygdala and hypothalamus, either from efferent output of the nucleus of the solitary tract via the parabrachial nucleus or through arguably one of the most important brain regions in nociceptive processing, the periaqueductal grey (PAG) (Hylden et al., 1986, Bernard et al., 1995) (see also section II.1.2 below for further information regarding the role of PAG in pain processing). The spinobulbar pathways described here therefore form the ascending branch of what may be termed a spino-bulbo-spinal loop – the descending component of which is now considered in greater detail.

II.1.2. Descending Influences

Greater insight into the role of descending pathways in the endogenous control of pain was obtained by electrophysiological studies of neural projections in cat and rat (Basbaum and Fields, 1979). Descending pathways were confirmed to influence endogenous analgesic mechanisms by electrically stimulating of regions of the brain with spinal projections, such as the raphe magnus nucleus, which via the dorsolateral funiculus, influences neurones in the dorsal horn (Bett and Sandkuhler, 1995). The combined knowledge that direct activation of brain stem neurones was able to reduce reflex responses to pain (for example, the tail-flick test in rats (Morgan and Franklin, 1988)) and that the spinal terminals of projections from neurones originating in the stimulated regions were located in the dorsal horn gave strong evidence for a descending influence being involved in controlling spinal cord excitability hence activity in nociceptive sensory pathways. Furthermore, the excitability of some NS cells was enhanced in the presence of a spinal block (Cervero et al., 1976, Hoheisel and Mense, 1990), indicating that cells of this type located in lamina I are subject to some degree of tonic descending inhibition.

The major descending pathways by which the brain controls spinal motor output, including hindlimb withdrawal reflexes, are the corticospinal, rubrospinal, and reticulospinal tracts. In the rat corticospinal (or pyramidal) tract (CST) cells synapse with MNs in the ventral horn in an indirect fashion, utilising di- or tri-synaptic pathways mediated by spinal interneurones (Alstermark et al., 2004, Al-Izki et al., 2008). Between 75% and 90% of CST axons decussate in the medulla oblongata to form the lateral CST with control over distal musculature, and the remaining 10% to 25% (now termed the anterior CST) influence proximal musculature and decussate at their terminal spinal segments. This tract has a

particular involvement in the control of fine voluntary movements such as those performed by the hand or digits (Whishaw et al., 1998). The rubrospinal tract descends adjacent to the corticospinal tract and controls more general or coarse actions of the limbs, such as locomotor movements (Muir and Whishaw, 2000). These axons have been shown to terminate in the spinal cord on the side contralateral to that from which they originate in the red nucleus, primarily in laminae IV-VII and IX (Küchler et al., 2002, Al-Izki et al., 2008). The reticulospinal tract is also implicated in locomotor control, posture and gait during locomotion, and posture and movement during targeted reaching (Ballermann and Fouad, 2006). Selective lesion studies coupled with stimulation of discrete brain nuclei demonstrate the critical role of the reticulospinal tract in the initiation and control of locomotor movements by its selective actions over flexor and extensor muscles (Orlovsky, 1972, Drew and Rossignol, 1984, Armstrong, 1988, Noga et al., 1991), though some degree of redundancy in the descending pathways is also evident (Loy et al., 2002).

The bulbospinal pathways briefly detailed above, whilst exerting a degree of control over motor output, are not integral in terms of a descending control of pain. The origin of supraspinal controls of sensitivity and normal responsiveness is the midbrain periaqueductal grey matter which projects to the rostroventral medulla (RVM) and other brainstem nuclei, from which projections descend through the dorsolateral funiculus to the dorsal horn of the spinal column (Basbaum and Fields, 1979, Mason and Fields, 1989). Stimulation of the periaqueductal grey by either electrical or chemical means generated an attenuation of both the responses of dorsal horn neurones and of spinally-mediated reflexes such as tail flick (Gray and Dostrovsky, 1983, Gao et al., 1997, Waters and Lumb, 1997), thus clearly indicating an important role for PAG in descending inhibitory controls. Furthermore, the reduced dorsal horn responsiveness to mechanical cutaneous inputs resulting from stimulation of the PAG has been shown to occur as a result of increased levels of neurotransmitter release (such as noradrenaline and serotonin) (Cui et al., 1999) thus providing a mechanistic insight into the observed changes. However, there are few direct axonal connections from the PAG to the dorsal horn, and so the numerous connections known to exist linking this region to brainstem nuclei such as the serotonergic raphe magnus nucleus (located within the RVM) and the noradrenergic locus coeruleus (Cedarbaum and Aghajanian, 1978, Luppi et al., 1995, Bajic et al., 2001, Odeh and Antal, 2001, Lee et al., 2005) act as a relay site through which alterations in spinal nociceptive responsiveness may be mediated. In addition to the well-documented analgesic effects

produced following activation of these descending pathways, there is substantial evidence for descending facilitatory pathways which also originate in the PAG (Urban and Gebhart, 1999, Porreca et al., 2002, Tillu et al., 2008). In particular, stimulation within the RVM was able to increase dorsal horn neuronal activity (Zhuo and Gebhart, 1992) whilst RVM lesions both attenuated the onset of hyperalgesia and increased the latency of withdrawal reflex responses (Urban et al., 1996, Urban et al., 1999).

Different classes of cell within the RVM, termed “on” or “off” cells (Fields et al., 1991), exhibit differential firing patterns that coincide with nociceptive withdrawal reflexes and therefore have roles in both descending inhibition and facilitation. In the rat tail-flick test, on-cells were shown to increase firing rate immediately before the movement was elicited, whereas off-type cells are tonically active up to approximately 400 ms prior to the occurrence of the reflex. Further classification of these cell types has categorised the on-cells as facilitatory for withdrawal reflexes (Neubert et al., 2004) while the action of off-cells has an inhibitory effect and therefore cessation of their firing has a disinhibitory effect. Experiments in which activity in the RVM was monitored during repeated noxious stimuli found an enhancement in activity co-presenting with enhanced motor reactions, which could indicate a role in hyperalgesic withdrawal reflexes (Foo and Mason, 2005). For a recent review of the role of medullary circuits in the modulation of nociception please see Mason (2012). Projections from these regions of the brain – and in particular those of noradrenergic and serotonergic nature – will now be the focus of the remainder of this review.

II.A.BULBOSPINAL NORADRENERGIC PATHWAYS

II.A.1 Biosynthesis and Metabolism of Noradrenaline

This section provides a brief overview of the biosynthetic pathway of noradrenaline, the enzymes catalysing the various stages of the process, and some of the key molecules involved. For a detailed review of this subject please refer to Eisenhofer et al. (2004).

Noradrenaline (NA), as well as adrenaline and dopamine, is a member of the catecholamine neurotransmitter family, in that their basic molecular structure is comprised of a benzene ring with two adjacent hydroxyl groups and a side-chain amine moiety. The precursor for the biosynthesis of these molecules is the L-enantiomer of the amino acid tyrosine which is hydroxylated to form L-3,4-dihydroxyphenylalanine (L-DOPA), a step catalysed by tyrosine hydroxylase (TH) (Nagatsu et al., 1964) (figure 1.4). TH is expressed in the ventral tegmental area of the brain in both nuclei and cytoplasm of perikarya and dendrites (Bayer and Pickel, 1990), in the locus coeruleus (see section II.A.2.1) associated with neurotubules in axons and dendrites but free in the cytoplasm in perikarya (Pickel et al., 1975), and in the raphe nuclei (Trulson et al., 1985). Decarboxylation of L-DOPA produces dopamine, which may be further hydroxylated to generate NA, reactions catalysed by DOPA decarboxylase (DDC) and dopamine-β-hydroxylase (DBH) respectively (Blaschko, 1939, Hagen, 1956, Holtz, 1959). In the presence of the methyl donor molecule *S*-adenosyl methionine, NA is methylated by phenylethanolamine-*N*-methyltransferase (PNMT) to form adrenaline (Kirshner and Goodall, 1957).

Both NA and adrenaline are degraded via an intermediate aldehyde molecule from a monoamine oxidase reaction (Richter, 1937) to produce the principal inactive end-stage metabolite of vanillyl mandelic acid (Armstrong et al., 1957, Armstrong and McMillan, 1959).

II.A.2 Noradrenergic and Adrenergic Cell Locations in the Central Nervous System

Catecholaminergic perikarya, visualized according to the histochemical protocol of Carlsson et al. (1962), were first extensively catalogued and mapped by Dahlström and Fuxe

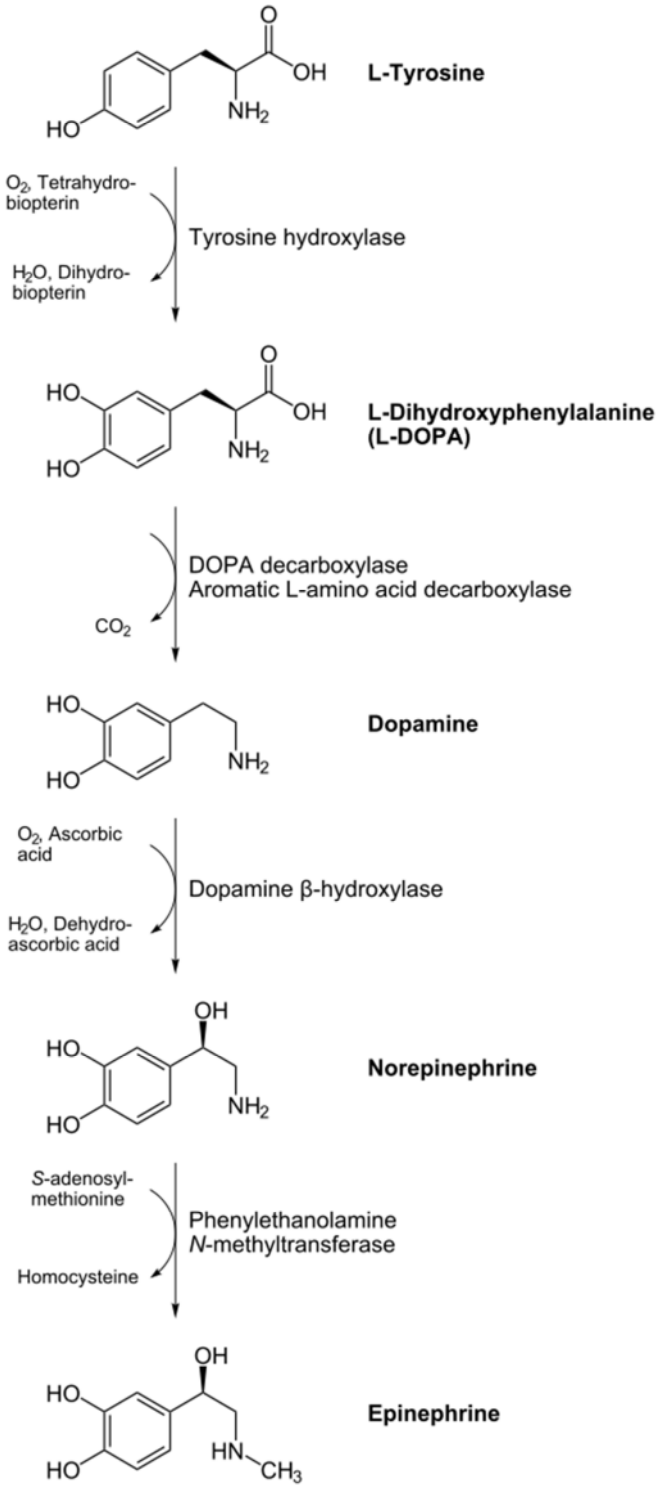


Figure 1.4: Biosynthetic pathway of catecholamine molecules.

(1964). In the brain, clusters of proposed noradrenergic cells responding to this formaldehyde- induced fluorescence treatment were assigned groups labelled A1-A12 from the caudal medulla to the rostral pons, of which A1-A7 are noradrenergic with the remainder forming the dopaminergic cell groups of the CNS. A13 in the hypothalamus was added by Fuxe (1965) and later extended further to A14 in the posterior hypothalamus and A15 in the paraventricular nucleus by Hökfelt et al. (1984b), A16 within the olfactory bulb (Baker et al., 1983) and A17 retinal cells (Hadjiconstantinou et al., 1984) (figure 1.5). Further adrenergic cell groups identified and named were C1 (in the ventrolateral medulla) and C2 (located in the dorsomedial medulla) (Fuxe et al., 1974) with the additional medullary adrenergic group C3 added subsequent to that study (Howe et al., 1980).

The original fluorescence method was subsequently improved upon using immunohistochemical technologies to allow even more precise identification of cells containing the different catecholamine neurotransmitters. The sequence of enzymatic reactions required in catecholamine biosynthesis allows investigators to differentiate between the individual neurotransmitters i.e. cells expressing DBH but not PNMT are presumed to be noradrenergic as opposed to dopaminergic or adrenergic. This section focuses on adrenergic and noradrenergic cell group distributions in the rat only. For a detailed review of the dopaminergic cell groups please refer to Björklund and Dunnett (2007).

II.A.2.1. Noradrenergic Cells in the Rat Brain

A1 cell group

Located in the ventrolateral part of the reticular formation of the medulla oblongata (Dahlström and Fuxe, 1964), the A1 cell group consists almost exclusively of noradrenergic perikarya (Kalia et al., 1985a, Smeets and González, 2000). Thus co-expression of TH and DBH in the absence of PNMT has been found throughout A1 (Dahlström and Fuxe, 1964, Swanson and Hartman, 1975, Ritchie et al., 1982, Kalia et al., 1985a, Kalia et al., 1985c, Halliday and McLachlan, 1991). Proceeding from caudal medulla to rostral pons, A1 cells transition from the dorsal and dorsomedial aspects of the lateral reticular nucleus to a more diffuse arrangement extending from the LRN's subtrigeminal portion laterally, to the LRN's magnocellular portion medially and to the retroambiguous nucleus dorsally (Dahlström and Fuxe, 1964, Armstrong et al., 1982).

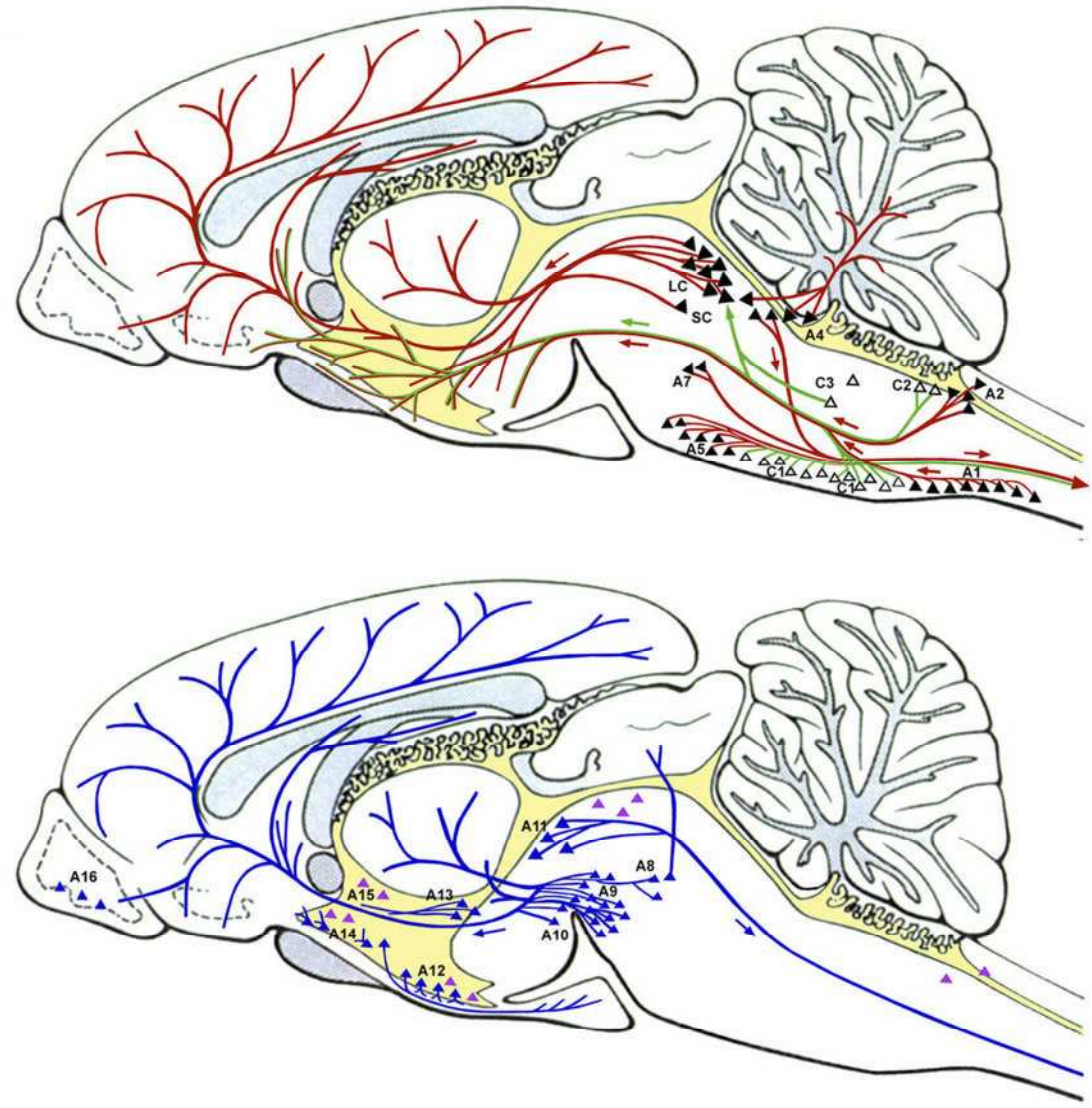


Figure 1.5: Noradrenergic and adrenergic cell groups in the rat brain (upper panel) and dopaminergic groups and projections (lower panel). Figure adapted from Kvetansky et al. (2009).

A2 cell group

Catecholaminergic cells have also been found in the caudal rhombencephalon in a dorsomedial position (Dahlström and Fuxe, 1964), which at the most caudal medullary levels exists as a single cluster in the ventromedial commissural nucleus of the nucleus of the solitary tract (Armstrong et al., 1982). In the coronal plane at a level corresponding to the area postrema (AP) numerous noradrenergic cell bodies have been found within the AP itself (Torack et al., 1973, Armstrong et al., 1982) and also in several loosely aggregated structures located ventral and lateral to the AP in the vicinity of the solitary tract and the area subpostrema (Dahlström and Fuxe, 1964, Torack et al., 1973, Armstrong et al., 1982). Beyond the rostral pole of the AP, clusters previously noted ventrolateral to AP are still evident and remain so up to the level of the rostral medullary sections. Very few fluorescent cells were found in the pontine levels (Armstrong et al., 1982). Whilst A2 is categorised primarily as a noradrenergic group, some clusters of cells here have been shown to express TH in the absence of DBH, in particular those within and dorsal to the dorsal motor nucleus of the vagus (i.e. dopaminergic in nature), and TH in the presence of DBH and PNMT (i.e. adrenergic) (Armstrong et al., 1982, Kalia et al., 1985a). However, some TH-expressing cells in the dorsal motor nucleus of the vagus have been shown to lack DDC (Jaeger et al., 1984) and therefore are not able to synthesise any catecholamine beyond the L-DOPA stage of the pathway. Jaeger and colleagues (1984)postulated that L-DOPA may be functioning as a neurotransmitter in these cells, a theory propounded by many others since (e.g. Misu et al., 1996, Weihe et al., 2006) .

A3 cell group

This group was identified by Dahlström and Fuxe (1964) as an area of weakly or very weakly fluorescent cells in the dorsal accessory nucleus of the inferior olive, but this region has not been identified by immunohistochemical methods (Swanson and Hartman, 1975) nor in species other than rodent (Tillet and Thibault, 1989).

A4 cell group

Although A4 was also originally identified as a distinct grouping (Dahlström and Fuxe, 1964), later authors have regarded it as a dorsolateral continuation of A6 rather than a cell group proper (Grzanna and Molliver, 1980, Foote et al., 1983, Moore and Card, 1984, Smeets and González, 2000). Cells in this region stained either for TH or DBH are clearly shown to extend from the dorsal portion of the locus coeruleus (LC, see A6 below) in the

pons in a caudal direction as far as the summit of the fourth ventricle and after which are situated in the lateral part of the roof of the ventricle (Swanson and Hartman, 1975, Grzanna and Molliver, 1980, Hökfelt et al., 1984b).

A5 cell group

Catecholaminergic cells have also been identified in the pons at the level of the superior olivary complex, in particular within the rubrospinal tract (Dahlström and Fuxe, 1964). These cells, shown to express TH and DBH, extend as a continuous column starting in the ventrolateral pons medial to the trigeminal and facial nerves as far caudally as the rostral edge of the adrenergic cell group C1 (see below) (Swanson and Hartman, 1975, Hökfelt et al., 1984b, Smeets and González, 2000).

A6 cell group

The largest of the noradrenergic groups (Smeets and González, 2000), Dahlström and Fuxe (1964) stated that the A6 cell group in the pons seemed to be "identical with the LC" and on the basis of results published in that paper was deemed almost purely catecholaminergic. Further investigation revealed that both TH and DBH were extensively expressed in LC (Swanson, 1976, Grzanna and Molliver, 1980, Westlund et al., 1983, Hökfelt et al., 1984b) in the absence of PNMT (Berod et al., 1984) and is therefore mostly if not entirely noradrenergic in nature.

On the basis of cytoarchitectural information, the LC may be divided into four sub-nuclei – the LC 'proper' or dorsal division, the nucleus subcoeruleus or ventral division, the rostral division which extends anteriorly from the LC, and the caudal division which is analogous to the A4 cell group (Grzanna and Molliver, 1980, Foote et al., 1983, Hökfelt et al., 1984b). The LC as a whole lies along the lateral margin of the pontine fourth ventricle and is bounded laterally by the mesencephalic nucleus of the trigeminal nerve and the superior vestibular nucleus (Dahlström and Fuxe, 1964, Palkovits and Jacobowitz, 1974, Swanson, 1976, Hökfelt et al., 1984b) with the greatest cross-sectional area located at the mid-pontine level (Grzanna and Molliver, 1980).

A7 cell group

The rostral-most noradrenergic cell group (A7), at the level of the pontine nuclei, is located within the lateral aspects of the reticular formation adjacent to or within the lateral

lemniscus (Dahlström and Fuxe, 1964, Hökfelt et al., 1984b, Moore and Card, 1984, Smeets and González, 2000). As the nucleus subcoeruleus forms the ventral-most extremity of A6 and is situated dorsal to the A7 cell group it is occasionally defined as an extension ofA7 (e.g. Caffé et al., 1985).

II.A.2.2. Adrenergic Cells in the Rat Brain

C1 cell group

The largest of the adrenergic cell groups (Howe et al., 1980, Minson et al., 1990), C1 is located in the ventrolateral medulla lateral to the olivary complex and caudal to the nucleus of the facial nerve (Fuxe et al., 1974) in a position rostral to A1 (Hökfelt et al., 1984a). This group occupies a very wide area of the ventral medullary tegmentum (Kalia et al., 1985b), extending from the caudal pole of the facial nucleus to the calamus scriptorius (Ruggiero et al., 1985, Tucker et al., 1987). By comparing the distribution of DBH and PNMT labelling, it is possible to segregate cells that are likely to synthesise noradrenaline from those capable of adrenaline synthesis. Using this technique, the caudal-most reaches of C1 were found to clearly overlap the rostral-most region of A1 (Hökfelt et al., 1984a) but also with a clear delineation of the two cell types at the level of the obex (Armstrong et al., 1982, Tucker et al., 1987).

C2 cell group

The dorsomedial adrenergic cell group is designated as group C2 (Fuxe et al., 1974). At the level of the area postrema, adrenergic cells occupy dorsomedial areas of the nucleus of the solitary tract and the dorsal motor nucleus of the vagus (Howe et al., 1980, Kalia et al., 1985b, Ruggiero et al., 1985, Minson et al., 1990, Smeets and González, 2000). C2 is notably smaller than C1 (accounting for around one-fifth of medullary adrenergic neurones whilst C1 contains greater than two-thirds of them (Howe et al., 1980)) but has also been shown to have an indistinct boundary with the similarly situated noradrenergic group, in this case group A2 (Hökfelt et al., 1984a).

C3 cell group

Representing less than one-tenth of the total adrenergic cell population of the medulla, C3 was not identified as a catecholaminergic cell group until 1980 (Howe et al., 1980) and is not found in all mammals (Smeets and González, 2000). This scattered group is located

between the dorsal raphe region and the intramedullary axons of the hypoglossal nerve, within and dorsal to the medial longitudinal fasciculus (Howe et al., 1980, Hökfelt et al., 1984b, Minson et al., 1990, Smeets and González, 2000).

II.A.2.3. Noradrenergic Projections to the Spinal Cord

Identification of which of the noradrenergic cell groups project to the spinal cord and whereabouts they terminate in reference to the laminar divisions of the cord was established using a combination of the techniques employed in earlier neuroanatomical studies. These included immunohistochemistry to label TH-, PNMT-, and DBH-containing neurones in both the spinal cord and the brain stem (Westlund et al., 1983, Mouchet et al., 1986, Hagihira et al., 1990), electrophysiological recording from proposed centres of noradrenergic control in response to spinal cord stimulation (Guyenet, 1980), mass-spectrometer coupled gas-chromatography to identify catecholamine-rich regions (Commissiong et al., 1978, Commissiong, 1981), formaldehyde-induced fluorescence and variations thereon (Carlsson et al., 1964, Commissiong et al., 1978, Loewy et al., 1979, Commissiong, 1981, Schrøder and Skagerberg, 1985), selective lesion studies (Carlsson et al., 1964, Nygren and Olson, 1977, Commissiong, 1981), retrograde HRP and DBH antibody transport studies (Loewy et al., 1979, Mason and Fibiger, 1979, Guyenet, 1980, Westlund et al., 1983), anterograde tritiated amino acid transport studies (Loewy et al., 1979), and immunohistochemistry utilising antibodies to noradrenaline itself (Mouchet et al., 1992).

One of the earliest studies which continues to be cited in reference to localisation of catecholaminergic terminals within the spinal cord is that of Carlsson et al. (1964), in which fluorescent cells at various levels of the spinal cord were noted to gradually decrease in number following an upper thoracic spinalization. This data thereby alludes to a supraspinal origin of those end terminals. Selective LC lesions also resulted in a significant decrease in the number of spinal cord cells labelled by this method (Nygren and Olson, 1977, Commissiong et al., 1978, Commissiong, 1981) though different degrees of this effect were noted between different laminae and between cell columns. Bilateral lesions of LC almost entirely abolished fluorescence in laminae IV-IX, only partially ablated the staining in laminae I-III, and had no effect on lamina X and the thoracic sympathetic lateral column (Nygren and Olson, 1977, Commissiong et al., 1978). Unilateral LC lesions or mid-thoracic

hemi-sectioning of the spinal cord also resulted in significantly reduced fluorescence on both sides of the cord, indicating a bilateral innervation (Commissiong, 1981).

Greater insight into the exact supraspinal location of the noradrenergic perikarya was gained via retrograde tracing studies, employing either the broad spectrum HRP technique or a more selective DBH-antibody approach. Injection of HRP or DBH-specific antibodies into cervical, thoracic, lumbar, or sacral-coccygeal spinal cord segments resulted in labelling of the ventral third of the LC along the majority of its anteroposterior length (Mason and Fibiger, 1979, Guyenet, 1980, Westlund et al., 1981, 1983), in addition to the nucleus subcoeruleus and cells located along the lateral edge of LC in the caudal pons (Guyenet, 1980, Westlund et al., 1981). HRP injected into the upper thoracic region also labelled cells within the pontine reticular formation and in A5, with almost every A5 cell with a positive catecholaminergic phenotype also labelled with HRP (Loewy et al., 1979). Retrograde transport of a DBH antibody also suggested A4 as a minor source of noradrenergic projections (Westlund et al., 1981).

Noradrenergic terminals are found throughout the grey matter at all spinal levels (Mouchet et al., 1992, Ko et al., 1997, Bruinstroop et al., 2011), but are most densely concentrated in the superficial dorsal horn (laminae I and IIo), in the intermediolateral nucleus (IML) within lamina VII, and in the area surrounding the central canal (i.e. lamina X) (Carlsson et al., 1964, Westlund et al., 1983, Schrøder and Skagerberg, 1985, Hagihira et al., 1990, Mouchet et al., 1992). Intense staining is also observed in the ventral horn, in particular in regions surrounding α-MNs at the levels of the cervical and lumbar enlargements (Carlsson et al., 1964, Commissiong et al., 1978, Westlund et al., 1983, Schrøder and Skagerberg, 1985, Mouchet et al., 1992). The greatest fluorescence in the thoracic and upper lumbar segments was localised to the IML and lamina X regions (Westlund et al., 1983, Schrøder and Skagerberg, 1985, Mouchet et al., 1992), though at lower lumbar levels the IML staining was no longer apparent (Schrøder and Skagerberg, 1985).

The noradrenergic cell groups projecting to spinal cord locations in the rat, determined by retrograde labelling studies, may therefore be summarised thusly: A1, A2 and A4 cell groups provide negligible contributions (Akeyson and Grzanna, 1983, Westlund et al., 1983); whilst the A5, A6, and A7 cell groups supply the majority of noradrenergic terminals, particularly those situated in the dorsal horn (Westlund et al., 1983, Maisky and

Doroshenko, 1991, Kwiat and Basbaum, 1992, Clark and Proudfit, 1993, Howorth et al., 2009).

II.A.3 Noradrenergic Receptors

Two classes of adrenoceptor, α and β, were first proposed by Ahlquist (1948) on the basis of their different response characteristics to endogenous and exogenous monoamines, in particular the propylated form of noradrenaline, isoprenaline. These differential agonist effects were confirmed by subsequent researchers, who by using selective antagonists such as yohimbine and piperoxan, proposed a further subdivision of adrenoceptors into α_1, α_2, β_1, and β_2 (Lands et al., 1967a, Langer, 1974, Drew, 1976, Berthelsen and Pettinger, 1977). Subsequently the development of highly selective agonists and antagonists (for examples see sections II.A.3.1 and II.A.3.2) coupled with advances in genomics and proteomics has identified three distinct α_1 adrenoceptors (α_{1A}, α_{1B}, and α_{1D}), three classes of α_2 receptor ($\alpha_{2A,}$ α_{2B}, and α_{2C}), and a further β subclass (β_3), which will be discussed briefly in the next section. For detailed reviews of these studies see Ruffolo et al. (1991), Minneman and Esbenshade (1994) and Coman et al. (2009).

II.A.3.1. Alpha Adrenoceptors

The α_1 class of receptor was initially distinguished from the α_2 subclass on the basis of their anatomical locations, with α_1 located post-synaptically and α_2 located pre-synaptically on peripheral sympathetic nerve terminals (Dubocovich and Langer, 1974, Langer, 1974). Contemporary studies discriminated between different α adrenoceptor subtypes based on a functional approach, citing the example that in frog skin, melanocyte stimulating hormone-induced melanin granule dispersion was inhibited via a post-synaptic but α_2-type receptor (Berthelsen and Pettinger, 1977), therefore supplanting the solely anatomical scheme of classification with one which defined α_1 receptors as mediating excitatory responses and α_2 as mediators of inhibitory effects. However, Drew and Whiting (1979) demonstrated that the post-synaptic vasoconstrictor effects of noradrenaline were inhibited in rat by both the α_1 receptor antagonist prazosin and the α_2 antagonist yohimbine, therefore illustrating that either anatomical or functional approaches alone were not sufficient to fully categorise the α family of adrenoceptors. With the advent of enhanced cloning technologies and increasingly selective ligands, a pharmacological

distinction now seems the most appropriate method by which to discriminate between the receptor subtypes and using these techniques the two α-adrenoceptor subtypes have been further sub-categorised.

Alpha-1-adrenoceptors

Pharmacological heterogeneity of α_1 receptors in the rat brain was first observed in competitive binding assays using radioligands, in which [^{3}H]prazosin was more readily displaced from one population of receptors (the α_{1A} subfamily) by α_1-antagonists WB4101 and phentolamine than another (the α_{1B} subfamily) (Morrow and Creese, 1986). Further confirmation of α_1 subtypes was provided by studies in which treatment of nervous tissue with an irreversible agonist (chloroethylclonidine, CEC) only inactivated around half of the available binding sites (Han et al., 1987, Johnson and Minneman, 1987) and those sites inactivated by CEC seemed to correspond to α_{1B} i.e. low affinity for WB4101, and thus those not inactivated by CEC were the same as those with high affinity for WB4101 from the previous study i.e. α_{1A}. A putative third subtype was identified by cDNA library screening and designated α_{1C} due to its deviations from the expected affinities to α_{1A} and α_{1B} ligands (Schwinn et al., 1990, Schwinn et al., 1991), but was later demonstrated to in fact be a recombinant form of the native α_{1A} receptor (Ford et al., 1994, Hieble et al., 1995). Further cDNA screening also identified an α_{1D} receptor in the rat (Perez et al., 1991) and therefore the currently recognised α_1 receptor family is composed of three members – α_{1A}, α_{1B}, and α_{1D} (Alexander et al., 2011).

Alpha-2-adrenoceptors

Evidence to suggest heterogeneity in the α_2 adrenoceptor family was originally supplied from competitive radioligand binding studies as with the α_1-adrenoceptor family. A comparison of the pharmacokinetics of the α_2-selective antagonist yohimbine and its diastereoisomer rauwolscine (in competition with the non-selective α-receptor antagonist phentolamine) in human platelet and rat cerebral cortex lysates led to the hypothesis that there were two variants of the α_2-adrenoceptor (Cheung et al., 1982), showing either species- or tissue-specificity. However, these authors urged caution in the interpretation of the results given that different tissues from different species were examined. A within-species study into the binding ratios of the partial agonist clonidine against yohimbine lent further credence to the theory, with rat cerebral cortex preferentially binding clonidine whilst rat cerebellum bound yohimbine more strongly (Bylund, 1985). The subclassification

was then proposed based on additional ligand-binding characteristics in rat, with receptors that had a relatively high affinity for the non-selective α-adrenoceptor agonist oxymetazoline and a low affinity for prazosin designated α_{2A}, and the inverse true for α_{2B} receptors (Bylund, 1985). Further subtype selective drugs were rapidly identified through screening assays, confirming oxymetazoline as selective for α_{2A} and identifying chlorpromazine, 7-hydroxychlorpromazine, and ARC-239 as selective for the α_{2B} subtype (Bylund et al., 1988), thus facilitating further characterisation of this family. A third member of the α_2 family was also identified via pharmacological assays. The immortalized opossum kidney (OK) epithelial cell line was found to express an α_2-adrenoceptor that displayed certain α_{2B} characteristics (i.e. prazosin binding) in addition to α_{2A}-like yohimbine binding (Murphy and Bylund, 1988) – a finding which was later confirmed in OK primary cells and rat kidney (Blaxall et al., 1991, Uhlén and Wikberg, 1991) and the receptor named α_{2C}. This subtype was also found during screening of the rat genomic DNA library for homologues to cloned human α_2-adrenoceptors (Lanier et al., 1991) – a procedure that also identified a fourth receptor subtype, which had previously been identified in the rat submaxillary gland as an α_2-adrenoceptor that did not possess a pharmacological profile congruous with any of the three subtypes already known to exist (Michel et al., 1989). It was suggested that this α_{2D} receptor was a member of an even further divided subfamily, and was an example of an α_{2A2}-adrenoceptor on the basis of the varying affinities of the receptors for the agonist guanoxabenz (Uhlén et al., 1993). However, analysis of amino acid identity in addition to comparisons of antagonist affinities determined that the α_{2D} receptor identified in rat was in fact a species homologue of α_{2A} (Kurose et al., 1993, Millan et al., 1993, O'Rourke et al., 1994a), so that in rat the α_2-adrenoceptor family consists of α_{2B}, α_{2C}, and α_{2D} subtypes (Starke, 2001).

II.A.3.2. Beta Adrenoceptors

A non-homogenous population of β-adrenoceptors was first proposed by Lands and co-workers (1967a, 1967b), who observed that different tissue preparations (rat diaphragm, rabbit jejunum, rat uterus, rat adipose tissue, rabbit heart, and guinea pig lung) treated with a series of sympathomimetic amines displayed one of two different response patterns. The first of those studies identified that lipolytic and cardiac *ex vivo* activity were mediated by a different receptor to bronchodilator and vasopressor activity (Lands et al., 1967a), with the second study defining the former category as β_1 and the latter as β_2, following the

observation that the two receptors had different affinities for catecholamine molecules (β_1: isoproterenol > noradrenaline ≥ adrenaline; β_2: isoproterenol > adrenaline > noradrenaline) (Lands et al., 1967b). Tissue-specific effects of subtype-selective ligands (e.g. the effect of salbutamol on bronchial smooth muscle vs. cardiac muscle) demonstrated further the heterogeneity of the β-adrenoceptor family (Cullum et al., 1969, Wasserman and Levy, 1972, Harms et al., 1977) and accelerated the pursuit of potent sub-type specific agonists and antagonists. Competitive radioligand binding assays, exploiting the fact that the two known classes of β-adrenoceptor were expressed in different tissues, identified prototype antagonists including CGP20712A, propranolol, metoprolol, and practolol for β_1 (Minneman et al., 1979, Daly, 1981, Dooley et al., 1986, Beer et al., 1988), with butoxamine and ICI 118551 regarded as β_2 antagonists (Minneman et al., 1979, O'Donnell and Wanstall, 1980, Beer et al., 1988).

Successful cloning of the β_2 receptor from hamster (Dixon et al., 1986) then human (Kobilka et al., 1987) and rat (Buckland et al., 1990), and the β_1 receptor from human (Frielle et al., 1987) and rat (Machida et al., 1990, Shimomura and Terada, 1990), allowed researchers to screen potential ligands for those with the most selectivity and potent functionality.

Investigations into the role of β-adrenoceptors in the lipolytic action of brown and white adipocytes revealed a third 'atypical' member of this family, not conforming to the β_1- or β_2-pharmacokinetics (Arch et al., 1984, Wilson et al., 1984), already named as β_3 (Tan and Curtis-Prior, 1983). The human (Emorine et al., 1989) and rat (Muzzin et al., 1991) genes for the β_3-adrenoceptor were successfully cloned and thus enabled comprehensive *in vitro* screens using known β_1 and β_2 ligands to further characterise this receptor.

II.A.3.3. Molecular Mechanisms in Noradrenergic Transmission

This section provides a brief overview of the molecular mechanisms implicated in noradrenergic signalling: for a detailed review on the subject please refer to Cotecchia (2010) for α_1 receptor mechanisms, Summers and McMartin (1993) for α_2 receptor signalling, and Hall (2004) for β-adrenoceptor actions.

Noradrenergic receptors are members of the guanine-nucleotide binding-protein coupled receptor (GPCR) superfamily, which are transmembrane metabotropic receptors. A detailed review of the G-protein mechanisms involved is beyond scope of this thesis; the reader is therefore directed to Fields and Casey (1997) and Kobilka (2007) for dedicated

reviews on the subject. Briefly, the G-proteins to which the receptors are coupled are heterotrimers, of which the α-subunit is most heavily implicated in the catalytic activity of the complex. Each of the adrenoceptor subfamilies (α_1, α_2, and β) is linked to a G-protein containing different classes of the α subunit after which the G-protein is named (G_q, G_i, and G_s respectively) and thus mediate different intracellular effects. Ligand binding to members of the α_1 family activates phospholipase C, thus increasing the intracellular concentrations of the second messenger molecules inositol 1,4,5-trisphosphate and diacyl glycerol by hydrolysis of phosphotidylinositol 4,5-bisphosphate (Berridge, 1984, Cotecchia et al., 1990). This in turn causes conformational changes in calcium channels towards an open configuration and thus raises levels of intracellular calcium ions. Activation of α_2- and β-adrenoceptors produce downstream signalling effects by adenyl cyclase inhibition and stimulation respectively, thus either lowering or raising the concentration of cAMP within the cell (De Lean et al., 1980, Cotecchia et al., 1990).

II.A.3.4. Adrenoceptor Expression in the Spinal Cord

Whilst adrenoceptor expression is by no means limited to nervous tissue, indeed they are expressed in one form or another in heart, lung, and kidney as well as taste buds, retina and smooth muscle (Drew and Whiting, 1979, Minneman et al., 1979, Hadjiconstantinou et al., 1984, Uhlén and Wikberg, 1991, Myslivecek et al., 2006, Zhang et al., 2010), the interest here lies in their contribution to spinally mediated hindlimb withdrawal reflexes, and so therefore this section with deal purely with the expression of adrenoceptors in the rat spinal cord. Representatives of the three adrenoceptor families (α_1, α_2, and β) are known to be expressed in the spinal cord (Jones et al., 1982) in a form capable of ligand binding, with their laminar and segmental distributions examined closely using in situ hybridization (ISH), autoradiography, electron microscopy, fluorescent labelling, and immunohistochemical techniques.

The α_1-adrenoceptors, located using [^{3}H]prazosin and ISH, are expressed throughout the spinal grey matter in rat along the full length of the cord (i.e. cervical, thoracic, lumbar, and sacral levels) (Roudet et al., 1993, Day et al., 1997). The highest expression occurs around the central canal (lamina X) and the lowest in laminae III to VI (Roudet et al., 1993). At the subtype level, expression of α_{1A} mRNA occurs in cervical, thoracic, and lumbar levels (Day et al., 1997) as well as in lumbar level DRGs (Nicholson et al., 2005), with expression most

concentrated in the intermediate and ventral horn (laminae VIII and IX) (Day et al., 1997, Nicholson et al., 2005). The location of α_{1B} receptors is similar to the distribution of α_{1A}, found throughout the grey matter at all spinal levels and in lumbar DRGs and most concentrated in lamina IX (Day et al., 1997, Nicholson et al., 2005), but at a lower level than the other members of the α_1 family (Day et al., 1997). In contrast, α_{1D} receptors were not found in the superficial dorsal horn or DRGs (Day et al., 1997, Nicholson et al., 2005) but were strongly expressed in lamina IX of the ventral horn (Day et al., 1997).

