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Plasticity of spinal circuits underlying chronic visceral pain

Mehreen Sana Arshi

This thesis is submitted in fulfillment of the requirements for the degree of Master of Philosophy (Medicine)

Sydney Medical School
The University of Sydney, August 2014
DECLARATION

I hereby declare this thesis to be an original work of my own composition. The material presented is due to original research undertaken for the degree of Master of Philosophy (Medicine) at the University of Sydney and has not been submitted for any other at this or any other institution. To the best of my knowledge, this treatise contains no material previously published, written by other persons or contains work by any other persons except where indicated through acknowledgment with the gratitude of this author.

Mehreen Arshi

August, 2014
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<tr>
<td>AITC</td>
<td>allylisothiocynate</td>
</tr>
<tr>
<td>CC</td>
<td>central canal</td>
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<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
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<td>ChAT</td>
<td>choline acetyltransferase</td>
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<td>CYP</td>
<td>cyclophosphamide</td>
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<td>DCM</td>
<td>dorsal commissure</td>
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<td>DH</td>
<td>dorsal horn</td>
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<td>DMSO</td>
<td>dimethylsulphoxide</td>
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<td>Hp</td>
<td>hind paw</td>
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<td>IC</td>
<td>interstitial cystitis</td>
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<td>IML</td>
<td>intermediolateral column</td>
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<td>IP</td>
<td>intraperitoneal</td>
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<td>IR</td>
<td>immunoreactivity</td>
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<td>IV</td>
<td>intravesical</td>
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<td>L5</td>
<td>lumbar spinal segment 5</td>
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<td>L6</td>
<td>lumbar spinal segment 6</td>
</tr>
<tr>
<td>LT</td>
<td>Lissauer’s tract</td>
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<tr>
<td>MO</td>
<td>mustard oil</td>
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<td>NK1R</td>
<td>neurokinin 1 receptor</td>
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<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PMC</td>
<td>pontine micturition centre</td>
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<td>PRV</td>
<td>pseudovirus rabies</td>
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<td>SP</td>
<td>substance P</td>
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<td>SPN</td>
<td>sacral parasympathetic nucleus</td>
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Abstract

Interstitial cystitis is a painful condition of bladder urgency and pain, often associated with inflammation with unknown etiology that commonly affects women. There are limited treatments available and no cure to date. This condition is known to affect two different pathways of the peripheral nervous system, the nociceptive pathway and the micturition reflex pathway. In this thesis, I have employed a key technique used to study somatosensory pain states to understand some of mechanisms underlying visceral pain by mimicking some primary symptoms of interstitial cystitis in rodents. In the somatosensory pain states, there is an increase in the release of the neurotransmitter SP in response to noxious stimulation of the sensory nociceptive terminals. The released SP binds to its receptor, the Neurokinin 1 receptor (NK1R) expressed on neurons in lamina I and outer lamina II of the lumbar spinal cord. The NK1R upon binding of its ligand shows internalisation in response to the noxious stimulation and thus identifies neurons that are activated. In this study we have applied this well-established methodology to a model of visceral pain to identify activated neurons in the sacral spinal cord in response to visceral noxious stimulation and inflammation.

Adult Female Sprague Dawley rats were used in this study and spinal cords were harvested following intracardial perfusions. In previous studies NK1R expression has been shown in the dorsal horn (DH) projection neurons and in the intermediolateral (IML) column of neurons, an area containing interneurons and bladder projecting autonomic preganglionic parasympathetic neurons. We have used a combination of immunohistochemical techniques and confocal microscopy to investigate NK1R expression and internalisation in cryosections of the sacral spinal cord DH and IML.
neurons. In the first part of the thesis NK1R expression was quantified in the IML region. We found that a majority of autonomic preganglionic neurons in the IML express the NK1R.

We next used capsaicin and mustard oil to induce noxious stimulation of the bladder afferent terminals. Capsaicin and mustard oil act on transient receptor potential vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1) channels (respectively) and are known to be involved in nociceptive processing. These TRP channels are expressed on the bladder afferents and are activated in response to their ligand binding. NK1R internalisation was analysed in cryosections of the sacral spinal cord DH and IML neurons following TRPV1 and TRPA1 activation. We found that neurons in both nociceptive and micturition reflex pathways were activated in response to noxious stimulation of the bladder afferents after TRPV1 activation but only in the IML after TRPA1 activation. Next we proceeded to establish cyclophosphamide (CYP) induced acute and chronic inflammation of the bladder. Both acute and chronic inflammations of the bladder caused activation (NK1R internalisation) in the DH and IML neurons. Bladder function was also assessed by performing continuous cystometry in control and inflammation groups and NK1R internalisation was measured. Although we did not see a statistical difference in the cystometrograms between the control and CYP treated groups, an increase in NK1R internalisation levels was observed in both DH and IML regions following inflammation.

This study has identified that the autonomic preganglionic neurons are a target of the bladder projecting peptidergic afferents. We have demonstrated that the NK1R internalisation technique established to study somatic inflammation can be used in a
visceral model of inflammation. The lower levels of NK1R internalisation seen after visceral inflammation compared to somatic inflammation indicate that there may be a difference in the amount of neuropeptide (substance P) released from the two types of afferents. The sparse innervation of the viscera by visceral afferents may also account for this lower level of internalisation compared to the response observed in the somatic model. The results indicate that there is a difference in neuronal activity in DH and IML neurons after TRPV1 and TRPA1 activation. This suggests that both the nociceptive and micturition reflex pathways should be studied and bladder hyperactivity alone should not be used as a surrogate marker to study bladder pain. Furthermore we have shown that non-noxious stimulation of the bladder afferents (cystometry) leads to an increase in NK1R internalisation after acute and chronic inflammation. These results provide an insight into some of the mechanisms that underlie the characteristic symptoms of cystitis and provide a valuable tool to further probe these mechanisms and establish a better understanding of this disease.
Chapter 1: An introduction to pelvic visceral pain.

1.1 Pain

Pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Loeser and Treede 2008). Nociceptors are primary sensory neurons that detect noxious stimuli (actual or potential tissue damaging events) at their peripheral ends and propagate the signals to their central terminals in the spinal dorsal horn. This detection of painful signals is a crucial component of the body’s protective mechanism that allows us to withdraw from the source of unpleasant sensation. In the case of acute pain a warning mechanism is triggered that involves initiation of reflexes that withdraw us from further damage. Pain can be classified as nociceptive, neuropathic or of inflammatory origin. Nociceptive pain is caused by activation of nociceptive pathways due to peripheral stimulation sufficient in intensity to lead or threaten to lead to tissue damage (Latremoliere and Woolf 2009). Neuropathic pain is caused by maladaptive plasticity of the nociceptive signaling that occurs as a result of disease or lesion of the nervous system, leading the somatosensory system to signal pain in the absence of a stimulus (Costigan et al. 2009). The pain that occurs in response to direct tissue damage and causes a subsequent inflammatory response is classified as inflammatory pain. It increases responsiveness of the nervous system to protect tissue against further damage while aiding the healing process (Juhl et al. 2008). Pain can be perceived as part of body’s defense mechanisms.
Acute pain is perceived when the body is under threat (e.g. due to cuts, burns or broken bones) and is relieved when the underlying cause is healed or treated. Chronic pain differs from acute pain in that it persists for periods much longer than the expected course of healing or the actual healing (Basbaum et al. 2009). It is also reported in the absence of a stimulus, a source of injury or any obvious damage to the tissue. Chronic pain is perceived not only from somatosensory tissues (skin, muscles, and joints) but also from the viscera (organ pain). Whereas it has not been easy to decipher the mechanisms underlying somatosensory pain, it has been even more difficult to understand the complexities of chronic visceral pain. This is due to some marked clinical differences between the anatomy and physiology of somatosensory and visceral afferents (see 1.1.2).