Autoradiographical analysis of α_2-adrenoceptor spinal cord distribution employing tritiated rauwolscine identified the superficial dorsal horn (laminae I and IIo) as the densest site of expression across spinal levels (Roudet et al., 1994). The receptor subtype localised to these laminae, identified via ISH studies, is both α_{2C} and α_{2D} (though expressed on different neuronal populations) (Stone et al., 1998) with lumbar DRGs also expressing α_{2C} (Nicholson et al., 2005) and α_{2D} found in lamina X and the IML in the thoracic cord (Stone et al., 1998). Further ISH targeting α_{2B} demonstrated that this receptor is also located within the superficial dorsal horn (Nicholson et al., 2005).

The spinal distribution of β-adrenoceptors was initially shown in fluorescent ligand experiments (using 9-aminoacridylpropranolol) to extend throughout the grey matter at all spinal levels with a particular localization to the ventral horn (Melamed et al., 1976). Neurones expressing β_1 are located in the intermediate grey matter (especially lamina VII) and in the motorneurone pools of lamina IX (Nicholas et al., 1993). These latter authors did not find any β_2 expression in the spinal cord, a finding contradicted by later investigations that found this receptor throughout the grey matter, especially in the superficial laminae I and IIo (Mizukami, 2004). The presence of β_3 adrenoceptors in the rat spinal cord has recently been investigated at the sacral level with distribution once again found throughout the grey matter, with an accumulation in the ventral horn (Füllhase et al., 2011).

From the literature reviewed above, the overall distribution of adrenoceptors in the rat spinal cord can therefore be broadly defined at dense accumulations in both the superficial dorsal horn and around the motorneurone pools in the ventral horn, with the dorsal group dominated by the α_2 subtype and the ventral group predominantly containing α_1 and β receptors.

II.B. BULBOSPINAL SEROTONERGIC PATHWAYS

II.B.1 Biosynthesis and Metabolism of Serotonin

The following is a brief description of the production and metabolism of the neurotransmitter serotonin, also known as 5-hydroxytryptamine (5-HT). For a detailed review of these processes the reader is referred to Jonnakuty and Gragnoli (2008), and for a history of their elucidation to Gershon (1977).

5-HT, as well as the hormone melatonin, are two further examples of monoamines and are collectively referred to as tryptamines. Both molecules are derived from the amino acid L-tryptophan that has a chemical structure comprising an indole ring functional group. The rate-limiting and first step in the conversion of L-tryptophan to 5-HT (Gal et al., 1964) is a hydroxylation at position 5 on the aromatic heterocyclic indole group of the amino acid, a process catalysed by tryptophan hydroxylase (TPH) (Grahame-Smith, 1964, Jéquier et al., 1967, Lovenberg et al., 1967) (see figure 1.6). The product of this reaction is 5-hydroxytryptophan (5-HTP). It has recently been demonstrated that two isoforms of this enzyme exist in vertebrates located at different chromosomal positions and are separately responsible for non-neuronal (TPH1) and neuronal (TPH2) serotonin biosynthesis (Walther and Bader, 2003, Walther et al., 2003, Zhang et al., 2004). Decarboxylation of 5-HTP to produce the active neurotransmitter is catalysed by an enzyme that was initially named 5-HTP decarboxylase (Udenfriend et al., 1953, Clark et al., 1954, Udenfriend et al., 1957), but was later demonstrated to in fact be the same enzyme as that known to be responsible for the synthesis of dopamine from L-DOPA (Westermann et al., 1958, Hagen, 1962, Hökfelt et al., 1973, Tison et al., 1991) (see section II.A.1). This enzyme is therefore now referred to as aromatic L-amino acid decarboxylase (AADC).

As with metabolism of catecholamine neurotransmitters, 5-HT is degraded via an intermediate aldehyde molecule (Weissbach et al., 1957) from a monoamine oxidase reaction to produce the metabolite for excretion, 5-hydroxyoindoleacetic acid (Udenfriend et al., 1956).

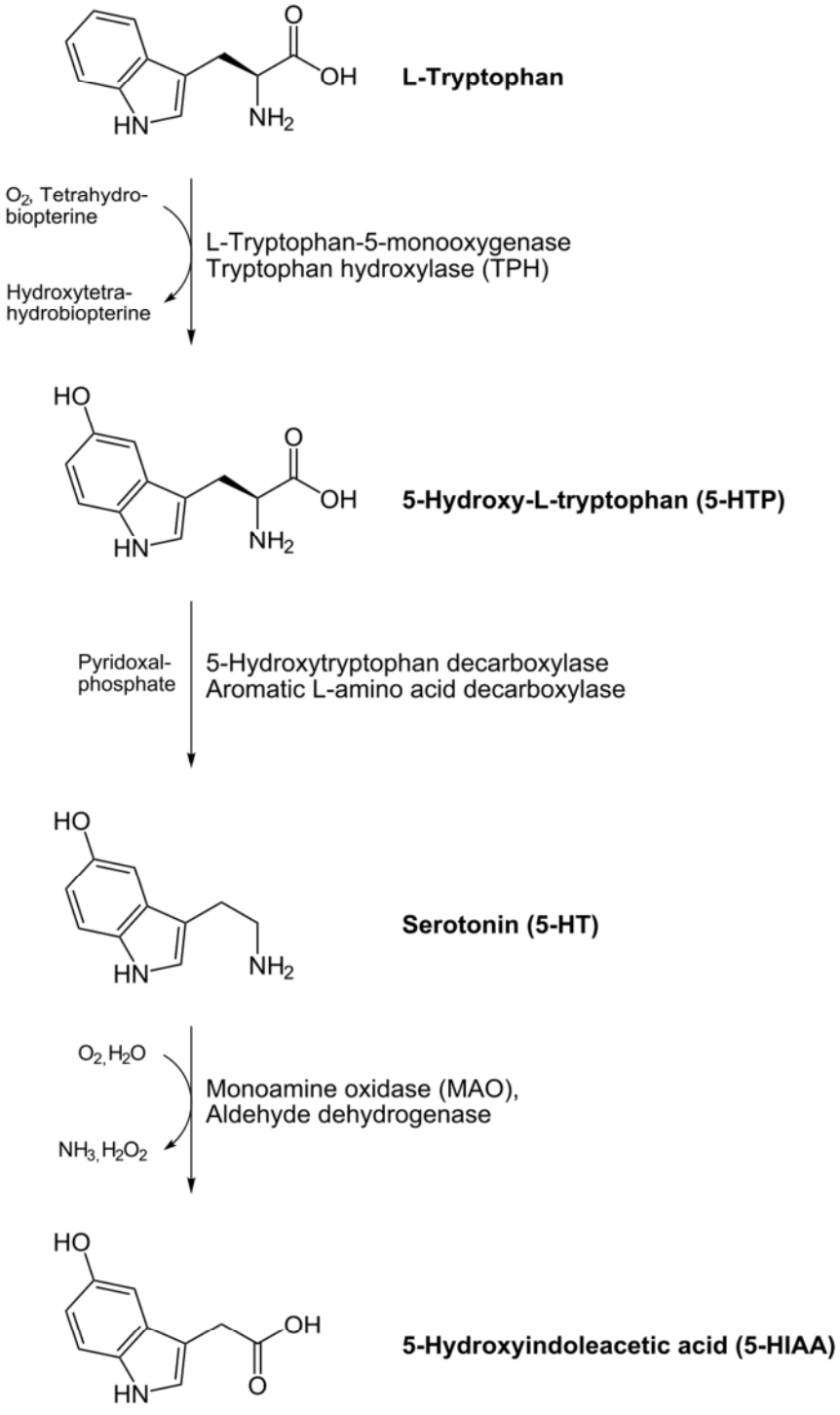


Figure 1.6: Biosynthetic pathway of serotonin.

II.B.2 Serotonergic Cell Locations in the Central Nervous System

Serotonergic perikarya, as with the catecholamine-containing cells detailed in section II.A.2, are monoaminergic and were therefore able to be visualized using the formaldehyde-induced fluorescence histochemical technique (Carlsson et al., 1964). The initial cataloguing of these cell groups was again performed by Dahlström and Fuxe (1964). The proposed serotonergic cell clusters in the brain have therefore been named according to the same principles as the catecholaminergic groups i.e. from B1 to B9, with B1 the caudal-most and B9 the rostral-most of the observed cell groups (figure 1.7). However, the formaldehyde-induced fluorescence method of monoamine visualization was found to be less robust for 5-HT than for NA due to faster decomposition of the fluorescent product upon exposure to ultraviolet light (Gershon, 1977, Steinbusch, 1981), and therefore the earlier documentations of serotonergic cell group locations have since been amended using newer technologies. These have included immunohistochemical techniques targeting either the enzymes required for the synthesis of 5-HT or the neurotransmitter itself (Pickel et al., 1976, Steinbusch, 1981, Weissmann et al., 1987, Grahn et al., 1999, Levin, 2004), protein expression analyses (Brownstein et al., 1975), and autoradiographical methods using either radio-labelled 5-HT or its precursors (Gal et al., 1964, Calas et al., 1976, Araneda et al., 1989).

II.B.2.1. Serotonergic Cells in the Rat Brain

<u>B1 cell group</u>

The caudal-most of the serotonergic cell groups, B1 is also referred to in the literature by its anatomical name – the raphe pallidus nucleus (Dahlström and Fuxe, 1964). Primarily situated in the caudal part of the medulla oblongata between the inferior olives and the pyramidal tract (Palkovits et al., 1974, Jacobs and Azmitia, 1992), the extremities of this cluster extend into the area dorsal to the dorsal accessory nucleus of the inferior olive and ventral to the lateral reticular nucleus in the rat (Dahlström and Fuxe, 1964, Steinbusch, 1981, Jaeger et al., 1984, Weissmann et al., 1987). Serotonergic cells of the raphe pallidus nucleus within the brain stem extend as far caudally as the pyramidal decussation and hypoglossal nerve, and rostrally to the rostral limit of the inferior olivary complex (Bowker et al., 1982, Jacobs and Azmitia, 1992). These anatomical definitions were assigned on the basis of fluorescence studies and immunohistochemistry using probes for TPH, AADC, and

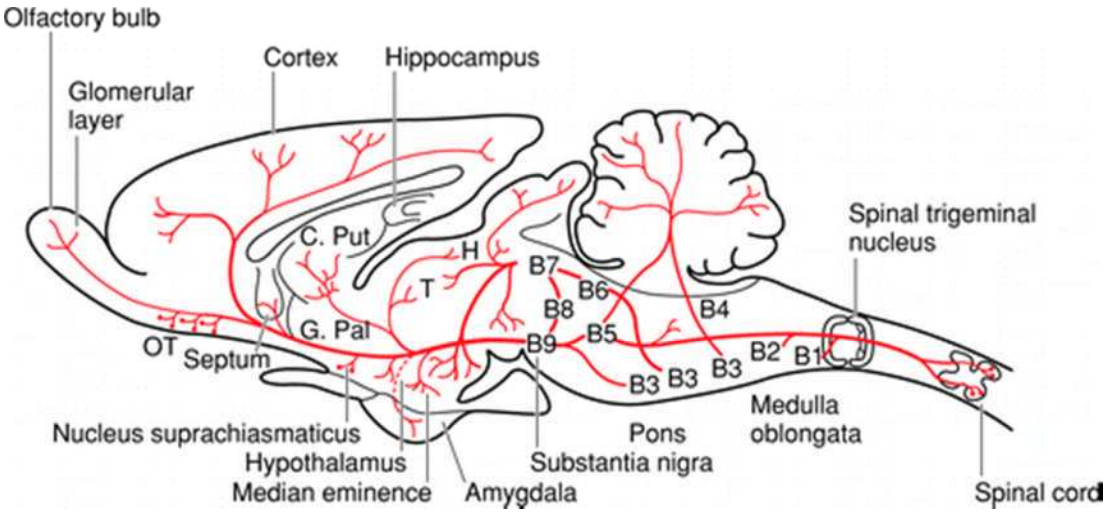


Figure 1.7: Major serotonergic pathways in the rat brain. C. Put, nucleus caudate-putamen; G. Pal, globus pallidus; H, habenula; OT, olfactory tuberculum; T, thalamus. From Pearl and Zigmond (2001).

5-HT, and have been corroborated by the uptake of tritiated 5-HT by cells situated on the median fissure (Gorcs et al., 1985), the ventral-most extent of the raphe pallidus nucleus.

B2 cell group

Serotonergic perikarya are also found in a further medullary cell group – B2, or the raphe obscurus nucleus – which is situated dorsal and rostral to B1 (Dahlström and Fuxe, 1964, Palkovits et al., 1974), at the midpoint of the dorsoventral axis of the medulla (Palkovits et al., 1974). Some cells assigned to this group are located in the most caudal part of the paramedian reticular nucleus at the level of the inferior olive (Steinbusch, 1981). The caudal pole of B2 extends as far as the pyramidal decussation, with some immunoreactive cells also observed just ventral to the central canal in the first cervical spinal cord segment (Bowker et al., 1982, Harding et al., 2004). The rostral limit of this group is level with the rostral inferior olive (Harding et al., 2004). TPH and AADC are expressed in the same cells of the raphe obscurus nucleus (Weissmann et al., 1987, Tison et al., 1991, Patel et al., 2004) which in addition to positive 5-HT-immunoreactivity (Jaeger et al., 1984) confirms the initial findings of Dahlström and Fuxe (1964). Of the three mid-line raphe nuclei found rostral to the area postrema (namely B1, B2, and B4, (Jaeger et al., 1984)), B2 contains the greatest population of AADC-/5-HT-immunopositive cells which are located in their highest numbers within the ventral and intermediate divisions of this nucleus (Palkovits et al., 1974, Bowker et al., 1982, Jaeger et al., 1984).

B3 cell group

Also within the medulla oblongata, serotonergic cell group B3, or the raphe magnus nucleus, was identified by Dahlström and Fuxe (1964) as a fairly large cell group surrounding the pyramidal tract at all levels of the nucleus of the facial nerve. The raphe magnus nucleus extends rostrocaudally from the rostral pole of the superior olive to the emergence of the roots of the hypoglossal nerve (Jaeger et al., 1984, Jacobs and Azmitia, 1992). However, the caudal limit of this nucleus is relatively indistinct and to some degree is intermingled with the rostral-most cells of the B1 group (Dahlström and Fuxe, 1964), therefore some studies refer to the two cells groups as a single entity. 5-HT expression in B3 also encompasses the region anterior to the inferior olivary complex in the ventral reticular formation (Bowker et al., 1982) and the trapezoid body (Steinbusch, 1981, Weissmann et al., 1987, Jacobs and Azmitia, 1992).

B4 cell group

In the formaldehyde-induced fluorescence studies that initially identified the nine separate clusterings of serotonergic neurones, B4 was defined as containing a few small to medium cells just under the fourth ventricle and dorsal to the vestibular and facial nerve nuclei (Dahlström and Fuxe, 1964). The group is located at the level of the medial vestibular nucleus, dorsal to the prepositus nucleus, and rostral to AP (Steinbusch, 1981, Jaeger et al., 1984, Weissmann et al., 1987). This cell group is not found in all species, for example the macaque (see Felten and Sladek, 1983), and does not correspond to any of the raphe nuclei.

B5 cell group

The caudal-most of the serotonergic cell groups located in the pons is B5, or the pontine raphe nucleus (Dahlström and Fuxe, 1964). This group exists as several aggregates along the midline, as far rostral as the mediodorsal border of the medial lemniscus (Jaeger et al., 1984, Weissmann et al., 1987). Since the original classification it has been proposed that B5 is not sufficiently well-defined to be termed a cell group proper, rather it may form the caudal border of B8 (Jacobs and Azmitia, 1992).

B6 cell group

The B6 cell group is not recognised as an independent cell group by many studies, which regard it as a caudal continuation of B7 (Jacobs and Azmitia, 1992, Harding et al., 2004). This group is a small cluster of cells located under the rostral part of the fourth ventricle at the level of, and dorsomedial to, the dorsal tegmental nucleus (Dahlström and Fuxe, 1964, Steinbusch, 1981, Weissmann et al., 1987), approximately at the same level as the noradrenergic LC nucleus (Bowker et al., 1982).

B7 cell group

The largest of the serotonergic cell groups, the B7 group occupies the majority of the dorsal raphe nucleus and extends into the medial longitudinal fasciculus (Dahlström and Fuxe, 1964, Bowker et al., 1982, Köhler and Steinbusch, 1982, Weissmann et al., 1987). The rostral pole of this nucleus reaches the level of the red nucleus and the nucleus of the oculomotor nerve (Bowker et al., 1982, Köhler and Steinbusch, 1982, Weissmann et al., 1987). The caudal boundary is less distinct, as a degree of fusion occurs between B7 and B6, but may be defined as reaching the caudal aspect of dorsal tegmental nucleus (Köhler

and Steinbusch, 1982). Labelling with tritiated 5-HT revealed that many immunoreactive perikarya of this group are located outside the confines of the dorsal raphe nucleus at a ventral position in the caudal periaqueductal grey (PAG) (Clements et al., 1985, Harding et al., 2004). The dorsal raphe nucleus may be considered as composed of three subgroups – dorsomedian, ventromedial, and lateral (Steinbusch, 1981, Kirifides et al., 2001) – the dorsomedian subgroup is located strictly on the midline, the ventromedial subgroup is the largest of the three, and the lateral subgroup is the smallest.

B8 cell group

The median raphe nucleus or B8 is a fairly large group of serotonergic neurones located in the rostral pons in the region of the dorsal part of the superior central nucleus and the dorsomedial portion of the interpeduncular nucleus (Dahlström and Fuxe, 1964, Steinbusch, 1981, Bowker et al., 1982, Singhaniyom et al., 1982, Jaeger et al., 1984). Some 5-HT immunopositive cells belonging to this cluster are found in the medial parts of the tegmental decussations, the medial parts of the area dorsal to linear nucleus, and the rostral part of the decussation of the superior cerebellar peduncle (Steinbusch, 1981).

B9 cell group

The rostral-most of the serotonergic cell groups, B9 does not readily identify with any of the raphe nuclei and as such was not synonymised in the same way, though has more recently been dubbed the supralemniscal nucleus (Vertes and Crane, 1997). B9 is located within and around the medial lemniscus in the mesencephalon from the rostral border of the superior olive to the level of the red nucleus (Dahlström and Fuxe, 1964, Steinbusch, 1981, Jacobs and Azmitia, 1992). Scattered immunoreactive cell bodies of this group are also located within the tegmental reticular formation (Dahlström and Fuxe, 1964, Bowker et al., 1982, Jaeger et al., 1984, Weissmann et al., 1987).

II.B.2.2. Serotonergic Projections to the Spinal Cord

Identification of which of the nine serotonergic cell groups project to the spinal cord, the funiculi through which their axons project, and whereabouts they terminate in reference to the laminar divisions of the cord was established using a combination of retrograde labelling and immunohistochemical techniques.

Supraspinal origins account for the majority of spinal serotonergic fibres and terminals, as in animals in which the spinal cord has been transected the number of serotonergic fibres and the levels of 5-HT itself were shown to be greatly reduced below the site of the lesion (Carlsson et al., 1963, Carlsson et al., 1964, Dahlström and Fuxe, 1965, Holets and Elde, 1982, Hadjiconstantinou et al., 1984, Newton et al., 1986, Newton and Hammill, 1989). Incomplete spinal transections severing only a particular region of the cord provided evidence that serotonergic terminals in different laminae had different supraspinal origins. For example, lesions of the ventral funiculus in the rat eradicated 5-HT immunofluorescence from the ventral horn only, whereas lesions of the lateral funiculi significantly reduced specific staining in the dorsal horn (Dahlström and Fuxe, 1965). Several of these studies noted that 5-HT-positive fibres were not completely lost from the spinal cord segments below the level of the lesion, and the small number that remained may be a result of either incomplete fibre degradation at the time of analysis, fibre regeneration, novel 5-HT synthesis in the remaining tissue, or the few intrinsic serotonergic neurones within the spinal cord which are known to be present (Holets and Elde, 1982, Hadjiconstantinou et al., 1984, Newton et al., 1986, Newton and Hammill, 1989).

Cell groups double-labelled for both HRP (via retrograde transport) and 5-HT in immunohistochemical staining procedures are the raphe pallidus nucleus (B1), the raphe obscurus nucleus (B2), the raphe magnus nucleus (B3), the pontine raphe nucleus (B5), the dorsal raphe nucleus (B7), and the supralemniscal nucleus (B9). Spinal projections do not therefore originate from B4, B6, or B8 (Bowker et al., 1981a, b). The caudal cluster of serotonergic nuclei (B1-B3) project to all spinal segments, whereas neurones located in the rostral groups (B7 and B9) are restricted to the cervical and rostral thoracic segments (Bowker et al., 1982, Kazakov et al., 1993).

The major tracts via which serotonergic neurones in the brain project to spinal regions are the dorsolateral funiculus (DLF) and ventral and ventrolateral funiculi (VLF) with each supplied by distinct cell groups (Bullitt and Light, 1989). Thus 5-HT neurones in B1 project to MNs in the ventral horn via the VLF, along with fibres originating in B2 (Basbaum and Fields, 1979, Skagerberg and Bjorklund, 1985). Cell group B3 primarily supplies terminals in the dorsal horn via the DLF, whilst very few cells double-label with retrogradely-transported HRP and 5-HT markers in B5 (Basbaum and Fields, 1979, Bowker et al., 1981a, Bowker et al., 1982, Skagerberg and Bjorklund, 1985, Bowker and Abbott, 1990). The

laminar terminations of these descending fibres in the dorsal horn aggregate mainly in laminae I and IIi (with a lower accumulation in lamina IIo) (Light et al., 1983, Müllner et al., 2008), with high terminal densities also found in lamina X in the area around the central canal, around the MNs in lamina IX, and in the intermediomedial and intermediolateral cell columns (Müllner et al., 2008).

The serotonergic cell groups projecting to spinal cord locations in the rat, determined by retrograde labelling studies, may therefore be summarised thusly: the medullary cell groups B1, B2, and B3 are the richest source of supraspinal serotonergic projections (Bowker et al., 1981a, b, Skagerberg and Bjorklund, 1985); whilst the B5, B7, and B9 nuclei are the origin of the remaining minority projections (Bowker et al., 1981a, b, Skagerberg and Bjorklund, 1985); and therefore no spinal serotonergic terminals originate from the B4, B6, and B8 cell groups (Bowker et al., 1981a, b).

II.B.3 Serotonergic Receptors

Heterogeneity within 5-HT receptors was initially hypothesised on the basis of functional studies by Gaddum and Picarelli (1957) using the guinea pig ileum as a model system. This work identified two sub-classes of tryptamine receptor, one with a high affinity for morphine, functioning in the depolarization of cholinergic nerves (named the 'M' subtype) and another with a high relative affinity for dibenzyline, involved in the contraction of smooth muscle (named the 'D' subtype). Radioligand binding studies also postulated the existence of multiple 5-HT receptor types in the rat cerebral cortex, in which the psychoactive exogenous antagonist spiroperidol and 5-HT exhibited inverse affinities for two binding sites which a second psychoactive exogenous ligand, lysergic acid diethylamide, did not discriminate between (Peroutka and Snyder, 1979). The receptor subtypes were hence named 5-HT_1 and 5-HT_2, with 5-HT_2 displaying preferential binding of spiroperidol. In order to reconcile the functional and radioligand studies, a review by Bradley et al. (1986) subsequently defined three sub-classes of 5-HT receptor: 5-HT_2 receptors correlated to the spiroperidol high-affinity receptor of Peroutka and Snyder (1979) and the functional 'D' class; 5-HT_3 now represented the 'M' subfamily; and a 5-HT_1-like receptors classification was suggested for any 5-HT-binding site not easily categorised as either of the former. Following advances in the synthesis of highly selective ligands and in molecular cloning technologies, the 5-HT receptor family has been comprehensively sub-

divided into seven different sub-families with at least fourteen distinct members – 5-HT_1 has five subtypes, 5-HT_2 has three, 5-HT_3 has two, 5-HT_5 also has two, and 5-HT_4, 5-HT_6, and 5-HT_7 currently have no subtypes identified. With the exception of 5-HT_3 (which is a ligand-gated ion channel), all of the receptors in this family are members of the GPCR superfamily. Many reviews on this subject are available such as Barnes and Sharp (1999) which provides a highly detailed account. For a more up-to-date though marginally less extensive review refer to Masson et al. (2012). For an exhaustive list of selective ligands for the various 5-HT receptor subtypes, the reader is referred to Barnes and Neumaier (2011). The nomenclature dictated by the most recent review will therefore be adhered to throughout the following sections.

II.B.3.1. 5-HT_1 Receptor Subtypes

The classification of a 5-HT_1 serotonergic receptor subtype occurred as a result of the radioligand binding studies described above (Peroutka and Snyder, 1979). Heterogeneity within that family was subsequently postulated as a result of selective binding assays with ligands such as spiroperidol, ketaserin, and 8-hydroxy-2-di-*n*-propylaminotetraline (8-OH-DPAT) which showed differential affinity for 5-HT_1 receptor subtypes (Pedigo et al., 1981, Middlemiss and Fozard, 1983, Pazos et al., 1984, Peroutka, 1988). There are currently five cloned subtypes of this receptor – A, B, D, E, and F, with all but E assigned to physiological functions (Barnes and Neumaier, 2011).

5-HT_{1A} receptors

8-OH-DPAT was among the first ligands employed in the differentiation of 5-HT_1 receptor subtypes (e.g. Pedigo et al., 1981, Gozlan et al., 1983, Middlemiss and Fozard, 1983) and remains one the most selective agonists for the 5-HT_{1A} receptor (Marcinkiewicz et al., 1984, Alexander et al., 2011). Recently, a new compound (F15599) has been shown to bind to this subtype in the rat with approximately the same affinity as 8-OH-DPAT but with a higher degree of selectivity (Newman-Tancredi et al., 2009), and due to this has since been employed as a radioligand in functional imaging studies, though even more highly selective ligands are currently being investigated (Lemoine et al., 2010). Some of the most selective antagonists for 5-HT_{1A} are robalzotan (NAD-299) and WAY-100635 (Forster et al., 1995, Ross et al., 1999), with robalzotan possibly preferable for use in *in vivo* studies due to the rapid metabolism of WAY-100635 (Larsson et al., 1998).

The rat homologue of the 5-HT_{1A} receptor when cloned and analysed functionally (Albert et al., 1990) demonstrated the mechanism by which it exerts its effects. Agonist binding (e.g. 5-HT) induced an inhibition of intracellular cAMP accumulation (Albert et al., 1990, Lanfumey and Hamon, 2000) indicating that the α subunit of the G-protein to which the receptor is coupled is of the adenyl cyclase-inhibitory subtype. *In vitro* antisense studies implied that 5-HT_{1A} was dominantly coupled to $G\alpha_{i1}$ (Liu et al., 1999) but more recent investigations revealed that in the rat brain the exact G-protein subunits involved in this activity are region-specific (Mannoury et al., 2006). In the cerebral cortex 5-HT_{1A} interacted equally with $G\alpha_o$ and $G\alpha_{i3}$, in the hippocampus mainly with $G\alpha_o$, purely with $G\alpha_{i3}$ in the anterior raphe area, and with $G\alpha_o$, $G\alpha_{i3}$, and $G\alpha_{i1}$ in the hypothalamus (Mannoury et al., 2006) thereby varying the specific downstream effects of 5-HT_{1A} receptor activation in these different brain regions.

5-HT_{1B} receptors

The 5-HT_{1B} receptor was originally differentiated from the other classes of serotonin receptor as a result of its relatively low affinity for spiperodol and 8-OH-DPAT (Pedigo et al., 1981, Middlemiss and Fozard, 1983). On the basis of pharmacological evidence, Heuring and Peroutka (1987) proposed that the previously identified receptor was possibly a rodent-specific homologue of a receptor they characterised in bovine brain membranes and named 5-HT_{1D}. Further subtypes of 5-HT_{1D} receptor were discovered by human cDNA library screening (Hartig et al., 1992), though one of those was proven to in fact be the human homologue of the previously rodent-specific 5-HT_{1B} receptor (Hartig et al., 1992). The currently employed nomenclature therefore recognises that both 5-HT_{1B} and 5-HT_{1D} subtypes are both present in all mammalian species examined, though with regional expression differences (Bruinvels et al., 1994).

Synthetic agonists for this receptor utilised the serotonin molecule itself as a template, leading to the development of the putative 5-HT_{1B} selective ligand RU-24969 and it's derivative compound CP-94253 (Macor et al., 1990), the latter being less potent but more subtype selective, particularly in the rat, than the former (Koe et al., 1992, Hoyer et al., 2002). Molecular cloning of the rat gene for 5-HT_{1B} (Voigt et al., 1991) facilitated the development of selective compounds, and allowed ligands reportedly to be highly specific in other species e.g. SB-224289 in human (Selkirk et al., 1998) to be screened. A compound

that performed well in *in vitro* screens as well as demonstrating reliable antagonist functionality *in vivo* was NAS-181 (Berg et al., 1998, Stenfors et al., 2000, de Groote et al., 2003) and as such is appropriate for investigations into the physiological role of 5-HT_{1B} receptors.

As with 5-HT_{1A} receptors, the downstream effects of the 5-HT_{1B} subtype are potentiated through negative coupling to adenyl cyclase and hence inhibition of intracellular cAMP concentrations, though primarily via the $G\alpha_{i2}$ and $G\alpha_{i3}$ catalytic routes (Lin et al., 2002, Newman-Tancredi et al., 2003).

5-HT_{1D} receptors

As stated above, this receptor subtype was initially postulated as an inter-species variant on the rodent 5-HT_{1B} receptor (Heuring and Peroutka, 1987), but following the identification of two human isoforms of the 5-HT_{1D} receptor (α and β) and subsequent genomic screening, it was confirmed as a 5-HT_1 receptor subclass in its own right (Hamblin et al., 1992). Due to relatively high sequence homology between 5-HT_{1B} and 5-HT_{1D} subtypes there are few truly selective ligands available (see Masson et al., 2012). The original cloning study by Hamblin et al. (1992) found that cyanopindolol and RU-24969 had a higher affinity for the rat 5-HT_{1B} receptor whilst sumatriptan and mianserin exhibited the reverse binding preference. More recent data however demonstrated that both of those putative 5-HT_{1D}-selective ligands are in fact capable of interaction with other receptor families, including adrenoceptors and other 5-HT receptors (Yoshio et al., 2001, Knight et al., 2004), and so whilst they provide useful tools for *in vitro* studies utilising recombinant cell types, the application of these compounds in *in vivo* studies seeking to resolve the role of 5-HT_{1D} receptors in physiological processes is somewhat limited. The 5-HT_{1D} receptor is known to play a role in pain associated with chronic headache (Goadsby et al., 2009, Ivanusic et al., 2011) and is therefore a target for pharmaceutical research. Ligands with known selectivity and affinity for the human orthologue of this receptor are therefore well-documented e.g. L-694247, PNU-109291, and SB-714786 (Beer et al., 1993, Cutrer et al., 1999, Ward et al., 2005, Alexander et al., 2011) though the literature regarding the subtype selectivity of these molecules with regard to rat receptors is sparse.

Analysis of cAMP accumulation in cells expressing the human variant of 5-HT_{1D} revealed that as with the first two members of this family, the downstream effects of agonist

binding are mediated via negative coupling to adenyl cyclase due to the presence of the $G\alpha_i$ subunit in the receptor structure (Hoyer and Schoeffter, 1988, Hamblin and Metcalf, 1991, Weinshank et al., 1992).

5-$HT_{1E/F}$ receptors

Following the successful molecular cloning of two further 5-HT receptors from the human genome – 5-HT_{1E} (Zgombick et al., 1992) and 5-HT_{1F} (Adham et al., 1993) – orthologues of these proteins were also characterised in the rat on the basis of pharmacological evidence (Lovenberg et al., 1993). Advances in molecular biological techniques have allowed the nature of these receptors to be examined further, and have in fact demonstrated that the 5-HT_{1E} receptor found in human tissues is absent from many of the commonly-used small laboratory animal species e.g. mouse, rat, and hamster (Bai et al., 2004), with the rat protein sharing the greatest sequence identity with the human 5-HT_{1F} receptor. *In vivo* studies in the guinea pig first highlighted the selectivity of the agonist LY-334370 for 5-HT_{1F} receptors (Johnson et al., 1997), a compound that has since been employed as a radioligand to identify regional expression patterns for this receptor (Lucaites et al., 2005, Wainscott et al., 2005). At the time of writing, no selective antagonists existed for this receptor (Barnes and Neumaier, 2011).

Again, as with the other members of the 5-HT_1 receptor family, 5-HT_{1F} is negatively coupled to adenyl cyclase though a $G\alpha_i$ subunit and therefore acts to inhibit intracellular cAMP accumulation (Amlaiky et al., 1992, Adham et al., 1993).

II.B.3.2. 5-HT_2 Receptor Subtypes

The 5-HT_2 class of receptors was first defined by their capability to bind spiroperidol with a higher affinity than the 5-HT_1 class (Peroutka and Snyder, 1979) and was subsequently subcategorised further on the basis of differential binding of the radiolabelled 5-HT_2 receptor antagonist ketanserin and agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) (McKenna and Peroutka, 1989).

5-HT_{2A} receptors

The 5-HT_{2A} subtype (previously known as 5-HT_2; see Humphrey et al., 1993, Baxter et al., 1995) was differentiated from other receptors in this family by the binding characteristics

observed for spiroperidol and DOI (Peroutka and Snyder, 1979, McKenna and Peroutka, 1989). Cloning of the rat gene for this receptor (Pritchett et al., 1988) enabled researchers to develop a pharmacological profile for the subtype in this species as a high affinity ketanserin and spiroperidol binding site with low affinity for the 5-HT_1 agonist 8-OH-DPAT (Pritchett et al., 1988). On the basis that ligands in the phenylethylamine class function as agonists for 5-HT_{2A} (e.g. DOI) (McKenna and Peroutka, 1989, Nelson et al., 1999, Monti and Jantos, 2006) a potent high affinity agonist in that class was developed. TCB-2 is capable of activating this receptor *in vivo* (Fox et al., 2010) and is selective for 5-HT_{2A} over the 5-HT_{2C} subtype (Kehne et al., 1996, McLean et al., 2006, Barnes and Neumaier, 2011), which will therefore enable future studies to address the individual roles of these receptors in physiological processes. Volinanserin, or MDL-100907, is a potent and selective antagonist for the 5-HT_{2A} receptor demonstrated through both *in vitro* and *in vivo* screening (Sorensen et al., 1993, Johnson et al., 1996, Kehne et al., 1996). Of particular note is the binding analysis performed in rat frontal cortex homogenates that found binding at a single site with sub-nanomolar affinity (Johnson et al., 1996) which emphasises the receptor specificity of this ligand. The functional activity of TCB-2 and MDL-100907, and hence the downstream effects of 5-HT_{2A} activation, was determined by quantification of phosphoinositide turnover (Kehne et al., 1996, McLean et al., 2006) which was either inhibited or promoted depending on the nature of the ligand investigated. Phosphoinositides are second messenger molecules, the formation of which is catalysed by phospholipase C, and therefore signalling via the activation of 5-HT_{2A} receptors is conducted through G-proteins containing the $G\alpha_q$ subunit (see Masson et al., 2012).

5-HT_{2B} receptors

The rat orthologue of this receptor was first cloned by Kursar and colleagues (1992) from the fundus of the stomach, and under the nomenclature favoured at the time was named 5-HT_{2F} due to the degree of similarity with receptors then known as 5-HT_{1C} (later 5-HT_{2C}) and 5-HT_2 (later 5-HT_{2A}). *In vitro* pharmacological profiling of this novel receptor subtype confirmed its dissimilarity from other members of the 5-HT receptor family, with low affinity for 8-OH-DPAT differentiating it from the 5-HT_{1A} sub-group, lower affinity for ketanserin than both 5-HT_{2A} and 5-HT_{2C}, and higher affinity for the pyridine-substituted indolic compound RU-24969 than 5-HT_{2C} (Wainscott et al., 1993). A reappraisal of the 5-HT receptor families based on structural, mechanistic, and pharmacological data led to this receptor being renamed as 5-HT_{2B} (Humphrey et al., 1993, Baxter et al., 1995).

There are few selective agonists characterised for 5-HT_{2B} receptors (Barnes and Neumaier, 2011), but one which has proven *in vivo* efficacy against this receptor is the 5-HT derivative BW-723C86. Originally demonstrated to be a potent agonist for the 5-HT_{2B} subtype but of unknown selectivity (Ellis et al., 1995, Kennett et al., 1996, Kennett et al., 1997), more extensive investigations and advances in pharmacological testing revealed a ten-fold selectivity over 5-$HT_{2A/C}$ subtypes (Baxter, 1996, Barnes and Sharp, 1999). Of the available antagonists for this receptor, SB-204741 is one of the most selective (Bonhaus et al., 1995, Baxter, 1996) and was first identified through a molecular screening programme (Forbes et al., 1995). This ligand does not seem to have any intrinsic biological activity *per se*, but is capable of blocking the effects of selective agonists (Knowles and Ramage, 1999) and is therefore valuable in determining the physiological role of the receptor. As with 5-HT_{2A} receptors, agonist binding at the 5-HT_{2B} subtype potentiates the activation of phospholipase C and an increase in phosphoinositide levels due to the activity of a $G\alpha_q$ catalytic monomer within the G-protein structure (Masson et al., 2012).

5-HT_{2C} receptors

This receptor subtype, first identified as the now defunct 5-HT_{1C} receptor on the basis of high affinity binding of 5-HT and low affinity for 8-OH-DPAT and RU-24969 (Pazos et al., 1984), was sequenced as a full length clone shortly after this identification (Julius et al., 1988). A number of agonists for 5-HT_{2C} receptors have been reported as selective under assay conditions, though on closer inspection many are capable of interacting with other subtypes at physiological concentrations. WAY-161503 was proposed as a potent full agonist for 5-HT_{2C} receptors with functional activity (Welmaker et al., 2000) but was later shown to bind the 5-HT_{2B} subtype with equal affinity (Rosenzweig-Lipson et al., 2006). Derived from the naturally-occurring ligand psilocin, 1-methylpsilocin showed twelve-fold selectivity for rat 5-HT_{2C} versus 5-HT_{2A} receptors (Sard et al., 2005), but when transferred to *in vivo* studies required the addition of 5-HT_{2A} receptor blockade in order to be efficacious (Halberstadt et al., 2011). Subsequent structure-activity relationship analyses proposed CP-809101 as a high-affinity and selective agonist (Siuciak et al., 2007), which also displayed *in vivo* dose-dependent effects (Strong et al., 2011), but was later found to be genotoxic (Kalgutkar et al., 2007) and therefore unsuitable for pre-clinical pharmaceutical trials. Most recently, the novel compound lorcaserin was shown to bind to rat 5-HT_{2C} receptors with at least five-times greater potency than to the other subtypes in the 5-HT_2 family (Smith et al.,

2008) and is also effective *in vivo* (Thomsen et al., 2008). At the time of writing, this drug was approved for clinical use and may therefore represent a truly selective 5-HT_{2C} agonist. The most selective 5-HT_{2C} antagonist currently available is RS-102221, which in human receptor pharmacological assays had nearly 100-fold selectivity for 5-HT_{2C} over 5-$HT_{2A/B}$ receptors (Bonhaus et al., 1997, Knight et al., 2004, Filip et al., 2012), though lower selectivity was documented for the rat orthologues (Bonhaus et al., 1997).

The signalling mechanism by which 5-HT_{2C} receptor activation initiates intracellular effector pathways is mediated, as with 5-HT_{2A} and 5-HT_{2B} subtypes, via a $G\alpha_q$ G-protein (Hartman and Northup, 1996, Chang et al., 2000, Cussac et al., 2002) to potentiate an increase in the turnover of phosphoinositides (Conn et al., 1986).

II.B.3.3. 5-HT_3 Receptors

Evidence for the third class of 5-HT receptors originates in the guinea pig ileum studies of Gaddum and Picarelli (1957), whose morphine-sensitive 'M' receptor was later reclassified as 5-HT_3 (Bradley et al., 1986). Unique amongst the 5-HT receptor family, the 5-HT_3 receptor is not a GPCR rather it is a cation-selective ligand-gated ion channel (Derkach et al., 1989, Maricq et al., 1991) composed of five subunits each consisting of four trans-membrane domains (for review see Barnes et al., 2009). Heterogeneity within the subunits has been demonstrated by molecular cloning techniques and electrophysiological evidence (Davies et al., 1999, Niesler et al., 2003), and reveals that in human there are five distinct subunits (A-E) of which only A and B are found in rodent (Karnovsky et al., 2003). The human 5-HT_3 receptor may exist as either a homopentamer composed entirely of A subunits or as one of a multitude of possible heteropentamers (Jensen et al., 2008), and the subunit composition of the receptor influences the biophysical and pharmacological characteristics of the channel (Dubin et al., 1999, Kelley et al., 2003, Das and Dillon, 2005), though does not necessarily impact ligand binding in all cases (Brady et al., 2001). Both the A and B subunits have been cloned in rat (Miyake et al., 1995, Hanna et al., 2000).

There are a number of well-established partial agonists for 5-HT_3, such as 2-methyl-5-HT and 3-chlorophenyl-biguanide (Richardson et al., 1985, Kilpatrick et al., 1990, Fozard et al., 1992, Humphrey et al., 1993), but the molecule closest to having full agonistic capabilities described so far is SR-57227A (Bachy et al., 1993). SR-57227A has been characterised

pharmacologically as well as exhibiting dose-dependent *in vivo* effects in the rat (Bachy et al., 1993, Edwards et al., 1996, Verheij et al., 2009, Alexander et al., 2011). Of the antagonists presently available, ondansetron (previously known as GR-38032F, Brittain et al., 1987) and Y-25130 are unrelated on a molecular level but bind to 5-HT_3 receptors with very similar potencies (Sato et al., 1992) and are both selective for the receptor (Brittain et al., 1987, Miyata et al., 1991). For greater detail regarding the effects of ondansetron *in vivo* please refer to Chapter 6.

Activation of 5-HT_3 receptors by endogenous or exogenous agonists does not initiate a complex downstream process comprising several enzymes and second messenger systems as with the G-protein coupled 5-HT receptors. As a ligand-gated ion channel, agonist binding instead triggers a conformational change within the receptor subunits thus opening the trans-membrane pore and allowing the efflux of potassium ions and influx of sodium and calcium ions (Yang, 1990, Hargreaves et al., 1994, Masson et al., 2012). Direct evidence for the conformational changes involved is somewhat lacking due to the methodological challenge of crystallising membrane-bound proteins in different conformational states, but indirect evidence of this process is available (Beene et al., 2004, Ilegems et al., 2005).

II.B.3.4. Other 5-HT Receptors

5-HT_4 receptors

The identification of a 5-HT_4 receptor occurred initially through functional studies in mouse and guinea pig tissue cultures as an atypical 5-HT receptor, that is, one which coupled positively to adenyl cyclase (Dumuis et al., 1988, Bockaert et al., 1990). The rat form of the receptor was cloned from cDNA as two splice variants, differing in their carboxy terminus sequence lengths (and therefore suffixed 'S' and 'L' for short and long, respectively) (Gerald et al., 1995). Following cloning of a greater number of splice variants from the human 5-HT4 gene which were designated a-I (Coupar et al., 2007), the rat isoforms were renamed to reflect the human nomenclature (Hoyer and Martin, 1997). The total number of splice variants of this gene currently identified in rat is three – 5-$HT_{4(a)}$, 5-$HT_{4(b)}$, and 5-$HT_{4(e)}$ (Claeysen et al., 1999), though as the pharmacological disparity between the variants is minimal (Pindon et al., 2004) the ligands discussed in this section relate to the 5-HT_4 family as a whole. There are currently no selective full agonists for 5-HT_4, though several selective partial agonists are available (Barnes and Neumaier, 2011). Of these, ML-10302 and RS

67506 both bind to 5-HT_4 with similar potencies and have almost identical intrinsic activity (Langlois et al., 1994, Eglen et al., 1995a, Eglen et al., 1995b). In addition to this, both ligands have proven activity *in vivo* (Fontana et al., 1997, Yang et al., 1997, Crema et al., 1999, Ishizuka et al., 2002). Antagonists with good potencies, high selectivity, and *in vivo* activity in rodents include GR-113808 and GR-125487 (Grossman et al., 1993, Gale et al., 1994, Schiavi et al., 1994, Castro et al., 2001, Cachard-Chastel et al., 2007, Gribovskaja-Rupp et al., 2012).

The primary sequence of 5-HT_4 receptors, as inferred from the cloned gene, displayed seven putative trans-membrane domains and thus suggested that this receptor also was a GPCR (Gerald et al., 1995). *In vitro* screens using cell-lines expressing the 5-HT_4 subtype from rat, mouse or human demonstrate that receptor activation by serotonin stimulates the formation of cAMP (Claeysen et al., 1996, Van den Wyngaert et al., 1997, Claeysen et al., 1999), and that the α subunit of the G-protein to which the receptor is coupled is therefore of the s-type i.e. adenyl cyclase stimulating (Becker et al., 1992).

5-HT_5 receptors

Two subtypes of 5-HT_5 receptor both with putative GPCR structures were identified in rat as a result of PCR amplification studies (Erlander et al., 1993) and named 5-$HT_{5\alpha}$ and 5-$HT_{5\beta}$. Human orthologues of both genes were later reported, though the 5-$HT_{5\beta}$ gene is in actuality a pseudogene that does not encode a functional protein given the incorporation of stop codons in the open reading frame (Grailhe et al., 2001). The rat 5-$HT_{5\beta}$ receptor is most closely related to the 5-HT_{1A} receptor (Erlander et al., 1993) and accordingly has high affinity with selective ligands for that receptor including 8-OH-DPAT (Wisden et al., 1993). The two are differentiated however by the potency of spiroperidol binding, which is significantly lower for 5-$HT_{5\beta}$ than for 5-HT_{1A} receptors (Wisden et al., 1993). Functional assessment of the rat 5-$HT_{5\beta}$ subtype failed to detect any alterations in cAMP or phosphoinositide levels upon agonist binding (Wisden et al., 1993) and so any physiological role for this subtype is as yet unknown. Conversely, activation of 5-$HT_{5\alpha}$ receptors cloned from both rat and human inhibits the action of adenyl cyclase and thus inhibits cAMP formation (Carson et al., 1996, Francken et al., 2000) and has been shown to function via $G\alpha_i$ and $G\alpha_o$ subunits equally (Francken et al., 2000). Further characterisation of these receptors has been somewhat hindered by a lack of selective agonists, with 5-carboxamidotryptamine currently the best available (Barnes and Neumaier, 2011). This

agonist is far from optimum however as it also binds as a full agonist to many of the other 5-HT receptor subtypes in rat, including 5-HT_{2B}, 5-HT_6, and 5-HT_7 receptors with similar potency or higher than is known for 5-$HT_{5\alpha}$ subtype (Erlander et al., 1993, Ruat et al., 1993b, Wainscott et al., 1993, Boess et al., 1997). A compound named SB-699551 was thought to be a selective competitive antagonist, and does show good selectivity in guinea pig and human, but in rat binds preferentially to the serotonin transporter protein and therefore it's application in studies in this species may be limited (Corbett et al., 2005, Thomas et al., 2006).

5-HT_6 receptors

Further cDNA library screening led to the cloning of another 5-HT receptor from the rat genome named 5-HT_6 (Ruat et al., 1993a) and was categorised as a seven-span trans-membrane receptor i.e. a GPCR based on hydropathy analysis (Monsma et al., 1993). In addition to rat, 5-HT_6 receptors have now been identified and sequenced in mouse, human, and non-human primates (Kohen et al., 1996, Kohen et al., 2001, Kroeze and Roth, 2006). Assays performed *in vitro* using cell expression systems and functionally active ligands including 5-HT demonstrated an increase in intracellular cAMP levels that was dependent on ligand binding and that the 5-HT_6 receptor must therefore positively couple to adenyl cyclase (Ruat et al., 1993a, Boess et al., 1997). Several compounds were proposed as selective agonists for the 5-HT_6 subtype, including EMD-386088, WAY-181187, and WAY-208466 and were tested *in vivo* (Mattsson et al., 2005, Cole et al., 2007, Schechter et al., 2007, Loiseau et al., 2008, Carr et al., 2011). However, the selectivity of EMD-386088, whilst acceptable for human receptor subtypes (Mattsson et al., 2005), has not been demonstrated in rat in an *in vitro* model (though has been employed as a test compound in psychotropic studies in this species, Nikiforuk et al., 2011). The WAY compounds also have good levels of selectivity for human receptor subtypes (Cole et al., 2007), but are currently proprietary drugs of Pfizer Inc. and are therefore not readily available for testing. A range of antagonists for this receptor however are accessible, and include the radioligand SB-258585 (Hirst et al., 2000), the non-functional though highly selective antagonist Ro-630563 (Boess et al., 1998, Sleight et al., 1998), and the functionally-active and selective antagonist SB-399885 (Boess et al., 1997, Hirst et al., 2006).

5-HT_7 receptors

The more recently discovered of the 5-HT receptor family, the 5-HT_7subtype is not a single entity, rather the gene in both rat and human encodes three splice variants (a, b, and c, in rat; a, b, and d in human) (Ruat et al., 1993b, Heidmann et al., 1997). The splice variants differ in the length of the carboxy-terminal sequences and the number of phosphorylation sites present (Vanhoenacker et al., 2000) but not in ligand binding profiles or intracellular events following activation (Heidmann et al., 1997, Krobert et al., 2001). To date, the availability of selective agonists is limited, with LP-44 one of the better characterised examples. This ligand displays high selectivity for 5-HT_7 receptors over the majority of other 5-HT receptors, though marginally less so in the case of 5-HT_{1A} receptors (Leopoldo et al., 2004). One of the better antagonists in terms of affinity and selectivity is SB-269970, though this does have a low but measurable affinity for the human variant for 5-$HT_{5\alpha}$ receptors (Lovell et al., 2000). However, this antagonist has been shown to populate a single binding site in the rat brain, and in *in vivo* measurements reveal that it is capable of penetrating the blood-brain barrier (Hagan et al., 2000, Thomas et al., 2002). The downstream signalling effects of activation of 5-HT_7 receptors is mediated through an increase in intracellular cAMP levels as a result of a positive coupling to adenyl cyclase (Ruat et al., 1993b, Barnes and Neumaier, 2011, Masson et al., 2012), thus the 5-HT_7 subtype is the third of the 5-HT receptor family to modulate cellular activity via this mechanism.

II.B.3.5. Serotonergic Receptor Expression in the Spinal Cord

Serotonergic receptors are expressed throughout the central and peripheral nervous systems as well as in smooth muscle, blood vessels, and the gastrointestinal tract, with receptors of different subtypes expressed in different somatic locations. For example, the 5-HT_{5A} receptor is expressed primarily within the CNS whilst 5-HT_3 receptors are found in both the peripheral and central nervous systems (Erlander et al., 1993, Morales et al., 1996, Fonseca et al., 2001), and 5-HT_{2B}, 5-HT_4, and 5-HT_7 receptors are all expressed in the gastrointestinal tract (Bard et al., 1993, Choi and Maroteaux, 1996, Liu et al., 2005). As with the adrenoceptors (section II.A.3.4), the interest here lies in the role of serotonergic receptors in modulating spinally mediated hindlimb withdrawal reflexes and sensitization of these reflexes, therefore this section with deal purely with the expression of these receptors in the rat spinal cord. The expression patterns discussed herein were ascertained

through a range of techniques, including radiolabelled ligand binding studies, immunohistochemistry, immunocytochemistry, *in situ* hybridization, and DNA amplification.

5-HT_1 receptors

The location of 5-HT_{1A} receptors has been frequently analysed via the means of radiolabelling with the selective and well-characterised tritiated agonist 8-OH-DPAT (Pazos and Palacios, 1985, Daval et al., 1987, Huang and Peroutka, 1987, Marlier et al., 1991, Pompeiano et al., 1992, Thor et al., 1993). Expression of this receptor in the rat spinal cord is concentrated in the dorsal horn, and in particular in the most superficial laminae i.e. laminae I and II (Pazos and Palacios, 1985, Daval et al., 1987, Marlier et al., 1991). The dorsal horn excepted, the densest staining and hence localisation of 5-HT_{1A} receptors is in the area of grey matter situated along the dorsal columns and above the central canal, which forms a characteristic 'V' pattern extending from the central canal to the dorsolateral tip of the superficial laminae in a symmetrical fashion (Marlier et al., 1991, Thor et al., 1993). Immunolabelling with specific antibodies and *in situ* hybridization corroborate the distribution observed in radioligand studies with the dorsal horn the most immunoreactive division of the spinal cord and the 'V' pattern again detected (Pompeiano et al., 1992, Kia et al., 1996). These tests have greater sensitivity than the ligand binding analyses and therefore also revealed that the expression of 5-HT_{1A} receptors in the superficial dorsal horn has a layered appearance, with a band of low immunoreactivity bounded on its dorsal and ventral aspects by regions of higher expression (Thor et al., 1993, Kia et al., 1996). Furthermore, expression of 5-HT_{1A} receptors in the rat spinal cord occurs along a rostrocaudal concentration gradient, with higher levels observed in the more caudal segments (Marlier et al., 1991, Thor et al., 1993).

Radiolabelling studies with either iodocyanopindolol or RU-24969 in addition to *in situ* hybridization data determined that 5-HT_{1B} receptors were also most highly expressed in the dorsal horn laminae (Pazos and Palacios, 1985, Marlier et al., 1991, Voigt et al., 1991), with the densest staining observed in laminae I, III, and IV (Thor et al., 1993). High density labelling was also found in the dorsal commissural grey matter and in the area dorsal to the central canal which formed the same 'V' pattern as described above for 5-HT_{1A} sites (Thor et al., 1993). A rostrocaudal expression gradient with a tendency to increase in the caudal

direction was observed as with 5-HT_{1A} receptors, in particular in the region bordering the central canal (Marlier et al., 1991).

Investigations into the spinal laminar localization of the 5-HT_{1D} receptor in the rat have not been widely performed, perhaps due to a lack of knowledge regarding ligand specificity in this species (see above). A recent study examining this issue via immunohistochemistry noted that this receptor is also most highly expressed in the superficial laminae of the dorsal horn, that is lamina I and IIo, with small numbers of receptors demonstrable in lamina V (Potrebic et al., 2003).

The 5-HT_{1F} receptor has also not been widely investigated in terms of its location in the rat central nervous system, perhaps again as a result of current limitations in the availability of selective ligands for this receptor. *In situ* hybridization studies have however been performed using dorsal root ganglion tissue from rats and have demonstrated that 5-HT_{1D} receptors are present at this site whereas 5-HT_{1F} receptors are absent (Pierce et al., 1996, Nicholson et al., 2003).

5-HT_2 receptors

Although selective ligands are currently available for receptors of this category, early radiographical studies were performed using ligands that are now known to lack selectivity, such as ketanserin and mesulergine (Pazos et al., 1985, Pranzatelli et al., 1992, Thor et al., 1993). The spinal localisation of 5-HT_2 receptors is therefore better established by the use of specific antibodies and mRNA probes.

The laminar arrangement of 5-HT_{2A} receptors in the rat is concentrated within the ventral horn, and in particular to the motorneurone pools of lamina IX (Pompeiano et al., 1994, Maeshima et al., 1998, Cornea-Hébert et al., 1999, Fonseca et al., 2001, Doly et al., 2004b). High density labelling is also found in the intermediolateral nucleus (Doly et al., 2004b), with moderate punctate staining occurring throughout laminae IV-VIII (Cornea-Hébert et al., 1999) and very occasional yet intense staining in the dorsal horn laminae (Maeshima et al., 1998). Most recently however, the inner zone of lamina II has also been shown to express the 5-HT_{2A} receptor in the rat (Doly et al., 2004b, Van Steenwinckel et al., 2009).

The 5-HT_{2B} receptor, as discussed above, was originally identified from rat stomach tissue and was therefore thought to be expressed primarily in gastrointestinal tissues (Kursar et al., 1992). However, central and peripheral nervous tissue have also been proven as sites of 5-HT_{2B} receptor expression in rat and human (Kursar et al., 1992, Helton et al., 1994, Duxon et al., 1997) with a potential role in regulating 5-HT release (Doly et al., 2008), though a spinal location has not yet been imaged for this receptor.

Studies investigating the spinal patterns of 5-HT_{2C} expression have been performed with radioligands (e.g. DOI or mesulergine, both previously thought to be selective for this subtype) and mRNA expression analyses. Specific labelling of 5-HT_{2C} receptors was found to occur in moderate levels across the spinal grey matter at all spinal segments (Pompeiano et al., 1994, Fonseca et al., 2001), with a slightly greater accumulation observed in the ventral horn (Thor et al., 1993). Intense staining was noted in lamina V, the intermediomedial and intermediolateral nuclei, and the lateral spinal nucleus (Molineaux et al., 1989, Fonseca et al., 2001) whilst relatively low hybridization occurred in lamina II (Fonseca et al., 2001).

5-HT_3 receptors

Significant expression of this receptor was first observed in the superficial dorsal horn, specifically in laminae I and II only, using the selective radioligands zacopride and LY-278584 (Hamon et al., 1989, Gehlert et al., 1991). The spinal segments with the densest staining were located in the cervical and lumbar regions, thus lacking the rostrocaudal expression gradient observed for receptors of the 5-HT_1 subtype (Doucet et al., 1999). Subsequent immunohistochemical studies confirmed these findings with moderate staining found throughout the grey matter and concentrated bands localised to laminae I and II (Kia et al., 1995, Doucet et al., 1999, Maxwell et al., 2003, Conte et al., 2005). However, in contradiction to the studies describing a preferential localisation of 5-HT_3 receptors to the dorsal horn in the rat, Fonseca et al. (2001) performed *in situ* hybridization analyses using selective RNA probes and found lower expression levels in the dorsal horn than in the intermediate, ventral, or central grey matter other than at sacral levels in which expression was generally uniform, and the highest expression localised to lamina IX. As no other study either prior to or following that publication has observed this inverse staining pattern i.e. greatest density in the ventral horn, it seems likely that the unusual pattern is a perhaps a result of methodological variation.

Other 5-HT receptors

Information regarding the rat spinal cord expression patterns of the remaining 5-HT receptor classes (5-HT_{4-7}) is not currently adequate to source a full and detailed account of differential expression from. A small number of studies have been published however, the findings of which are summarised here.

The cervical spinal cord expression of 5-HT_4–like binding sites has in the rat been investigated using the tritiated antagonist GR-113808, which demonstrated that the greatest binding densities occur in laminae I and II particularly when compared to the levels found in the ventral horn (Waeber et al., 1994). The receptor subtype 5-$HT_{5\alpha}$ is also most highly expressed in the superficial dorsal laminae at all spinal levels, and is also present in relative abundance in the dorsolateral nucleus of lamina IX within the lumbar spinal cord (Doly et al., 2004a). 5-$HT_{5\alpha}$ receptors were also present to a moderate degree in lamina X (Doly et al., 2004a). With regard to the expression of 5-HT_6 receptors in the rat spinal cord, the published literature on the subject is contradictory. Immunohistochemistry and reverse-transcription PCR analyses indicated that this receptor is expressed at both the transcript and protein levels (Gérard et al., 1996, Gérard et al., 1997), whereas *in situ* hybridization and real-time PCR studies found evidence to the contrary (Ward et al., 1995, Hirst et al., 2003). Ligands for this receptor, when administered intrathecally in *in vivo* tests of nociceptive processing, have been shown to have either pro-nociceptive effects or to have no effect, and so the role of these receptors and indeed even their presence in the rat spinal cord remains unknown (Castañeda-Corral et al., 2009). The distribution of 5-HT_7 receptors is similar to 5-HT_1 receptors in that the densest localisation is found in the superficial laminae, in this case in laminae I and IIi but less so in lamina IIo (Doly et al., 2005).

2. MATERIALS & METHODS

Studies were performed under the UK Animals (Scientific Procedures) Act of 1986 and following approval from the Local Ethical Review Committee. Experiments were performed on adult male Wistar rats weighing between 250 and 370 g, which were obtained from Harlan Animal Laboratories, UK. Animals were housed on a 12 hour light-dark cycle at 19-23°C and 55% ± 10% relative humidity, and had ad libitum access to water and food (Teklad Global 2018 rodent maintenance pelleted diet, Harlan UK). Cages had a floor area of 1820 cm^2 (Techniplast 1354G Eurostandard Type IV) fitted with raised lids, and animals were supplied with environmental enrichment in the form of play tunnels and chew bricks as standard (Datesand).

2.1 Surgical Preparation

All procedures and protocols were carried out in accordance with, and under the approval of, the UK Animals (Scientific Procedures) Act of 1986, and following approval from the local Ethical Review Committee. Experiments were performed on three surgically distinct preparations, for which details of the surgical procedures are given below. Briefly, the three preparations were:

i) anaesthetized – trachea, carotid, and jugular cannulated; intact neuraxis
ii) decerebrate – as above, with the addition of the opposing carotid temporarily occluded; decerebrated at the pre-collicular level
iii) spinalized – surgery as for the decerebrate preparation, with the addition of a spinal cord transection following a laminectomy procedure.

The detailed surgical methodology which now follows applies to all three preparations unless otherwise stated.

2.1.1. Anaesthesia

General anaesthesia was induced in an anaesthetic chamber by isoflurane inhalation (IsoFlo, Abbott Laboratories Ltd). Carrier gases of nitrous oxide and oxygen were administered in a ratio of two to one i.e. flow rates of 1.2 L min^{-1} and 0.6 L min^{-1}

respectively, with isoflurane at 3-3.5%. Sufficient depth of anaesthesia was assessed by the absence of a righting reflex and decreased respiratory rate from approximately 110-120 breaths per minute in awake animals to 60-70 breaths per minute (Duong, 2007). At this stage the animal was removed from the induction chamber and transferred to a face mask with reduced flow rates of 0.6 and 0.3 L min^{-1} respectively and with the level of isoflurane also reduced to between 2.0 and 2.75%. Thermoregulation of the animal was artificially maintained throughout using a Harvard thermostatically-controlled heating blanket set to 37.5 ± 0.5^{o}C which received feedback from a rectal probe.

2.1.2. Cannulation

With the animal in a supine position, the hair overlying the throat was removed and local anaesthetic (2% lignocaine hydrochloride, Lignol, Dechra Ltd) injected subcutaneously to minimise nociceptive input at the site of the incision. Depth of anaesthesia was assessed by pinching the skin over the trachea: if no reaction was observed to this stimulus then surgery commenced. An incision was made in the skin along the line of the trachea, with the underlying fat and connective tissue separated by blunt dissection. Further Lignol was then injected into the sternohyoid muscle before it was bisected to expose the trachea beneath. This was then cannulated (Portex; fine bore polythene cannula, outside diameter 2.42 mm, inside diameter 1.67 mm) so that anaesthesia could be continued via to and fro re-breathing of 2 to 3 % isoflurane in nitrous oxide and oxygen (2:1).

Approximately 0.5 to 1 cm of the left carotid artery was cleared of surrounding tissue and ligated at its rostral end, with particular care taken to preserve the integrity of the associated vagus nerve. To allow constant measurement of arterial blood pressure, a polythene cannula (Portex; outside diameter 1.0 mm) was then inserted into the vessel and firmly tied into position. The cannula had previously been filled with heparinised Ringer's solution (10 IU mL^{-1}) to prevent the formation of blood clots. In one experiment the left carotid artery was not able to be cannulated, therefore the vessel was tied off and the cannula was placed in the right artery. The left jugular vein was also cleared of surrounding tissue and ligated in the same way as the artery, to allow for the insertion of two Ringer-filled polythene cannulae (Portex; outside diameter, 0.63 mm). This intravenous (i.v.) line allowed rapid systemic application of anaesthetic agents and also provided an additional route for administration of anaesthetic and other fluids. In five experiments the left jugular

was not able to be cannulated, and so the vessel was tied off and the i.v. lines were placed in the right jugular vein.

In the anaesthetized preparations (i.e. intact neuraxis), anaesthesia was maintained throughout recordings by continuous i.v. administration of 10 mg mL^{-1} alfaxalone (Alfaxan, Vetoquinol UK).

2.1.3. Decerebration

For decerebrate and decerebrate-spinal preparations, in addition to the cannulation procedures above, the right carotid artery was exposed and carefully dissected away from the vagus nerve branches and then reversibly occluded using a temporary vessel clip in order to reduce bleeding during the decerebration process. A detailed methodology for the decerebration procedure is provided in Chapter 3. Briefly, a bilateral craniectomy was performed to allow ligation of the superior sagittal sinus before the central portion of the parietal bone was removed. A coronal section of the brain was performed immediately rostral to the colliculi and the forebrain and regions of the cortex lateral to the colliculi removed by suction. Haemostasis was achieved using haemostatic sponge and tissue adhesive (Spongostan and Vetbond). The cranial cavity was then loosely filled with cotton wool which was subsequently soaked with paraffin oil to prevent tissue dehydration. To maintain blood volume during decerebration, animals were continuously infused i.v. with a solution of D-glucose and sodium hydrogen carbonate (both at 100 mM) prepared in reverse osmosis water.

Following completion of surgery the animal was secured to magnetic bases by means of Plaster of Paris bandages and custom-made plastic supports to guard against excessive motor activity often seen in decerebrate animals (Woolf, 1984) and the isoflurane anaesthesia discontinued, which was approximately 30 minutes following completion of decerebration. The vessel clip securing the intact carotid artery was then carefully opened and withdrawn.

2.1.4. Spinalization

Animals to be spinalized underwent a laminectomy prior to decerebration. The hair overlying the lower thoracic regions of the spinal column was removed and a midline

incision made from between the scapulæ to the upper lumbar vertebrae. A blunt dissection of the superficial fascia was performed to expose the latissimi dorsi muscles which were then incised down either side of the spinous processes using a scalpel. The vertebral bones were exposed by removal of muscle from around the spinal segments using rongeurs, with care taken not to extend the myectomy further rostral than T8 due to the potential proximity of the azygos vein (de Medinaceli, 1986). The T8 and T9 vertebrae themselves were cut away beginning with the spinous process of T9, enlarged from the facet joint between T9/10, and extended in a rostral direction so as to expose the dorsal surface of the spinal cord. The spinal cord was then transected at spinal segment T9/10 (the rostral-most region exposed by the laminectomy) via a transverse cut made with surgical scissors which was enlarged rostro-caudally by aspiration to form a 1-2 mm cavity thereby ensuring a complete section. The remaining void was loosely filled with Spongostan which was then moistened with Ringer's solution to prevent tissue dehydration at the site of transection. Ringer-soaked cotton wool was placed over the exposed surface of the cord to keep the tissues moist and the bisected skin and muscle were then sutured together over the laminectomy.

Both decerebrate and decerebrate-spinal preparations were maintained on a sedative level of Alfaxan (1 mg mL^{-1} prepared in a solution of D-glucose and sodium hydrogen carbonate both at 100 mM) to prevent excessive destructive movements following the completion of all surgical procedures. All animals of all three preparations were also maintained on room air enriched with 0.1 L min^{-1} oxygen.

2.1.5. Stimulation and Recording

Most of the hair was removed from the left hind limb and paired, percutaneous varnish-insulated copper wire electrodes were implanted into TA, MG, and BF (knee flexor biceps femoris) muscles using a 23 gauge needle. EMG reflex responses in these muscles were evoked by electrical stimulation of the plantar skin of the foot at the heel and at the metatarsophalangeal joints of the two most lateral toes using paired, stainless steel 27 gauge needle electrodes separated by 2 mm. A silver-silver chloride earthing pellet inserted into an exposed muscle group through a small midline incision in the skin, approximately overlying the region of the upper lumbar vertebrae. In the spinalized preparation this was placed via the laminectomy incision. Electrical stimuli were delivered as constant current

pulses of 1 ms duration from AMPI Isoflex stimulators. The stimulus was typically set to a higher intensity than the threshold value for evoking reflexes up to a maximum of 10 mA. On the basis of previous studies (Clarke et al., 1989) MG and BF reflexes were recorded in response to heel stimulation and TA and BF reflexes were recorded after stimulation at the toes for the establishment of control values. The signals were amplified (NeuroLog NL104A, x5000), filtered (NeuroLog NL125), digitised (Cambridge Electronic Design (CED) analogue-digital converter, 1401), and sent to a personal computer running Signal v.2.08 (CED) to be averaged and integrated (see Figure 2.1).

The electrical stimulus employed throughout these studies as the method by which EMG responses were evoked may be defined as 'non-natural' i.e. unlike chemical or mechanical stimuli encountered in nature, which as such simultaneously and non-selectively evoke responses in all classes of peripheral afferent fibres and therefore precludes the identification of different population responses (Plaghki and Mouraux, 2003). However, electrical stimulation enables the strength and location of that stimulus to be very tightly controlled and maintained during long duration experimental protocols - a factor of critical importance in the studies within this thesis, and is also easily adjusted should experimental conditions require it.

2.1.6. Cardiovascular Measurements

An electrocardiogram (ECG) was recorded by subcutaneous insertion of two needle (length 16 mm, 25 gauge) electrodes either side of the chest. The signals from these electrodes were amplified and used to trigger an instantaneous rate meter (NeuroLog NL253), and arterial blood pressure measurements were made via a pressure transducer (SensoNor 840, Horten, Norway) connected to the carotid cannula. Both measurements were recorded by connecting to a second computer running Spike2 for Windows v.3 (CED), via a CED micro1401 interface.

2.1.7. Sensitizing Stimuli

The acute conditioning stimulus used for the initial mapping of hind limb sensitization fields was 5 µL 20% mustard oil (Aldrich) in paraffin oil, applied to the surface of the skin using a blunted needle, or injected directly into deeper tissues using a 27g needle. This was only applied when three consecutive readings from both the flexor muscles (TA and BF) and

extensor muscle (MG) varied by less than 10% of one another. Mustard oil was applied to a maximum of four sites per animal; the details of site locations are provided within each of the following results chapters (chapters 4, 5, and 6). Treatments were separated by a minimum of 63 minutes.

2.2 Statistical Analyses

Detailed descriptions of the statistical analyses performed are provided within each individual results chapter (sections 4.2.3, 5.2.3, and 6.2.3). In all experiments reflex responses were normalised to produce the mean of the pre-drug control, control levels being expressed as 100%. In each experimental group data are presented as medians and the scatter of responses are indicated by the interquartile ranges of the median values. Analyses of reflex data therefore use non-parametric tests. To compare the effect of a single treatment, such as MO application or a dose-response test, one-way ANOVA were used – either Friedman's ANOVA or Kruskal-Wallis tests were appropriate. Paired data within a given group, such as stimulation parameters, were analysed with Wilcoxon's Matched Pairs test. For comparisons between groups (e.g. treated vs. untreated) in which the time course of the experiment was also a factor, the non-parametric two-way ANOVA Scheirer-Ray-Hare test was used.

Cardiovascular responses were also not normally distributed (assessed using the Kolmogorov-Smirnov normality test) and were therefore analysed using the same non-parametric one-way ANOVA analyses as used for the reflex data.

All statistical analyses were performed in Prism 5 (v.5.0.2, GraphPad Software), except for the Scheirer-Ray-Hare tests which were performed in PASW Statistics 17.0 (SPSS) and Excel 2007 (Microsoft Office).

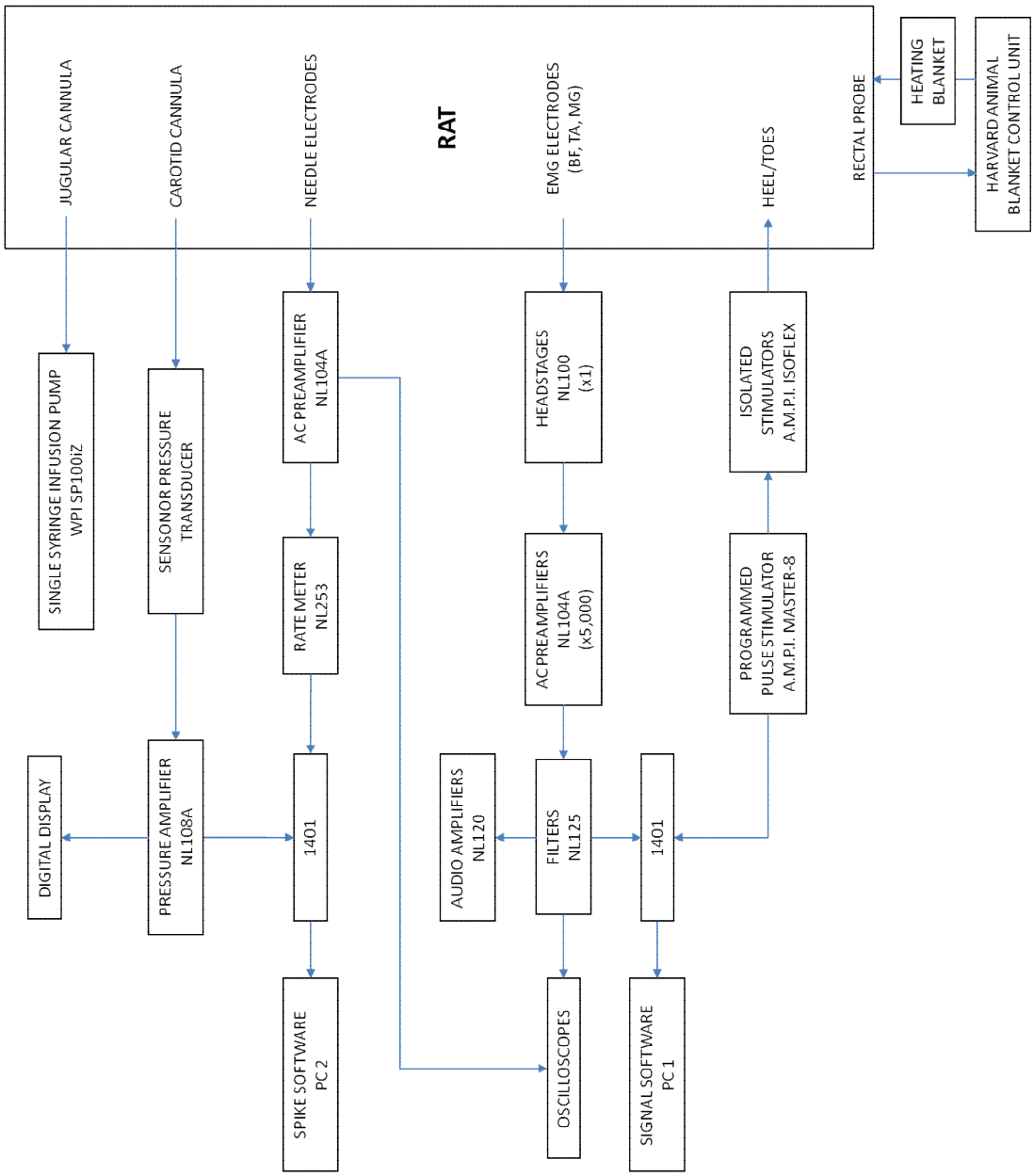


Figure 2.1: Flow diagram of the recording apparatus.

3. A DETAILED SURGICAL METHOD FOR MECHANICAL DECEREBRATION OF THE RAT

3.1 Introduction

Pre-clinical neurological studies which aim to increase understanding of mechanisms involved in chronic pain, such as the present studies, are frequently undertaken in whole animal models given the complexity of the underlying systems. To this end, electrophysiological studies on single spinal neurones or spinal reflexes are routinely performed in deeply anaesthetized animals. However, high levels of anaesthetic inhibit spinal excitability, particularly with respect to reflex responses (Jinks et al., 2003), and interpretation of pharmacological data may be confounded by an interaction between the drug and the anaesthetic agent; the facility to compare causal mechanisms or drug effects in an unanaesthetized model is therefore invaluable.

Decerebration is the removal of higher brain structures or severance of the sensory centres of the brain from peripheral inputs, and may be achieved either surgically via mechanical removal or destruction of specific neural tissues (e.g. Sapru and Krieger, 1978), or ischaemically by oxygen deprivation and hence loss of functionality of targeted sites (e.g. Fouad and Bennett, 1998). Decerebration severs the pathways connecting the sensory processing regions of the brainstem and spinal cord from more rostral structures responsible for the transformation of that stimulus into a perceived event i.e. pain from a nociceptive input. As a result of this, the decerebrated animal preparation does not require anaesthesia (although low level sedation may be required) and can be studied as a neurologically anaesthetized animal model (Yang et al., 1990) as opposed to the chemically anaesthetized alternative (Silverman et al., 2005).

The use of decerebrate animal preparations to gain insight into how the forebrain influences motor outputs without the confound of anaesthetic-induced hypotonia goes back to the early 19th century. Among these early studies was the work undertaken by Rolando (published 1809, later translated by Flourens (1824)), who in experiments performed on a wide range of different species (including but not limited to tortoise, goat, chicken, and sheep) found that by varying the extent of injury to the cerebellum differing effects were observed in terms of loss of movement. These ranged from total paralysis to gradual loss of function of a particular limb dependent on the scale and site of the injury

induced. Later investigations into cerebral control of proprioception and voluntary and involuntary reflexes were performed in decerebrate dogs, with differential effects in terms of motor control occurring dependent on the level of brain transection performed (Longet, 1842, Goltz, 1892) demonstrating the role of the brainstem and spinal cord in control of reflexes. In one such case detailed by Sharpley-Schäfer (1898), the decerebrated dog was able to both walk and eat, and reacted to both noxious and non-noxious stimuli accordingly. Furthermore, bi- or unilateral sectioning of the brain to sever connections between the cerebral hemispheres and the brainstem provided evidence for the selective functionality of those regions in reference to awareness and somatosensing. Hemispherectomised animals exhibited loss of withdrawal reflexes to noxious stimuli (Boyce, 1895) and tonic muscular contractions and rigidity on the opposing side of the body (experiments by Probst (1904) translated in Luciani (1915)), whilst those undergoing bilateral decerebration displayed uniform decerebrate rigidity (Sherrington, 1898).

Critical to the outcome of such experiments is the level at which the transection of the brain is performed. In classical terminology, frequently referred to in contemporary literature, a mesencephalic section separating the forebrain from more caudal structures at the mid-point of the colliculi is the *cerveau isolé* preparation (which translates as isolated forebrain/telencephalon). Alternatively a post-medullary section at either the C1 or C2 segment of the spinal cord is termed the *encephale isolé* preparation (which translates as isolated brain; Gottesmann, 1988). The decerebrate rigidity mentioned above is only observed in the intercollicularly decerebrated animal, whereas the posture of a post-medullary decerebrate animal may be described as drooped or flaccid (Sherrington, 1898) due to sectioning caudally beyond the level of the pontomedullary reticular formation (Katayama et al., 1988). This observation provides preliminary evidence for the role of the aforementioned reticular formation (RFo) in descending control of muscle tone and posture. Efferents from the RFo extend rostrally to thalamic and cortical regions, as well as caudally to the cerebellum and spinal cord (Jones and Yang, 1985, Jacobs and Azmitia, 1992). The RFo is therefore central to sensorimotor integration, encompassing the assimilation of noxious stimuli as well as the co-ordination of withdrawal reflexes. Ascending projections to the medial thalamus and limbic system from the functional component of the RFo known as the reticular activating system (RAS), are crucial to the perception of pain and to consciousness in general (Moruzzi and Magoun, 1949, Muir,

2008), therefore physical interruption of these pathways abolishes that perception (Silverman et al., 2005).

Existing methods of decerebration in the rat typically fall into one of two broad categories: mechanical decerebration by physical transection of the brain; or ischaemic decerebration, in which targeted brain regions are destroyed by cessation of blood supply. These strategies may be further subdivided into mechanical decerebration with or without total removal of the forebrain, and ischaemic decerebration by either surgical ligation of specific blood vessels or by injection of embolitic agents (Fukuda et al., 1974, Tian and Duffin, 1996, Fouad and Bennett, 1998, Lee et al., 2002, Smith et al., 2010, Tsuchimochi et al., 2010). However, although rarely reported, decerebration of rats carries a high risk of haemorrhagic death (Woolf, 1984) hence mortality rates even for the experienced operator can be at least as high as 50% (Fouad and Bennett, 1998). In order to carry out the intended experiments within this thesis, initial attempts at creating the decerebrate model therefore proved problematic and it was clear that procedures previously employed to near perfection in this laboratory in the rabbit were not transferable to the rat. As published papers on the decerebration technique lacked any detailed methodological procedures (and expertise in the UK appeared to be lacking), the decision was taken to perfect the technique within the laboratory. The following details how this was achieved.

3.2 Method Development

Previously, this laboratory has employed a decerebrate rabbit model as a means to test pharmacological agents against hind-limb withdrawal reflexes as well as their intrinsic physiological organization (Clarke et al., 1988) and hence elucidate the nature of supraspinal controls governing sensitization of those reflexes. These animals were rendered decerebrate using a mechanical technique, in which the forebrain was removed by aspiration as far as the superior colliculi, and the basal cranial vessels were occluded by way of aluminium clips (Harris, 1995). This technique, in conjunction with occlusion of both carotid arteries, resulted in minimal haemorrhaging, and that which did occur was easily staunched by application of activated cellulose to the necessary regions e.g. at the rostral face of the superior colliculi. The overall success rate was virtually 100% (Harris, unpublished data). Using the same surgical technique in a rat, the animal rarely survived the surgery and never for periods greater than 1 hour (Fig. 3.1).

3.2.1. Trialled Amendments to Mechanical Decerebration

Preparatory surgery undertaken to cannulate the trachea and blood vessels for these experiments is detailed in section 2.1. All details of the technique used to decerebrate rabbits are based on descriptions in Harris (1995). Note that throughout the development of the technique, due in particular to heavy initial animal losses, there was an ongoing dialogue with the UK Home Office Inspectorate in order to ensure compliance with the terms of the project license in addition to receiving further guidance on the technique.

In order to perform a mechanical decerebration, the operator must first gain access to the cranial cavity via a craniectomy procedure. In rabbits, this was achieved by trephining a small hole in the cranium which was then enlarged using rongeur forceps to expose the cerebral hemispheres. Attempting this procedure in rats (n = 7) caused massive blood loss, and either resulted in the animal's death prior to the completion of decerebration or during the hour following completion (Fig. 3.1). Aluminium clips were applied to the basilar artery (as in the rabbit) in an effort to prevent catastrophic bleeding, but these were discarded as the shearing force associated with their application proved great enough to tear the vessels and resulted in further haemorrhage.