1.1.1 Nociceptive afferents and their pattern of termination

Among various populations of somatosensory afferents there are two major classes of nociceptive afferents, the medium diameter thinly myelinated Aδ afferents that mediate acute or fast pain and the small diameter unmyelinated C fibres that convey poorly localized slow pain (Basbaum et al. 2009). As can be seen in Figure 1.1, the spinal cord is divided into different laminae, and nociceptive fibres terminate specifically in the superficial dorsal horn, in laminae I and II (outer) (Todd 2010). Superficial lamina I neurons of the spinal dorsal horn that receive nociceptive inputs transmit signals to the brain and receive descending inputs from the brainstem. Due to their involvement in processing nociceptive signaling, it has been proposed that lamina I neurons in the superficial spinal dorsal horn are involved in neuroplastic changes and peripheral and central desensitization (see 1.5) (Latremoliere and Woolf 2009).
Primary afferents arborize within the dorsal horn in an orderly way: a laminar termination pattern based on fibre diameter and function is superimposed on a somatotopic distribution that determines mediolateral and rostrocaudal location. The grey matter of the spinal cord is divided into a series of laminae based on variations in the size and density of neurons. This illustration shows specific sites of central terminations of the major types of primary afferent fibres in the spinal dorsal horn. Peptidergic primary afferents (A\(\delta\) and C fibres) terminate mainly in lamina I and lamina II (outer) with some fibres penetrating more deeply. Most non-peptidergic C fibres occupy the central part of lamina II. The A\(\delta\) hair follicle afferents are known to terminate mainly in lamina II (inner) and lamina III. The A\(\beta\) tactile and hair afferents end mainly in lamina III-V. (Todd 2010).

**Figure 1.1. Overview of the pattern of afferent nerve termination in the spinal dorsal horn.** The grey matter of the spinal cord is divided into a series of laminae based on variations in the size and density of neurons. This illustration shows specific sites of central terminations of the major types of primary afferent fibres in the spinal dorsal horn. Peptidergic primary afferents (A\(\delta\) and C fibres) terminate mainly in lamina I and lamina II (outer) with some fibres penetrating more deeply. Most non-peptidergic C fibres occupy the central part of lamina II. The A\(\delta\) hair follicle afferents are known to terminate mainly in lamina II (inner) and lamina III. The A\(\beta\) tactile and hair afferents end mainly in lamina III-V. (Todd 2010).
1.1.2 Differences between somatic and visceral sensory afferents

Visceral sensory afferent projections in the spinal cord are extremely complicated as reviewed by (Robinson and Gebhart 2008). As depicted in Figure 1.2 a, somatosensory afferents terminate in specific localized region of the spinal dorsal horn. In contrast each visceral afferent (Fig. 1.2 b) synapses in multiple laminae of the dorsal horn and in many rostro-caudal segments. This causes the nociceptive signals to be diffused and poorly localized (Robinson and Gebhart 2008). In addition, some somatic and visceral afferents converge onto the same neurons, leading to the sensation of pain emanating from non-visceral structures even when there is a visceral source. Compared to the skin, muscles and joints, innervation of viscera is sparse. Moreover, somatic afferents have one of the many different types of sensory endings (e.g. Merkel cells, Ruffini endings and Pacinian corpuscles) that are used to detect and transduce stimuli. On the other hand, the majority of visceral afferent endings are unspecialized and only two types of specialized peripheral terminal endings have been identified, intraganglionic laminar endings and intramuscular arrays (Robinson and Gebhart 2008). Lack of specialized endings and sparse innervation of the viscera also cause the sensation to be poorly localized. These differences in neuro-anatomy between somatic and visceral afferents pose a great challenge to the diagnosis of visceral pain. Measuring visceral pain is also especially difficult in animal models of pain, making development and assessment of potential new therapies difficult. In somatosensory pain models, behavioral responses such as tail flick and paw withdrawal are believed to be good indicators of a nociceptive response in an animal. In the case of visceral pain, animal posture and level of activity may reflect part of the visceral pain experienced by animals. This however is not a
sufficient or clear indication of the actual magnitude of pain and can be difficult to quantify.

Figure 1.2. Schematic representation of sensory afferent projections to the spinal dorsal horn. a) The somatosensory afferents have a focused field of termination in the spinal dorsal horn. b) In contrast, visceral sensory afferents have many branches that arborise and terminate on multiple laminae of the dorsal horn and in rostro-caudal segments of the spinal cord. They also cross over to the contralateral side and terminate on multiple segments of the spinal cord (Pasricha et al, 2007). This complicated arborisation of the visceral sensory afferent terminals is thought to account for the difference between the perceived visceral and somatosensory pain sensations.
1.2. Chronic pelvic pain

A very common example of chronic pain is chronic pelvic pain that can lead to a poor quality of life, loss of productivity, low self esteem and adds to medical costs. It is often considered that pelvic pain is generated from a gynecological source. However recent studies indicate that there are several commonly diagnosed etiologies of chronic pelvic pain that include endometriosis, adhesions, irritable bowel syndrome, lower urinary tract infection and interstitial cystitis (Zondervan et al. 1999; Howard 2003; Bogart et al. 2007; Ortiz 2008). These pain conditions have been referred to as dysfunctional pain conditions in which substantial pain is experienced with no or minimal inflammatory pathology (Woolf 2010). Often symptoms of one diagnosed condition are common to other diseases of the lower urinary tract, for example, interstitial cystitis has many features in common with lower urinary tract infection and endometriosis (Dell 2007). Here I will discuss interstitial cystitis in detail as an example of chronic pelvic pain.