A phenomenon observed during these initial experiments was the tendency for blood loss to occur during the craniectomy itself as opposed to any of the latter stages of the procedure, and with reference to the cerebral vasculature of a rat (Scremin, 2004), this stage of the method was adapted accordingly. In order to preserve the venous sinus (superior sagittal sinus, SSS) which lies in parallel with the sagittal suture, a bilateral craniectomy was devised and implemented. By leaving this central region of the parietal bones intact, the SSS is not ruptured, and the operator is able to apply ligatures at the rostral- and caudal- most points of the craniectomy in order to occlude the vessel.
This adaptation resulted in a complete decerebration being achieved in 2 of the following 3 experiments, but survival times following completion were still less than one hour, and ligating the SSS did nothing to staunch the haemorrhaging generated by severance of the basilar vessels (the basilar artery and cavernous sinus) leaving the success rate still well below the rate that was achieved in the rabbit (Fig. 3.1).

Subsequent experiments which attempted to halt blood loss from those vessels adapted a technique that had been employed previously in the rabbit model as a method to stem residual bleeding – the addition of a haemostatic gelatine sponge (Spongostan, Ferosan). In one experiment, the sponge was also soaked in a bovine thrombin solution (50 U mL^{-1}) in an attempt to encourage thrombosis from the site of bleeding (see Woods, 1964, Tian and Duffin, 1996, Pickering et al., 2002), but this was found to be unsuccessful in fully preventing further blood loss from that site. Three further experiments also utilised a combination of Spongostan and thrombin solution, with the additional application of a tissue adhesive (3M Vetbond, Animal Care Products) to secure the sponge over the ruptured vessels, as it was noted that the rate of haemorrhage was great enough to displace the sponge if an adhesive was not incorporated. As before, this technique was trialled in three experiments, with 2 of the 3 animals surviving decerebration but with no improvement on duration of survival. A subtle modification in which the adhesive was applied to one surface of the sponge prior to it's placement (with thrombin administered *pro re nata*) was carried out in the subsequent 15 experiments. Using this methodology, all animals survived the decerebration procedure, with a median survival time of 0.5 hours (range 0 – 6; Fig. 3.1). Of the 15 animals that underwent decerebration in the manner described above, 10 showed a gradual decline in cardiovascular output or evoked reflexes (7 of which did not survive long enough to enable EMG recordings to commence), 2 manifested extreme decerebrate posturing to the degree that vessel cannulae were unintentionally removed, and 3 suffered terminal blood loss during surgery.

With the surgical procedure optimised to some degree (over 50% surviving to the recording phase, but 100% of animals survived the surgery itself), the focus was shifted to improving the survival times. Many other laboratories utilising decerebrate animal models ligate at least some of the arteries supplying the brain. All the experiments detailed above were performed in rats that had a cannula inserted into the left carotid artery, and a permanent ligature applied to the right carotid artery. Further development of the method with respect to stabilising the preparation and rendering it suitable for a study of this nature (i.e. one in which responses are recorded for a period of at least 5 hours) was therefore undertaken. By occluding both carotid arteries in the manner described, and thereby restricting circulation through the carotid sinuses, homeostatic regulation of systemic blood pressure would be impeded given the relative importance of baroceptors innervating those structures (in conjunction with the baroceptors in the aortic arch) in the feedback-

EXPERIMENTS (inclusive)	PROCEDURE MODIFICATION	PERCENTAGE SURVIVING	SURVIVAL TIME (Hrs)		
			1st QUARTILE	MEDIAN	3rd QUARTILE
1-7	Mechanical decerebration	0%	n/a	n/a	n/a
8-10	Bilateral craniectomy	67%	0	0	0.5
12-15	Thrombin/Spongostan plus Vetbond	25%	0	0	0.25
16-30	Vetbond/Spongostan	53%	0	0.5	2.5
31-85	Right carotid artery reversibly clipped	82%	4.5	7	9

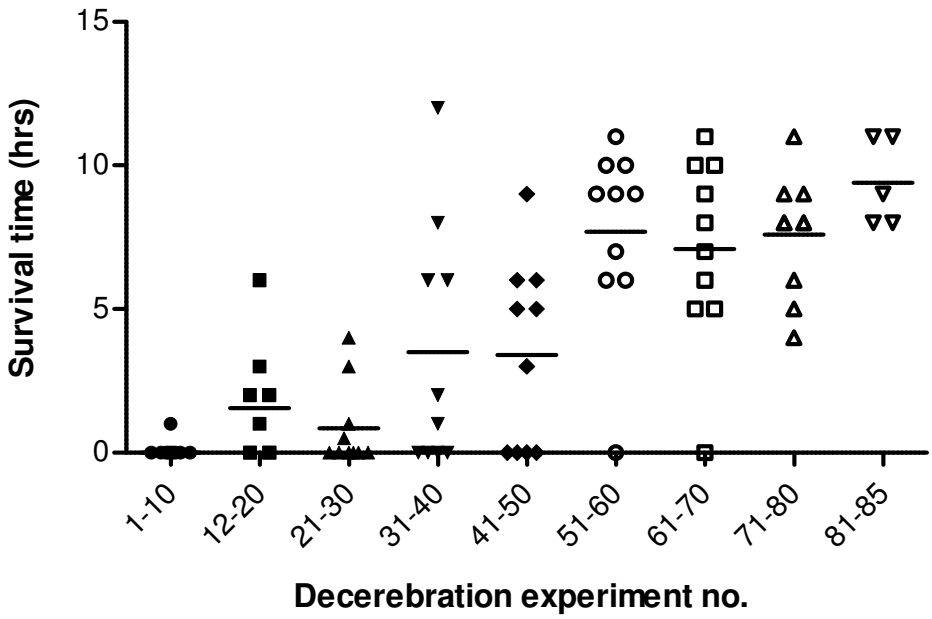


Figure 3.1: Post-decerebration survival times during the course of method development. Percentage surviving is defined as an experiment in which some recording took place, the duration of which is detailed as median values with upper and lower quartiles. These data are expressed graphically with experiments arbitrarily divided into cohorts of 10, average bars are median survival times per group. N.B. Experiment 11 was excluded on the basis that it was a sole experiment in which one carotid artery was left free and therefore is not comparable.

control of that physiological parameter (Tang and Hu, 2011). Theoretically, reversibly occluding one or both carotid arteries so that blood loss during decerebration could be minimised, such that following completion of the procedure circulation to these vessels could be restored, would improve the likelihood that the decerebrate preparation could maintain a stable blood pressure and potentially increase survival times. In larger species, carotid circulation has been occluded via loosely tied ligatures weighed down with haemostatic forceps (e.g. Bazett and Penfield, 1922), but given the friability of these vessels in the rat, the use of a reversible artery clip seemed more appropriate. The subsequent 55 rats in which decerebration was performed received intra-arterial cannulae in the left carotid artery as before, with the right carotid artery carefully clamped for the duration of the remaining surgical procedures and removed following withdrawal of anaesthesia. All 55 survived decerebration, with varying degrees of success. Of this cohort, 8 animals survived less than 1 hour following the completion of surgery, with the most likely explanation a failure to effectively staunch cranial bleeding rapidly enough to maintain a viable blood volume. A further 3 went on to produce reliable evoked reflex responses for periods of less than 5 hours i.e. insufficient time for the experimental protocol to be completed, which cannot be definitively attributed to volume of blood lost during decerebration – the cause of death is therefore unknown in these animals. In 4 of the 55 experiments the cause of death was removal of cannulae during decerebrate posturing and therefore did not occur as a result of a flawed decerebration procedure. The remaining 40 experiments all produced reliable reflexes for periods of greater than 5 hours (the median survival time was 8 hours, inter-quartile range 3.25 hours; Fig 3.1.) and were therefore deemed fully successful. Data obtained from these experiments is detailed in Chapter 4.

3.2.2. Final Method

The mechanical decerebration method was therefore optimised to the following protocol. Most of the scalp hair was removed, an incision made along the mid-line of the head (Fig. 3.2a), and the pericranium scraped back to expose the sagittal, coronal, and transverse sutures in the bone beneath (Fig. 3.2b). Two 3 mm diameter holes were trephined in the cranium either side of the sagittal suture approximately midway between the coronal and transverse sutures using a micro-drill (Fig. 3.2c). These were enlarged to form a large bilateral craniectomy using rongeur forceps with a 1.0 mm bite width leaving the suture itself intact and thereby maintaining the integrity of the SSS (Fig. 3.2d). The craniectomy

therefore extended from just ventral to the temporal line on either side, approximately 5 mm rostral of the coronal suture, and as far caudal as possible whilst preserving the lambdoid suture. The remaining central portion of the parietal bones was ligated at its rostral- and caudal-most points using silk braided suture (USP size 3-0) carefully passed under the bone and sinus with a surgical needle (Fig. 3.2e). With the blood flow in the SSS occluded, the central bone was carefully removed (Fig. 3.2f). Any remaining dura mater was cut and retracted in a caudal direction to expose the dorsal surface of the cerebral hemispheres.

Mechanical decerebration was then initiated by slow careful aspiration of the dorsal-most structures of the cerebral cortices using a 1.65 mm O.D. cannula attached to a vacuum pump (Eschmann DV110) in a caudal to rostral stroking motion. Sufficient layers were removed to the point where the rostral edge of the colliculi was clearly visible (Fig. 3.2g). This point was identified using the posterior cerebral artery as a reliable landmark (which has the appearance of an inverted lowercase Greek letter omega when viewed from a caudal aspect). A complete coronal transection of the brain was then carried out using a microspatula leaving the colliculi intact, with all structures rostral to the point of section then rapidly aspirated (Fig. 3.2h). With the suction still in place to remove any blood caused by rupture of the basilar artery and/or cavernous sinus, a cuboidal piece of haemostatic gelatine sponge (Spongostan, Ferosan) soaked in tissue adhesive (3M Vetbond, Animal Care Products) was pressed firmly downwards at the rostral edge of the superior colliculi to occlude any ruptured vessels and prevent further blood loss from this site (Fig. 3.2i). A further piece of Spongostan was pressed into the olfactory cavity to extrude any portion of the olfactory bulbs not aspirated prior to this point, which also assisted with general haemostasis. Decerebration was completed by gentle suction of all structures lateral and dorsal to the colliculi (Fig. 3.2j). Rupture of the transverse sinuses during this part of the procedure was almost inevitable, and therefore additional pieces of Vetbond-soaked Spongostan were firmly pressed into these cavities to encourage haemostasis (Fig. 3.2k).

Cotton wool was loosely packed into the remaining cavity which was then soaked with paraffin oil to prevent dehydration of exposed brain tissues (Fig. 3.2l). The skin incision was clipped together to further protect the underlying tissues from dehydration. The vessel clip securing the intact carotid artery was removed after cessation of anaesthesia.

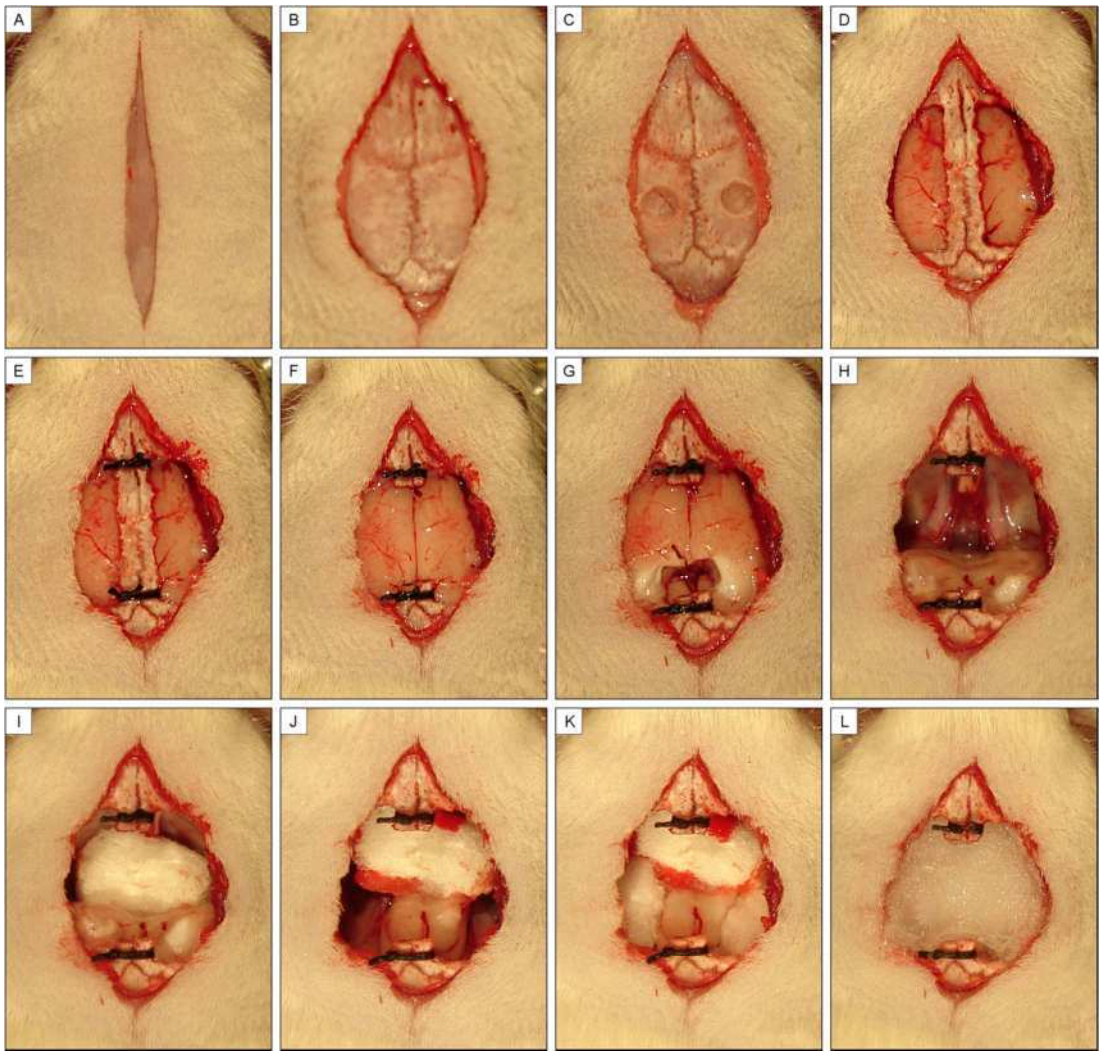


Figure 3.2: Photo series of the stages of the decerebration procedure in a euthanized rat. (A) Dorsal view of the head with a mid-line incision. (B) Exposure of the cranial vertex by scraping back the pericranium. Transverse, coronal, and sagittal sutures are now visible. (C) Bore holes trephined into the parietal bones approximately midway between coronal and transverse sutures. (D) Bilateral craniectomy enlarged using rongeur forceps leaving the sagittal and transverse sutures intact. (E) Rostral- and caudal-most points of remaining portion of parietal bones ligated to occlude the underlying superior sagittal sinus. (F) Medial portion of parietal bones removed using rongeur forceps. (G) Aspiration of dorsal-most layers of the cerebral cortex until the posterior cerebral artery is visible at the rostral edge of the colliculi. (H) Blunt coronal transection at rostral edge of the colliculi and rapid aspiration of all tissue rostral to that point, including olfactory bulbs. (I) Vetbond-soaked Spongostan pressed firmly onto basal vessels at the cut surface. (J) Aspiration of all structures lateral to the colliculi and hindbrain. (K) Additional pieces of Vetbond-soaked Spongostan pressed into lateral cranial cavities to occlude transverse sinuses ruptured during aspiration of brain tissues. (L) Cotton wool loosely packed into main cranial cavity and subsequently saturated with paraffin oil.

At this point removal of brain tissue was complete and the continued presence of isoflurane served solely to inhibit excessive motor activity to which decerebrate rats are prone (Woolf, 1984). Thus following completion of decerebration, animals may either be paralysed for recordings direct from muscle nerves (Marchenko et al., 2002) or, as in the present studies, lightly sedated and restrained for recording electromyograms (Pickering et al., 2002).

3.3 Cardiovascular Effects

Blood pressure fluctuations were monitored in all animals undergoing decerebration, with detailed analysis performed on those in which decerebration was carried out according to the finalised methodology (n = 55) and in which the animal survived for greater than 1 hour following the completion of surgery (n = 47). MAP was recorded as described in section 2.1.6 and heart rate (HR) calculated from this trace using an instantaneous frequency event memory channel system (Spike2, CED). Empirical data is given in Table 3.1. Changes in cardiovascular output between stages of the procedure were assessed for statistical significance using a one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

3.3.1. Blood Pressure

Aspiration of the dorsal-most cortical tissue to expose the posterior communicating artery reduced MAP with a mean decrease of 2.3% ± 4.7% compared to pre-decerebration values – this stage typically resulted in very little or no blood loss from the site of decortication (see Table 3.1). On the other hand a stereotyped decrease occurred coincidental with the pre-collicular coronal section, with a mean drop in MAP by, on average, 27.1% ± 8.8%. In the 30 minute period between the immediate completion of surgery, and prior to the commencement of recordings (during which isoflurane anaesthesia was continued and the artery clip *in situ*), the mean MAP remained close to the level reached during the coronal section stage (25.2% ± 15.7% lower than pre-decerebration values). The first 2000 seconds of recording were selected as a period representative of post-decerebration cardiovascular parameters, given that isoflurane anaesthesia was discontinued a minimum of 1 hour prior to this point, the artery clip removed coincidental with the cessation of anaesthesia, and as recordings occurring during this period were exclusively control readings with no

Procedure	MAP (mmHg)		HR (bpm)	
	Mean	S.E.M.	Mean	S.E.M.
Pre-decerebration	95.7	1.5	434.3	3.6
Cortical aspiration	93.3	1.5	436.6	3.6
Coronal section	67.1***	1.3	441.3	4.7
Post-decerebration	68.4***	2.1	448.4	4.6
Recording (first 2000s)	87.7	3.4	455.5*	8.0

Table 3.1: Mean MAP and HR values recorded during decerebration (n = 47). Statistical significance was tested using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. Asterisks denote a significant difference compared to pre-decerebration values (*** $p < 0.001$, * $p < 0.05$). Drops in MAP were countered by decreasing the inspired percentage of isoflurane.

conditioning stimuli applied (which may alter both MAP and HR). Mean MAP recorded during this phase of the experiment was 93% ± 27% of control values.

Statistical analysis shows that MAP determined during the coronal sectioning procedure and post-decerebration (pre-recording) was significantly ($p < 0.001$) different to control values, but that there was no significant ($p > 0.05$) change from control values during either the initial aspiration of cortical tissues or during the first 2000 seconds of recording.

3.3.2. Heart Rate

HR increased gradually throughout decerebration, with an increase from pre-decerebration levels of 0.5% ± 1.2% during initial aspiration, of 1.6% ± 4.5% during the coronal section, of 3.3% ± 4.7% post-decerebration, and of 4.9% ± 9.7% during recording (Table 3.1). The increase found between pre-decerebration control HR and that measured during recording was statistically significant ($p < 0.05$) with all other changes not significant ($p > 0.05$).

3.4 Discussion

The use of decerebrate animal preparations has been reported in a variety of species including monkey, cat, dog, mouse, rat, and rabbit (Sherrington, 1898, Liu, 1979, Clarke et al., 1988, Tonkovic-Capin et al., 1998, Smith et al., 2010, Meehan et al., 2011). Methods employed in each species can be broadly categorised as either mechanical or ischaemic decerebration. Mechanical methods physically transect the brain and either leave the forebrain intact (Faber et al., 1982) or remove it entirely (Hayashi, 2003), and ischaemic decerebration induces destruction of particular brain regions by the occlusion of specific blood vessels, either by surgical ligation (Pollock and Davis, 1930, Kniffki et al., 1981, Bennett et al., 1998) or by injection of embolitic agents (Fouad and Bennett, 1998). The following discussion places this new method in the context of pre-existing strategies for rodent decerebration.

The simplest conceptual form of decerebrating an animal is to mechanically transect the brain and thus sever reticulothalamic pathways rendering the preparation insentient. The majority of studies employing a mechanical decerebration technique perform the primary transection by application of either a fine blunt instrument such as a microspatula (Lee et

al., 2002), or by aspiration alone (Woolf, 1984). However blood loss due to severance of the major cranial blood vessels is the primary cause of death during decerebration (Woolf, 1984) therefore addressing this is critical to the success of the procedure. With respect to this there seems to be a considerable variation in cranial bleeding between some species. Decerebration of rabbits is associated with relatively little bleeding (even with one carotid artery left open), that is easily and effectively staunched by application of aluminium clips to the ruptured blood vessels (Clarke et al., 1988). On the other hand, this approach proved to be impractical in the rat due to the friability of the vessels and the rapid nature of the blood loss which, unabated, completely fills the cranial space in approximately 5 - 10s. Published techniques to combat this bleeding in the rat have included physical staunching by packing the cranial cavity or covering the exposed brainstem with gelatine sponge and/or cotton balls (de Almeida et al., 2010, Smith et al., 2010, Tsuchimochi et al., 2010), the addition of pro-clotting agents such as bovine thrombin (e.g. Woods, 1964; Tian and Duffin, 1996) or thromboplastin (Pickering et al., 2002), and minimal tissue removal to reduce the likelihood of rupturing a major vessel (Faber et al., 1982). However the success/mortality rates associated with these approaches have not been specifically reported. In developing this methodology, physically packing the cranial cavity with cotton balls/sponge was not effective in completely preventing ongoing blood loss. On the other hand, the combination of gelatine sponge and tissue adhesive completely staunched bleeding when applied to the base of the cranial vault which consequently reduces the likelihood that subsequent elevations in MAP following anaesthetic withdrawal will generate further haemorrhage. The use of tissue adhesive also eliminated the necessity of applying an antihaemorrhagic solution.

Although removal of significant amounts of brain tissue increased the likelihood of significant bleeding from ruptured blood vessels, this is preferable to a more minimalist approach. The method of ‘chronic decerebration’, in which a coronal section is performed around the level of the colliculi but with no removal of tissue rostrally and tissue lateral and dorsal to the colliculi remains intact (Faber et al., 1982), means that it is difficult to ascertain with any certainty the completeness of the decerebration. If the forebrain is to be left *in situ* then other confirmations of the totality of decerebration must be observed e.g. by the recording of electroencephalograms (EEGs) (Huang et al., 1992) or by visual inspection performed post-mortem (Liu, 1979, Faber et al., 1982, Lee et al., 2004). Removal of brain tissue, as in the present methodology, allows visual verification during surgery as

opposed to posthumously; this provides assurances that the animal has been rendered completely insensate. An alternative approach in rats has been developed by Ono et al. (1987) which utilises a radiofrequency lesioning system targeted to the desired stereotaxic coordinates with a fine electrode. This then achieves a dual-outcome: the mechanical decerebration of the rat as the primary objective, with the secondary benefit of cauterising ruptured vessels and minimising blood loss. Hindering the wider adoption of this technique however is the expense of the required equipment relative to the alternatives. Recently, a novel *in situ* decerebrate preparation has been described in which the animal is transcardially perfused immediately following decerebration and artificially maintained via an intra-aortic warmed modified Ringer's solution perfusate (Pickering and Paton, 2006). This technique therefore eradicates the issue of haemorrhage in decerebration and is reported to provide a stable recording environment for cardiac, respiratory, and spinal nerve recordings (Koganezawa et al., 2011, Sadananda et al., 2011, Moraes et al., 2012). Whilst the *in situ* decerebrate provides a valuable alternative method by which to investigate molecular mechanisms or single-cell responses, in its current form it cannot be applied to the measurement of EMGs (due to neuromuscular blockade) or immunological studies given that the animal is exsanguinated.

As previously mentioned, there are clear differences between species with respect to cranial bleeding during the mechanical decerebration process. The variation in blood supply to the brain is in fact highlighted by studies which have performed decerebration via ischaemic rather than mechanical techniques. The original method by which ischaemic decerebration was carried out was via ligation of those vessels which supply oxygenated blood to the cerebral hemispheres, as even a temporary cessation cranial circulation of 5 to 10 minutes was sufficient to cause permanent damage (Kabat et al., 1941, Symon, 1993). The complexity of the surgical procedure required to produce an effective decerebration was dependent upon the anatomy of the species involved in the study and therefore which/how many vessels supply the forebrain. Variation between individual animals must also be taken into account. In the late nineteenth century Hill (1896) published details of his experiments into cerebral blood flow, incorporating findings from other researchers. Ligation of both common carotid arteries resulted in death in horses and goats, suggesting no secondary arterial supply to the brain. In cats and rabbits, he demonstrated that both vertebral arteries must be ligated in addition to the carotids to fully abolish the cranial blood supply, though two-thirds of the cats tested survived even this invasive procedure.

All of the dogs and monkeys examined survived this four-artery occlusion technique, implying a tertiary arterial supply in these species.

The first example of an animal decerebrated by selectively induced ischaemia was described by Pollock and Davis (1930), who developed a method in the cat in order to further study the role of the forebrain in certain reflex responses, including nociceptive withdrawal reflexes. The surgical preparation involved dissection and ligation of the basilar artery through the buccal cavity, followed by ligation of both the internal and external branches of both common carotid arteries. These processes restricted cerebral circulation to the inferior half of the cerebellum, the caudal half of the pons, the medulla, and the spinal cord only, resulting in the same recognisable rigidity described previously. Both methodological variants of ischaemic decerebration of a cat (selective and total) are still in use (Geertsen et al., 2011, Schomburg et al., 2011). The selective ligation model has since been adapted for the rat (Windle and Minear, 1933, Fukuda et al., 1974), with the basilar artery being accessed ventrally through the same incision as the carotids. The effect of these procedures is a lower decerebration (i.e. the termination of the blood supply occurs in a more caudal position than in the cat) but the same extensor rigidity is still apparent. A less surgically invasive approach has since been developed for ischaemic decerebration of the rat, due to the smaller size of the animal increasing the risk associated with any incidental blood loss. Injection of a viscous embolitic agent, such as polyvinylsiloxane (PVS) (Fouad and Bennett, 1998), into both common carotid arteries has been shown to efficiently restrict cerebral circulation to the cerebellum and the hindbrain and again precipitates the onset of decerebrate rigidity.

In addition to the possibility of subtle anatomical differences in terms of cranial vasculature, the differences encountered with respect to decerebration of the rabbit and rat in this laboratory may also have been compounded by slight differences in anaesthetic regimes. Decerebration in rabbits was typically performed on animals maintained in a state of general anaesthesia using the inhalational agent halothane, whereas all of the rat experiments were performed under isoflurane anaesthesia. Several published studies have investigated the specific cerebrovascular effects of various inhalational anaesthetics, and in rabbit and cat, isoflurane elevated intracranial pressure (ICP) to a greater extent than the equivalent dose of halothane (Todd and Drummond, 1984, Kaieda et al., 1989b), whereas in dog and human the reverse was true (Adams et al., 1981, Artru, 1984). Studies

investigating these parameters in rats tend to focus on the newer-generation anaesthetics such as desflurane or sevoflurane therefore the most relevant comparison for the purposes of this discussion i.e. one comparing the effects halothane and isoflurane on ICP in a rat model, has not been published. There is therefore the potential that the use of isoflurane instead of halothane resulted in an elevated ICP and a reduced intracranial space in the rats, making rupture of the SSS during the craniectomy more likely and thereby reducing the possibility of a favourable outcome to the surgery as a whole. Under the experimental conditions employed here however, the choice of anaesthetic agent is likely of lesser importance than the inclusion of nitrous oxide as a carrier gas, as nitrous oxide significantly elevates ICP regardless of the anaesthetic used (Kaieda et al., 1989a).

The critical factor in the rat mechanical decerebration procedure developed here is the adequate management of blood loss, by undertaking both the necessary surgical preparation (temporary occlusion of the carotid artery and permanent occlusion of the SSS), the correct preparation of the surgical kit to reduce delays during the procedure to an absolute minimum, and by careful maintenance of anaesthetic levels. The nature of a mechanical decerebration however means that total avoidance of blood loss is exceedingly difficult, and loss throughout the procedure is reflected in MAP measurements.Bilateral ligation of the carotid arteries is commonly employed prior to decerebration in order to limit intracranial haemorrhaging (Sapru and Krieger, 1978, Hayashi, 2003, Smith et al., 2010, Tsuchimochi et al., 2010). Leaving one carotid artery intact, certainly in rabbits, reduced the frequency of spontaneous blood pressure surges to which decerebrates are particularly prone to in this species (Taylor et al., 1991) which is potentially attributable to maintaining circulation to the carotid baroceptors. Hence the approach of reversibly occluding a carotid artery not only minimises blood loss during decerebration but also maintains circulation to the right carotid sinus and likely preserves baroreflexes from this site to some degree.

The drops in MAP that were observed during mechanical decerebration can be largely attributed to severance of blood vessels and the blood lost therefrom and/or the interruption of projections to the hypothalamus and it's tonic autonomic effects (Gellhorn et al., 1956). Hence aspiration of the neocortex to expose the posterior communicating artery has minimal impact on MAP and HR, however these factors are likely to be particularly important with respect to the coronal section, which separates the cerebellum

and hind-brain from all forebrain structures and also typically results in rupture of the basilar vessels. The decrease in MAP from pre-decerebration values following completion of surgery showed a higher degree of variability than that observed with the coronal section, which is likely due to the variable nature of volume of blood lost during the aspiration of lateral regions of the cortex and concomitant rupture of the transverse sinuses. Blood loss from this site tended to occur with the same level of rapidity as with rupture of the basilar vessels, but demanded greater dexterity on the part of the operator to successfully staunch it due to the acute angle between the remaining brain tissue and cranium and poor visibility of the region as a result of this. Occasionally this process required several attempts before haemorrhaging was adequately stemmed, and in lengthening the time taken to complete this step a greater volume of blood was lost, thereby impacting further on MAP. However, values obtained in the early recording phase were not significantly different to those pre-decerebration, which (although this does not take into account the effect of anaesthetic withdrawal) implies that the impact of the blood loss and severance to tonic controls is not so great as to have a deleterious effect on the overall cardiovascular stability of the preparation. The significant change recorded in HR is most likely a result of the transition from an anaesthetized to an unanaesthetized state rather than as a side-effect of decerebration given that isoflurane is known to decrease basal MAP (Goren et al., 2001, Lee et al., 2002) and that HR is controlled by structures in or below the pons (Blake and Korner, 1982).

Several groups give blood pressure information either pre- or post-decerebration in rats (Sapru and Krieger, 1978, Faber et al., 1982) but have reported little in terms of peri-surgical measurements. Monitoring mean arterial pressure (MAP) during surgery allows any precipitous drops in this parameter, which may otherwise prove fatal or deleterious to the preparation, to be rapidly corrected by modulation of anaesthetic levels and thereby improve survival rate. Cessation of isoflurane anaesthesia in rats leads to restoration of both the righting reflex and withdrawal reflexes to noxious stimulation in less than 5 minutes (Lysko et al., 1994, Solt et al., 2011), therefore reducing the percentage of inspired isoflurane in response to drops in MAP during the decerebration procedure rapidly alters the anaesthetic plane. Given that isoflurane reduces MAP in a dose-dependent manner (Conzen et al., 1992, Goren et al., 1999), the survival rate for mechanical decerebration is improved by this additional control over the cardiovascular output which then assists in preventing hypovolaemic shock.

3.5 Conclusions

Details of surgical methods currently employed for the mechanical decerebration of a rat are poorly described in the literature, hence an information void exists for researchers wishing to successfully adopt this technique. Decerebration can lead to a high rate of mortality due to cranial bleeding but the methodology developed in the present studies indicates effective approaches such as reversible occlusion of a carotid artery, the combined use of tissue adhesive and haemostatic sponge, and peri-surgical monitoring of blood pressure, by which to control blood loss and hence maintain mean post-surgical blood pressure within acceptable physiological parameters. Success rates of greater than 80% were obtained using the methodology described however any determination of a success rate is very subjective; indeed excluding those experiments that could reasonably be said to still be a part of the method development phase the survival rate is greater than 94%. In support of this, the success rate achieved in experiments for pharmacological studies in Chapters 5 and 6 (that also included the additional surgery of intrathecal cannulation prior to decerebration) was 98% (n = 96). Operator experience is therefore a critical factor in the outcome of a challenging surgical technique such as that described herein, and a near 100% success rate - comparable to that previously achieved in rabbit – is eminently attainable using this technique.

4. ORGANIZATION AND CONTROL OF SENSITIZATION OF WITHDRAWAL REFLEXES

4.1 Introduction

There is a great deal of evidence to indicate that withdrawal reflexes are organized in a 'modular' fashion such that a noxious or potentially damaging stimulus applied to the hindlimb activates muscles best suited to withdrawal the limb away from the site of stimulation, whilst those muscles which may move the limb towards the stimulus are inhibited (Schouenborg et al., 1994, Clarke and Harris, 2004; see section I.3). Intense noxious stimuli however can also affect subsequent reflex responses due to sensitization of the nervous system both peripherally and centrally (Woolf, 1983, Cook et al., 1987, Clarke et al., 1992; see section I.4). Previous novel studies in this laboratory have looked at this facilitatory (and also inhibitory) effect on the pattern of organization of reflex responses in the rabbit using the noxious chemogenic agent mustard oil (MO) (Harris and Clarke, 2003) which showed that the size of "sensitization fields" for withdrawal in hindlimb flexor muscles is powerfully controlled from the brain (Harris and Clarke, 2003). Thus in decerebrate, spinal animals, reflexes evoked in the knee flexor semitendinosus and the ankle flexor tibialis anterior were enhanced after the application of MO to anywhere on the ipsilateral limb. In contrast, in decerebrate non-spinal animals, sensitization could be evoked only from plantar areas that the muscle would withdraw from contact with the ground. For the knee flexor semitendinosus (ST) this was the whole of the plantar surface of the ipsilateral hind paw whilst for the ankle flexor tibialis anterior (TA) it was only the anterior portion of the plantar surface. In intact pentobarbitone-anaesthetized animals, sensitization fields for reflexes to these flexor muscles lay between the spinal and non-spinal states, with ST showing a spinal-like field and TA behaving more like the non-spinal condition. Therefore the area from which flexor withdrawal reflexes can be sensitized is determined by activity in descending pathways and appears to be a dynamic process. In contrast, organization of sensitization of reflexes in the ankle extensor medial gastrocnemius (MG) appeared to be more at the spinal level, as the pattern of sensitization for this response was similar in all three preparations, only occurring when MO was applied to the heel (Harris and Clarke, 2003). Interestingly, this extensor reflex was the only one of the three responses to be inhibited by MO applied to the ipsilateral hindlimb; an effect that was maintained in the spinalized animal.

The fact that these studies were performed in rabbit however raises the question as to whether these findings apply to other species hence whether important species differences exist, in particular with respect to pre-clinical studies, whether the brain control of the sensitization is similar in the rat, a species widely employed in pain research. Certainly there is prior evidence to suggest that descending pathways in rats have a role in promoting sensitization (Porreca et al., 2002) whereas no such facilitatory effects were seen in the rabbit (Harris and Clarke, 2003). The present studies have therefore studied the sensitizing effects on hindlimb reflexes of mustard oil applied to, and into, a number of locations on the body in Alfaxan-anaesthetized and decerebrated (with and without an intact spinal column) rats in order to determine sensitization fields for reflexes in individual muscles and the nature of any supraspinal modulatory controls in this species.

4.2 Methods

Experiments were performed on a total of 155 male Wistar rats assigned to one of three surgical preparations: - anaesthetized (n = 56), decerebrate, non-spinalized (n = 60), and decerebrate, spinalized (n = 39). The mean weight of the total cohort was 311 g ± 19 g.

4.2.1. Surgical Preparation

The surgical procedures performed are described in detail in section 2.1. Briefly, in all animals, the trachea, left carotid artery, and left jugular vein were cannulated under isoflurane anaesthesia for airway maintenance, blood pressure monitoring, and drug administration respectively, and rats undergoing decerebration also had their right carotid artery reversibly occluded to prevent blood loss during this procedure. From this point no further surgery was undertaken in the anaesthetized group of animals however rats in the decerebrate, spinalized group (39 animals) underwent a laminectomy and the spinal cord was transected at T9/10 (see section 2.1.4) prior to decerebration. Decerebration was performed by suction to the pre-collicular level (see chapter 3) to render the animal insensate and anaesthesia discontinued. ECG and earth electrodes were implanted in all animals. EMG recording electrodes were inserted in to the TA, BF and MG muscles and paired stimulating electrodes were placed in the heel and toes (see section 2.1.5). Decerebrate animals (spinal and non-spinal) were maintained on a sub-anaesthetic i.v. infusion of Alfaxan (1 mg mL^{-1}, mean rate 1.1 mL hr^{-1}) to prevent excessive movements

during recording and intact anaesthetized preparations on an infusion of 10 mg mL^{-1} Alfaxan (mean rate 1.6 mL hr^{-1}).

4.2.2. Experimental Protocol

Hindlimb reflexes were evoked using electrical stimulation up to a maximum of 10 mA alternately at either the heel (MG and BF reflexes) or toes (TA and BF reflexes). Control values were defined as three consecutive readings (within 10% of one another) from heel-MG, toes-TA, and toes-BF reflexes following which 5 µL 20% mustard oil was applied to either ipsilateral toes (plantar), metatarsophalangeal joints (plantar), midsole, heel, toes (dorsal), flexion of the ankle (dorsal), knee, ankle joint or lateral gastrocnemius muscle; contralateral heel, toes (plantar) or ankle joint; ipsilateral forelimb footpad; contralateral forelimb footpad, snout, or tail tip (see details in section 2.1.7). Reflexes were then recorded every 2 mins for a minimum of 63 mins (i.e. a total of 32 stimulations, 16 heel and 16 toes) before another MO stimulus was applied following establishment of stable control responses. A maximum of four mustard oil sites were tested in a single animal.

4.2.3. Statistical Analyses

Comparisons between stimulation parameters and the magnitude of raw control reflex responses were performed using Wilcoxon's Matched Pairs tests or Kruskal-Wallis one-way ANOVA's where appropriate.

To determine the effect of each application of MO, the mean of the three control reflex responses immediately prior to its administration was determined, then values were expressed as a percentage of that mean control period. Duration of changes was taken as the time for reflexes to recover to within 2 standard deviations of the mean pre-MO application control level for two successive readings; due to the design of the experiment, the maximum possible duration that could be attributed to MO-induced effects on extensor and flexor responses was 61 mins and 63 mins, respectively. Pooled values for reflexes and durations are then expressed as medians and interquartile ranges. The statistical significance of the changes was assessed using Friedman's ANOVA on ranks over the time period indicated from the duration analysis.

Blood pressure and heart rate data were measured as a series of average values from one minute time bins and are expressed as a percentage of the mean of ten pre-MO control values. In cases where heart rate exceeded 500 bpm (the saturation point of the ADC), instantaneous frequency was calculated from the blood pressure trace using Spike2 software. The cardiovascular parameters were compared between the three preparations using Kruskal-Wallis tests followed by Dunn's Multiple Comparison test, and changes in MAP and HR induced by MO application were assessed using Friedman's ANOVA.

4.3 Results

4.3.1. Electrical Stimulation Parameters

Median electrical stimulation parameters are recorded in table 4.1. For each stimulation site, the minimum stimulation amplitude required to evoke a reflex EMG response in one of the appropriate muscles was recorded as the stimulation threshold. In each of the three preparations, the heel evoked reflexes had a significantly higher threshold than the toes reflexes ($p < 0.001$, Wilcoxon matched pairs tests). There were also significant differences between preparations for stimulation thresholds for the heel and for the toes ($p < 0.001$, Kruskal-Wallis tests). With respect to the heel, thresholds for evoking reflex responses were significantly higher ($p < 0.001$, Dunn's Multiple Comparison Test) in anaesthetized compared to the other two preparations, with thresholds in the decerebrate model being significantly higher ($p < 0.01$, Dunn's Multiple Comparison Test) in the spinally intact preparation. For the toes, there was no significant difference in stimulation thresholds between anaesthetized and decerebrate, non-spinal animals but thresholds in both of these were significantly higher ($p < 0.001$, Dunn's Multiple Comparison Test) than in the decerebrate, spinalized model.

The stimulus strengths used are to some degree subjective as the amplitude was increased until the evoked responses were deemed sufficient to commence the experiment. However in the anaesthetized preparation the median stimulus strength used to evoke responses throughout the experiments was significantly higher for the heel reflexes relative to that required at the toes ($p < 0.01$, Wilcoxon matched pairs test). No significant difference between heel and toes was found for stimulus strengths employed during the decerebrate experiments, while the decerebrate spinalized preparations required a significantly

Preparation	Median Threshold (mA) (IQR)		Median Stimulation strength (mA) (IQR)	
	Heel	Toes	Heel	Toes
Anaesthetized	2.07 (0.96 – 3.40)	0.57 (0.39 – 0.70)	5.70 (3.48 – 7.49)	3.88 (2.01 – 6.63)
Decerebrate	0.65 (0.40 – 1.02)	0.40 (0.30 – 0.52)	2.50 (1.60 – 4.31)	2.00 (1.05 – 4.00)
Decerebrate Spinalized	0.38 (0.28 – 0.62)	0.22 (0.18 – 0.32)	2.00 (1.38 – 2.89)	3.00 (0.98 – 5.60)

Table 4.1: Median electrical stimulation parameters for the heel and toes sites in each preparation. Statistical analyses performed on this data and significant differences are reported in the main text of this chapter.

Preparation	Control reflex amplitudes (median and IQR; µV.ms)			
	Heel-MG	Heel-BF	Toes-TA	Toes-BF
Anaesthetized	117 (80 - 180)	84 (57 - 189)	88 (66 - 120)	83 (61 - 115)
Decerebrate	379 (222-910)	409 (214-751)	151 (121-229)	240 (151-403)
Decerebrate Spinalized	117 (88-156)	295 (157-573)	106 (76-161)	120 (91-179)

Table 4.2: Median raw reflex responses by preparation. As above, statistical analyses performed on this data and significant differences are reported in the main text of this chapter.

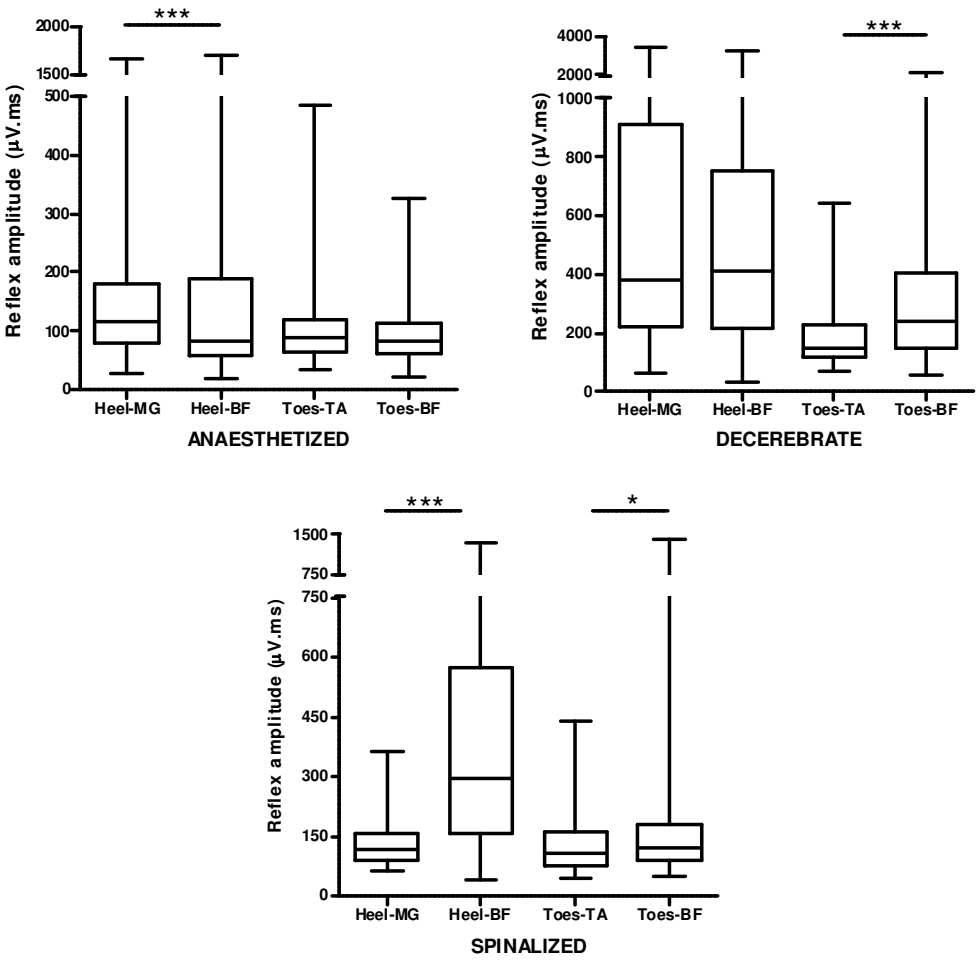


Figure 4.1: Median raw reflex responses by preparation. Box-and-whisker plots are used to depict statistical differences between the control responses. Statistical differences were assessed using Kruskal-Wallis tests, with asterisks highlighting significant differences as indicated by Dunn's Post Test ($p < 0.05$= *, $p < 0.001$ = ***).

stronger stimulus applied at the toes relative to the heel ($p < 0.01$, Wilcoxon matched pairs test). Comparison between preparations indicated significant ($p < 0.01$, Kruskal-Wallis tests) differences such that heel stimulation strengths were significantly higher ($p < 0.001$, Dunn's Multiple Comparison Test) in the anaesthetized compared to the decerebrate models and toe stimulation amplitudes were significantly different ($p < 0.01$, Dunn's Multiple Comparison Test) between anaesthetized and decerebrate, non-spinalized animals. It should be noted that these determinations are for the stimulus amplitudes employed at the start of the experiment, and if on some occasions the size of the evoked reflexes reduced too greatly, the stimulus parameters were altered in order to establish reliable control reflexes ready for the next MO treatment.

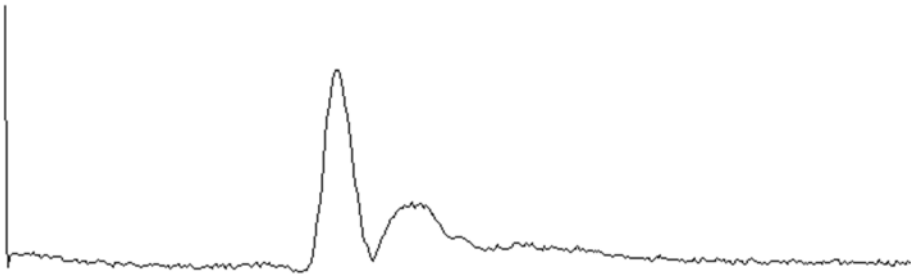
4.3.2. Control Reflex Responses

Median pre-mustard oil reflexes were significantly different both between individual reflexes within a preparation and between the different preparations. Median values are given in table 4.2. All comparisons of reflexes between the preparations i.e. heel-BF in anaesthetized vs. heel-BF in decerebrate revealed significant variation ($p < 0.001$, Kruskal-Wallis test followed by Dunn's multiple comparison test) with the singular exception of heel-MG between anaesthetized and decerebrate spinalized preparations. Average responses in the decerebrate preparation were universally larger than in the other surgical preparations, with responses in the anaesthetized preparation the lowest in magnitude.

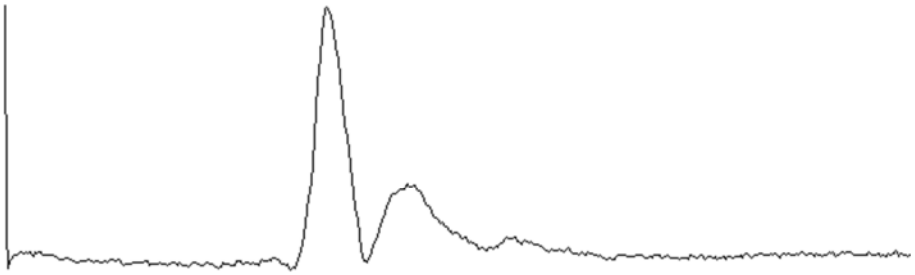
4.3.3. Effect of Mustard Oil on Reflexes

For the sake of conciseness and clarity, the data described in the following section are summarised diagrammatically in figure 4.4 with all the changes described quantified in terms of magnitude, duration, and significance in table 4.3 (located at the end of this results section). In general (but not absolutely), mustard oil can be summarised as producing relatively small, short duration facilitations in the magnitude of reflexes or slower developing inhibitions depending on the preparation studied. In addition, selected graphs of this data are provided in figure 4.3.

Control Heel-BF reflex - anaesthetized



Heel-BF reflex following MO application to IL heel



Heel-BF reflex following MO application to IL knee

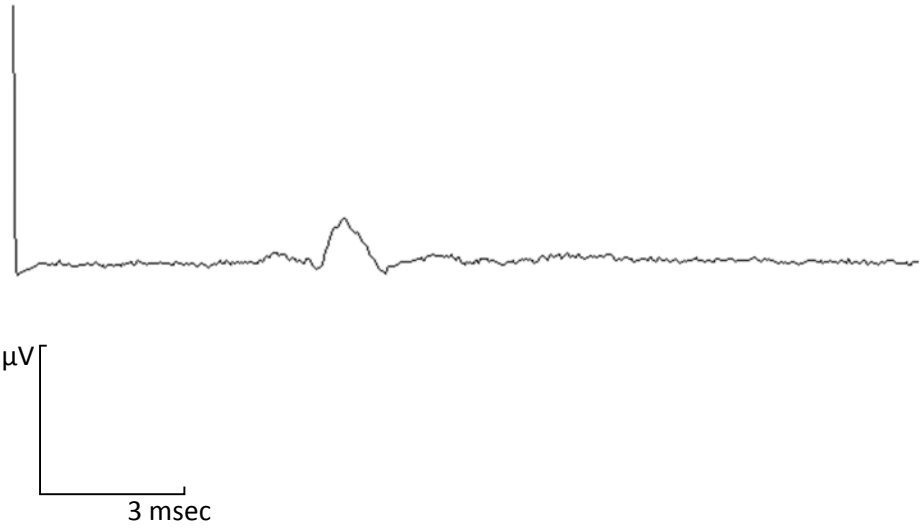


Figure 4.2: Raw data traces showing the responses of BF to heel stimulation under control conditions (upper), following MO application to the IL heel (centre), and following MO application to the IL knee (lower). Each plot is an average of eight sweeps and the stimulus was applied at the beginning of each sweep, represented here at the large upward-deflected stimulus artefact.

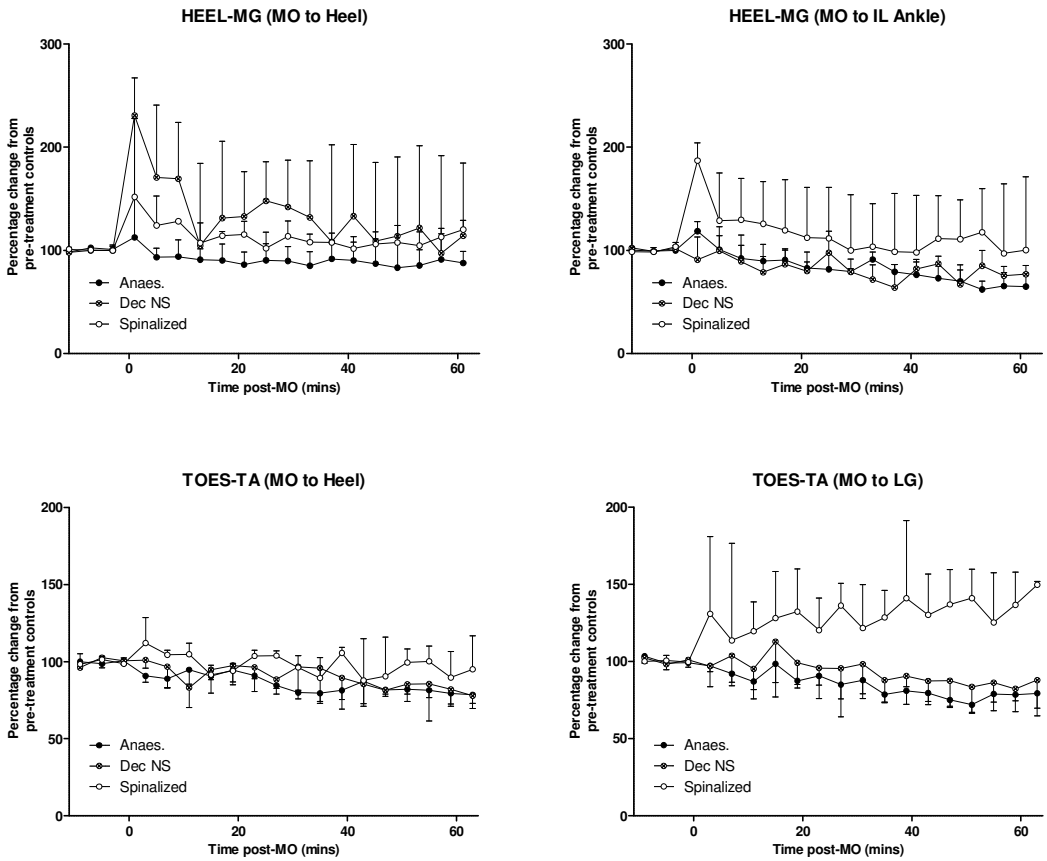
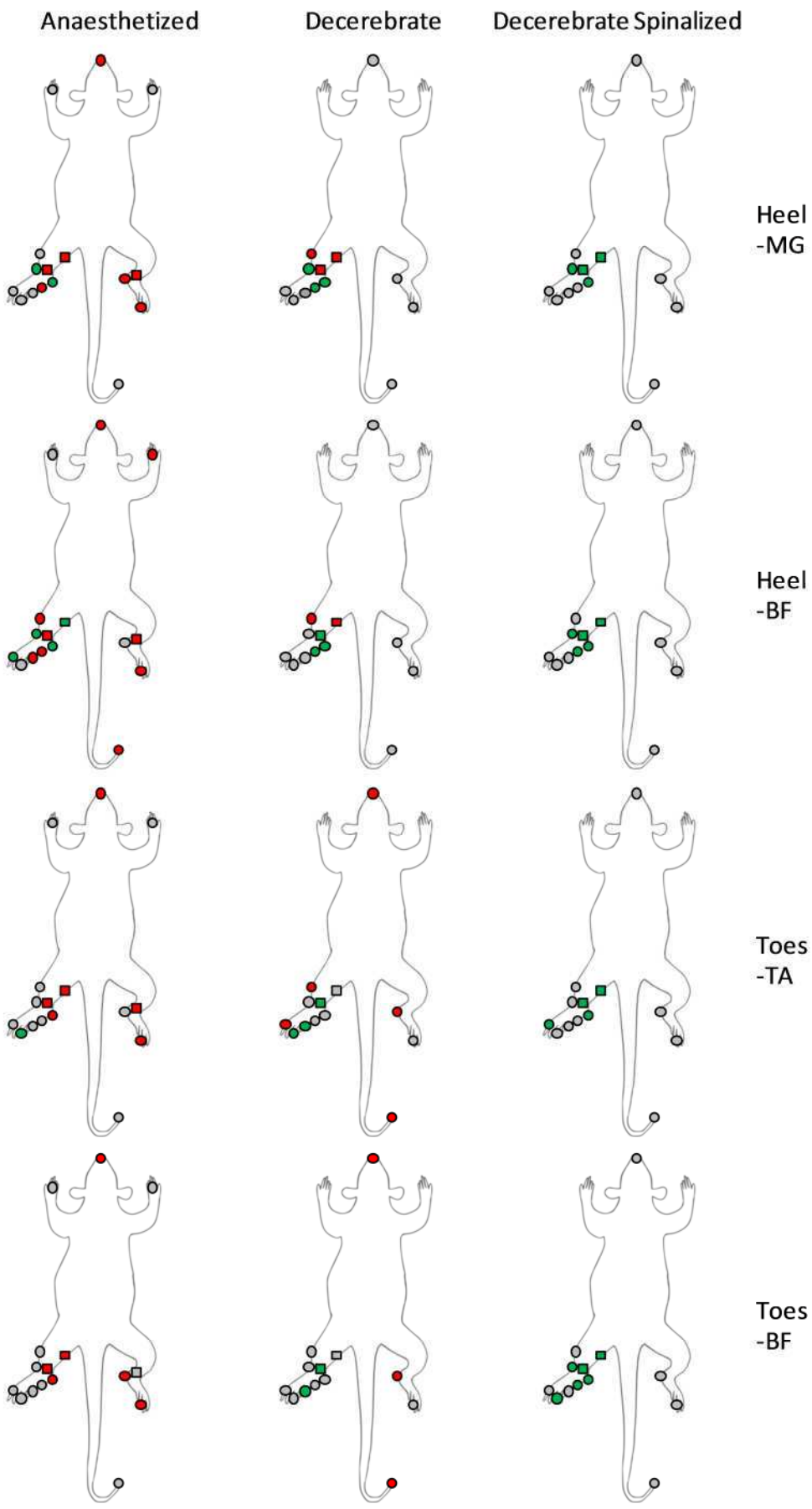


Figure 4.3: Example graphs to show the differential effects of MO application to different sites between the three preparations. Values plotted are medians ± upper/lower quartiles. The three preparations are referred to in the figure legend thusly: Anaes. – anaesthetized; Dec NS – decerebrate non-spinal; and Spinalized – decerebrate spinalized rats.

In the decerebrate spinalized preparation the effect of MO was to cause only facilitation of a reflex response (or no change), with inhibition of reflexes not seen from any site (figure 4.4). The heel-MG reflex was significantly facilitated ($p < 0.01$, Friedman's ANOVAs) to greater than 150% of control values by MO treatment applied to the IL heel, flexion of the ankle, the ankle joint, and LG, with changes of the greatest magnitude obtained from the deep tissue treatment sites. A similar pattern of MO-induced facilitation was found for heel-evoked BF responses ($p < 0.01$, Friedman's ANOVAs), with significant facilitation obtained from the same sites as for heel-MG with the addition of IL mid-sole. The pattern of reflex sensitization was slightly different for the toes-TA reflex, in which significant facilitation was again generated in response to MO application to the two deep tissue sites as well as IL heel but with no significant change induced by MO application to the flexion of the ankle and a significant ($p < 0.05$, Friedman's ANOVA) facilitation produced by MO applied to the dorsal aspect of the IL toes. The toes-BF response was the most widely modulated reflex in the decerebrate spinalized preparation, with a significant enhancement ($p < 0.05$, Friedman's ANOVAs) of responses found from MO applied to the same five sites as for heel-BF as well as when it was given to the plantar aspect of the IL toes. As would be predicted in a spinalized preparation, MO had no effect on reflex responses when applied to off limb sites.

The decerebrate spinally-intact preparation was generally less amenable to excitation than the spinalized counterpart, with MO producing reflex facilitation from fewer sites and inhibition obtained in several instances. For heel-MG reflexes, significant MO-induced facilitation ($p < 0.05$, Friedman's ANOVAs) was induced from the IL heel, mid-sole, and flexion of the ankle, whilst in contrast to the spinalized model, significant inhibition was produced following MO application to the IL knee, ankle and LG. The sites from which the heel-BF reflex was significantly modulated by MO were almost the same as those that impacted on the heel-MG response, although MO injected into the IL ankle caused facilitation not inhibition, no significant change ($p > 0.05$, Friedman's ANOVA) occurred following MO application to the flexion of the ankle and a significant inhibition ($p < 0.05$, Friedman's ANOVA) was produced from MO treatment of an off-limb site, the CL toes. The two toes-evoked reflexes were significantly modulated from several of the same treatment sites, with facilitation of toes-TA and toes-BF reflexes generated by MO application to the IL MTJ and ankle and significant inhibition found following treatment of the CL heel, snout,

Figure 4.4: MO effects on hindlimb reflexes in the three preparations. Circles indicate sites to which MO was applied topically, with squares representing deeper sites at which MO was injected. Green filled shapes indicate sites from which significant facilitation was obtained, red filled shapes indicate sites from which significant inhibition was obtained, grey filled shapes indicate no significant effect. Significance was assessed using Friedman's ANOVA (instances where $p < 0.05$ are shown here).


Anaesthetized
Decerebrate
Decerebrate Spinalized
Heel
-MG
Heel
-BF
Toes
-TA
Toes
-BF

and tail tip. Additionally, toes-TA responsiveness was significantly enhanced by MO applied to the IL toes and reduced by treatment of the knee and the dorsal aspect of the IL toes. Inhibitory effects of MO were by far the most prevalent in the anaesthetized preparation. Significant facilitation ($p < 0.05$, Friedman's ANOVA) following MO application was therefore only obtained from IL heel and flexion of the ankle for heel-MG reflexes, from those two sites plus the dorsal toes and LG for heel-BF responses, from the IL toes only for toes-TA reflexes, whilst for toes-BF responses MO did not produce facilitation from any site. In contrast, all reflexes were significantly inhibited ($p < 0.05$, Friedman's ANOVAs) by MO application to sites on the IL limb as well as the CL limb and other off-limb sites, including the IL ankle, CL toes, and snout. Heel-MG was also significantly inhibited ($p < 0.05$, Friedman's ANOVA) by MO applied to mid-sole, CL heel, and CL ankle. The greatest inhibition of the heel-BF reflex was obtained following MO application to the CL toes with responses significantly inhibited, on average, by over 30% relative to control values. Significant inhibition of this reflex was also produced by MO applied to the IL MTJ, knee, CL ankle, and CL forelimb. Additionally toes-TA reflexes were significantly attenuated by MO application at the IL heel, LG, and CL ankle; and toes-BF inhibited by treatment at IL heel, LG, and CL heel.

4.3.4. Duration of Reflex Effects

Median durations of the effect of mustard oil on reflexes are also given in table 4.3.
In all three preparations, the median duration of reflex modulation by MO application was generally less than 15 minutes i.e. within 15 minutes following the treatment responses had returned to within 2 standard deviations of the control responses. A notable effect also observed across all three preparations was the prolonged effect of MO treatment when it was given as an intra-muscular or intra-articular injection to the LG or IL ankle. In the case of the spinalized preparation the four reflex responses were all potentiated for upwards of 30 minutes after deep tissue MO treatments, whereas in the decerebrate non-spinal and anaesthetized preparations the effects were more varied. For example, the enhancement of the toes-BF reflex in decerebrate animals which occurred as a result of MO injected into the IL ankle had the longest duration (a median of 43 minutes) of all changes observed in this preparation whereas injection of MO at the same site in anaesthetized animals produced a long lasting inhibition (median duration 63 mins) In fact in the anaesthetized group, MO injections into the IL limb mostly caused a rapid-onset inhibition, the effect

typically persisting for greater than 30 minutes. Whilst the majority of effects generated by cutaneous MO treatment were of short duration (see above), there were a number of examples in which treatments of this type produced longer-lasting changes. These include facilitation of the heel-MG and heel-BF reflexes by MO application to the IL heel in decerebrate non-spinal rats (medians of 35 and 29 minutes respectively), and inhibition of the toes-TA reflex by the same treatment in anaesthetized animals, in which the median duration of the change was 63 minutes.

In decerebrate non-spinal and anaesthetized preparations, a number of treatment sites resulted in a delayed onset inhibition (table 4.3), in particular the snout. The duration of the delay observed prior to the onset of significant inhibition ($p < 0.05$, Friedman's ANOVA) ranged from 4 to 38 minutes, with the longest delays found in the anaesthetized group. However, whilst reflex inhibition was delayed in these instances, the effect typically persisted until the cut-off time of either 61 minutes (heel-evoked reflexes) or 63 minutes (toes-evoked reflexes), and so these attenuations may potentially endure for durations of up to or greater than one hour.

4.3.5. Cardiovascular Effects

Median control MAP values i.e. prior to the first MO treatment for the anaesthetized, decerebrate non-spinal, and decerebrate spinal preparations were 124 mmHg (IQR 114 – 133mmHg), 93 mmHg (IQR 83 – 105 mmHg), and 61 mmHg (IQR 51 – 69 mmHg), which were all found to be significantly different to one another ($p < 0.001$, Kruskal-Wallis test with Dunn's Multiple Comparison post-test). The equivalent values for median HR were 398 bpm (IQR 374 – 428 bpm), 487 bpm (IQR 449 – 516 bpm), and 468 bpm (IQR 442 – 498bpm), respectively. In this case, the HR in the anaesthetized preparation was significantly ($p < 0.001$, Kruskal-Wallis test with Dunn's Multiple Comparison post-test) lower than in the two decerebrated groups.

In the anaesthetized preparation every site of mustard oil treatment resulted in a significant decrease in MAP measured in the 34 minute period immediately following MO treatment ($p < 0.001$, Friedman's ANOVA) compared to control pre-MO values. This hypotensive effect was preceded by a transient (< 5 mins) elevation in MAP, though this component of the biphasic change was not found to be significantly different from control

values ($p > 0.05$, Dunn's Multiple Comparison post-hoc test). However this post-hoc test did find significant differences between controls and decreases in MAP at later time points following MO treatment at several sites (see table 4.3). In the decerebrate group, mustard oil altered MAP when applied to IL toe tips, IL MTJ, IL flexion, IL ankle, IL LG, and CL toes ($p < 0.05$, Friedman's ANOVA) with no post-test significance observed. The effect of MO on MAP in this preparation was characterised as one of three modulations: a transient increase followed by a return to basal levels (IL MTJ), a transient increase followed by a sustained decrease (IL toes, flexion, ankle, and CL toes), or a sustained decrease with no transient effect in either direction (IL LG). The only treatment sites producing an effect on MAP in decerebrate spinalized animals were IL ankle and snout. In this preparation the change in MAP initiated by MO application to the IL ankle was that of a sustained decrease, and to the snout was of a transient increase followed by a sustained decrease.

All sites, with the exception of tail tip, had a significant effect on HR in the anaesthetized intact neuraxis preparation ($p < 0.02$, Friedman's ANOVA). HR in decerebrate non-spinalized animals was significantly affected by MO treatment at IL flexion, IL ankle, CL heel, and snout ($p < 0.04$, Friedman's ANOVA), whilst in decerebrate spinalized animals a significant effect on HR was found when MO was applied to IL toe tips, IL MTJ, IL dorsal toes, IL flexion, and snout ($p < 0.02$, Friedman's ANOVA).

Table 4.3: Effects of MO on hindlimb reflexes.
This table is composed of three sections, each relating to a different surgical preparation (spinalized, decerebrate non-spinal, and anaesthetized). Further subdivision details the outcome of MO application to the sites listed in the left-most column on the four evoked reflex responses.
Each cell includes the following information: firstly, the maximum alteration in the reflex response expressed as a percentage relative to normalised control values as a median with the interquartile range; secondly, the median duration of that change as determined by the length of time taken for responses to recover to within 2 standard deviations of the mean control for each reflex (also with interquartile ranges); thirdly, the p-value generated from Friedman's one-way ANOVA analyses on the median responses for the period dictated by duration analyses (ns = not significant, $p > 0.05$); and finally, the number of times treatment resulted in an elevation, depression, or no change in evoked responses (stated as x/y/z i.e. facilitation/inhibition/no change).
Entries preceded by an obelisk † indicate a delayed onset effect, with time of onset in **bold** typeface with duration given from that timepoint. The numbers of experiments showing an increase/decrease/no change are therefore also calculated from that timepoint.

SPINALIZED

SITE	HEEL-MG	TOES-BF	TOES-TA	HEEL-BF
IL toes	105 (102 - 114) 5 (0 – 13) ns (6/1/2)	111 (106 – 119) 7 (3 – 51) p < 0.05 (7/2/0)	110 (98 – 119) 3 (0 – 15) ns (5/2/2)	94 (91 – 110) 0 (0 – 61) ns (3/1/5)
IL MTJ	96 (92 – 151) 0 (0 – 5) ns (3/2/2)	96 (96 - 100) 0 (0 – 7) ns (3/1/3)	100 (88 - 109) 0 (0 - 3) ns (3/3/1)	105 (95 - 107) 0 (0 - 4) ns (2/2/2)
IL midsole	113 (93 - 133) 5 (0 – 13) ns (7/2/2)	119 (105 - 140) 3 (0 – 11) p < 0.001 (9/1/3)	112 (95 - 119) 3 (0 – 11) ns (9/3/1)	110 (95 – 122) 1 (0 – 5) p < 0.01 (7/1/3)
IL heel	152 (117 – 228) 37 (5 – 61) p < 0.01 (8/1/0)	166 (144 – 229) 7 (3 – 11) p < 0.001 (8/0/1)	112 (103 – 129) 3 (0 – 11) p < 0.05 (5/0/4)	172 (150 – 249) 17(9 – 29) p < 0.001 (8/0/0)
IL toes (dorsal)	117 (99 – 122) 1 (0 - 1) ns (4/2/1)	97 (88 – 121) 0 (0 – 37) ns (3/3/1)	114 (111 – 121) 3 (0 – 35) p < 0.05 (4/0/3)	106 (106 – 131) 0 (0 – 1) ns (2/0/3)
IL flexion of ankle	153 (116 – 181) 19 (4 – 49) p < 0.001 (9/0/0)	128 (120 – 150) 19 (4 – 61) p < 0.01 (8/1/1)	114 (94 – 125) 7 (0 – 17) ns (6/4/0)	125 (118 – 132) 1 (1 – 61) p < 0.01 (7/0/2)
IL knee	108 (100 – 128) 0 (0 – 1) ns (4/0/5)	105 (94 – 109) 3 (0 – 11) ns (6/1/2)	101 (99 – 103) 0 (0 – 7) ns (3/4/2)	103 (99 – 129) 0 (0 – 1) ns (3/4/2)
IL ankle (injected)	165 (120 – 379) 49 (33 – 55) p < 0.01 (6/1/0)	145 (124 – 175) 31 (23 – 43) p < 0.01 (6/0/1)	144 (119 – 170) 63 (19 – 63) p < 0.01 (7/0/0)	181 (165 – 385) 49 (27 – 61) p < 0.01 (7/0/0)
IL LG (injected)	187 (123 – 204) 33 (2 – 61) p < 0.01 (8/2/0)	199 (154 – 217) 63 (63 – 63) p < 0.01 (9/1/0)	150 (106 – 152) 63 (3 – 63) p < 0.05 (9/1/0)	197 (168 – 224) 61 (13 – 61) p < 0.001 (8/0/0)

CL heel	92 (91 – 95) 0 (0 – 10) ns (0/2/4)	100 (91 – 101) 2 (0 – 48) ns (1/3/2)	100 (87 – 112) 0 (0 – 2) ns (2/2/2)	88 (79 – 92) 0 (0 – 5) ns (0/2/3)
CL toes	93 (88 – 98) 1 (0 – 1) ns (1/3/2)	91 (81 – 92) 0 (0 – 8) ns (2/2/2)	104 (94 – 117) 0 (0 – 5) ns (2/2/2)	96 (92 – 99) 0 (0 – 4) ns (1/2/3)
Snout	97 (92 – 101) 0 (0 – 0) ns (2/3/3)	100 (91 – 115) 0 (0 – 10) ns (3/1/4)	106 (92 – 115) 2 (0 – 3) ns (4/3/1)	95 (90 – 104) 0 (0 – 3) ns (2/1/4)
Tail tip	103 (95 – 107) 0 (0 – 33) ns (2/0/3)	96 (92 – 100) 0 (0 – 3) ns (1/2/2)	80 (78 – 101) 3 (0 – 11) ns (1/3/1)	110 (107 – 112) 1 (1 – 1) ns (1/1/2)

DECEREBRATE NON-SPINAL

SITE	HEEL-MG	TOES-BF	TOES-TA	HEEL-BF
IL toes	62 (80 – 84) 5 (0 – 21) ns (2/6/1)	111 (95 – 114) 3 (0 – 7) ns (5/1/3)	106 (103 – 108) 3 (0 – 11) p < 0.01 (4/0/5)	97 (72 – 108) 0 (0 – 1) ns (2/3/3)
IL MTJ	79 (45 – 88) 9 (1 – 61) ns (4/4/1)	118 (111 – 178) 11 (0 – 39) p < 0.05 (7/1/1)	121 (116 – 178) 11 (3 – 39) p < 0.01 (7/1/1)	99 (95 – 128) 5 (1 – 21) ns (3/2/2)
IL midsole	116 (109 – 143) 5 (3 – 33) p < 0.05 (6/0/2)	100 (92 – 121) 6 (0 – 24) ns (4/2/2)	103 (97 – 115) 4 (0 – 27) ns (4/2/2)	115 (98 – 131) 7 (1 – 19) p < 0.05 (6/1/1)
IL heel	230 (181 – 267) 35 (9 – 61) p < 0.001 (5/1/0)	111 (79 – 145) 2 (0 – 12) ns (3/2/1)	101 (96 – 117) 2 (0 – 3) ns (3/2/1)	196 (186 – 521) 29 (9 – 61) p < 0.01 (5/0/0)

IL toes (dorsal)	138 (75 – 145) 13 (0 – 37) ns (4/3/0)	111 (103 – 112) 0 (0 – 13) ns (3/2/2)	†66 (60 – 86) **39** 24 (0 – 28) p < 0.05 (1/5/1)	110 (101 – 135) 1 (1 – 16) ns (3/3/0)
IL flexion of ankle	139 (106 – 176) 19 (4 – 35) p < 0.05 (7/3/0)	104 (93 – 122) 5 (0 – 10) ns (6/3/1)	113 (97 – 122) 9 (0 – 15) ns (5/4/1)	127 (110 – 158) 7 (1 – 26) ns (6/0/2)
IL knee	†78 (58 – 105) **28** 23 (5 – 33) p < 0.05 (1/9/1)	105 (91 – 111) 0 (0 – 7) ns (3/5/3)	87 (78 - 102) 3 (0 – 17) p < 0.05 (2/6/3)	69 (49 – 106) 1 (0 – 35) p < 0.05 (3/4/3)
IL ankle (injected)	61 (45 – 121) 13 (0 – 61) p < 0.05 (3/6/0)	139 (106 – 158) 43 (11 – 55) p < 0.01 (6/3/0)	129 (89 – 155) 11 (3 – 63) p < 0.05 (7/2/0)	167 (99 – 204) 17 (1 – 22) p < 0.01 (7/2/0)
IL LG (injected)	64 (56 – 86) 1 (0 – 61) p < 0.01 (2/5/1)	117 (98 – 122) 13 (8 – 48) ns (5/3/0)	95 (82 – 115) 20 (0 – 63) ns (2/4/2)	55 (49 – 155) 1 (0 – 31) p < 0.05 (3/4/0)
CL heel	77 (60 – 89) 21 (0 – 46) ns (3/4/1)	95 (88 – 98) 9 (0 – 17) p < 0.05 (2/5/1)	†83 (78 – 101) **18** 47 (7 – 47) p < 0.05 (1/5/2)	133 (99 – 170) 1 (0 – 21) ns (4/2/1)
CL toes	95 (87 – 102) 0 (0 – 11) ns (2/2/3)	103 (95 – 110) 0 (0 – 43) ns (2/2/3)	113 (95 – 114) 0 (0 – 3) ns (3/2/2)	†97 (66 – 101) **53** 8 (0 – 24) ns (1/2/3)
Snout	93 (83 – 116) 5 (0 – 29) ns (4/5/0)	†69 (49 – 97) **26** 35 (35 – 35) p < 0.05 (4/4/1)	†68 (65 – 81) **10** 55 (55 – 55) p < 0.05 (2/5/2)	171 (111 – 204) 1 (1 – 3) ns (5/0/2)
Tail tip	93 (84 – 100) 1 (0 – 49) ns (2/5/2)	94 (80 – 97) 11 (0 – 39) p < 0.01 (1/6/2)	91 (90 – 94) 7 (0 – 63) p < 0.001 (1/6/2)	105 (98 – 113) 0 (0 – 1) ns (3/2/4)

ANAESTHETIZED

SITE	HEEL-MG	TOES-BF	TOES-TA	HEEL-BF
IL toes	84 (46 – 107) 9 (1 – 61) ns (4/12/3)	97 (93 – 106) 7 (0 – 63) ns (4/5/10)	108 (99 – 119) 7 (0 – 19) p < 0.05 (8/6/5)	94 (83 – 109) 1 (0 – 9) ns (4/5/7)
IL MTJ	84 (76 – 111) 1 (0 – 12) ns (4/10/0)	101 (95 – 107) 0 (0 – 23) ns (4/3/7)	96 (92 – 98) 6 (0 – 27) ns (4/4/6)	92 (91 – 99) 0 (0 – 3) p < 0.05 (6/4/4)
IL midsole	†77 (71 – 96) **20** 41 (25 – 41) p < 0.05 (4/11/1)	95 (90 – 102) 3 (0 – 7) ns (2/4/10)	96 (91 – 101) 3 (0 – 5) ns (2/6/8)	†77 (73 – 87) **16** 45 (41 – 45) p < 0.001 1/5/6
IL heel	112 (90 – 228) 1 (0 – 11) p < 0.05 (8/3/4)	†83 (79 – 96) **26** 37 (20 – 39) p < 0.01 (4/8/3)	80 (73 – 87) 63 (11 – 63) p < 0.001 (1/12/2)	164 (103 – 212) 1 (1 – 1) p < 0.01 (9/0/4)
IL toes (dorsal)	90 (47 – 146) 1 (0 – 11) ns (5/5/0)	100 (93 – 106) 4 (0 – 10) ns (2/2/6)	114 (97 – 123) 5 (0 – 20) ns (6/4/0)	129 (115 – 170) 1 (1 – 9) p < 0.05 (5/0/2)
IL flexion of ankle	131 (108 – 179) 3 (1 – 37) p < 0.01 (13/3/0)	98 (93 – 103) 3 (0 – 15) ns (4/5/7)	103 (92 – 109) 3 (0 – 3) ns (4/4/8)	144 (132 – 169) 17 (1 – 37) p < 0.001 (10/0/3)
IL knee	89 (84 – 108) 9 (1 – 61) ns (4/8/2)	95 (90 – 98) 11 (0 – 63) ns (1/5/8)	93 (87 – 100) 7 (3 – 35) ns (2/6/6)	87 (77 – 91) 23 (4 – 46) p < 0.01 (1/344)
IL ankle (injected)	53 (46 - 87) 61 (0 – 61) p < 0.001 (2/11/0)	77 (70 – 96) 63 (4 – 63) p < 0.001 (1/10/2)	84 (72 – 89) 41 (4 – 63) p < 0.05 (3/8/2)	72 (56 – 83) 33 (1 – 61) p < 0.001 (0/8/4)

IL LG (injected)	†62 (55 – 70) **26** 33 (13 – 33) p < 0.001 (0/9/1)	94 (82 – 115) 3 (0 – 63) p < 0.001 (4/5/1)	72 (67 – 86) 63 (0 – 63) p < 0.001 (0/9/0)	118 (111 – 140) 5 (2 – 5) p < 0.05 (6/0/2)
CL heel	94 (93 – 96) 15 (1 – 35) p < 0.05 (2/3/2)	91 (88 – 95) 19 (3 – 54) p < 0.05 (1/4/2)	96 (87 – 102) 7 (4 – 11) ns (0/2/5)	103 (93 – 110) 1 (1 – 1) ns (2/1/0)
CL toes	81 (38 – 90) 39 (1 – 61) p < 0.001 (1/10/1)	94 (90 – 103) 7 (5 – 11) p < 0.01 (0/7/5)	97 (90 – 101) 9 (3 – 51) p < 0.001 (1/5/6)	68 (58 – 77) 61 (31 – 61) p < 0.001 (0/7/2)
CL ankle (injected)	†80 (70 – 100) 5 (0 – 61) p < 0.05 (3/2/1)	102 (95 – 107) 3 (0 – 3) ns (2/1/3)	86 (83 – 89) 31 (16 – 47) p < 0.05 (0/3/3)	†85 (85 – 86) **52** 8 (4 – 16) p < 0.01 (0/4/1)
IL forelimb	101 (91 – 120) 1 (0 – 9) ns (3/2/3)	98 (92 – 104) 3 (2 – 4) ns (1/2/5)	95 (87 – 109) 3 (0 – 11) ns (2/2/4)	105 (98 – 110) 9 (9 – 9) ns (1/0/5)
CL forelimb	104 (102 – 112) 1 (1 – 4) ns (5/1/1)	99 (94 – 103) 5 (2 – 8) ns (2/2/3)	92 (90 – 104) 5 (3 – 10) ns (1/5/1)	†79 (79 – 87) **16** 45 (36 – 45) p < 0.001 (1/5/0)
Snout	†54 (36 – 90) **4** 57 (1 – 57) p < 0.001 (1/9/2)	†78 (69 – 97) **38** 27 (27 – 27) p < 0.01 (2/10/0)	†82 (69 – 92) **26** 30 (30 – 39) p < 0.001 (1/8/3)	†70 (60 – 85) **36** 25 (25 – 25) p < 0.001 (1/6/2)
Tail tip	93 (90 – 105) 1 (0 – 9) ns (3/2/2)	99 (97 – 103) 3 (1.5 – 7) ns (2/1/4)	108 (97 – 126) 11 (9 – 13) ns (4/1/2)	84 (78 – 86) 11 (8 – 17) p < 0.001 (0/3/3)

4.4 Discussion

Previous studies performed in rabbit have indicated that areas of the hindlimb from which individual reflex responses can be sensitized by MO is dependent on descending pathways, particularly with respect to flexor muscle responses (Harris and Clarke, 2003). The present studies therefore built on those findings by investigating the role of descending controls over sensitization of hindlimb withdrawal reflexes in the rat, not only by way of comparison to the previous studies, but also to establish the nature of these controls in a preclinical species with a different locomotor pattern that is more extensively used as a model of chronic pain and other hyperalgesic states (for reviews on this subject see Le Bars et al., 2001, Mogil, 2009, Berge, 2011). Furthermore, the range of reflexes measured has been extended from those studied in rabbit in order to gain a wider understanding of descending controls over the limb as a whole.

This study has revealed that mustard-oil sensitization of withdrawal reflexes in the rat is under both tonic inhibitory and facilitatory descending control mechanisms. This contrasts to findings in the rabbit which only obtained evidence for descending inhibitory controls of reflex sensitization fields (Harris and Clarke, 2003) and raises an interesting difference in this respect between these two and potentially other species including humans. The recognition of a contribution by descending facilitatory pathways in the modulation of reflex responses to a noxious conditioning stimulus was perhaps to be anticipated given the evidence for descending pathways promoting sensitization in the rat (Porreca et al., 2002) and highlights the complex balance that exists between supraspinal inhibitory and facilitatory pathways in influencing spinal excitability. The differences in the extent to which reflexes are facilitated by MO could potentially be attributed to differing characteristics of descending pathways. It has been well established for many years that descending inhibitory pathways are tonically active and disruption of the spinal cord by anaesthetic or cold block or by surgical transection releases this inhibition and results in an increase in excitability of withdrawal reflexes (Eccles and Lundberg, 1959a, Duggan and Morton, 1988). For example, dorsal horn cells in anaesthetized cats displayed “substantially greater spontaneous activity” (Handwerker et al., 1975), which therefore implies that when the spinal cord is intact, activity in dorsal horn cells is suppressed and the whole reflex pathway is less excitable overall. Further evidence supporting a supraspinal origin of tonic inhibition can be demonstrated by electrically stimulating specific brain regions in the

rostral medulla (e.g. lateral reticular nucleus or gigantocellular reticular nucleus, GRN) and observing the effects on other spinally-mediated reflexes such as the tail-flick test (Janss et al., 1987, Zhuo and Gebhart, 1990). Supraspinal centres also provide tonic facilitatory controls of spinally organized reflexes, shown by an increase in excitability of spinothalamic neurones with electrical stimulation of the rostral ventromedial medulla (McCreery et al., 1979) or inversely by destruction the GRN and inhibiting the onset of hyperalgesia (Wei et al., 1999). Influence of supraspinal centres on control of hind-limb withdrawal reflexes has been shown to vary dependent upon the specific nature of the stimulation used to evoke these reflexes. Heat-evoked responses were facilitated following spinalization, whereas responses to noxious cold or mechanical stimuli were inhibited (Kauppila et al., 1998). The exact nature of the descending systems and the supraspinal centres at their origin may account for the difficulty in generating central sensitization using MO in the rat relative to the rabbit (see below).