1.2.1 Interstitial cystitis

The urinary bladder is a hollow organ that acts as a reservoir of urine until a suitable time for it to be excreted from the body. It is primarily composed of three layers; the mucosal lining that includes the epithelium and urothelium, suburothelial connective tissue and the detrusor (smooth muscle) (Skryma et al. 2011). Interstitial cystitis (IC) is a chronic disorder of bladder pain and irritative symptoms (Dawson and Jamison 2007; Theoharides et al. 2008). It is characterized by pain in the bladder and pelvic region, urinary urgency, frequency and nocturia (waking up during the night one or more times with a strong desire to void)(Abrams et al. 2002; Dell 2007; Theoharides 2007;
Histological analysis of bladders from IC patients reveals chronic infiltration of mast cells, vasodilation and edema (Yoshimura and de Groat 1999). There are no known causes of IC to this date and in some patients symptoms of chronic inflammation continue to persist for more than 6 months in absence of any urinary tract infection. Although extensive research is being conducted to investigate etiology of the pathophysiology of IC and some patients can undergo treatments to manage the disease, there is still no known cure (Lukban et al. 2002; Phatak and Foster 2006; Theoharides 2007).

Initiating a treatment or management plan for IC can prove difficult and patients can suffer for a number of years before IC is clinically diagnosed. This is because it is very common for IC patients to be misdiagnosed with recurring urinary tract infection (UTI) and because of the overlapping symptoms of IC with UTI and other diseases leading to chronic pelvic pain (Dell 2007). Once IC is diagnosed, a treatment plan is provided according to the severity of every individual case. The many treatments for IC include bladder hydrodistension, oral and intravesical pharmacological interventions, a change in life style, dietary modifications and exercise (Moldwin et al. 2007; Theoharides 2007).

A considerable amount of research has been conducted to study IC using questionnaires that examine severity of symptoms, co–existence of IC with other diseases leading to pelvic pain and assessment of quality of life. The epidemiological studies prove to be very useful in understanding prevalence of IC in the society and managing treatment plans for patients. Bladder biopsies are helpful in investigating morphological changes in the bladder. However, as there is no known etiology of IC, it is not possible to
establish an animal model of the condition. Nevertheless, to further improve our understanding of IC, there are animal models that *mimic* some of the symptoms of IC. These use chronic bladder inflammation to affect bladder function and can cause nociceptive behavior. This gives us insight into the mechanisms underlying some aspects of the disease and allows us to manipulate the condition to explore therapeutic targets. These will be discussed further below in the context of plasticity of nerves innervating the urinary bladder.

1.2.2 Innervation of the urinary bladder

The urinary bladder of a rat receives its sensory input from neurons that have their cell bodies in the dorsal root ganglia, with peripheral terminals reaching the bladder and central ends terminating in the spinal cord dorsal horn. From the dorsal horn, sensory signal is sent to higher centres in the brain. The motor output from the brain travels via descending pathways back to the autonomic centres in the sacral cord. Sensory neurons innervating the bladder project their axons in pelvic and hypogastric nerves (Applebaum et al. 1980; Blok 2002; de Groat 2006). In rodents, the hypogastric nerve carries projections to the rostral lumbar (L1/2) and pelvic nerve projections from the lumbosacral (L6/S1) spinal cord segments (Gabella and Davis 1998). Peripheral endings of sensory afferents terminate suburothelially and in the detrusor (Gosling and Dixon 1974; Maggi 1995; Birder et al. 2010). The suburothelial sensory nerves form a plexus that becomes particularly dense around the bladder neck and trigone compared to the dome (Andersson 2002). These afferents detect bladder pressure and chemical stimuli.
Figure 1.3 shows the sensory and motor connection between the spinal cord and the higher brain centers involved in the micturition reflex pathways (Fowler et al. 2008). As mentioned above, afferents arising from the bladder and the urethra terminate on the lumbar and sacral segments of the spinal cord dorsal horn. Some also appear to terminate in the intermediolateral column or nucleus (IML), where cell bodies of efferent preganglionic fibres are known to be located (Nadelhaft and Booth 1984). The spinal projection neurons signal to the brain and lead to activation of the thalamus, cortex and the periaqueductal gray (PAG). From the PAG, information regarding micturition and continence is relayed to other brain regions. Information from the higher brain regions is transmitted via the spinal cord preganglionic neurons to the sympathetic and parasympathetic ganglion neurons that make up the motor component of the bladder innervation. The sympathetic and parasympathetic preganglionic neurons are located in the upper lumbar and sacral cord respectively. The location of preganglionic parasympathetic neurons that form a nucleus in the L6 and S1 spinal segments in rodents has been confirmed by several studies (Morgan et al. 1981; Nadelhaft and Booth 1984; Hwang et al. 2005).

The parasympathetic preganglionic fibres travel with sensory axons from sacral DRG in the pelvic nerve to the pelvic ganglion (Blok 2002). The sympathetic preganglionic fibres have their cell bodies in the upper lumbar cord (Vaughan and Satchell 1992) and their axons project with sensory axons from upper lumbar DRG in the hypogastric nerve and synapse on postganglionic neurons of the pelvic ganglion (de Groat 2006). From the pelvic ganglion, postganglionic fibres travel in the accessory nerve and terminate within the bladder.
Figure 1.3. A simplified flow chart of the connections between the bladder and the spinal cord, and the brain regions. 

a) The physical stimuli in the bladder are detected by the peripheral terminals of the sensory afferents and the signal is propagated via the sensory afferents to the spinal dorsal horn and the IML neurons. Sensory signals are then received in the higher brain regions via the ascending pathways. Some of these regions include periaqueductal grey (PAG), the pontine micturition centre (PMC) and the hypothalamus.

b) The motor signal is received from the higher brain regions including the PAG, PMC, raphe nuclei, the red nucleus, and the locus coeruleus (LC) via the descending fibres. The signal is projected to the spinal interneurons and preganglionic neurons of the intermediolateral column of neurons of the sacral cord and the postganglionic neurons before reaching the bladder.
1.2.3 Models of lower urinary tract inflammation

Various approaches using intravesical, systemic or environmental stimuli have been taken to mimic some of the symptoms of IC in animals to better understand the disease. Intravesical application of varying concentrations of acetone in many species (rats, rabbits and monkeys) has shown alteration in micturition pattern, reduced bladder capacity and morphological changes in bladder urothelium (Westropp and Buffington 2002). Acute and chronic studies using noxious stimuli such as turpentine, mustard oil and croton oil have been conducted to study the effects on plasma extravasation (Habler et al. 1990). Intravesical injections of substance P are also known to induce cystitis and increase plasma extravasation (Bjorling et al. 1999). Naturally occurring feline interstitial cystitis has been used to compare symptoms with the condition in humans (Westropp and Buffington 2002) and especially to understand changes in bladder capacity, changes in substance P immunoreactivity and environmentally induced stress related changes (Westropp and Buffington 2002)

More recently cyclophosphamide induced inflammation of the lower urinary tract has been commonly used to study cystitis. Cyclophosphamide (CYP) is a pro-inflammatory agent that was initially administered as an anti-tumour agent for bladder cancer patients. Later on it was observed to be a cause of haemorrhagic cystitis of the bladder. Further research into causation of this cystitis led to the discovery of acrolein (a metabolite of CYP produced by the kidneys) that accumulated in urine and caused haemorrhage by being in contact with the bladder wall (Cox 1979; LaBerge et al. 2006). With establishment of the role of acrolein, it has been possible to use it as a pro-inflammatory agent to cause inflammation of the lower urinary tract in animals. The symptoms of
CYP induced bladder inflammation very closely mimic some of the characteristic symptoms of IC and this is also confirmed by histological evidence (Lanteri-Minet et al. 1995). Intraperitoneal administration of CYP has been shown to have its effects from as little as 4 hours after injection and reaches maximum severity from 24-48 hours (Lanteri-Minet et al. 1995). This model offers an opportunity to induce inflammation without any surgical procedures, catheter balloon insertions or any other experimental manipulations that could contribute as confounding factors. Intraperitoneal injection of CYP is also viscera specific. It is unlike other visceral pain models that are either invasive or also affect parts of the somatosensory system.