Of the two influences it seems that descending inhibitory pathways are predominant in the spinally intact animal and therefore when this influence is removed by spinalization, thresholds for evoking reflex responses are lower, hence stimuli required to evoke responses are also reduced. In addition, MO application to the limb always led to facilitation of all four reflexes. This is a similar finding to that seen for the effect of MO on flexor reflexes in the spinalized rabbit (Harris and Clarke, 2003) however a noticeable difference is that inhibition of the heel-MG extensor response seen in this species was not seen in the rat; in fact the pattern of facilitation of heel-MG (and heel-BF) responses in the spinalized rat model was very similar to the flexor responses therefore this reflex appears to be more obviously modulated by descending influences than in the rabbit. A subtle difference may be that spinalization in the rabbit induced an expansion of the sensitization fields across the entire plantar surface of the ipsilateral limb, whereas this effect in the rat is more restricted. Expansion of receptive fields *per se* following spinalization has been reported in other rat studies utilising a range of peripheral stimulus modalities (Cook et al., 1987, Schouenborg et al., 1992) as well as in other species including man (Andersen et al., 1999). A decrease in the cutaneous receptive field of dorsal horn WDR neurones has also been documented (Laird and Cervero, 1990) lending further weight to the argument in favour of descending facilitatory control over spinally mediated withdrawal reflexes.

In spinally intact preparations (decerebrated or anaesthetized) MO was able to facilitate the toes-TA reflex on application to the ipsilateral toe ends, and the heel-MG reflex (as well as the heel-BF response) when applied to the heel, which corresponds with the protective role of these spinal withdrawal reflexes which is to lift the toes and heel (or both in the case of BF) from ground contact respectively. In addition, in the anaesthetized model inhibition of the heel-evoked responses was seen by mustard oil stimuli applied to more distal parts of the plantar surface of the foot whereas toe-evoked responses were inhibited from the heel. Taken together, this reflects the 'modular' pattern of sensitization found in previous studies in the anaesthetized rabbit (Harris and Clarke, 2003) and hence the organization of excitatory cutaneous RFs for reflexes in the rat *per se* (Schouenborg and Kalliomaki, 1990), as well as other species including man (Andersen et al., 1999). Interestingly, although sensitization fields for the two toes-evoked reflexes seemed to expand in the decerebrate, non-spinalized model (whilst the two heel-evoked reflexes displayed a moderate reduction in MO-induced sensitization) the above inhibitory effects seen on MO application to plantar sites were not observed in this preparation suggesting that the mechanism(s) responsible may be present at an anatomical level rostral to the colliculi.

Overall, depression of reflex responses by MO was more readily obtained than sensitization in this study and with longer median durations, in particular in the anaesthetized preparation, which displayed MO-induced inhibition of all reflexes from a diffuse range of treatment sites. As noted above a more restricted attenuation was found in the decerebrate non-spinal group, and a total loss of inhibition was seen in the spinalized preparation. Mustard oil applied to the IL hindlimb was not capable of inhibiting either the knee- or ankle-flexor withdrawal reflexes in the rabbit across the three preparations, rather the opposite effect was achieved – contradictory to the findings presented here from the anaesthetized rat model in which both of these reflexes are inhibited by MO application to the IL heel. In further contrast to the rat findings, inhibition of heel-MG was observed in decerebrate spinalized rabbits when the sensitizing stimulus was applied to distal ipsilateral sites. Taken together, these details highlight the dissimilarity between both the propensity for mustard oil to sensitize reflex responses and the balance between descending facilitatory and inhibitory influences in the two species.

Inhibition of hind-limb withdrawal reflexes by applying a noxious stimulus to off-limb sites (e.g. heel-MG with MO to tail tip or toes-TA with MO to snout) is likely to be related to a phenomenon known as diffuse noxious inhibitory controls (DNIC), a term coined by Le Bars and colleagues (1979). Stimulation of Aδ- or C- fibres at remote sites such as tail tip or snout is capable of inhibiting the outputs from the interneurones onto which the primary afferents converge, and has been shown in rat, rabbit, and man (Schouenborg and Dickenson, 1985, Willer et al., 1989, Kalliomäki et al., 1992, Gjerstad et al., 2000, Harris and Clarke, 2003). In spinally-transected animals this effect is abolished, indicating that this effect is controlled by supraspinal structures (Morton et al., 1987).

The reflexes examined in this study may therefore be subdivided by function into flexors (toes-TA and toes-BF) and extensors (heel-MG and heel-BF), given that BF has dual functionality: in rat this muscle has a biarticular role as both a knee flexor and a hip extensor. The above findings imply that the dominant action of descending pathways on hindlimb extensors in the rat is inhibitory, whereas the action on flexors is a balance of inhibitory and facilitatory effects, an outcome not observed in the rabbit studies. Descending modulation of flexors and extensors, mediated via the reticulospinal tract, has been shown to be differentially controlled at the level of the brainstem with low medullary lesions inhibiting extensor function while also releasing inhibition of flexors, possibly due to lesions at the level of the rostral ventromedial medulla (RVM) (Holmqvist and Lundberg, 1961, Zhuo and Gebhart, 1997, Wei et al., 1999). The spinalized animal, having the neuraxis disrupted at a more caudal location, is therefore also subject to this effect. Heterogeneous effects of spinalization have also been shown to be dependent on the nature of the test stimulus employed, with noxious cold and mechanical responses suppressed following spinalization and radiant heat responses facilitated (Kauppila et al., 1998). This suggests a possible divergence between the control of reflexes with myelinated and unmyelinated afferent fibre sensory inputs, and that under hyperalgesic conditions spinal cord excitability is enhanced so that when descending pathways are interrupted only disinhibition is apparent. Pharmacological evidence for a differential control of these differently evoked reflexes has been observed previously in rabbit, in which the cannabinoid agonist HU 210 was found to have a more potent inhibitory effect on the heel-MG reflex relative to anything evoked from the toes (Jenkins et al., 2004) thereby raising the possibility of other differences existing between the control mechanisms underlying the sensitization of these

two reflex categories. However, this example only describes varying levels of inhibition rather than a split inhibitory/facilitatory effect.

Of particular relevance to hind-limb withdrawal reflex studies is the natural gait of the species in question, as different locomotor patterns require different patterns of muscle flexion and extension. In the case of the rat, the hind-limbs flex and extend alternately around the pelvic girdle, whereas the rabbit locomotes with a hopping motion, generating force from both hind-limbs synchronistically. Central pattern generator (CPG) neuronal networks in isolated rat spinal cord preparations exhibit rhythmic firing that correlates to limb motion (Cazalets et al., 1990) and thereby suggests a spinal organization for stereotyped locomotor behaviour. This implies a limited input or control of these actions by supraspinal structures other than during voluntary motion. Despite the variation in the specifics of their individual gaits, both species locomote in a primarily digitigrade manner with plantigrade adopted during rest or standing (Viala, 2006, Bennett et al., 2012), though gait may still account for some of the differences in sensitization pattern between the species. For example, in decerebrate non-spinal rabbits, the knee flexor reflex was sensitized from across the plantar surface, whereas in the same rat preparation sensitization of the equivalent reflex was restricted to mid-sole and ankle only, and the gait difference may also contribute to the shift in sensitization pattern seen in the rat flexor reflexes as opposed to the expansion seen in the rabbit. A comparative study between decerebrate and decerebrate spinalized rabbits found that the spinally intact animal still displayed the hopping-type motion whereas spinally-transected animals adopted a bilateral alternating motion as in the rat (Viala, 2006). This differing supraspinal control of reflexes may be a critical factor in the relatively narrow sensitization observed in the rat relative to the rabbit.

A prominent finding of the current studies is that with the exception of injection into deeper tissues, MO induced enhancement exhibited a much shorter duration in this model than was found in the rabbit (median duration 5 minutes compared to greater than 40 minutes) and with lower median peak increases (112-108% vs. 493-343% respectively). The longer duration of MO-induced reflex enhancement that occurred following treatment of deeper sites is in agreement with previous observations in rat, in which noxious stimulation of the gastrocnemius muscle or the ankle joint generated long-lasting increases in withdrawal reflexes (Wall and Woolf, 1984, Woolf and Wall, 1986).

The reduced duration from topical sites in rat relative to rabbit may be a result of a different expression profile of TRPA1 limiting the afferent barrage transmitted to the spinal cord during MO application and thereby decreasing the duration of sensitization. TRPA1 is widely reported to be expressed on primary afferent C-fibres in several species (Story et al., 2003, Kobayashi et al., 2005, Atoyan et al., 2009), but recent studies have also revealed localisation in epidermal keratinocytes in mouse and human (Anand et al., 2008, Denda et al., 2010). While these investigations are still in their infancy, it is feasible that the differences in MO-induced enhancement of reflexes between rat and rabbit are related to variations in epidermal TRPA1 expression. Ion channel binding characteristics may also be responsible for the differences observed. It is known that MO binds to TRPA1 via an electrophilic interaction with specific cysteine resides (Hinman et al., 2006, Macpherson et al., 2007a) and a simple point mutation in the gene resulting in a missense mutation in the primary sequence of the protein could dramatically alter the receptor's affinity for the agonist. In addition, a recent study performed in knock-out mice strongly indicated that MO is also an agonist for TRPV1, and that in patch clamping experiments MO activation of TRPA1 occurs rapidly and at low concentrations while TRPV1 activation requires a higher concentration of MO but persists for a longer period of time (Everaerts et al., 2011). Coupled with the fact that TRPA1 and TRPV1 are frequently co-expressed on sensory neurones (Story et al., 2003, Kobayashi et al., 2005) it is possible that the longer duration sensitization found in the rabbit studies is due to MO acting at both TRPA1 and TRPV1, while the transient effects found here in the rat are more purely mediated via TRPA1.

The relative ease with which sensitization of withdrawal reflexes is induced by MO in the rabbit relative to the rat may also be due to other anatomical/physiological factors, in particular the fact that the rabbit hind-limb is almost exclusively covered by hairy skin, including the plantar surface (apart from a small region at the heel known as the calcaneus) whilst the rat has glabrous skin at the plantar surface and hairy skin elsewhere on the foot and limb. TRPA1 is expressed in both skin types in mice, (Kwan et al., 2009) and in hairy skin in humans at least (Anand et al., 2008, Atoyan et al., 2009) therefore a reasonable assumption would be that this channel is also expressed in both skin types in rat and rabbit and would not necessarily explain the discrepancy in sensitization field or duration. However, MO has been shown in anaesthetized rats to excite DH neurones with receptive fields located on hairy skin to a greater degree than those with glabrous skin RFs (Harris and Ryall, 1988), possibly mediated by Aδ fibre activation. The transient nature of those

effects is corroborated by others (Heapy et al., 1987) as a result of Aδ vs. C fibre activation, and therefore, relating to the findings presented in this chapter, may partially explain why the duration of reflex facilitation is much shorter in rat than in rabbit. From these data, it may be postulated that the mechanism behind this sensitization is peripherally-driven in the rat given the relatively short recovery times compared to the longer-term potentiation observed in the rabbit.

4.5 Conclusions

Modulation of hindlimb withdrawal reflexes by MO is more strongly influenced by descending facilitatory pathways in the rat than in the rabbit, with a shift in sensitization fields observed following spinalization. However, the effects tended to be of a more transient nature indicating either that the MO stimulus is not potent enough in the rat to generate central sensitization or that descending inhibitory controls are acting to suppress that response. The dominant effect of MO in the intact anaesthetized preparation was inhibition of reflex responses, with the decerebrate spinalized preparation demonstrating reflex facilitation only. Decerebrate non-spinal animals represented a more balanced pattern with a combination of facilitation and inhibition, though the anaesthetized preparation best reflected a modular organization of reflex modulation. This study also emphasises that in order to relate findings from one pre-clinical species or another to clinical conditions the idiosyncrasies of that species need to be carefully scrutinized with regard to any translatable conclusions.

5. NORADRENERGIC MODULATION OF SPINAL REFLEXES

5.1 Introduction

Modulation of hindlimb reflexes by mustard oil application to either superficial or deep tissue sites is influenced by supraspinal control mechanisms, with site- and reflex-specific actions (see chapter 4). Transection of the spinal cord of decerebrate rats resulted in an increase in MO-induced reflex excitability in several instances, with facilitation produced by treatment at IL sites including cutaneous application at the flexion of the ankle and heel, and deeper application at the ankle and LG, which was not observed in the corresponding non-spinal preparation; this enhancement of facilitation in spinalized animals occurred in each of the reflexes examined. These findings therefore demonstrate that surgically transecting the spinal cord has removed a modulatory inhibitory control and that reflex excitability is influenced by the actions of descending pathways (see section II.1.2). Analogous studies from this laboratory performed in the rabbit also found an increase in reflex enhancement by MO in the spinalized preparation compared to the corresponding spinally-intact model (Harris and Clarke, 2003). For example, the sensitization field of the toes-TA reflex showed significant expansion post-spinalization, encompassing proximal plantar and lower limb sites from which MO-induced facilitation was absent in the non-spinalized preparation.

The brain nucleus most readily implicated as the origin of this descending inhibitory control mechanism is the noradrenergic cell group A6 – the locus coeruleus (see section II.A.2.1), which has been proven to be a source of noradrenergic terminals located within the spinal cord (Loewy et al., 1979, Westlund et al., 1981, Tavares et al., 1996).

In particular, noradrenergic influences over spinal cord excitability and hence spinally-mediated reflex responses occur primarily via the α_2-adrenoceptor subtype expressed therein (see sections II.A.3.1 and II.A.3.4). Blockade of these receptors in the spinal cord has a facilitatory effect on spinal responses measured *in vivo* (Ogilvie et al., 1999, Rank et al., 2011) whilst spinal application of selective agonists such as bromonidine increases withdrawal latency and inhibits dorsal horn responsiveness (Stone et al., 1997, Chen et al., 2011), thus confirming the role of noradrenergic α_2-adrenoceptors in modulation of responses of this nature.

Spinal reflexes in the rabbit have been shown to be subject to a tonic inhibitory noradrenergic control mediated by spinal α_2-adrenoceptors (Harris and Clarke, 1992, Harris and Clarke, 1993). In addition, in this species preliminary studies of MO-induced inhibition of reflexes from off limb sites found that spinal α_2-adrenoceptors mediated at least some of this effect (Harris et al., 2003). However an investigation of the possible mediators of MO-induced inhibition at sites directly on the limb has not been previously performed. This study has therefore investigated the effects in the decerebrated rat of spinal application of the selective α_2-adrenoceptor antagonist RX 821002 (Hudson et al., 1992, Mallard et al., 1992, Clarke and Harris, 2002) on reflexes *per se* and on sensitization of those reflexes by mustard oil.

5.2 Methods

Experiments were performed on a total of 38 rats with a mean weight of 313 g ± 16 g. Two groups of experiments were performed. The first was a dose-response study for intrathecal application of RX 821002 (n = 6 for a vehicle control study and n = 7 for the antagonist). The data obtained from those experiments then informed the dose selection for the second group (n = 25), in which the effects of intrathecal RX 821002 on MO-induced modulation of hindlimb reflexes was examined.

5.2.1. Surgical preparation

The surgical procedures performed are described in detail in section 2.1. Briefly, in all animals, the trachea, left carotid artery, and left jugular vein were cannulated under isoflurane anaesthesia (2.5 – 3%) for airway maintenance, blood pressure monitoring, and anaesthetic/replacement fluid administration respectively. Post-decerebration to the pre-collicular level using the method described in chapter 3, a sub-anaesthetic dose of alfaxalone (1 mg mL^{-1} prepared in a solution of D-glucose and sodium hydrogen carbonate both at 100 mM) was administered i.v. at a rate of 1 ml hr^{-1}. As all animals in this cohort were decerebrated, the right carotid artery was also reversibly occluded. A laminectomy was performed between vertebrae T8 and T9 to permit placement of an intrathecal (i.t.) cannula. The cannula was inserted from a small incision in the meninges at the caudal most point of the laminectomy, and was inserted 11 mm caudal from the point of insertion so that the tip lay at the level of the L3/L4 segment of the spinal cord. The cannula was

sutured into position via ligatures secured in the muscle tissue overlying the T8 vertebra to prevent movement during drug administration. Ringer-soaked cotton wool was placed over the exposed surface of the cord to prevent tissue dehydration, a silver-silver chloride earthing pellet inserted into an exposed muscle group, and the bisected skin and muscle were then sutured together over the laminectomy. Confirmation of cannula placement was carried out at the end of each experiment by injecting 10 µL of potassium chloride solution (saturated) i.t. with a vehicle flush. A successful cannula placement resulted in activation of the muscles of interest within a few seconds of application. Post-mortem examinations were carried out in instances where KCl failed to produce a timely activation of those muscles. In some experiments 10 µL pontamine sky blue was applied intrathecally followed by the standard vehicle flush to ascertain the regions of the spinal cord reached by the antagonist when administered in these volumes. The staining typically extended from the rostral edge of the T11 vertebra to the caudal edge of the T13 vertebra corresponding to spinal cord segments T13 to L5 inclusively (Hebel and Stromberg, 1976) therefore encompassing the dermatomes of the muscles of interest (Takahashi et al., 1994). This also indicated that the antagonist was applied at spinal levels corresponding to the primary afferent terminations of fibres innervating the MO treatment sites (Molander and Grant, 1986).

Needle electrodes were situated either side of the thorax to enable recording of an ECG. EMG electrodes were implanted into the left MG, BF and TA with responses evoked by alternate electrical stimulation at the heel and toes every 2 mins using bipolar needle electrodes (see section 2.1.5).

5.2.2. Experimental protocol

i) Dose-response studies

Following attainment of consistent control reflexes (i.e. three consecutive readings within 10% of one another), a series of seven doses of either vehicle (Ringer's solution; n = 6) or increasing concentrations of antagonist (n = 7) were administered intrathecally separated by an interval of 24 minutes (i.e. six pairs of readings from both heel- and toes-evoked reflexes between doses). Vehicle was applied as a 20 µL bolus on each occasion and RX 821002 was applied in a volume of 10 µL followed by a 10 µL vehicle flush of the i.t.

cannula using a needle-tipped 50 µL Hamilton syringe (22S gauge). RX 821002 hydrochloride (Tocris Bioscience) was dissolved in rat Ringer's solution to give a stock solution of 10 mg mL^{-1} (36.9 mM). The stock was serially diluted to give four solutions of (1) 0.0$\dot{3}$ mg mL^{-1}, (2) 0.1 mg mL^{-1}, (3) 0.$\dot{3}$ mg mL^{-1}, and (4) 1 mg mL^{-1}. A cumulative i.t. dosing regime was employed, in which the total mass of drug applied refers to the antagonist as the hydrochloride salt. The cumulative doses applied were (in µg): 0.3, 1, 5, 10, 20, 30, and 100.

ii) MO in the presence of i.t. RX 821002

To examine the effect of i.t. RX 821002 on the effects of MO applied at different sites on the hindlimb on reflex responses, experiments were conducted according to the following protocol, with stable control reflexes attained prior to each drug and each mustard oil treatment: 20 µL vehicle (Ringer's solution) was applied intrathecally; 2.5 µL of 20% mustard oil was then applied to the lateral or medial aspect of the IL heel, IL MTJ, or IL flexion of the ankle and reflexes followed for a minimum of 63 minutes; 10 µg RX 821002 in a volume of 10 µL plus a 10 µL Ringer flush was applied intrathecally; finally 2.5 µL of 20% mustard oil was applied to the same site at that treated previously but to the aspect as yet untreated (i.e. lateral if medial was treated initially), and reflexes again followed for a minimum of 63 minutes. The dose of antagonist used was chosen on the basis of data obtained in the dose-response study. The volume of mustard oil applied was half that used during the organization of sensitization study (chapter 4) in order to minimise spread of the solution across the skin and thereby allow a second treatment to be applied to the same 'site' (MTJ, heel, flexion) but to adjacent skin. The order in which the adjacent skin areas were treated i.e. lateral region vs. medial region was alternated between experiments. Sites for MO treatment were selected based on the results of the previous chapter into the organization of reflex sensitization.

5.2.3. Statistical analysis

Electrical thresholds and stimulation intensities were assessed for significant differences between the heel and toes sites using Wilcoxon's matched pairs tests. Raw control reflex responses were compared using Kruskal-Wallis one-way ANOVA tests.

Within individual experiments, reflexes were normalised such that each response is a percentage of the mean of three pre-treatment control readings per reflex. Values for reflexes for pooled data for all experiments are then expressed as medians and inter-quartile ranges. For cardiovascular parameters, within individual experiments the difference from the mean of ten pre-drug control readings was calculated for each minute following drug administration and the median and inter-quartile ranges calculated for the pooled data. Non-parametric statistical tests have been performed throughout for reflex and cardiovascular data, as every data set was tested and failed the Kolmogorov-Smirnov test for a Normal distribution.

For dose-response experiments, in order to determine the effect of either vehicle or RX 821002 on reflexes or cardiovascular measurements compared to pre-treatment controls, non-parametric one-way ANOVAs were used; for vehicle data these were Friedman's ANOVAs whilst, due to missing values at the highest dose in one experiment, the effect of cumulative RX 821002 i.t. was assessed using Kruskal-Wallis one-way ANOVAs – both were followed by Dunn's Multiple Comparison tests. Significance between vehicle and RX 821002 dose-response curves was assessed using Scheirer-Ray-Hare non-parametric two-way ANOVAs.

Assessment of the effect of mustard-oil on reflexes in the presence of vehicle or RX 821002 compared to pre-MO controls was assessed using Friedman's ANOVA on ranks delimited by the duration of the effect, determined as when post-MO values had returned to within two standard deviations from the mean pre-MO control reflex responses. Comparisons between the effect of MO with/without RX 821002 were made using Scheirer-Ray-Hare non-parametric two-way ANOVAs over the time period indicated by the outcome of the Friedman's ANOVAs.

5.3 Results

The median electrical threshold for evoking heel-MG reflexes was 0.53 mA (IQR 0.42 – 0.75) with a subsequent stimulation strength of 1.80 mA (IQR 1.25 – 2.54), and the threshold for toes-evoked reflexes was 0.29 mA (IQR 0.26 – 0.35) with a stimulation strength throughout recording of 1.05 mA (IQR 0.76 - 1.96 mA). The thresholds were significantly different to one another ($p < 0.001$, Wilcoxon's Matched Pairs Test), with the stimulation parameters

employed throughout the recording period also significantly different ($p < 0.05$, Wilcoxon's Matched Pairs Test).

Median control raw reflex responses were 265 μV.ms (IQR 142 - 593) for heel-MG, 515 μV.ms (IQR 325 - 921) for heel-BF, 125 μV.ms (IQR 93 - 186) for toes-TA, and 284 μV.ms (IQR 182 - 438) for toes-BF. These were significantly different from one another ($p < 0.01$, Kruskal-Wallis test) with the subsequent post-test indicating that the differences lay between toes-TA and each of the other reflexes ($p < 0.01$, Dunn's Multiple Comparison test).

5.3.1. Effect of RX 821002 on reflex responses per se

Repeated i.t. vehicle application generally had no significant effect on reflex responses ($p > 0.05$, Friedman's one-way ANOVAs, $n = 6$) although a small significant ($p < 0.05$, Friedman's ANOVA) decrease in toes-BF responses did occur in these particular studies (figure 5.1). In contrast, cumulative application of the noradrenergic α_2-adrenoceptor antagonist RX 821002 to the spinal cord resulted in significant facilitation of all four reflex responses ($p < 0.05$, Kruskal-Wallis one-way ANOVAs, $n = 7$) (figure 5.1, raw traces provided in figure 5.2). The effect of the antagonist compared to vehicle was also confirmed as significant by the non-parametric two-way ANOVA tests ($p < 0.02$ for all reflexes). Heel-MG responses showed the greatest facilitation in the presence of the antagonist, first displaying a significant increase ($p < 0.05$, Dunn's Multiple Comparison Test) after an i.t. dose of 5 μg and a maximum median increase of 275% (IQR 178 - 396%) of controls following a 10 μg cumulative dose. The two BF reflexes were also greatly enhanced by application of the antagonist, with potentiation of heel-BF and toes-BF first becoming significant ($p < 0.05$ in each case, Dunn's Multiple Comparison Test) after 5 μg and 20 μg cumulative doses and maximum median increases of 213% (IQR 186 - 319%) and 220% (IQR 183 - 268%) respectively being obtained after a 30 μg cumulative dose in each case. Finally reflex responses evoked in TA from the toes first became significantly ($p < 0.05$, Dunn's Multiple Comparison Test) facilitated after a 20 μg i.t. dose of RX 821002, with a maximum median increase of 172% (IQR 153 - 254%) above controls after a 30 μg cumulative dose.

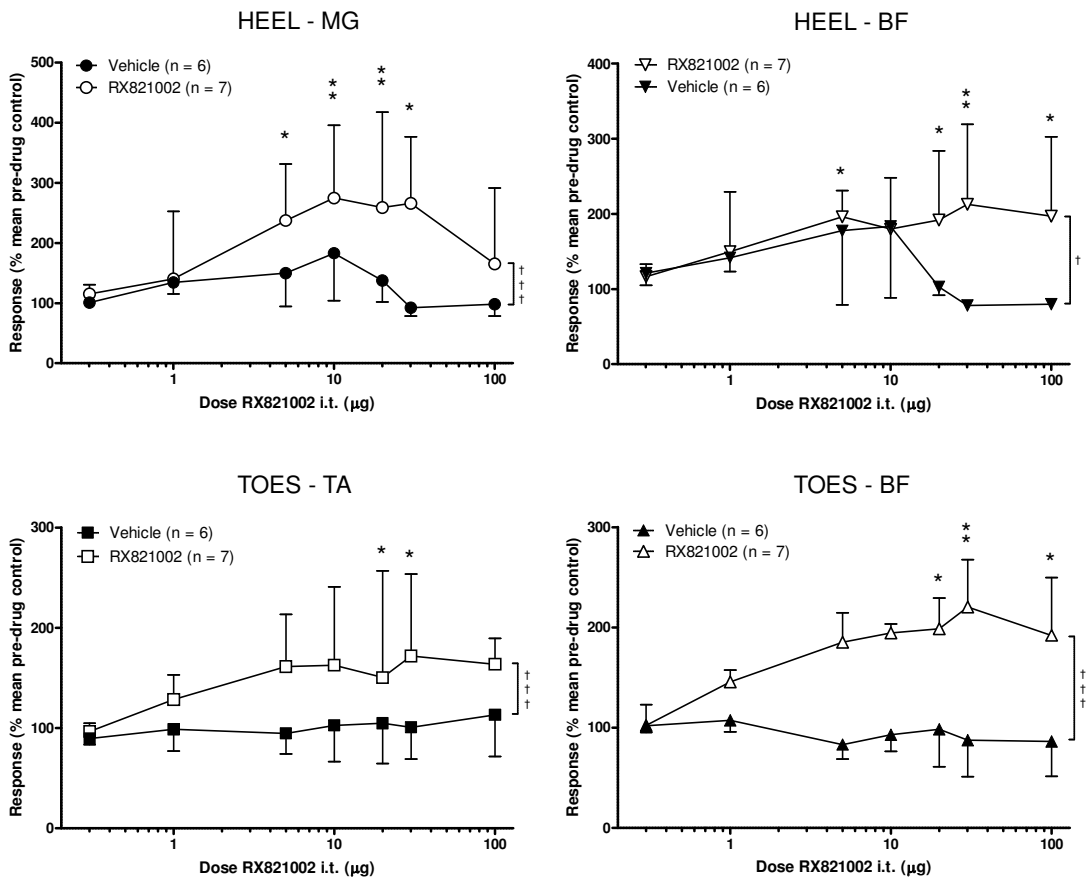


Figure 5.1: Effect of cumulative intrathecal application of vehicle or the noradrenergic α_2-adrenoceptor antagonist RX 821002 on each of the four hindlimb reflexes. All responses were significantly enhanced by RX 821002 administration (Friedman's ANOVAs, see body text). Asterisks indicate significant differences compared to pre-treatment controls (* $p < 0.05$, ** $p < 0.01$, Dunn's multiple comparisons post-test) and obelisks denote significant differences between vehicle and ondansetron dose-response curves († $p < 0.05$, ††† $p < 0.001$, Scheirer-Ray-Hare two-way ANOVA).

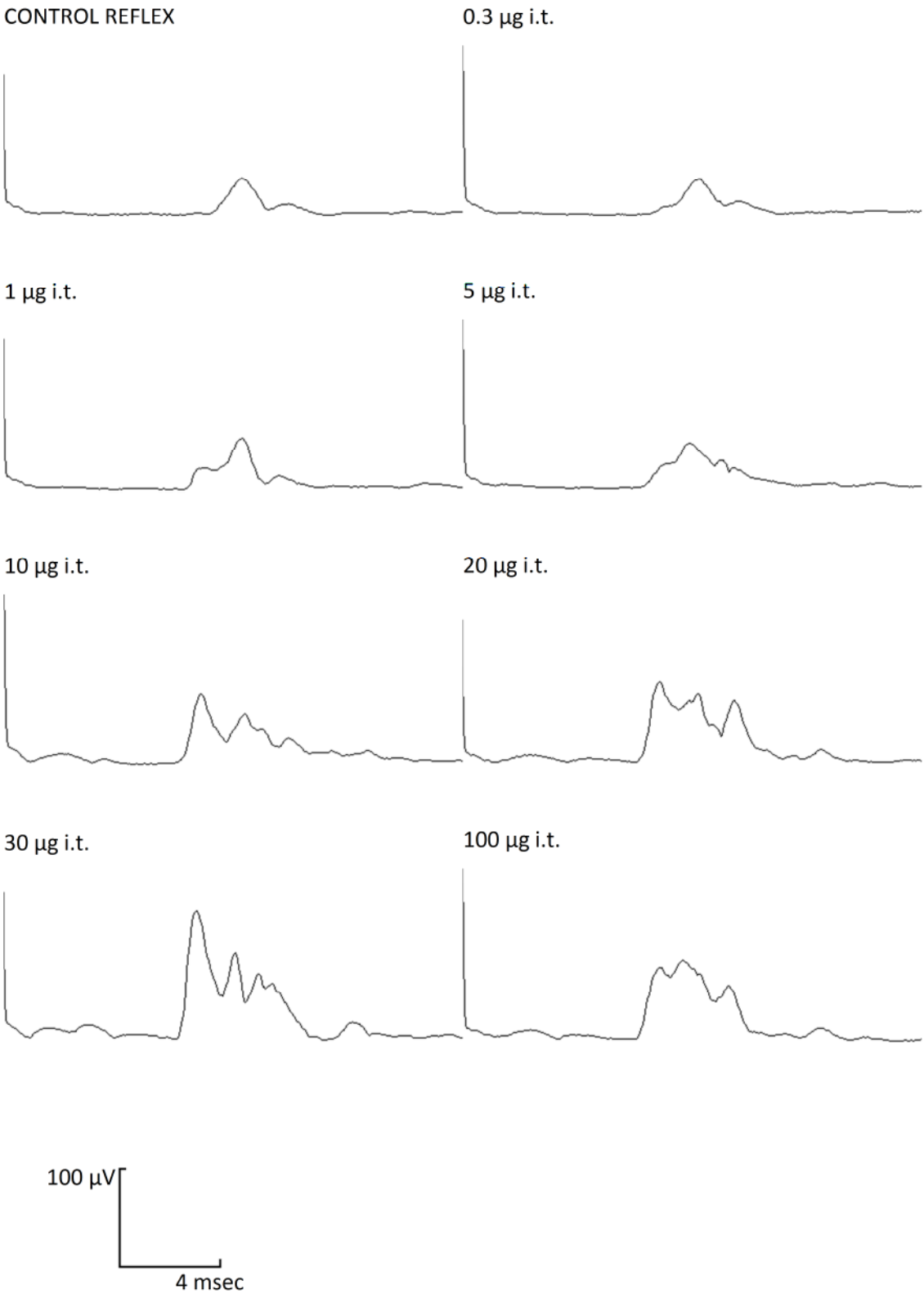


Figure 5.2: Raw data traces showing the facilitatory effect of RX 821002 on the heel-MG reflex. Data are averages of 8 sweeps.

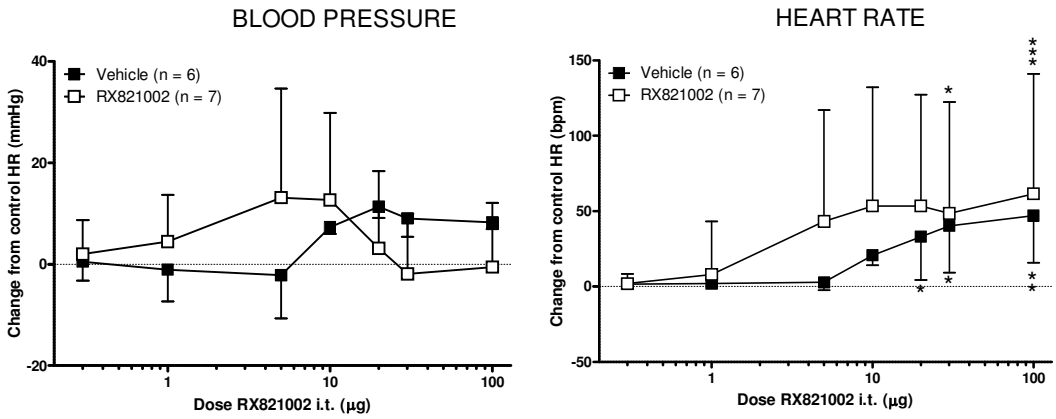


Figure 5.3: Effect of cumulative intrathecal application of vehicle or the noradrenergic α_2-adrenoceptor antagonist RX 821002 on cardiovascular responses (median ± upper/lower quartile). Blood pressure was significantly altered from control values in a biphasic fashion whereas HR also showed significant increases (Friedman's ANOVAs, see body text). Asterisks indicate significant differences compared to pre-treatment controls (* $p < 0.05$, *** $p < 0.001$, Dunn's multiple comparisons post-test).

The median control values (i.e. pre-drug) for MAP and HR were 79 (IQR 62 – 83) mmHg and 374 (IQR 357 – 431) bpm for the vehicle group and 69 (IQR 63 – 86) mmHg and 428 (IQR 403 – 448) bpm for the drug group. MAP changed significantly from control values throughout the dosing study for both vehicle and drug treatments ($p < 0.05$, Friedman's ANOVA), whilst HR also showed a significant increase from basal levels ($p < 0.001$, Friedman's ANOVA), in particular at the latter two doses for both treatments ($p < 0.05$, Dunn's Multiple Comparison test). The alteration in MAP observed as a result of RX 821002 dosing was biphasic in character, and although not found to be significantly different from the change in MAP observed during the vehicle dose response study ($p > 0.05$, Scheirer-Ray-Hare test), this still demonstrated a peak elevation at the cumulative dose of 5 μg to a median maximum of 95 (IQR 61 – 106) mmHg. The peak elevation in HR for both vehicle and treated groups occurred at the final dose (100 μg RX 821002 and the seventh dose of vehicle), with median maxima of 499 (IQR 484 – 500) bpm and 441 (IQR 407 – 461) bpm for drug- and vehicle-treated cohorts, respectively.

5.3.2. Mustard oil-induced changes in reflexes in the presence of RX 821002

i. Effect of MO applied to IL flexion of the ankle (figure 5.4)

In the presence of vehicle, mustard oil application at this site significantly (Friedman's ANOVA, $p < 0.04$, $n = 8$) potentiated heel-MG reflexes to a peak of 160% (IQR 143 - 231%) of controls (after 1 min) for a median duration of 33 min (IQR 23 - 53 min; figure 5.4). In contrast toes-TA responses displayed a small but significant (Friedman's ANOVA, $p < 0.04$) decrease following MO application to the IL flexion of the ankle (peak median inhibition was to 85% (IQR 82 - 92%) of controls 11 mins after MO application) of median duration 23 mins (IQR 2 - 63 mins).

In the presence of 10 μg i.t. RX 821002, no significant alteration of reflex responses was induced by MO application. Thus compared to pre-MO control responses, heel-MG reflexes did not significantly increase and toes-TA responses were not significantly inhibited (Friedman's ANOVAs, $p > 0.05$).

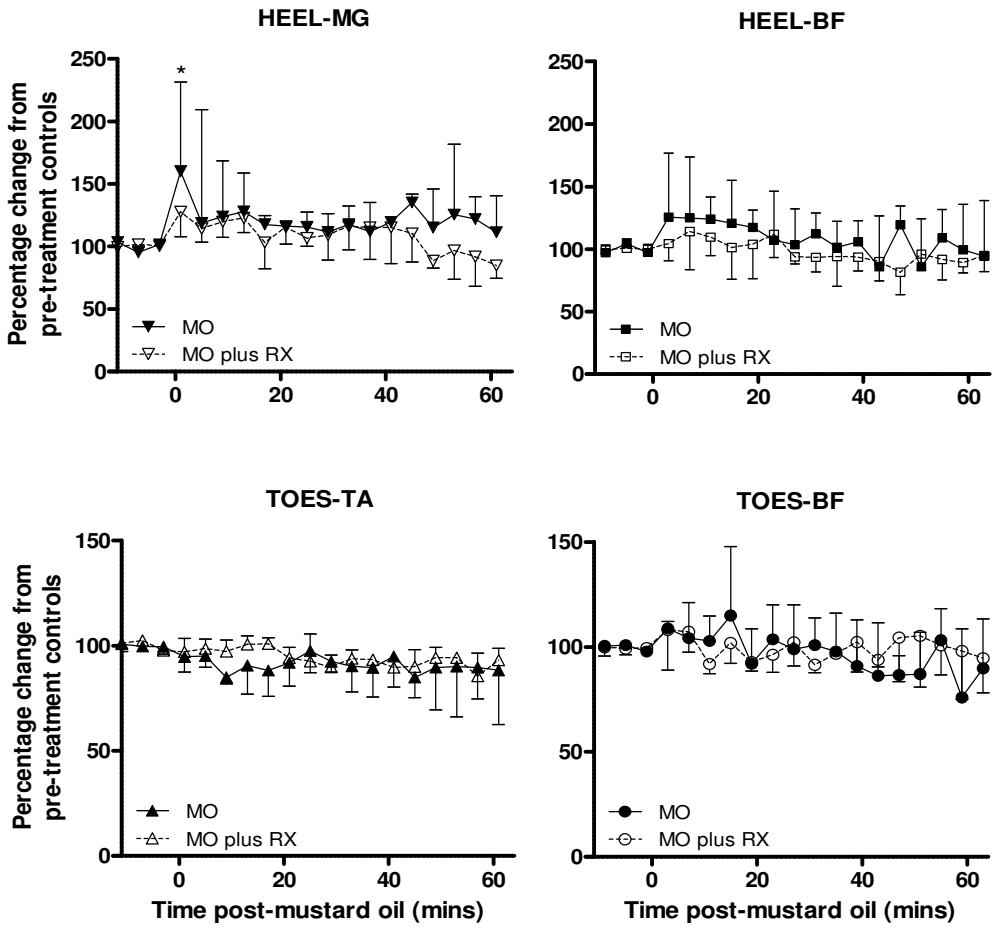


Figure 5.4: Effect of mustard oil application to the ipsilateral flexion of the ankle alone i.e. following vehicle (MO; filled symbols) and in the presence of i.t. RX 821002 (MO plus RX; open symbols). Upper left graph (inverted triangles) shows heel-MG reflex responses, upper right graph (squares) shows heel-BF responses, lower left graph (triangles) shows toes-TA reflex responses, and lower right graph (circles) shows toes-BF responses. Data are median values displayed with either upper or lower quartiles. Mustard oil was applied at time 0. Asterisks denote significant differences from pre-MO control values (* $p < 0.05$, Dunn's Multiple Comparison test after Friedman's ANOVA).

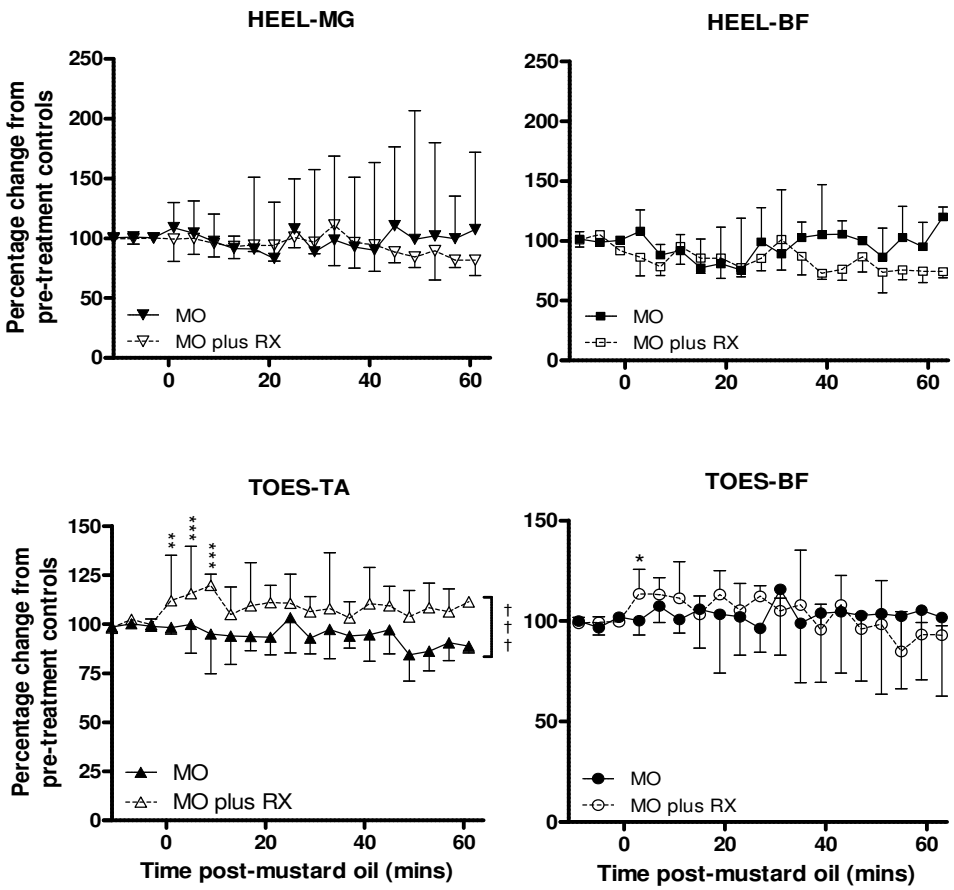


Figure 5.5: Effect of mustard oil application to the ipsilateral metatarsophalangeal joints alone i.e. following vehicle (MO; filled symbols) and in the presence of i.t. RX 821002 (MO plus RX; open symbols). Upper left graph (inverted triangles) shows heel-MG reflex responses, upper right graph (squares) shows heel-BF responses, lower left graph (triangles) shows toes-TA reflex responses, and lower right graph (circles) shows toes-BF responses. Data are median values displayed with either upper or lower quartiles. Mustard oil was applied at time 0. Asterisks denote significant differences from pre-MO control values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Dunn's Multiple Comparison test after Friedman's ANOVA), whilst obelisks denote significant differences between MO and MO plus RX 821002curves (††† $p < 0.001$, Scheirer-Ray-Hare two-way ANOVA).

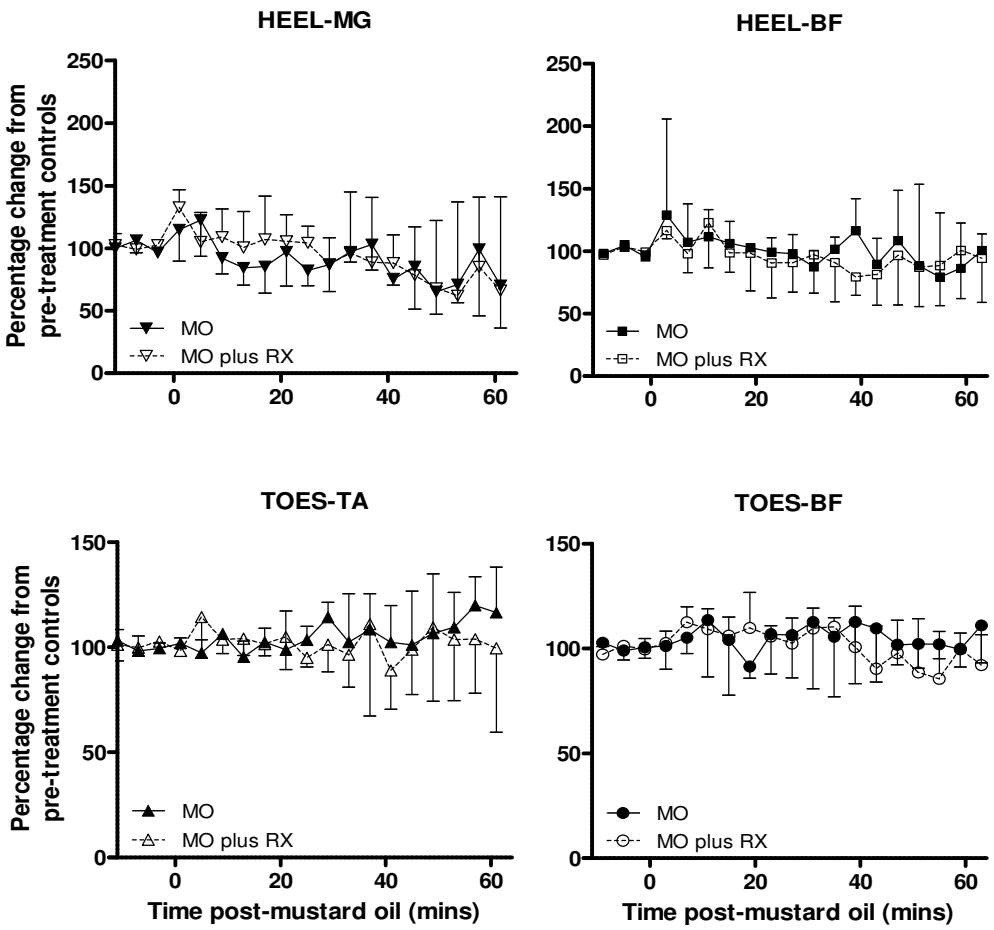


Figure 5.6: Effect of mustard oil application to the ipsilateral heel alone i.e. following vehicle (MO; filled symbols) and in the presence of i.t. RX 821002 (MO plus RX; open symbols). Upper left graph (inverted triangles) shows heel-MG reflex responses, upper right graph (squares) shows heel-BF responses, lower left graph (triangles) shows toes-TA reflex responses, and lower right graph (circles) shows toes-BF responses. Data are median values displayed with either upper or lower quartiles. Mustard oil was applied at time 0.

ii. Effect of MO applied to IL MTJ (figure 5.5)

MO application (following i.t. vehicle) to the IL MTJ produced no significant changes in all four reflexes (Friedman's ANOVAs, $p > 0.05$). In contrast, a small but significant enhancement was seen for reflexes evoked from the toes when MO was applied in the presence of RX 821002. For toes-TA responses, this increase (Friedman's ANOVA, $p < 0.003$, $n = 10$) was to a median maximum of 120% (IQR 112 - 125%) of controls at 11 min post-MO for a median duration of 19 min (IQR 11 – 63 min), whilst the enhancement (Friedman's ANOVA, $p < 0.05$) of toes-BF responses was to a median maximum of 113% (IQR 102 - 126%) of controls at 3 min post-MO for a median duration of 7 min (IQR 0 – 31 min).

iii. Effect of MO applied to the IL heel (figure 5.6)

Mustard oil applied to the ipsilateral heel produced transient (< 5 min) increases in heel-MG and heel-BF reflexes, however these increases were only significant (Wilcoxon matched pairs tests, $p < 0.04$ for both reflexes) when RX 821002 was present; responses 3 min after MO application being 133% (IQR 114 – 147%) and 117% (IQR 110 – 143%) of controls for heel-MG and heel-BF respectively. Reflexes evoked from the toes were similarly not significantly changed by MO application (following vehicle) to the heel, however following antagonist treatment, MO did induce significant (Friedman's ANOVA, $p < 0.04$) facilitation of the toes-BF reflexes to a median peak of 113% (IQR 110 – 120%) of controls 7 min after its application, for a median duration of 13 min (IQR 5 – 21 min).

5.4 Discussion

The present studies have shown that intrathecal application of the selective α_2-adrenoceptor antagonist RX 821002 to the spinal cord of the decerebrated rat facilitates spinally-mediated reflex responses, thereby implying that they are subject to tonic noradrenergic inhibition mediated by spinally located α_2-adrenoceptors.

The influence of noradrenaline (NA) on spinal processing is something that has been studied for a number of decades. NA and its biosynthetic precursor L-DOPA have both been investigated in terms of their actions on the excitability of reflex pathways *in vivo* in studies

looking at either neuronal firing rates or EMG responses. When applied systemically (i.v.) L-DOPA depressed transmission from primary afferents to MNs in spinalized animals (Andén et al., 1964, Andén et al., 1966, Schomburg and Steffens, 1988), an action confirmed as noradrenergic as opposed to dopaminergic by pre-treatment with α_2-adrenoceptor antagonists (Andén et al., 1966). When applied directly to the spinal cord, NA exhibited a dose- and site-dependent modulation of reflex excitability. At low doses or when injected into the dorsal horn only, NA had an inhibitory effect on reflex activity (Bell and Matsumiya, 1981, Wiesenfeld-Hallin, 1987, Sakitama, 1993) and at higher doses or given into the ventral horn, the opposite effect was observed i.e. excitation (Dhawan and Sharma, 1970, Bell and Matsumiya, 1981, Wiesenfeld-Hallin, 1987, Sakitama, 1993). These contradictory observations were hypothesized to relate to the differential effects of NA on dorsal horn sensory neurones compared to ventral horn MNs (Andén et al., 1964), along with the potential for the highest doses to act as inhibitors of inhibitory pathways (Wiesenfeld-Hallin, 1987). More recent evidence has shown that dorsal horn interneurones receiving inputs from different subtypes of muscle afferent fibres are differentially modulated by NA application to the spinal cord, specifically with depression of responses evoked from group II afferents but facilitation of responses evoked from group I afferents (Jankowska et al., 2000). The differential effects of NA can in fact be broadly attributed to mediation by the two main α-adrenoceptor subtypes: α_2-adrenoceptors mediating inhibitory effects are most densely expressed in the superficial dorsal horn i.e. lamina I and IIo (Uhlén et al., 1993, Roudet et al., 1994, Uhlén et al., 1997, Stone et al., 1998, Nicholson et al., 2005, Chen et al., 2007) and α_1-adrenoceptors mediating facilitatory effects in the ventral horn (Day et al., 1997, Nicholson et al., 2005; see also section II.A.3.4). As NA is a non-subtype selective agonist, a proportion of the pronociceptive findings may be attributed to activation of α_1-adrenoceptors (Nuseir and Proudfit, 2000, Nalepa et al., 2005). Therefore in addition to endogenous molecules, the improvement of the specificity and affinity of adrenoceptor subtype-selective ligands has enhanced our understanding of the underlying mechanisms of noradrenergic-mediated depression (and facilitation) of reflex excitability.

Yohimbine (Spiegel, 1896) and rauwolscine (Chatterjee, 1941), both derived from natural sources, are regarded as antagonists selective for the α_2-adrenoceptor subtype (see section II.A.3.1). Synthetic ligands for this receptor family were later developed using the molecular template of benzodioxan (also referred to as piperoxan), a known adrenolytic agent (Goldenberg et al., 1947, Gifford et al., 1952). The substitution of the piperidine functional

group of benzodioxan for a imidazole moiety produced a ligand with higher selectivity for the α_2 subtype known as idazoxan (Chapleo et al., 1981, Chapleo et al., 1983, Doxey et al., 1984), which was further modified with a methoxy group to form the even more potent compound RX 821002 (chemical name 2-(2,3-Dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1H-imidazole hydrochloride) (Stillings et al., 1985, Welbourn et al., 1986). This modification produced a molecule with high selectivity for α_2 compared to α_1 adrenoceptors (O'Rourke et al., 1994b) but with relatively low selectivity for different members of that sub-family (Uhlén et al., 1992, Hudson et al., 1999). In particular, high affinity binding by this ligand has been reported in rat nervous tissue (Hudson et al., 1992, Mallard et al., 1992), and therefore RX 821002 is an appropriate antagonist to apply to the investigation of the contribution of α_2-adrenoceptors in reflex excitability. Blockade of spinal α_2-adrenoceptors by ligands such as idazoxan, yohimbine, or RX 821002 is known to have a facilitatory effect on spinal responses measured *in vivo* e.g. EMGs or MN firing rates, which includes previous studies performed in this laboratory in the rabbit (Harris and Clarke, 1993, Ogilvie et al., 1999, Clarke et al., 2001, Clarke et al., 2002). The significant facilitatory effect of intrathecal RX 821002 on hindlimb reflexes reported in the present studies is therefore consistent with this group's previous work in a different species as well as from other research groups using the rat (Jones and Gebhart, 1986a, Danzebrink and Gebhart, 1990, Mansikka et al., 1996, Onttonen et al., 2000, Rank et al., 2011).

The selectivity ratio of RX 821002 for NA α_2-adrenoceptors over the 5-HT_{1A} receptor is more than one hundred and fifty-fold in favour of the adrenoceptor (Newman-Tancredi et al., 1998), a marked improvement over its molecular predecessors such as yohimbine, idazoxan, and rauwolscine, which all bind to these receptor subtypes with affinity ratios in the range of one- to fifty-fold selectivity for the adrenoceptor (Vauquelin et al., 1990, Newman-Tancredi et al., 1998). At the highest doses used in the dose-response studies, some proportion of the facilitatory effects on reflexes may occur via the 5-HT_{1A} receptor as opposed to purely through the NA α_2 receptor, as RX 821002 and other NA α_2 receptor antagonists have been proposed as ligands for this serotonergic receptor subtype (Meana et al., 1996). *In vivo* studies examining the role of 5-HT_{1A} in descending control of reflex pathway excitability using, for example, the selective antagonist WAY-100635, demonstrate the potential for tonic inhibition mediated by this receptor (Clarke et al., 1996, Ogilvie and Clarke, 1998). WAY-100635 has also been reported to have potent agonist activity at the dopamine D_4 receptor (Chemel et al., 2006), thus demonstrating one of the challenges

involved in undertaking such pharmacological studies *in vivo*: true ligand selectivity. It is therefore difficult to fully elucidate the underlying processes and specify with complete certainty the full range of receptors that may be involved, however the high level of selectivity of RX 821002 for α_2-adrenoceptors and the dose selected for these studies ensures the absolute minimum of non-specific activity.

The present studies have found that the inhibitory noradrenergic modulation of spinal reflexes is tonically active in the decerebrate model. Some investigations have suggested that reflex facilitation by antagonism of α_2-adrenoceptors only occurs when the drug is applied to an animal in which inflammation is established (Green et al., 1998, Herrero and Solano, 1999) and that α_2 adrenergic controls do not therefore play a role in tonic control of spinal excitability in normal animals. Whilst the enhancement of neuronal activity in models of inflammation is clearly exacerbated by α_2-adrenoceptor antagonism in those studies, numerous others have reported facilitation following α_2-adrenoceptor blockade in non-inflamed animals (e.g. Janss et al., 1987, Clarke et al., 1988, Liu and Zhao, 1992, Mansikka and Pertovaara, 1995) and it therefore seems reasonable to conclude that tonic α_2-mediated inhibition exists. Given that blockade of α_2-adrenoceptors in the spinal cord facilitates both mechanically-evoked activity of dorsal horn WDR neurones and thermally-evoked tail-flick reflexes in anaesthetized rats (Gebhart and Ossipov, 1986, Rahman et al., 2008), the facilitation of electrically-evoked hindlimb EMG responses in decerebrate rats described here fits well with the established knowledge in the field. Further support for the tonic nature of the noradrenergic influence on spinal reflexes could come from future studies in anaesthetized animals.

In general, the facilitation caused by intrathecal RX 821002 was non-selective in that all four of the hindlimb reflexes showed increases in magnitude with increasing dose whether they were extensor or flexor responses. It would therefore appear that the tonic suppression of spinal reflexes mediated by the α_2-adrenoceptor subtype in this preparation is a global phenomenon not distinguishing between different functionality. Differential noradrenergic control of the heel- and toes-evoked reflexes may however be inferred from these results, with significant facilitation of the two heel-evoked reflexes first occurring at a lower dose of RX 821002 than was required for the toes-evoked equivalent. The stimulation strength utilized to evoke each pair of reflexes was statistically the same hence there appears to be no methodological bias in the activation of responses from the heel vs.

the toes, therefore this difference may relate to some aspect of the anatomy of the respective pathways, or indeed their control by descending influences.

In support of studies in the previous chapter, application of MO to hindlimb sites caused a differential effect on reflex responses depending on the site of application and the reflex studied. Although it should be noted that the effects of MO in the rat seem to be far less profound than previously observed in the rabbit (i.e. of smaller amplitude and duration as well as sometimes variable at a given site; see section 4.3.3), some of the present data suggest that the inhibitory effects of MO on spinal reflexes can be prevented by intrathecal application of RX 821002, evident here as either a block on MO-induced inhibition or a facilitation that was not present in the absence of the antagonist. The latter is particularly notable in the shift to facilitation observed in the response of both of the toes-evoked reflexes to MO application at the IL MTJ. However evidence from the organization of sensitization study (Chapter 4) indicated that facilitation of these particular reflexes should occur in response to MO applied topically at the MTJ when the spinal cord is intact i.e. before RX 821002 is administered; this was not found to be the case in the present study. A possible factor in the failure of MO to generate the effects predicted from the previous study, such as the heel-MG reflex facilitation by MO application to the IL heel, is the necessity of a reduction in the volume of MO applied to enable a second application to the same site. It is plausible that by halving the number of MO molecules available to bind to the specific receptors that the sensory barrage was also reduced and thus the evoked reflexes were not modulated to the same degree, and that the greater volume utilised in the previous study provided sufficient sensory input to overcome the modulatory effects of the noradrenergic pathways to the extent that reflex facilitation was observed. Thus, by halving the stimulus, the inhibitory influence was not overcome, but that when the α_2-adrenoceptor antagonist was applied the noradrenergic control was removed and the MO stimulus therefore facilitated the flexor reflexes.

These findings also imply that blockade of spinal α_2-adrenoceptors enhances the effect of MO by either removing tonic noradrenergic inhibition so that the noxious stimulus is more effective or that it prevents binding of NA which is known to be released in the spinal cord in response to noxious peripheral stimulation i.e. phasic (Tyce and Yaksh, 1981, Men and Matsui, 1994, Hitoto et al., 1998). The reflex pathway is therefore more readily excited by cutaneous MO application due to a reduction in α_2-adrenoceptor activation by NA and the

associated suppressive effects on spinal activity of that event. A more involved mechanism behind the shift to facilitation may occur through a release of NA-mediated inhibition of excitatory interneurones, as noradrenergic α_2-adrenoceptors are known to be involved in inhibiting the activity of glutamatergic cells in the dorsal horn (Pan et al., 2002, Kawasaki et al., 2003), the excitatory action of which are well-characterized (Schneider and Perl, 1988, Yoshimura and Jessell, 1990, Lu and Perl, 2005, Maxwell et al., 2007). The antagonist would therefore prevent NA binding to the adrenoceptor, thereby promoting the excitatory influence of the glutamatergic interneurones, and increasing the excitability of the reflex, observed here as increased responsiveness to MO application. Activation of receptors of this noradrenergic subtype have also been shown to inhibit glutamatergic inputs to motorneurones (Tartas et al., 2010), and thereby impinge directly onto motor output of the reflex arc. Blockade of the α_2-adrenoceptors would thence allow greater activity in the excitatory glutamatergic neurones, which could potentiate heightened responsiveness within the motorneurone pools to result in reflexes of greater magnitude.

Although most of the observed data can be explained by a reduction in the direct inhibitory influence of noradrenergic pathways via α_2-adrenoceptors, the reduction in the facilitatory effects of MO (such as by MO application to the ankle flexion site in the heel-MG reflex) following α_2-adrenoceptor blockade is at odds with direct block of α_2-mediated inhibition. However it must be taken into account that this effect was quite small and variable therefore should be treated with some caution, however this finding does correlate with observations made in the rabbit in this laboratory indicating that RX 821002 can block MO-induced facilitation of spinal reflexes (Harris et al., 2003) and suggests that descending control of mustard-oil induced alterations is potentially much more complex than the simplistic view that noradrenergic pathways have purely inhibitory effects. One possible route by which reduced facilitation could occur is via blockade of a descending α_2-mediated disinhibitory effect i.e. the antagonist is acting at α_2-adrenoceptors expressed on tonically active inhibitory interneurones that suppress the excitability of the reflex arc. Intrathecal administration of RX 821002 could therefore prevent NA binding at these α_2-adrenoceptors following its release in response to MO stimulus, thus perpetuating an inhibition of the reflex pathway. NA is known to facilitate the inhibitory actions of γ-amino butyric acid (GABA)-ergic and glycinergic inhibitory interneurones by enhancing the firing rate of those cell types (Baba et al., 2000), though that these effects were not blocked by application of yohimbine in spinal cord slice studies implies that the catecholamine is not eliciting this

effect via α_2-adrenoceptors (Gassner et al., 2009). However, in the study by Gassner et al. (2009), the focus of the investigation was the mechanism by which NA influences the activity of GABAergic cells, and the neurotransmitter was also found to have a hyperpolarizing effect on non-GABAergic dorsal horn cells i.e. a reduction in their excitability caused by NA. Most compelling with regard to this speculation that suppression of MO-induced facilitation by RX 821002 may be occurring through a blockade of disinhibition is recent evidence that confirms the link between inhibitory interneurones in lamina II and noradrenaline-induced hyperpolarization. Different morphological classes of dorsal horn neurones relate to transmitter content e.g. islet form cells are GABAergic inhibitory cells (Lu and Perl, 2003, Yasaka et al., 2010), and thus the hyperpolarizing effect of noradrenaline on some dorsal horn neurones can be categorically stated to occur at inhibitory interneurones (Lu and Perl, 2007, Yasaka et al., 2010).

Changes in reflex excitability could also be attributed to the binding of RX 821002 to presynaptic α_2-adrenoceptors on noradrenergic terminals themselves (autoreceptors) or other neuronal terminals (heteroceptors) i.e. sites at which NA inhibits its own release or that of other neurotransmitters (Russell, 1987, Wortley et al., 1999, Gilsbach and Hein, 2012). Presynaptic receptor blockade would therefore prevent inhibition of transmitter release, with the subsequent greater synaptic concentrations potentially diminishing or increasing reflex excitability depending on the nature of the transmitter and its postsynaptic receptors.

Whilst the selectivity ratio for RX 821002 at presynaptic receptors is 2.8-fold in favour of α_2-adrenoceptors versus α_1-adrenoceptors (Stillings et al., 1985), it should also be considered that there is a small possibility that at the dose selected this antagonist may have some activity at the less-favoured receptor subtype. As discussed earlier, the differential effects of exogenously applied NA have been shown to be mediated by these two main α-adrenoceptor subtypes with the α_1-adrenoceptor mediating excitation (Bell and Matsumiya, 1981, Wiesenfeld-Hallin, 1987, Sakitama, 1993). As α_1-adrenoceptors are expressed predominantly in the ventral horn (see section II.A.3.4) the contribution of non-subtype selective binding, in this case to the α_1-adrenoceptor, may therefore be implicated in reflex potentiation by MO given that actions mediated via this receptor include the enhancement of motor responses (Chan et al., 1986, Fung et al., 1991). In addition, application of α_1-adrenoceptor antagonists has been found to attenuate NA-induced firing

of inhibitory interneurones imply that α_1-mediated excitation can also be seen in the dorsal (Baba et al., 2000) and ventral horns (Wada et al., 1997, Harvey et al., 2006, Rank et al., 2011).

Finally some consideration can be given to the source of this noradrenergic modulation of spinal reflexes. The origin of terminals in the lumbar segments of the rat spinal cord are derived virtually entirely from supraspinal locations, in this case the noradrenergic cells group of the pons (A5, A6, and A7), particularly A6, the locus coeruleus (see section II.A.2.3), which was determined primarily by the use of retrograde and double-labelling techniques (Loewy et al., 1979, Westlund et al., 1981, Tavares et al., 1996). Selective destruction of this nucleus has a hyperalgesic effect in rodent models of both acute and chronic nociception, including measures such as the tail-flick and formalin tests (Tsuruoka and Willis, 1996, Martin et al., 1999, Jasmin et al., 2003); stimulation of the LC has in opposite result – an antinociceptive or analgesic effect (Jones and Gebhart, 1986b, West et al., 1993, Rojas-Piloni et al., 2012). Furthermore, disruption of this pathway by pharmacological means, often through antagonism of spinal noradrenergic receptors, also reduces noxious response thresholds and increases pain-related behaviour in rodent models (Tjølsen et al., 1990, Uchihashi et al., 2000, Li et al., 2011), and thus the LC can be definitively classified as a major supraspinal source of control of spinally mediated reflexes.

5.5 Conclusions

The results of the present study indicate that tonic descending control of hindlimb reflexes in the decerebrate non-spinal rat, is at least partly mediated by NA α_2-adrenoceptors and is inhibitory in nature. These findings echo extensive studies in this laboratory examining the role of noradrenergic descending control of reflex excitability in the rabbit which demonstrated that blockade of α_2-adrenoceptors by intrathecal idazoxan or RX 821002 facilitates hindlimb reflexes to both electrical and natural stimuli (Harris and Clarke, 1993, Ogilvie et al., 1999, Clarke et al., 2001, Clarke et al., 2002). Stimulus evoked inhibition (and possibly facilitation) of reflex responses by cutaneous MO application was also prevented by intrathecal RX 821002 treatment, suggesting the involvement of the α_2-adrenoceptor subtype in mediating these effects in this model.

6. SEROTONERGIC MODULATION OF SPINAL REFLEXES

6.1 Introduction

In addition to the presence of descending inhibitory influences on spinal excitability there is also some evidence to suggest that facilitatory bulbospinal pathways may contribute to the effects of MO on reflexes (see chapter 4). Hence in the decerebrated rat, MO-induced increases in reflex excitability from certain sites in the spinally intact animal were not seen from the same sites in the spinalized preparation. Specifically there appeared to be a loss of facilitation in both the toes-TA and toes-BF reflexes by MO application to the IL MTJ, as well as a further loss of facilitation in toes-TA responses to MO applied to the distal IL toe tips. Interestingly studies from this laboratory performed in rabbit found no evidence for facilitatory bulbospinal pathways contributing to reflex enhancement by MO (Harris and Clarke, 2003), suggesting a possible inter-species difference. However studies in the rabbit have shown that analogous spinal reflexes are influenced by tonic descending facilitatory pathways and that a balance exists between descending inhibitory and facilitatory controls (Harris and Clarke, 1992, Clarke et al., 1996).

Prime origins for descending facilitatory control of spinal excitability are the serotonergic nuclei of the medulla – and in particular the B1, B2, and B3 cell groups (Bowker et al., 1981a, b, Kazakov et al., 1993; see also section II.B.2.2). Of these, B3 (the raphe magnus nucleus) is thought to be among the primary sources of descending serotonergic modulation of spinal nociceptive processing. As bulbospinal fibres originating in B3 project via the DLF to supply the serotonergic terminals of the superficial dorsal horn (Light et al., 1983, Müllner et al., 2008; see also section II.B.2.2), it can therefore be presumed that this facilitatory influence acts at this site of sensory integration.

Of the many heterogeneous 5-HT receptor subtypes (section II.B.3), the ligand-gated ion channel receptor 5-HT_3 has been repeatedly demonstrated to play a role in the transmission of descending facilitatory control of spinal neuronal excitability (Suzuki et al., 2002, Rahman et al., 2009, Asante and Dickenson, 2010; see also D'Mello and Dickenson, 2008 for a review of 5-HT_3 receptors in nociceptive processing). The present study therefore investigated potential serotonergic facilitatory influences on reflexes *per se* and on sensitization of those reflexes by mustard oil in the decerebrated rat by using spinal

application of the selective 5-HT_3-receptor antagonist ondansetron (Brittain et al., 1987, Butler et al., 1988).

6.2 Methods

Experiments were performed on a total of 36 rats with a mean weight of 314 g ± 15 g. Two groups of experiments were performed. The first was a dose-response study for intrathecal application of ondansetron (n = 9). The data obtained from those experiments then informed the dose selection for the second group (n = 27), in which the effects of intrathecal ondansetron on MO-induced modulation of hindlimb reflexes was examined.

6.2.1. Surgical Preparation

The surgical procedures performed are described in detail in section 5.2.1. Briefly, in all animals, the trachea, left carotid artery, left jugular vein and spinal cord were cannulated under isoflurane anaesthesia (2.5 – 3%) for airway maintenance, blood pressure monitoring, anaesthetic/replacement fluid administration and intrathecal dosing of the antagonist, respectively. All animals were decerebrated to the pre-collicular level as described in chapter 3.

Needle electrodes were situated either side of the thorax to enable recording of an ECG. EMG electrodes were implanted into the left MG, BF and TA with responses evoked by alternate electrical stimulation at the heel and toes using bipolar needle electrodes (see section 2.1.5).

6.2.2. Experimental Protocol

i) Dose-response studies

Following attainment of consistent control reflexes (i.e. three consecutive readings within 10% of one another), increasing concentrations of antagonist (seven in total) were administered intrathecally separated by an interval of 24 minutes (i.e. six pairs of readings from both heel- and toes- evoked reflexes between doses). Ondansetron hydrochloride (Tocris Bioscience) was dissolved in rat Ringer's solution to give a stock solution of 3.3$\dot{3}$ mg

mL^{-1} (10.1 mM). The stock was serially diluted to give four solutions of (1) 0.03̇ mg mL^{-1}, (2) 0.1 mg mL^{-1}, (3) 0.3̇ mg mL^{-1}, and (4) 1 mg mL^{-1}. A cumulative i.t. dosing regime was employed, in which the total mass of drug applied refers to the antagonist as the hydrochloride salt. The cumulative doses applied were (in µg): 0.3, 1, 5, 10, 20, 30, and 70. On each occasion ondansetron was applied in a volume of 10 µL vehicle (Ringer's solution) followed by a 10 µL vehicle flush of the i.t. cannula using a needle-tipped 50 µL Hamilton syringe (22S gauge). As the vehicle used was identical to RX 821002 studies, the vehicle dose-response data from chapter 5 (n = 6) has been included here as well to compare with the effects of cumulative ondansetron.

ii) MO in the presence of i.t. ondansetron

To examine the effect of i.t. ondansetron on the effects of MO applied at different sites on the hindlimb on reflex responses, experiments were conducted according to the following protocol, with stable control reflexes attained prior to each drug and each mustard oil treatment: 20 µL vehicle (Ringer's solution) was applied intrathecally; 2.5 µL of 20% mustard oil was then applied to the lateral or medial aspect of the IL heel, IL MTJ, or IL flexion of the ankle and reflexes followed for a minimum of 63 minutes; 10 µg ondansetron in a volume of 10 µL plus a 10 µL Ringer flush was applied intrathecally; finally 2.5 µL of 20% mustard oil was applied to the same site as that treated previously but to the aspect as yet untreated (i.e. lateral if medial was treated initially), and reflexes again followed for a minimum of 63 minutes. The dose of antagonist selected was chosen on the basis of data obtained in the dose-response study.

The volume of mustard oil applied was the same as for RX 821002 studies in chapter 5 i.e. half that used during the organization of sensitization study (chapter 4); this was to minimise spread of the solution across the skin and thereby allow a second treatment to be applied to the same 'site' (MTJ, heel, flexion) but to adjacent skin. As before, the order in which the adjacent skin areas were treated i.e. lateral region vs. medial region was alternated between experiments. Sites for MO treatment were the same as in the RX 821002 studies and selected based on the changes induced by MO in chapter 4.

6.2.3. Statistical Analysis

Electrical thresholds and stimulation intensities were assessed for significant differences between heel and toes sites using Wilcoxon's matched pairs tests. Raw control reflex responses were compared using Kruskal-Wallis one-way ANOVA tests.

Within individual experiments, reflexes were normalised such that each response is a percentage of the mean of three pre-treatment control readings per reflex. Values for reflexes for pooled data for all experiments are then expressed as medians and inter-quartile ranges. For cardiovascular parameters, within individual experiments the difference from the mean of ten pre-drug control readings was calculated for each minute following drug administration and the median and interquartile ranges calculated for the pooled data. Non-parametric statistical tests have been performed throughout for reflex and cardiovascular data, as every data set was tested and failed the Kolmogorov-Smirnov test for a Normal distribution.

For dose-response experiments, in order to determine the effect of ondansetron on reflexes or cardiovascular measurements compared to pre-treatment controls, Friedman's ANOVA on ranks followed by Dunn's multiple comparisons tests have been used. Significance differences between vehicle and ondansetron dose-response curves were assessed using Scheirer-Ray-Hare non-parametric two-way ANOVAs.

Assessment of the effect of mustard-oil on reflexes in the presence of vehicle or ondansetron compared to pre-MO controls was assessed using Friedman's ANOVA on ranks delimited by the duration of the effect, determined as when post-MO values had returned to within two standard deviations from the mean pre-MO control reflex responses. Comparisons between the effect of MO with/without ondansetron were made using Scheirer-Ray-Hare non-parametric two-way ANOVAs over the time period indicated by the outcome of the Friedman's ANOVAs.

6.3 Results

The median threshold for heel stimulation was 0.74 mA (IQR 0.53 - 0.97 mA) and for toes stimulation was 0.32 mA (IQR 0.28 - 0.39 mA). The median stimulus amplitudes employed throughout reflex recording were2.04 mA (IQR 1.36 – 3.15 mA) and 1.54 mA (IQR 1.17 - 1.87 mA) for heel and toes stimulation respectively. The thresholds were significantly

different to one another ($p < 0.001$, Wilcoxon's Matched Pairs test), as were the stimulation intensities employed throughout the recording period ($p < 0.05$, Wilcoxon's Matched Pairs test).

Median control raw reflex responses were 188 µV.ms (IQR 114 - 360) for heel-MG, 483 µV.ms (IQR 267 - 992) for heel-BF, 134 µV.ms (IQR 99 - 169) for toes-TA, and 253 µV.ms (IQR 151 - 470) for toes-BF. These were significantly different from one another ($p < 0.05$, Kruskal-Wallis test) with the subsequent post-test revealing significant differences in all pairwise comparisons of reflex magnitude with the singular exception of heel-MG *cf* toes-BF ($p < 0.05$, Dunn's Multiple Comparison Test).

6.3.1. Effect of Ondansetron on Reflex Responses per se

The vehicle employed for this study was the same as that used in the RX 821002 study (rat Ringer's solution) which, with the exception of a small decrease occurring in the toes-BF response ($p < 0.05$, Friedman's ANOVA), had no effect on reflex responses (see section 5.3.1). In contrast, cumulative application of the serotonergic 5-HT_3 receptor antagonist ondansetron to the spinal cord resulted in significant decreases in three out of the four reflex responses with only heel-BF reflexes not being significantly altered ($p > 0.05$, Friedman's ANOVA; figure 6.1, raw traces provided in figure 6.2). Post-tests indicated that toes-TA reflexes were first significantly depressed relative to pre-drug controls at cumulative doses equal to or greater than 5 µg ondansetron ($p < 0.05$, Dunn's Multiple Comparison Test) with a maximum median decrease to 73% (IQR 57 - 77%) of controls following a 30 µg cumulative dose. Toes-BF and heel-MG responses were significantly reduced after cumulative doses of 10 µg and 20 µg, with maximum attenuations occurring following the cumulative 20 µg (median 58%, IQR 46 – 65%) and 70 µg dose (median 46%, IQR 24 – 67%) respectively. The effect of the antagonist compared to vehicle was significantly different for all reflexes according to the non-parametric two-way ANOVA tests ($p < 0.02$ for all reflexes).

The median control values (i.e. pre-drug) for MAP and HR were 79 (IQR 62 – 83) mmHg and 374 (IQR 357 – 431) bpm for the vehicle group and 92 (IQR 72 – 103) mmHg and 430 (IQR 404 – 461) bpm for the drug group. The effect of i.t. ondansetron on MAP and HR was not found to be significantly different from the effect of i.t. vehicle administration ($p > 0.05$,

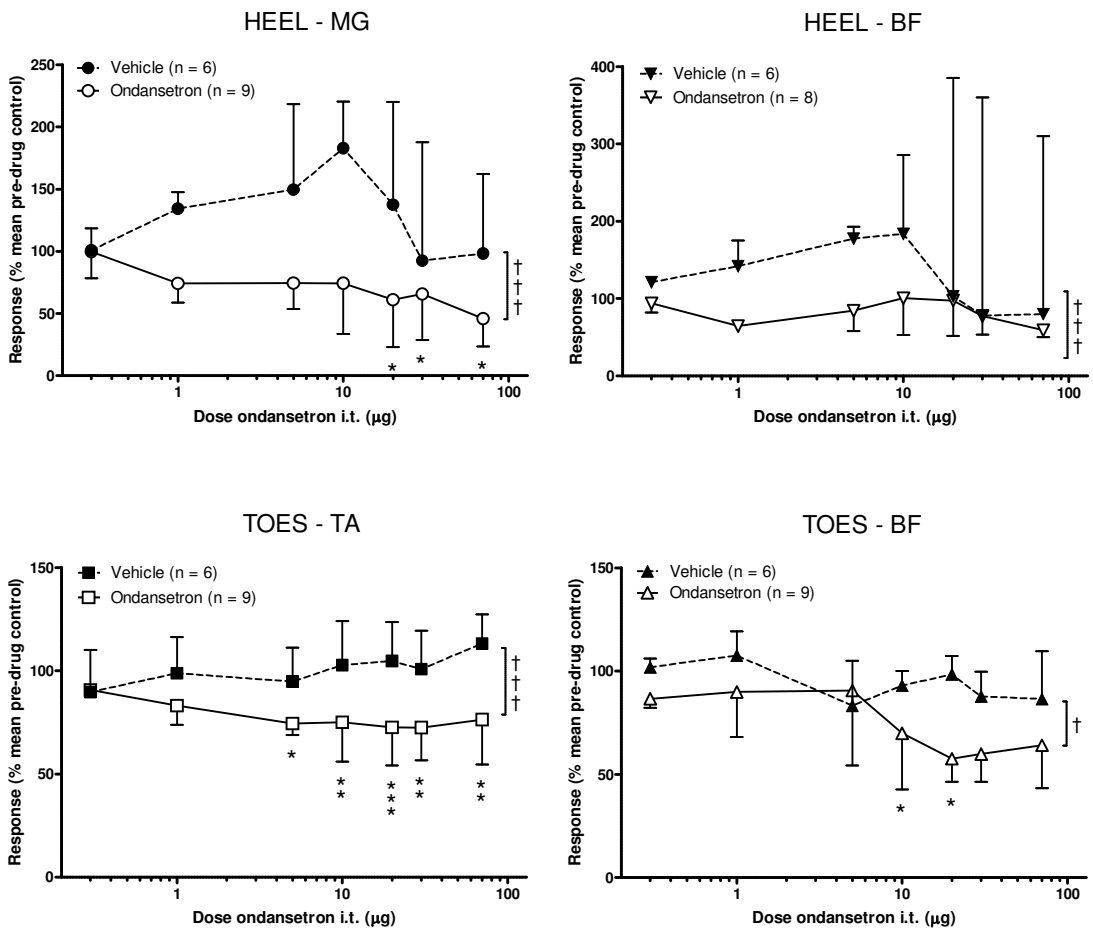


Figure 6.1: Effect of cumulative intrathecal application of vehicle or the 5-HT_3 receptor antagonist ondansetron on each of the four hindlimb reflexes. All but heel-BF responses were significantly reduced by ondansetron administration (Friedman's ANOVAs, see body text). Asterisks indicate significant differences compared to pre-treatment controls (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Dunn's multiple comparisons post-test) and obelisks denote significant differences between vehicle and ondansetron dose-response curves († $p < 0.05$, ††† $p < 0.001$, Scheirer-Ray-Hare two-way ANOVA).

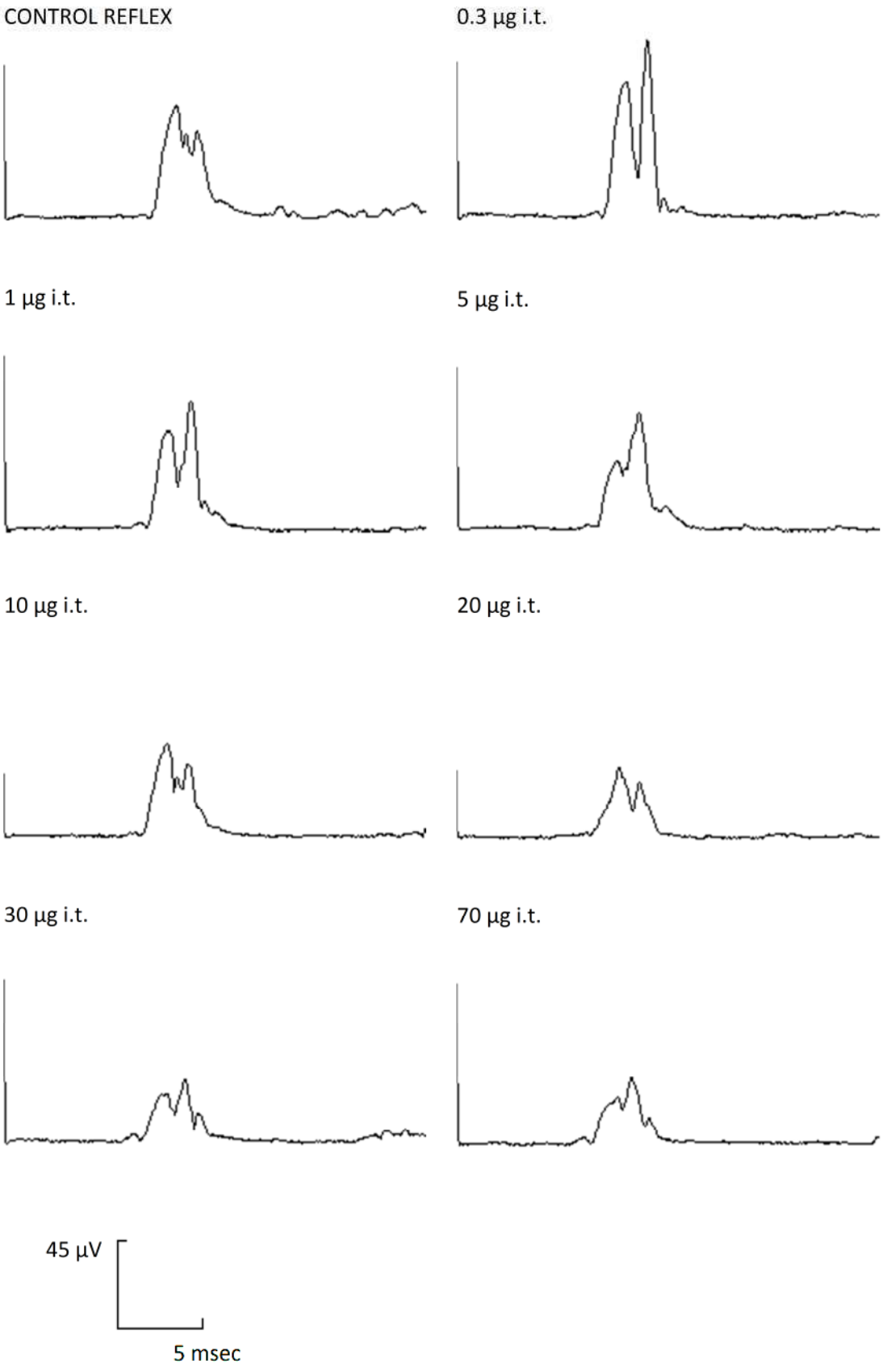


Figure 6.2: Raw data traces showing the inhibitory effect of ondansetron on the heel-MG reflex. Data are averages of 8 sweeps.