Studies mainly conducted in rodents reveal that the properties of bladder afferents play a crucial role in regulating bladder function. The bladder sensory afferents are responsible for detecting pressure and distension. Inflammation and disease of the bladder may suggest that this sensory component of bladder innervation has been compromised. In the case of bladder inflammation, sensitivity of afferent nerves is increased and that in turn causes altered bladder activity such as decreased threshold for voiding. This increase in afferent nerve sensitivity has been confirmed in animal electrophysiological studies of isolated bladder sensory afferent neurons after chronic inflammation (Yoshimura and de Groat 1999). CYP administration has several effects on the urinary bladder, including increased micturition frequency and a decrease in the volume threshold for micturition (Lecci et al. 1994) that could be a result of a reduction in bladder capacity. More recently, CYP-induced cystitis has been demonstrated to cause an increase in many neuropeptides, transcription factors and second messengers in bladder afferents and spinal pathways (Vizzard 2000; Vizzard 2000). It has also been shown that CYP treatment in rats causes macrophage infiltration, increased frequency
of voiding in awake rats. Maggi and colleagues have demonstrated bladder hyperactivity in anaesthetized rats (Maggi et al. 1992).

Release of inflammatory mediators associated with cystitis is thought to be an important factor in the development of persistent bladder pain (Cervero and Laird 2004). This has been indicated in behavioral studies that are conducted to measure referred pain in rodents with chemically induced cystitis. Such studies assessed referred mechanical hyperalgesia in the hindpaw at different time points after inflammation and confirmed a reduced threshold to mechanical stimulation in animals with bladder inflammation (Jaggar et al. 1999; Guerios et al. 2009; Merriam et al. 2011). In my study, I have used systemic CYP treatment to induce acute and chronic inflammation of the lower urinary tract.

1.3. General properties of visceral afferents

The physiological properties of visceral afferents are different from non-visceral (i.e. somatic) afferents. The somatosensory afferents conduct sensations of touch, temperature, pinch and cutting. Visceral afferents on the other hand, conduct sensations such as bloating and distension. The primary function of visceral afferents is to transduce signals of stimuli in the normal physiological range. For example, in the bladder, low threshold stretch mechanosensory afferents transmit the sensation of stretch with a rise in intravesical pressure within the low physiological range. A very small number of visceral afferents are recruited to conduct signals for these visceral sensations. A majority of the visceral afferents cannot be activated by stimuli in the
normal physiological range. These are a special class of afferents called silent afferents that are recruited only under special conditions (Michaelis et al. 1996). It is reported that a majority (90%) of visceral afferents are of this type and during bladder inflammation, almost half of these afferents become responsive (Michaelis et al. 1996). Somatic silent afferents have also been reported but a much smaller number of the somatic afferents are silent. Due to the involvement of visceral afferents in autonomic reflexes (as conductors of information in the normal physiological range) as well as in detection of noxious stimuli, it becomes difficult to study their role in nociception exclusively in conditions of bladder pain. However with an abnormal increase in pressure these afferents also have an ability to encode this increase in intensity in the noxious range and become active.

There are 3 types of sensory afferents that project to the bladder, including stretch sensitive mechanoreceptors, chemoreceptors and volume receptors (Zagorodnyuk et al. 2006). These afferents are small diameter thinly myelinated A-δ and unmyelinated C-fibres that consist of both mechanosensitive and chemosensitive receptors in bladder wall and central terminals in the superficial dorsal horn of the upper lumbar and sacral spinal cord. The majority of visceral afferents are A-δ or C-fibres. A very small proportion of visceral afferent is comprised of large diameter Aα/β-fibres (Robinson and Gebhart 2008).

The fine diameter primary afferent fibres densely innervate the superficial dorsal horn in the sacral spinal cord (Todd et al. 2002). These afferents can be further classified into groups on the basis of the neurochemical markers they synthesize. The peptide producing fine diameter primary afferents are known as peptidergic fibres. The
peptidergic population expresses calcitonin gene-related peptide (CGRP) and a majority of these also contain substance P (SP) (Fowler 2002; Hwang et al. 2005; Arms and Vizzard 2011). Most of these peptidergic neurons also express the capsaicin receptor, transient receptor potential vanilloid 1 (TRPV1) (Ghatei et al. 1985; Jancso and Maggi 1987; Gabella and Davis 1998; Avelino et al. 2002). The second class of primary afferents is identified by its ability to bind the plant lectin, isolectin B4 (IB4) (Silverman and Kruger 1990).

A vast majority of the rat bladder afferents neurons are peptidergic and a small proportion are non-peptidergic (IB4) binding afferents (Robinson and Gebhart 2008). It is proposed that in the rat bladder, capsaicin-sensitive primary afferents fibres use SP as an excitatory neurotransmitter (Maggi 1991), along with glutamate. Increase in SP release in the rat bladder after acute administration of capsaicin triggers the micturition reflex, suggesting involvement of TRPV1 in the micturition reflex (Maggi 1991).

Studies also show that the number of bladder afferents expressing SP increases after CYP induced cystitis (Ishigooka et al. 2001) and the immunoreactivity of SP and CGRP is also known to increase in the lumbosacral spinal cord after chronic inflammation (Vizzard 2001). These changes in neuropeptide release by activation of bladder nociceptive afferents provides a potentially valuable tool to further explore activation of their central targets.

1.3.1 Synaptic plasticity in the dorsal horn induced by inflammation

Synaptic plasticity refers to functional and chemical changes that occur in neurons to alter synaptic communication following a change in their environment. This can include changes in transduction and conduction properties of neurons to cause an altered state of
sensitivity (Woolf and Costigan 1999). Spinal dorsal horn neurons activated by nociceptive afferents have been a focus of studies to determine how peripheral inflammation impacts on spinal signaling. Plasticity at these synapses in the superficial dorsal horn neurons has been studied using intracellular, extracellular and whole cell patch clamp recordings. Electrophysiological studies have revealed involvement of the Neurokinin 1 receptor (NK1R; the preferred receptor for SP) expressing nociceptive neurons of lamina 1 in mediating inflammation and nerve injury induced hyperalgesia (Ikeda et al. 2003). Due to their ability to undergo changes in response to inflammation, the superficial dorsal horn projection neurons will be a focus of our study in investigating effects of noxious stimulation of visceral afferents in bladder inflammation.

Changes in neuropeptide expression (CGRP and SP) in afferents of animals with CYP-induced cystitis have been implicated to synaptic plasticity. The immunoreactivity of CGRP and SP has been shown to increase in the superficial dorsal horn of the sacral spinal cord in animals with bladder inflammation (Vizzard 2001). This increase was also observed in the sacral parasympathetic nucleus, an area that contains cells bodies of the autonomic preganglionic neurons. In an arthritic pain model, CGRP is also known to enhance synaptic transmission of superficial dorsal horn neurons by increasing the excitatory post-synaptic currents (Bird et al. 2006). These studies suggest that changes in activity of visceral afferents lead to changes in synaptic activity of the dorsal horn neurons that are involved in processing nociceptive information. However to my knowledge, individual spinal neurons activated in response to peripheral stimulation of peptidergic visceral nociceptive afferents have not been visualised directly. In this study we will aim to visualize the activation of neurons in a visceral model of pain by
investigating a region of the spinal cord that is a known target of synaptic plasticity in response to noxious stimulation and inflammation.

1.4. TRP channel expression by visceral nociceptors

Transient receptor potential (TRP) channels comprise a superfamily of ion channels that are permeable mainly to calcium but also to other monovalent ions such as sodium and magnesium. An increase in intracellular calcium can lead to an increase in firing frequency of neurons, as we will see can occur in nociceptors. There are currently 7 known subfamilies of the TRP channels, only some of which are expressed in neurons, and many of these are expressed in nociceptors. TRPs are involved in signal transduction of pain, taste, vision, heat, cold and pressure. We will now discuss the two TRP channels that are most relevant to this study.

1.4.1 TRPV1

The first TRPV1 receptor was cloned in 1997 (Caterina et al. 1997). It is now known that capsaicin, the active ‘hot’ ingredient in chilli peppers mediates its actions by binding to the TRPV1 receptor that is expressed on small to medium diameter nociceptive afferents. TRPV1 is activated by low pH, heat in the noxious range (> 43°C) and vanilloids such as capsaicin or resiniferatoxin (Szallasi and Blumberg 1999; Birder et al. 2001; Avelino et al. 2002; Avelino and Cruz 2006; Birder 2007).

The role of capsaicin-sensitive peptidergic afferents in regulating bladder function has been demonstrated in both human and animal based studies. Intravesical application of capsaicin is known to cause contraction of the detrusor muscle and lowering of the
volume required to trigger the micturition reflex in humans. It also causes hyperactivity of the bladder (Maggi et al. 1989; Cruz et al. 1997; Lecci and Maggi 2001). Studies conducted in animals reveal that during inflammation, capsaicin-sensitive afferents lower the volume threshold for the micturition reflex (Ishizuka et al. 1994). Using TRPV1 knockout mice, Birder and colleagues (Birder et al. 2002) demonstrated that the lack of TRPV1 receptors leads to non-voiding contractions. Defective voiding responses have also been reported in these TRPV1 deficient mice during cystometry with an increase in bladder capacity at micturition threshold and overflow incontinence. These results suggest that presence of TRPV1 on bladder afferents contributes to the mechanisms responsible for normal bladder function. Further studies on TRPV1 knockout mice conducted by Grundy and colleagues (Daly et al. 2007) investigating mechanosensitivity of the bladder afferents confirmed a reduced sensitivity to distension in TRPV1 mutant mice.

Hyperalgesia is an increased response to a painful stimulus (Keizer et al. 2008). Cruz and colleagues (Dinis et al. 2004) used CYP induced inflammation to study the contribution of the TRPV1 receptor not only in bladder hyperactivity but also in inflammatory hyperalgesia. Their results demonstrated an increase in anandamide (an endogenous ligand of TRPV1) concentration after inflammation of the bladder that indicates a possible increase in activation of TRPV1 receptor. These studies suggest a role for TRPV1 in bladder inflammatory pain states. We will use agonist-induced activation of TRPV1 channel to investigate central targets of TRP-expressing visceral afferents in sacral spinal cord.
1.4.2 TRPA1

Transient receptor potential ankyrin 1 (TRPA1) is a cation channel that is activated by pungent chemicals such as mustard oil, cinnamon oil, allicin and acrolein (Story et al. 2003; Bandell et al. 2004; Jordt et al. 2004; Fujita et al. 2007). It is also known as a receptor sensitive to cold temperatures but not to menthol and responds to temperatures in the cold noxious range (i.e. < 17 °C).

Noguchi and colleagues discovered co-expression of TRPV1 and TRPA1 in primary sensory neurons in the rat dorsal root ganglion that gave rise to Aδ and small diameter C-fibres (Kobayashi et al. 2005). Studies also indicate that nociceptive behavioral responses are abolished in TRPA1 knockout mice. In rats, TRPA1 is co-expressed with TRPV1 in unmyelinated C-fibres located near the urothelium, in the muscle layer and around blood vessels throughout the bladder (Sun et al. 2010). Furthermore in mice TRPA1 mRNA is expressed in dorsal root ganglion neurons that innervate the bladder (Du et al. 2007). The involvement of TRPV1 in nociceptive signaling, and the co-expression of TRPA1 with TRPV1 in Aδ and C fibres, suggests the possibility of TRPA1 also being involved in maintenance of hyperalgesia in inflammation. TRPA1 expression in bladder DRG neurons and their co-expression in the TRPV1 expressing nociceptive afferents, suggests a role for them in bladder sensory innervation and nociceptive responses. In this study we will use agonists for TRPV1 and TRPA1 channels to study the effects of noxious stimulation on peripheral afferent terminals on sacral dorsal horn and IML preganglionic neurons.
1.5. Neurokinin 1 receptor (Substance P receptor)

Substance P (SP) is a neuropeptide belonging to the tachykinin family (Garland et al. 1996). It is widely distributed in central and peripheral nervous systems and performs various functions. SP is known to stimulate smooth muscle contraction and exocrine secretions, act as a neurotransmitter in the brain, spinal cord and the enteric nervous system, and mediates neurogenic inflammation. It mediates these processes by binding to the Neurokinin 1 receptor (NK1R) (Grady et al. 1995). The NK1R is a G-protein coupled receptor with 7 hydrophobic transmembrane domains that is activated by SP release (E.F. Grady 1996). Released SP acts on second order neurons of the spinal dorsal horn and changes their threshold of excitability to subsequent stimuli (Ishigooka et al. 2001). An increase in SP release can lead to lowering of firing threshold of the afferents and can increase excitability of their targets, and cause synaptic changes at their central targets (Latremoliere and Woolf 2009).