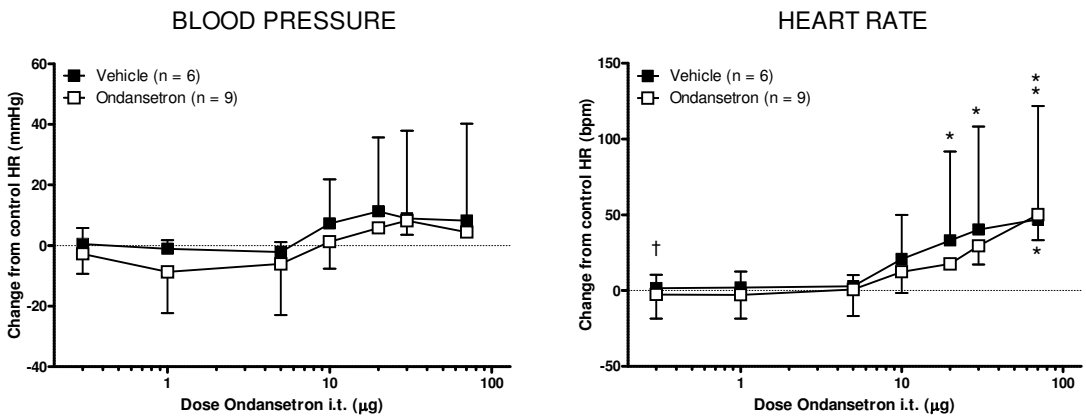


Figure 6.3: Effect of cumulative intrathecal application of vehicle or the 5-HT_3 receptor antagonist ondansetron on cardiovascular responses (median ± upper/lower quartile). Blood pressure was not significantly altered from control values whereas HR showed significant increases (Friedman's ANOVAs, see body text). Asterisks indicate significant differences compared to pre-treatment controls (* $p < 0.05$, ** $p < 0.01$, Dunn's multiple comparisons post-test).

Mann-Whitney tests), with the exception of a small depression in HR at the 0.3 µg dose (figure 6.3). MAP did not change from control values throughout the dosing study for both vehicle and drug treatments ($p > 0.05$, Dunn's Multiple Comparison test), whereas HR showed a significant increase from basal levels at the latter three doses for vehicle only and at the highest dose for ondansetron ($p < 0.05$, Dunn's Multiple Comparison test).

6.3.2. Mustard Oil-induced Changes in Reflexes in the Presence of Ondansetron

i. Effect of MO applied to IL flexion of the ankle (figure 6.4)

In the presence of vehicle, mustard oil application at this site did not significantly modulate any of the four reflexes measured (Friedman's ANOVA, $p > 0.05$, $n = 11$). In the presence of 10 µg i.t. ondansetron however, MO application caused significant facilitations in both the heel-MG and heel-BF reflexes relative to the pre-MO control responses. For the heel-MG response, this took the form of an initial increase ($p < 0.01$, Wilcoxon signed rank test) to a median of 122% (IQR 108 – 171%) of controls at 1 min post-MO which then developed over time to reach a maximum median increase of 170% (IQR 111 – 213%) of controls at 63 min ($p < 0.001$, Friedman's ANOVA). The enhancement ($p < 0.02$, Friedman's ANOVA) of heel-BF responses was to a median maximum of 125% (IQR 107 – 159%) of controls, also at 1 min post-MO, with a median duration of 9 min (IQR 1 – 48 min). In addition to the enhanced facilitation seen in the heel-evoked reflexes in the presence to ondansetron, MO applied to the flexion of the ankle subtly depressed the toes-BF reflex when the antagonist was present. The inhibition ($p < 0.03$, Friedman's ANOVA) was to a median maximum of 92% (IQR 84 – 108%) relative to pre-MO controls, which occurred 7 min post-MO with a median duration of 7 min (IQR 0 – 63 min). Non-parametric two-way ANOVA analyses indicated that the effect of MO on the heel-MG, heel-BF, and toes-TA reflexes was significantly different ($p < 0.05$, Scheirer-Ray-Hare test) when applied in the presence of vehicle relative to in the presence of ondansetron.

ii. Effect of MO applied to IL MTJ (figure 6.5)

Significant enhancement was seen only for reflexes evoked in BF from the toes when MO was applied in the absence of ondansetron. This increase ($p < 0.05$, Friedman's ANOVA) was to a median maximum of 114% (IQR 93 – 120%) of control values after 11 min and had

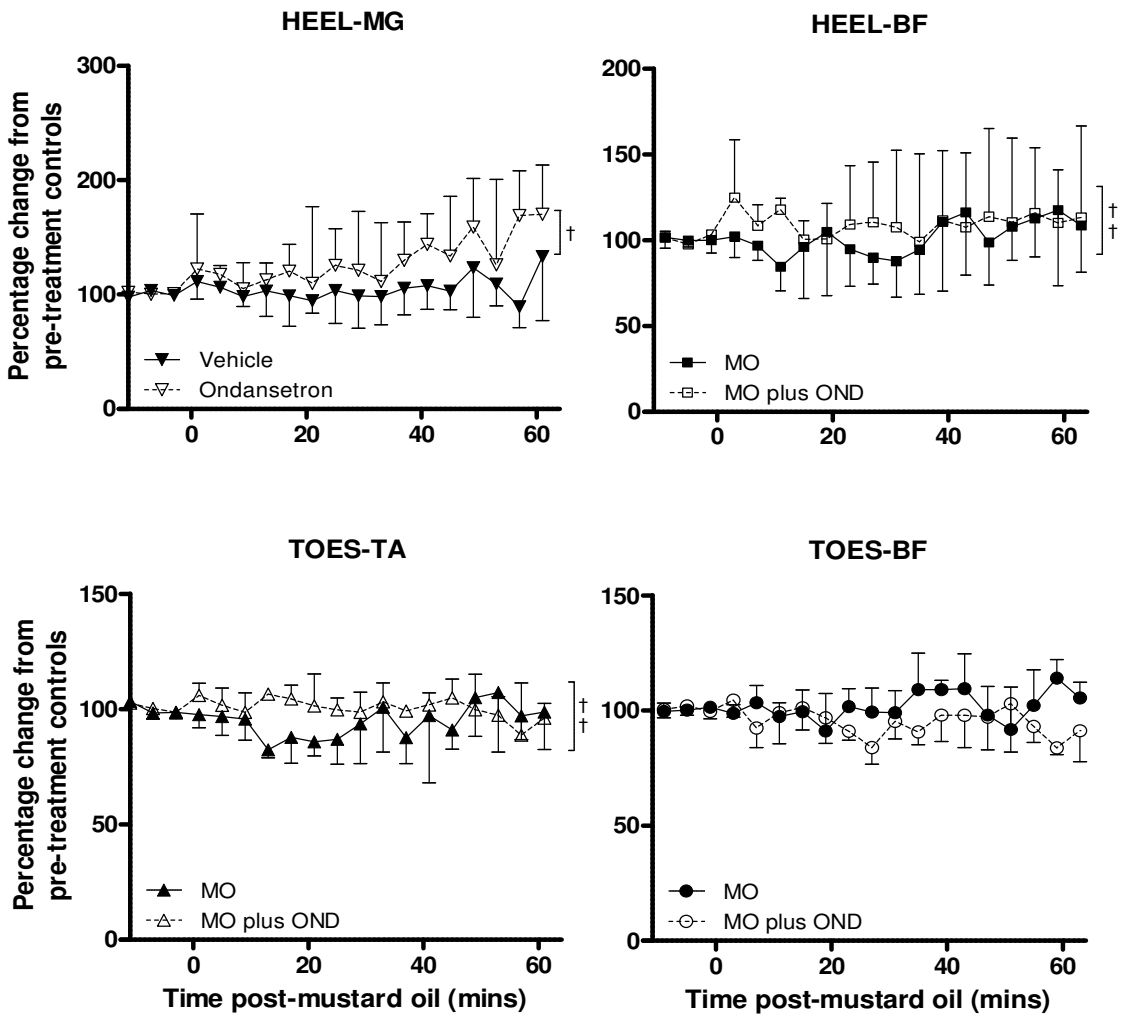


Figure 6.4: Effect of mustard oil application to the ipsilateral flexion of the ankle alone i.e. following vehicle (MO; filled symbols) and in the presence of i.t. ondansetron (MO plus OND; open symbols). Upper left graph (inverted triangles) shows heel-MG reflex responses, upper right graph (squares) shows heel-BF responses, lower left graph (triangles) shows toes-TA reflex responses, and lower right graph (circles) shows toes-BF responses. Data are median values displayed with either upper or lower quartiles. Mustard oil was applied at time 0. Obelisks denote significant differences between MO and MO plus ondansetron curves († $p < 0.05$, †† $p < 0.01$, Scheirer-Ray-Hare two-way ANOVA).

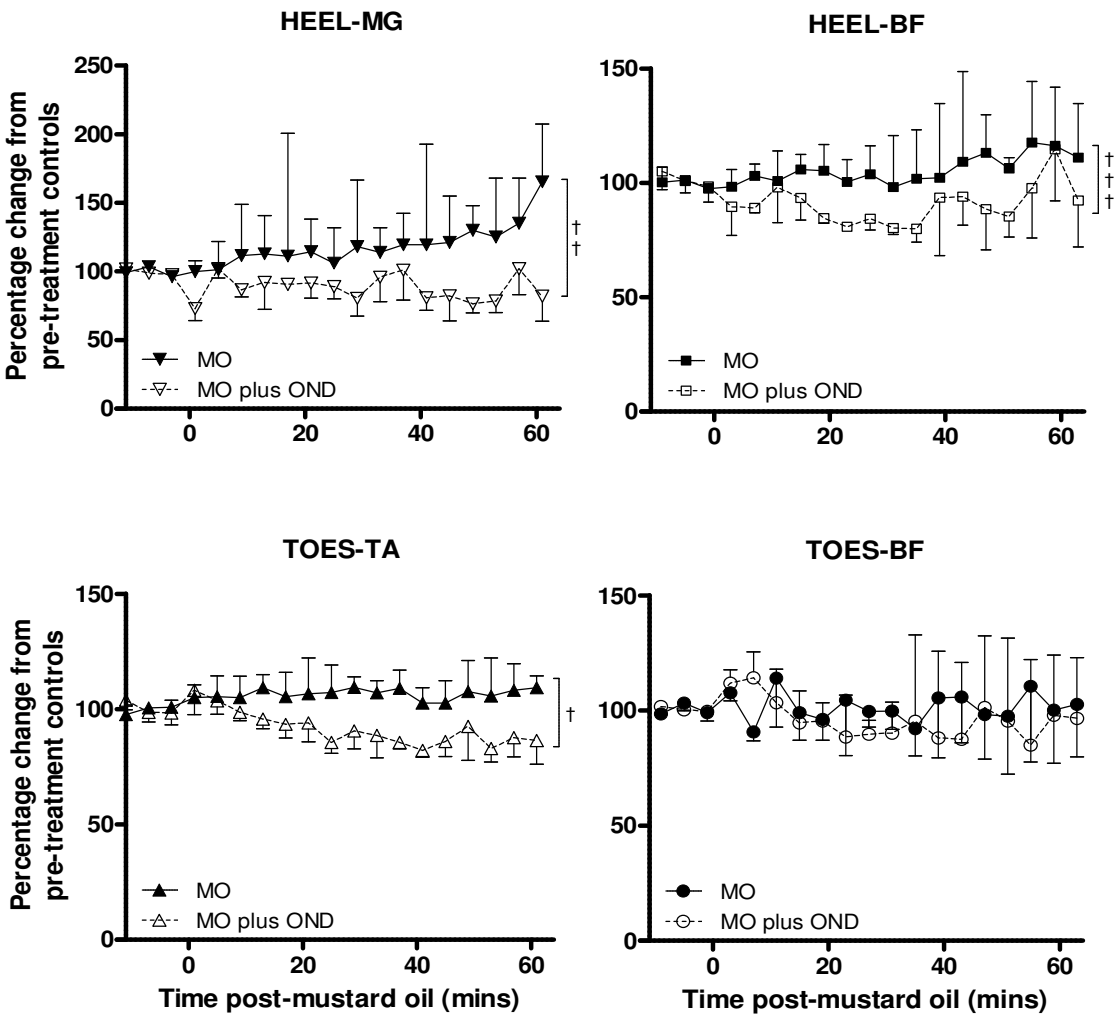


Figure 6.5: Effect of mustard oil application to the ipsilateral metatarsophalangeal joints alone i.e. following vehicle (MO; filled symbols) and in the presence of i.t. ondansetron (MO plus OND; open symbols). Upper left graph (inverted triangles) shows heel-MG reflex responses, upper right graph (squares) shows heel-BF responses, lower left graph (triangles) shows toes-TA reflex responses, and lower right graph (circles) shows toes-BF responses. Data are median values displayed with either upper or lower quartiles. Mustard oil was applied at time 0. Obelisks denote significant differences between MO and MO plus ondansetron curves († $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$, Scheirer-Ray-Hare two-way ANOVA).

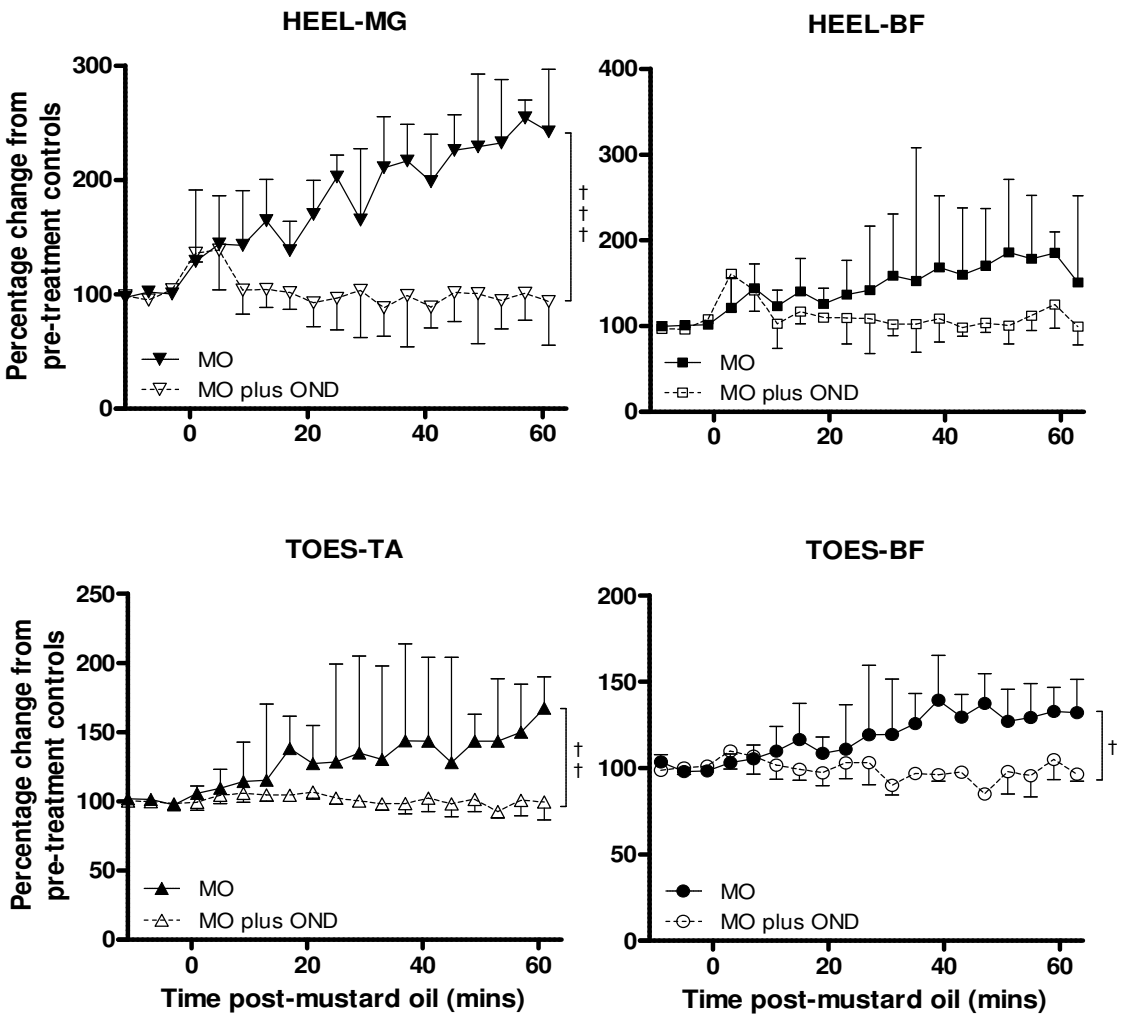


Figure 6.6: Effect of mustard oil application to the ipsilateral heel alone i.e. following vehicle (MO; filled symbols) and in the presence of i.t. ondansetron (MO plus OND; open symbols). Upper left graph (inverted triangles) shows heel-MG reflex responses, upper right graph (squares) shows heel-BF responses, lower left graph (triangles) shows toes-TA reflex responses, and lower right graph (circles) shows toes-BF responses. Data are median values displayed with either upper or lower quartiles. Mustard oil was applied at time 0. Obelisks denote significant differences between MO and MO plus ondansetron curves († $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$, Scheirer-Ray-Hare two-way ANOVA).

a median duration of 11 min (IQR 3 – 19 min). In the presence of ondansetron, a significant increase in toes- BF reflexes was no longer detected (although there was still a tendency to cause a short latency increase in this response). Perhaps more apparent was the fact that MO application to the MTJ had inhibitory effects on heel-MG and toes-TA reflexes when applied in the presence of ondansetron. In the case of the heel-MG response this inhibition was rapid and relatively short lasting ($p < 0.05$, Friedman's ANOVA), within 1 min post-MO decreasing responses to a median of 73% (IQR 64 – 101%) of controls, for a duration of 9 min (IQR 1 -17 min). In the case of the toes-TA reflex, MO-induced inhibition ($p < 0.03$, Friedman's ANOVA) was slower in onset, reaching a maximum median inhibition of 82% (IQR 79 – 93%) of the pre-MO control values, 43 min after MO application. The effect of MO applied in the presence of vehicle relative to application following i.t. ondansetron was significantly ($p < 0.05$, Scheirer-Ray-Hare test) different for the heel-MG, heel-BF, and toes-TA reflexes.

iii. Effect of MO applied to the IL heel (figure 6.6)

Mustard oil applied to the ipsilateral heel produced long-lasting (>30 min) increases in all reflexes. Hence in the presence of vehicle alone, heel-evoked reflexes were enhanced ($p < 0.05$, Friedman's ANOVA) to median maxima above control values of 254% (IQR 215 – 270%) at 57 min post-MO and 186% (IQR 100 – 271%) at 49 min post-MO for the MG and BF responses, respectively. Facilitation of the toes-evoked responses ($p < 0.01$, Friedman's ANOVA) was of slightly lesser magnitude, with toes-TA reflexes elevated to a median of 167% (IQR 132 – 190%) and toes-BF responses to 139% (IQR 117 – 165%) relative to their respective controls, 63 and 39 min post-MO, respectively. Following i.t. ondansetron treatment this facilitation was abolished (toe-evoked responses) or greatly reduced (heel-evoked responses). Thus when MO was applied to the heel, toes-TA, and toes-BF reflexes displayed no significant alteration in magnitude relative to controls ($p > 0.05$, Friedman's ANOVA) whilst heel-MG and heel-BF responses were initially significantly ($p < 0.05$, Friedman's ANOVA) facilitated to a median of 139% (IQR 104 – 146%) and 161% (IQR 117 – 186%) of controls at 5 and 1 min post-MO for relatively brief median durations of 13 min (IQR 5 – 33 min) and 15 min (IQR 2 – 52 min) respectively i.e. there was no long duration component to these responses. The heel-MG, toes-TA, and toes-BF reflexes responded significantly differently to MO application depending on whether vehicle or ondansetron was present ($p < 0.05$, Scheirer-Ray-Hare test).

6.4 Discussion

The present studies have shown that intrathecal application of the selective 5-HT_3 receptor antagonist ondansetron to the spinal cord of the decerebrated rat is able to dose-dependently inhibit spinally-mediated reflex responses, thereby implying that they are subject to tonic serotonergic facilitation mediated by spinally located 5-HT_3 receptors. This modulation does not appear to differentiate between flexor (e.g. toes-TA) and extensor (e.g. heel-MG) reflexes which were both suppressed by the 5-HT_3 antagonist. Interestingly, the same antagonist applied intrathecally to decerebrated non-spinalized rabbits facilitated responses in the medial gastrocnemius muscle nerve to electrical stimulation of the sural nerve (Clarke et al., 1996) i.e. the opposite to current findings implying a tonically active 5-HT_3 mediated inhibition was present. This may be explained by a species difference or the fact that differential effects of 5-HT_3 receptor antagonists such as ondansetron have been observed in response to different stimulus modalities, such that the response to a natural stimulus in the form of mechanical or thermal input is attenuated by the antagonist whilst electrically evoked responses remain unchanged (Rahman et al., 2004). The contrasting effects observed in rat and rabbit dose-response studies detailed above were both a result of reflexes evoked by electrical stimulation, albeit with different stimulation sites and end-point measurements, but the possibility raised by Rahman et al. (2004) that variations in stimulus modality can alter the perceived physiological action of a drug may still be applicable here.

5-HT_3 receptors are ligand-gated ion channels and as such are therefore able to rapidly influence the excitability of nervous tissue (Barnes et al., 2009), compared to the relatively slow impact of GPCRs that constitute the remainder of the 5-HT receptor family (see section II.B.3). Previous studies have therefore sought to elucidate the specific nature of the role of the 5-HT_3 receptor in neuronal excitability and altered pain states such as hyperalgesia. It seems that in these studies as well, there is data to suggest that 5-HT_3 receptors are mediators of both inhibitory and facilitatory effects. Use of selective exogenous agonists, including 2-methyl-5-HT and m-chlorophenylbiguanide (mCPBG) implied that activation of this receptor leads to an increase in nociceptive threshold, an effect that has been measured *in vivo* using paw withdrawal latencies and EMG activity (Bardin et al., 2000, Seo et al., 2002) and *in vitro* by dorsal horn neuronal firing in an

isolated spinal cord preparation (Khasabov et al., 1999). However, mCPBG has also been demonstrated to have pro-nociceptive functionality by increasing the responsiveness of dorsal neurones during the rat tail flick test (Ali et al., 1996b), thus raising doubt over the outcome of 5-HT_3 receptor activation with regards to pain processing. 5-HT_3 receptor antagonists employed in similar investigations have included tropisetron, zacopride, bemesetron (MDL-72222), ondansetron, and alosetron, and as with the agonists have led to conflicting findings. Several groups have shown that either intrathecal or systemic application of these antagonist compounds leads to either a reduction or no change in nociceptive thresholds in tail flick and paw withdrawal tests in rodents (Giordano and Dyche, 1989, Glaum et al., 1990, Alhaider et al., 1993, Xu et al., 1994, Bardin et al., 2000), thus implying that 5-HT_3 antagonism has a pro-nociceptive effect overall whilst other groups using similar methodologies have found primarily anti-nociceptive effects (Giordano and Dyche, 1989, Ali et al., 1996b, Ye et al., 1997, Green et al., 2000, Miranda et al., 2006). The work of Giordano and Dyche (1989) is of particular note due to the comparison made between the modulating effect of 5-HT_3 antagonists and the noxious stimuli employed to evoke a response, in which they showed no effect of the antagonists when thermal or mechanical stimulation was used, but a significant anti-nociceptive effect when the stimulation was chemical in nature.

Ondansetron was selected as the antagonist employed in the present study on the basis of a high level of selectivity for the receptor subtype of interest (i.e. 5-HT_3) over other non-specific binding sites. *In vitro* competitive radioligand binding assays have demonstrated that this ligand exhibits weak or no affinity for other subtypes of the 5-HT receptor family including 5-HT_{1A} and 5-HT_2 receptors, as well as non-serotonergic receptor classes such as α_2- and β-adrenoceptors, $GABA_A$ receptors, and glycine receptors (Kilpatrick et al., 1987, van Wijngaarden et al., 1990, van Wijngaarden et al., 1993). Receptors other than 5-HT_3 that have appreciable affinity for ondansetron are 5-HT_{1B} and 5-HT_{2C} receptors, α_1-adrenoceptors, and μ-opioid receptors (van Wijngaarden et al., 1990), though in each case the affinity at the non-5-HT_3 receptor is several orders of magnitude lower. Antagonism at each of these receptor subtypes has been shown to impact on spinal nociceptive processing – that is by blocking any of the four binding sites mentioned, the antinociceptive effect of endogenous ligand binding are prevented, thus illustrated in dorsal horn recordings or withdrawal reflex measurements as an increased responsiveness in the presence of the respective antagonists (Sagen and Proudfit, 1984, Uchihashi et al., 2000,

Jeong et al., 2004, Obata et al., 2004, Chen and Pan, 2006, Liu et al., 2007, Bee et al., 2011). However, at the dose utilised in this study, ondansetron is unlikely to exert any meaningful physiological effects via receptors other than 5-HT_3 and thus any significant impact on spinal excitability as measured by hindlimb reflex responses can be said to occur through specific binding at this receptor site.

The findings of this study have suggested differential effects of spinal 5-HT_3 receptor antagonism on mustard-oil induced modulation of hindlimb reflexes in the decerebrate non-spinalized rat. For the most part, when MO was applied to either the IL heel or MTJ following ondansetron administration a reduction of facilitation was observed. This suggests that facilitation of some hindlimb reflexes in the rat caused by chemogenic noxious stimulation occurs as a result of descending facilitatory controls acting at 5-HT_3 receptors. There is some precedent for the involvement of descending facilitatory pathways in mediating stimulus-evoked changes in withdrawal reflexes in the rat. For example in the formalin model of inflammatory pain, ondansetron dose-dependently reduced dorsal horn activity during both the acute and inflammatory phases of that test (Green et al., 2000). However, i.t. application of ondansetron had no inhibitory properties with regard to the hyperalgesia manifested in spinal nerve ligation models of neuropathic pain (Peters et al., 2010) indicating that the role of 5-HT_3 mediated antinociception does not have universal applications in alleviating different classifications of pain. This conclusion has been further consolidated by studies into the pain behaviours of 5-HT_3 receptor knock-out mice, which also displayed greatly reduced responsiveness to inflammatory pain but normal responses to acute pain (Zeitz et al., 2002, Kayser et al., 2007). In the present studies there was also a suggestion that 5-HT_3 receptors may also mediate stimulus-evoked inhibition (i.e. in experiments where MO was applied to the flexion of the ankle). As the sole difference between these experiments and those in which ondansetron attenuated reflex facilitation was the site of MO application, it seems that the location of the noxious insult could be a determining factor with respect to the nature of the pathways activated (inhibitory or facilitatory).

Both the attenuation and enhancement of MO-induced inhibition of hindlimb reflexes, as well as the tonic effects of serotonergic pathways on reflexes *per se* can be mediated by 5-HT_3-receptors via several possible mechanisms. This receptor subtype is most densely expressed in the superficial dorsal horn i.e. lamina I and IIi (Hamon et al., 1989, Gehlert et

al., 1991, Maxwell et al., 2003, Conte et al., 2005, Peters et al., 2010; see also section II.B.3.5), and radioligand binding has confirmed this area of the dorsal horn as the target for ondansetron (Doucet et al., 1999). A direct mechanism by which 5-HT_3 receptor mediated facilitation can occur is therefore via an action of endogenously released serotonin (either tonically or evoked by noxious stimulation of peripheral tissues (Tyce and Yaksh, 1981, Zhang et al., 2000)) on dorsal neurones in the reflex pathway, blockade of which by ondansetron diminishes the facilitatory effect (Khasabov et al., 1999, Bardin et al., 2000, Seo et al., 2002). More involved mechanisms would incorporate excitatory or inhibitory interneurones. To this end activation of 5-HT_3 receptors in rat neuronal cultures by application of the selective agonist phenylbiguanide has been shown to enhance the release of the excitatory neurotransmitter glutamate (Funahashi et al., 2004) and *in vitro* electrophysiological studies examining the relationship between activation of 5-HT_3 receptors in the spinal cord and the activity of GABA- and glycinergic-inhibitory neurones have shown an increase in firing of the inhibitory cell in the presence of selective 5-HT_3 receptor agonists – an effect blocked by the addition of selective antagonists such as ondansetron and tropisetron (Fukushima et al., 2009, Xie et al., 2012).

Finally where might be the origin of these serotonergic influences? The supraspinal origin of serotonergic terminals in the thoracic and lumbar segments of the rat spinal cord are the serotonergic cells group of the pons (B1, B2, and B3), particularly B3, the raphe magnus nucleus (see section II.B.2.2), established using a combination of retrograde labelling and immunohistochemical techniques (Bowker et al., 1981a, b, Skagerberg and Bjorklund, 1985). Of these, the raphe magnus nucleus is thought to be among the primary sources of descending serotonergic modulation of spinal nociceptive processing. Infiltration of this cell group by local anaesthetic attenuates the onset and development of cutaneous hyperalgesia (Tillu et al., 2008), indicating an overall facilitatory action of these neurones; a view consolidated by studies in which stimulation of the raphe magnus nucleus facilitates dorsal horn firing in response to peripheral noxious stimulation (Zhuo and Gebhart, 1997). As bulbospinal fibres originating in B3 project via the DLF to supply the serotonergic terminals of the superficial dorsal horn (laminae I and II) (Light et al., 1983, Müllner et al., 2008; see also section II.B.2.2), it can therefore be presumed that this facilitatory influence acts at this site of sensory integration.

6.5 Conclusions

The present studies have shown that in the decerebrated rat, reflex responses are tonically facilitated by serotonergic pathways and at least a part of this control is mediated by spinal 5-HT_3 receptors. Potentiation (and possibly inhibition) of reflexes following an acute chemogenic insult also appears to involve the actions of serotonin at 5-HT_3 receptors in the spinal cord.

7. GENERAL DISCUSSION

The nociceptive withdrawal reflexes are a group of spinally mediated muscle actions that are selectively activated in response to a noxious stimulus and serve to limit tissue damage at the site of stimulation. These reflexes were originally characterised by *in vivo* work carried out at the end of the 19th century and were collectively described as the "flexion reflex" (Sherrington, 1910). This protective withdrawal function was proposed to act to remove the stimulated limb away from the origin of the stimulus by excitation of muscles that result in flexion at the hip, knee, and ankle joints with concomitant inhibition of extensor muscles about the same joints. The effects of the noxious input also affected the limb contralateral to the site of stimulus such that the opposite pattern of muscle responses was seen i.e. an excitation of extensors with inhibition of flexors, termed the "crossed-extension reflex" which was thought to maintain the overall balance of the animal. Later work evolved the flexion reflex theory further to indicate that limb withdrawal was not a stereotypical response and included the idea of a detailed modular withdrawal reflex with characteristic excitatory and inhibitory receptive fields for each muscle promoting or inhibiting movement accordingly (Schouenborg, 2002). Previous work from this laboratory in the rabbit has shown a similarly organized 'modular' pattern of hindlimb withdrawal reflexes (Clarke and Harris, 2004), and that sensitization of reflexes follows a similar modular pattern that is controlled from supraspinal sites via bulbospinal projections (Harris and Clarke, 2003).

The first part of the present study sought to translate the observations from the rabbit into the rat, a preclinical species more typically employed in investigations into nociceptive processing and therefore with a greater pool of published literature with which to draw comparisons. Utilising decerebrate spinalized, decerebrate non-spinal, and anaesthetized preparations provided a model in which to directly observe the influence of supraspinal structures on reflex sensitization (and inhibition) and from which to draw conclusions regarding the relative importance of these controls in rat compared to rabbit. Mustard oil application to cutaneous sites, regardless of the preparation, typically generated a much small alteration in the magnitude of the evoked reflexes in the rat relative to that found in the rabbit, and that which did occur tended to be of a shorter duration even in spinalized animals when the potential to generate excitability in spinal systems or reflexes is at its greatest (Schouenborg et al., 1992). Intramuscular or intra-articular application however

induced a longer-term modulation of reflexes, a phenomenon previously reported in rats (Wall and Woolf, 1984; Woolf and Wall, 1986) but not in rabbits (Harris and Clarke, 2003) possibly indicating a differential expression of the receptors targeted by MO between the sites or indeed between the species. Previously, MO was thought to function as a selective agonist at TRPA1 receptors expressed by keratinocytes and sensory neurones (Anand et al., 2008), but has recently been postulated as a TRPV1 receptor agonist, with different temporal characteristics resulting from activation of each receptor type (Everaerts et al., 2011). This evidence may go some way toward explaining why the alteration of reflex responses by MO in the rat was both more transient and less dramatic than in the rabbit, and could be investigated further through receptor expression studies using such techniques as *in situ* hybridization or immunohistochemistry. These techniques could also be used to explore other possible influential factors governing the differential MO effects, such as an examination of receptor expression in glabrous and hairy skin from both species as well as in epidermis relative to deeper tissues.

The present studies have provided some evidence to suggest that the spatial organization of inhibitory and facilitatory sensitization fields in rats with intact spinal cords conforms to the modular organization of withdrawal reflexes *per se* in the rat described in detail by Schouenborg et al. (1994). MO application to sites that would be moved toward the source of noxious stimulation induced inhibition in the muscles integrated in the generation of that movement, with the complementary reflex facilitation observed from MO applied at sites moved away. As in the rabbit (Harris and Clarke, 2003) this modular pattern for sensitizing stimuli is dependent upon descending pathways, as spinal cord transection at the lower thoracic level resulted in a modest expansion of sensitization fields for some reflexes and a total absence of inhibition. Unlike the rabbit however, in which the ankle extensor heel-MG reflex appeared to be controlled at the level of the spinal cord, this reflex in the rat conformed more to a flexor-like organization i.e. organized in a modular fashion imposed by descending pathways. The greater potential for sensitization of reflexes in the decerebrate spinally-intact rat preparation relative to the anaesthetized animal (in which inhibition was the predominant effect of MO application) cannot conclusively be attributed to the removal of forebrain structures, which though this difference may impact on reflex excitability, the concurrent reduction in potentially confounding anaesthetic is also a prevailing factor.

Although the pattern of sensitization seen in the current studies reflects the modular organization of sensitization of hindlimb withdrawal reflexes reported from this laboratory in the rabbit to some degree (Harris and Clarke, 2003), one notable exception to the parity of MO-induced modulation between these two species is that of inhibition produced by MO application to the ipsilateral limb i.e. the limb in which reflexes were evoked and recorded. Inhibition of reflex responses in the anaesthetized rabbit by IL MO application was rare, indeed it was found only in the ankle flexor TA and ankle extensor MG after MO injection into the MG muscle itself. In contrast, MO applied to the IL limb in anaesthetized rats was capable of inducing reflex inhibition from both deep and topical application and was evident in all four reflexes. The implication from this is that a difference exists between rat and rabbit with regard to somatomotor integration, either in relation to the afferent inputs generated by MO, the intraspinal networks impinging on the processing of those inputs, or the threshold required to generate an effect on the motor output, be it facilitatory or inhibitory in nature.

The alterations in the spatial organization of reflex excitability following spinalization, in conjunction with the observation that the effect of intrathecal pharmacological manipulation was dependent on the location of MO application, implies that the descending control of these changes exhibits a degree of site specificity. There is evidence in the literature to show that inputs from cutaneous sites are differently modulated by descending control pathways originating in the periaqueductal grey depending on the functional implications of that stimulus in a behaving animal (Heinricher et al., 2009), such that “distracting” C-fibre-mediated inputs are inhibited with those mediated primarily by sensory-discriminative A-fibres preserved. This differential descending control governing the responsiveness of dorsal horn neurones to peripheral stimulation (Waters and Lumb, 2008, Heinricher et al., 2009) may provide some explanation for the contrast in the effects of MO following spinal drug administration when comparing the dorsal flexion of the ankle treatment site with the plantar heel and MTJ sites if one considers the plantar sites as providing greater A-fibre-mediated sensory-discriminative information than the dorsal site.

In spite of the findings in the present study, it is worthy of note that due to the variable and often subtle effects of MO on evoked reflex responses, (particularly when compared to the rabbit) that a true elucidation of modulatory receptive fields in this model was somewhat complicated. Whether this was due to the nature of the stimulus (a reduced volume of MO

was employed relative to the rabbit studies to account for size differential between the species and the need to localise the sensitizing stimulus to a discrete site) or due to anatomical and/or physiological differences is unknown. It may be postulated however that by reducing the volume of the chemogen the sensory barrage was also reduced, and therefore employing a more potent chemical stimulus such as capsaicin or simply a higher concentration of MO (20% was used here) might assist in clarifying the modulatory patterns. However, by using a less potent conditioning stimulus it was possible to apply multiple treatments to a single animal and thereby reduce the numbers required to fulfil the study – a more potent stimulus would prohibit this due to ongoing and inflammatory effects and the number of animals required would rise as a result.

In addition to site- and reflex-specific effects, the overall response of spinally organized hindlimb reflexes is under the critical control of descending pathways, demonstrated here by the differential sensitization patterns for certain reflexes in spinally transected compared to 'intact' preparations. This manifested as both significant reflex facilitation from MO sites that in the non-spinal preparation had no effect, and conversely MO-induced reflex facilitation in non-spinal rats that was lost following spinalization. These findings illustrate that supraspinal control of MO-induced reflex sensitization in the rat has both facilitatory and inhibitory components, whilst the comparable study in rabbit found no evidence of a facilitatory descending control in that species (Harris and Clarke, 2003). The second part of these studies therefore aimed to begin to investigate the nature of those descending controls by way of pharmacological blockade of spinally-expressed receptors that mediate effects the two principal bulbospinal pathways known to affect spinal cord excitability; G-protein coupled noradrenergic α_2-adrenoceptors and serotonergic ligand-gated 5-HT_3 receptor ion channels.

Descending noradrenergic controls originate primarily in the pons, and in particular from the locus coeruleus (see section II.A.2.3) which has a tonic inhibitory role in the control of nociceptive processing in the spinal cord (Jasmin et al., 2003, Rojas-Piloni et al., 2012). Antagonism of the α_2-adrenoceptor at the level of the spinal cord by intrathecal administration of RX 821002 could indicate to what degree the descending inhibitory effects were mediated via this receptor subtype. Blockade of α_2-adrenoceptors by this highly selective antagonist enhanced reflex responses, thus demonstrating the tonic nature of the descending inhibitory control in this model. The impact of this pharmacological

intervention on MO-induced alteration of reflex responses was much more subtle, possibly indicating a role for α_2-adrenoceptors in the descending inhibition of sensitization fields.

Descending serotonergic influences over spinal excitability are medullary in origin (rather than pontine as with the noradrenergic control pathways) with the raphe magnus nucleus a primary source (Bowker et al., 1981b, Skagerberg and Bjorklund, 1985), and given the heterogeneity with serotonergic receptor classes (see section II.B.3) are implicated in both pro- and anti-nociceptive modulatory effects. As the 5-HT_3 receptor has been linked with the facilitatory element of these opposing actions, spinal blockade of this receptor subtype was utilised as a method by which to assess its contribution to descending facilitation. Intrathecal administration of the selective antagonist ondansetron attenuated reflex responses *per se*, implying that tonic descending facilitation is indeed mediated via the 5-HT_3 receptor subtype in the rat. Enhancement of hindlimb reflexes by MO was also attenuated by spinal application of this antagonist, reinforcing the idea that where MO-induced facilitation was reduced following spinalization was at least partly due to disruption of bulbospinal serotonergic pathways acting via spinal 5-HT_3 receptors (Zhuo and Gebhart, 1997, Tillu et al., 2008).

The decerebrate rat model as employed throughout these studies provides a useful model in which to investigate the organization of spinally-mediated hindlimb reflex responses but is not limited to such measurements. Greater exploration of this organization may be undertaken in such a model, with the potential to investigate spinal neuronal responsiveness directly through extracellular recording techniques, or to investigate reflexes as a function of peripheral motorneurone firing rather than through EMGs. The pattern of reflex organization presented here not only correlates with findings from other pre-clinical species such as the rabbit but also with the organization of lower limb reflexes in the human (Sonnenborg et al., 2000). This knowledge, as well as the evidence for descending modulatory controls that influence spinal cord excitability, could aid the development of future analgesic or antihyperalgesic strategies.

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