On a subcellular level, binding of SP to NK1R leads to activation of second messenger systems followed by desensitization of the activated receptor. Desensitization of the receptor leads to a reduction in its ligand binding affinity. This loss of binding ability is recovered as the receptor is resensitised (Grady et al. 1996). The second messenger system involves G-protein receptor kinases (GRKs) and protein kinases A and C (PKA, PKC). Binding of SP to NK1R causes the GRKs, PKA and PKC to translocate from the cytosol to the plasma membrane and phosphorylate the activated receptor. The uncoupling of the phosphorylated receptor is followed by clathrin-mediated receptor endocytosis and internalisation (Bowden et al. 1994). The NK1R internalisation depletes the number of SP receptors from the cell membrane and down-regulates
responsiveness of the cell to SP (Garland et al. 1994). Upon internalization, the ligand and receptor dissociate and the receptor is relocated to the plasma membrane. SP on the other hand is degraded in lysosomes (Grady et al. 1995). A wide range of studies have been conducted to study NK1R expression in the spinal cord. The NK1R internalises in the form of small endosomes through endocytosis and has been of interest due to its involvement in various SP induced cellular responses.

Lamina I neurons receive nociceptive signals and transmit this information to the brain via ascending pathways. Approximately 80% of lamina I spinal dorsal horn neurons that project to the brain express NK1R (Al Ghamdi et al. 2009). These neurons undergo neurochemical plasticity during noxious stimulation and acute inflammation as described above (see 1.3.1). Hence, these dorsal horn projection neurons have been a target for investigating NK1R internalisation in nociception due to their involvement in nociceptive signaling and their ability to undergo neurochemical changes during inflammation. Some of the studies involving NK1R are summarised in Table 1.1. The studies referred to in Table 1.1 have all been conducted in rodents and a majority in the lumbar spinal cord. Major advances have been made using this methodological approach. NK1R internalisation has been used to understand the mechanisms of internalisation and as a measure of SP release and neuronal activation by peptidergic afferents. However, despite the availability of this powerful method, most of the research conducted to study NK1R internalisation has been conducted in the lumbar spinal cord in models of somatosensory noxious stimulation and inflammation. In the case of visceral inflammation, NK1R internalisation has been used to study sacral spinal cord following inflammation of the colon. NK1R immunoreactivity in sacral spinal cord has also been investigated after chronic bladder inflammation in sacral spinal cord.
(Ishigooka et al. 2001). In this later study, Ishigooka and colleagues reported an increase in NK1R immunoreactivity after chronic bladder inflammation. The results reported an increase in intensity of NK1R immunoreactivity in the sacral spinal cord. However this study does not comment on properties of individual spinal neurons or include an analysis of the subcellular changes that take place in the dorsal horn neurons in response to an increase in SP release. To our knowledge, the NK1R internalisation approach has not been used to investigate at the cellular level spinal cord activation following experimental manipulation of the bladder or other pelvic viscera. In this study, quantification of NK1R internalization will be used to visualize individual neurons in the sacral cord that are activated by SP. We will apply this approach to investigate changes in the spinal cord following various types of bladder manipulations relevant to visceral inflammatory pain.
1.6. Thesis synopsis and aims

The general aim of this study is to define potential sites of plasticity within the sacral spinal cord that are targeted by peptidergic nociceptors in a model of visceral inflammation and following various bladder manipulations relevant to bladder pain.

The specific aims of this study are:

1. **To investigate if preganglionic autonomic neurons in the sacral cord IML are a functional target of peptidergic bladder afferents by characterising NK1R expression and internalization in response to intra-vesical capsaicin.** We will study the co-expression of NK1R with preganglionic neuronal markers choline acetyltransferase (ChAT) and nitric oxide synthase (NOS) in the IML.

2. **To determine if NK1R internalisation can be used as a tool to visualize activation of sacral spinal neurons innervated by peptidergic bladder nociceptors after visceral noxious stimulation.** Previous reports show that noxious stimulation of somatic afferents causes SP release and NK1R internalisation in dorsal horn neurons. We will activate visceral afferents using the TRPV1 agonist capsaicin and use immunofluorescence to visualize and quantify NK1R internalisation in the dorsal horn nociceptive projection neurons.

3. **To determine if intra-vesical activation of TRPV1 receptor can activate the population of sacral preganglionic parasympathetic neurons of the IML.** TRPV1 expressing afferents project to both dorsal horn and IML regions. We will analyse the
levels of cellular activation in both regions by noxious stimulation using intra-vesical administration of the TRPV1 agonist, capsaicin.

4. To determine if intravesical activation of TRPV1 and TRPA1 channels activate similar populations of neurons in the sacral IML and dorsal horn. We will analyse the levels of cellular activation in both regions by noxious stimulation using intra-vesical administration of the TRPA1 agonist, mustard oil and compare it to the cellular activation in response to TRPV1 agonist.

5. To define the level of basal activation of peptidergic bladder afferent neurons after acute and chronic bladder inflammation. Bladder inflammation may stimulate NK1R internalisation in both nociceptive (dorsal horn) and preganglionic neurons (IML); this will be quantified in both regions. The effects of mustard oil induced noxious stimulation will also be investigated in animals with chronic bladder inflammation.

6. To determine if bladder voiding (continuous cystometry) activates NK1R internalisation in dorsal horn and IML neurons and if this internalisation is influenced by prior bladder inflammation. Voiding behavior is altered by inflammation, manifest as increased voiding frequency. We will compare levels of NK1R internalisation after acute and chronic bladder inflammation.
Table 1.1 Summary of studies using NK1R internalisation

<table>
<thead>
<tr>
<th>Objective</th>
<th>Treatment</th>
<th>Spinal level</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>To visualize NK1 receptor Internalisation in vivo in response to somatosensory stimulation</td>
<td>Noxious mechanical stimulation or intraplantar injection of capsaicin</td>
<td>L4</td>
<td>(Mantyh et al. 1995)</td>
</tr>
<tr>
<td>To determine the distribution of NK1 receptor internalizing neurons in response to inflammation</td>
<td>Mechanical stimulation of hindpaw in rats with CFA induced inflammation</td>
<td>L2-L6</td>
<td>(Abbadie et al. 1997)</td>
</tr>
<tr>
<td>To investigate the role of NK1 expressing lamina 1 neurons in ascending nociceptive signaling</td>
<td>Intrathecal injection of cytotoxin/SP</td>
<td>L4</td>
<td>(Mantyh et al. 1997)</td>
</tr>
<tr>
<td>To determine if endogenous Neurokinins are released upon electrical stimulation of the dorsal root</td>
<td>Electrophysiological stimulation of the dorsal root, intraplantar injection of capsaicin</td>
<td>L2-L5</td>
<td>(Marvizon et al. 1997)</td>
</tr>
<tr>
<td>Identification of afferents that contribute to NK1 receptor internalisation after sciatic nerve transaction and inflammation</td>
<td>Electrical stimulation of different classes of afferents to measure magnitude of NK1 receptor internalisation</td>
<td>L4</td>
<td>(Allen et al. 1999)</td>
</tr>
<tr>
<td>To understand the mechanisms of peripheral and central sensitization during inflammatory pain</td>
<td>Intraplantar injection of: Formalin, carrageenan and CFA. Bacterial induced polyarthritis</td>
<td>L4</td>
<td>(Honor et al. 1999)</td>
</tr>
<tr>
<td>To investigate a change in NK1 receptor immunoreactivity in spinal cord after chronic bladder irritation</td>
<td>Cyclophosphamide induced bladder inflammation (CYP administered i.p.)</td>
<td>L1-5, L6-S1</td>
<td>(Ishigooka et al. 2001)</td>
</tr>
<tr>
<td>To investigate change in number of NK1 expressing dorsal horn neurons in acute and chronic colonic inflammation</td>
<td>Zymosan induced colonic inflammation.</td>
<td>T13, L4,S1</td>
<td>(Honore et al. 2002)</td>
</tr>
<tr>
<td>Use of spinal cord slices to characterize the time course of NK1 receptor internalisation and recycling in the dorsal horn lamina 1 neurons</td>
<td>Incubation of harvested spinal cord slices in SP for different time points</td>
<td>L1-L5</td>
<td>(Wang and Marvizon 2002)</td>
</tr>
<tr>
<td>Use of electrophysiology to study capsaicin evoked SP and NK1 receptor internalisation in spinal cord slices</td>
<td>Superfusion of spinal cord slices with capsaicin</td>
<td>L1-L5</td>
<td>(Marvizon et al. 2003)</td>
</tr>
<tr>
<td>To confirm the release of SP as a marker of peptidergic afferent activation during colonic inflammation.</td>
<td>Zymosan induced colonic inflammation</td>
<td>T13-L2, L6-S2</td>
<td>(Landau et al. 2007)</td>
</tr>
<tr>
<td>To study the effect of different modes of primary afferent firing on SP release in spinal cord slices</td>
<td>Electrophysiological stimulation of the dorsal root</td>
<td>L2-L4</td>
<td>(Adelson et al. 2009)</td>
</tr>
</tbody>
</table>
Chapter 2: Peptidergic afferent targets in the sacral cord and their activation in response to noxious stimulation

2.1 Introduction:

The first sets of experiments were conducted to observe neuronal activation in the sacral spinal cord in response to noxious stimulation of visceral nociceptive afferents. This part of the study also verifies the efficacy of using Neurokinin 1 receptor (NK1R) expression and internalisation in studying visceral noxious stimulation. This series of experiments were conducted to address three main aims. 1) We first wanted to investigate if preganglionic autonomic neurons in the sacral IML are a functional target of peptidergic bladder afferents. To address this aim, we first conducted a qualitative analysis of the NK1R immunoreactivity in the dorsal horn lamina I neurons and IML preganglionic neurons of the sacral spinal cord. Next we made a comparison of NK1R immunoreactivity in the sacral cord to that in the lumbar cord. This was followed by quantification of NK1R expressing preganglionic neurons of the IML. 2) We wished to determine if NK1R internalisation can be used as a tool to visualise activation of spinal neurons innervated by peptidergic bladder afferents. The NK1R is known to internalise in superficial dorsal horn lamina I neurons of the rat lumbar spinal cord. This is observed after acute noxious stimulation of the hindpaw (somatosensory stimulation) with intraplantar capsaicin injection. In our experiments we replicated this finding and tested its application in visceral noxious stimulation. We applied this strategy to identify activated spinal neurons in response to noxious visceral afferent stimulation specifically in the sacral spinal cord. We investigated this in lamina I neurons (likely projection neurons) after intravesical
application of TRPV1 receptor agonist. 3) To determine if intravesical activation of TRPV1 receptor can activate the population of preganglionic parasympathetic neurons of the IML. NK1R immunoreactivity has been reported in the IML region of the sacral spinal cord (Ishigooka et al. 2001). Many of the preganglionic neurons involved in this area are involved in the micturition reflex. We used capsaicin, a TRPV1 receptor agonist to induce noxious stimulation of the bladder afferents to observe cellular activation of IML neurons.

2.2 Methods:

Adult female Sprague-Dawley rats aged 8-11 weeks (weighing 200-250g) were used in this study. Population based studies conducted have found that IC is more prevalent in women than in men. It is known that approximately 90% of IC patients are female (Jones and Nyberg 1997), hence female rats were used in this study. Animals were purchased from the Animals Resource Centre (ARC, Murdoch, WA Australia) and housed in groups of 3 in a 12-hour light/dark cycle. All procedures carried out were approved and in accordance with the Royal North Shore Hospital and University of Sydney Animal Ethics Committees. Animals were allowed to feed and drink ad libitum. Estrous cycle was monitored in each animal but not controlled for. Four animals were used for each treatment group unless stated otherwise.
2.2.1 Acute noxious stimulation

All procedures were performed under ketamine (80mg/kg) and xylazine (60mg/kg) anaesthesia administered intraperitoneally. In one group of animals, 50µl capsaicin (1mM in ethanol) was injected in the plantar surface of one hindpaw to activate peripheral terminals of TRPV1 expressing nociceptive afferents. A separate group of animals was injected intra-vesically with capsaicin (100 µl) using an insulin syringe with a 30G needle. To do this, a lower midline abdominal incision was made to expose the urinary bladder. The bladder was emptied manually by applying gentle pressure before injection of capsaicin or the same volume of saline. Animals were perfused intracardially with fixative10 minutes after the injection (see below).

2.2.2 Tissue harvesting and immunohistochemistry

After each treatment animals were perfused with ~ 250ml of 0.9% saline solution (containing 1% sodium nitrite and 5000IU/ml heparin), followed by ~ 500ml of 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. (PFA; Sigma, catalogue # 44122). Spinal cords were harvested at appropriate levels and processed for immunohistochemistry: at L6/S1 segment for intra-vesical capsaicin and L4/5 for hindpaw capsaicin. The dissected cords were post-fixed overnight in the same fixative and then washed in 0.1 M phosphate buffered saline (PBS) and stored in 0.1M PBS containing 1% azide until cryosectioning. Spinal cord were cryoprotected in PBS containing 30% sucrose overnight before sectioning and embedded in an inert mounting medium (OCT Tissue Tek, Sakura, Torrence, CA) prior to sectioning. Transverse sections (40µm) were collected in a 1:4 series and processed for immunohistochemistry.
with the free floating method (Peddie and Keast 2011). Sections were incubated in a blocking solution containing 10% normal horse serum (NHS) and 0.5% triton X-100 for two hours, followed by primary antisera for 48 hours at room temperature on a shaker. For L6/S1 spinal segments, the following antibody combinations were used: NK1R (1:10000, host rabbit, Sigma catalogue # S8305) with ChAT (1:500, host goat, Millipore catalogue # AB144P), NOS (1:5000, host sheep, gift from Piers Emson, Babraham, UK), CGRP (1:2000, host goat, Biogenesis catalogue # 1720-9007) and SP (1:800, host rat, Seralab catalogue # MAS035). Working dilutions of antibodies were made in PBS containing 2% NHS, 0.5% triton X-100 and 0.1% sodium azide. At the end of the 48 hour incubation period, unbound antibody was washed off (3 x 10 minutes in PBS) before incubation in host specific secondary antibodies: donkey anti-rabbit Cy3 (1:1500 Jackson catalogue # 711-165-152), donkey anti sheep AF488 (1:1000 Invitrogen, catalogue # A11015) and donkey anti rat FITC (1:400 Jackson, catalogue # 712-096-150) for 4 hours. The sections were incubated at room temperature on a shaker, protected from light. Sections were mounted on 0.1% gelatinised slides and cover slipped in Vecta shield mounting medium (Vector laboratories Inc. Burlingame, CA, catalogue # H-1000). Sections of L4/5 spinal cord were processed same as above and stained with NK1, SP and CGRP antisera (see above for antisera detail).
2.2.3 Antibodies:

**Neurokinin 1 receptor antibody**: The NK1 receptor (NK1R) antibody was raised in rabbit against a synthetic peptide corresponding to amino acids 393-407 at the C terminus of the rat NK1R. Whole antiserum was purified to provide an IgG fraction of antiserum. We found a similar pattern of NK1 immunoreactivity as previously seen (Al-Khater et al. 2008)

**Calcitonin gene-related peptide**: The CGRP antiserum was raised in goat against synthetic rat Tyr-CGRP (23-27) globulin and is specific for the whole molecule (1-37) and 23-37 fragments. The immunoreactivity pattern to CGRP was similar as demonstrated in other published works (Alvarez et al. 2004).

**Substance P**: The substance P antibody was raised in rat and the immunoreactivity pattern was similar to other published works (Timmermans et al. 1990).

**Choline acetyltransferase (ChAT)**: ChAT antiserum (immunoaffinity purified polyclonal) was raised in goat against the human placental enzyme. The immunoreactivity pattern was similar as demonstrated in previously published work (Peddie and Keast 2011)

2.2.4 Fluorescence microscopy and neuronal counts

An Olympus BX51 fluorescence microscope was used to visualise expression of NK1R in autonomic preganglionic neurons of the IML. The proportion of preganglionic
neurons also positive for NK1R was manually quantified. Preganglionic neurons were
identified by staining for the enzyme choline acetyltransferase (ChAT) that joins acetyl-
CoA to choline, which results in formation of the neurotransmitter acetylcholine that
characterises preganglionic parasympathetic neurons of IML. Neurons were also
identified by staining for nitric oxide synthase, an enzyme that catalyzes production of
nitric oxide (NOS, a cell-signaling molecule). A minimum of 50 IML neurons were
counted under a 40x objective in randomly selected 40µm sections of the sacral spinal
cord. The ChAT neurons that co-stained for NK1R in the IML were counted as positive
neurons and the number was expressed as a percentage. Only neurons that were
sectioned through the centre with a visible nucleus were included in the count to avoid
double counting of the cells.

2.2.5 Confocal microscopy and endosomal analysis

A Leica SP5 confocal microscope was used to acquire images, using argon and krypton
lasers with excitation wavelengths of 488nm and 561nm respectively and using an
objective of 63 x. Neurons were chosen at random for imaging in the dorsal horn lamina
I and outer II. Only neurons that showed immunoreactivity for NK1 were imaged. In the
IML only neurons that showed immunoreactivity for both ChAT and NK1 were imaged
to ensure that endosomes were counted specifically in the cholinergic autonomic
neurons. For each image, stacks of optical sections were obtained at an interval of
0.5µm. Images were acquired at a size of 2048x 2048 pixels. Each image was averaged
twice (frame average) to reduce noise and a zoom factor of two was used to improve
pixel resolution of the image. Images were viewed using Bitplane Imaris 6.21 software.
Early endosomes were manually counted in alternate optical sections of the z-stack to
avoid double counts. As per previously established criteria (Hughes et al. 2007), endosomes were identified as intensely stained intracellular organelles of 0.2-0.8µm in diameter and not in contact with the plasma membrane or the nucleus. Approximately 30 neurons were counted from each treatment group for endosome quantification. The endosomes were only counted in the soma of the dorsal horn and IML neurons. Endosomes in the dendrites were excluded from all quantifications (See discussion for more comments).

2.2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0. Results are given as Mean ± SEM. A paired Student’s t-test was used to test the effect of capsaicin injection in the hindpaw. An unpaired Student’s t-test was used on samples to test the effect of capsaicin treatment on NK1R in preganglionic autonomic neurons (proportion of NK1 positive neurons). An unpaired student’s t-test was also used to test the difference in internalisation of endosomes between the intravesical saline and capsaicin treatment groups.
2.3 Results:

2.3.1 Neurokinin 1 receptor and CGRP immunoreactivity in sacral spinal cord

Substance P is released from the peptidergic fibres that terminate in laminae I and II (outer) of the dorsal horn and the majority of these fibres also contain CGRP (Todd 2002). We labelled transverse sections of the lumbar and sacral cord with CGRP and NK1R antisera. We visualised the CGRP immunoreactivity in relationship to the NK1R expressing preganglionic neurons in the IML region. Most of the CGRP and NK1R staining pattern was similar between the lumbar and sacral segments. However some CGRP and NK1R expression was unique to the sacral cord as described below.

NK1R immunoreactivity was observed in neuronal somata in the lumbar cord superficial dorsal horn (medial and lateral) and deeper in laminae III and IV (Fig. 2.1 a). The area around the central canal (CC) and the dorsal commissure (DCM) also contained cell bodies and dendrites labelled with the NK1R. Within the lumbar cord, the most intense and prominent staining of the CGRP was observed in the superficial medial and lateral dorsal horn (Fig. 2.1 b). Some CGRP fibres were seen in the DCM, but very few CGRP positive fibres could be seen in the deeper laminae. The NK1R- and CGRP- immunoreactive structures showed a close relationship only in the superficial dorsal horn (Fig. 2.1 c ).

NK1R immunoreactivity has not previously been studied in detail in the sacral cord. As in the lumbar cord, in the sacral spinal cord the NK1R immunoreactivity was present in the superficial dorsal horn. NK1R positive cell bodies and dendrites were seen in the
medial and lateral dorsal horn and also in the deeper laminae (Fig. 2.1 d). Intense immunoreactivity was also observed in the DCM and in the area around the central canal. From the lateral edge of the dorsal horn, the NK1R immunoreactivity was observed to extend ventrally from Lissauer’s tract. Along the lateral collateral pathway (LCP) of Lissauer’s tract, the NK1R positive fibres reached an area known as the sacral parasympathetic nucleus.
Figure 2.1. Low power (10x) confocal images of CGRP and NK1R immunoreactivity (IR) in the lumbar and sacral spinal cord. Panels a-c show L5 and d-f L6 spinal segments. a: In the lumbar cord, NKIR-IR is seen in superficial dorsal horn in laminae I-II, in deeper laminae, around the central canal (CC) in lamina X and in the dorsal commissure (DCM). b: CGRP in the L5 segment is distributed primarily in the superficial laminae,(I-II) of the DH. c: Merged images of NK1R and CGRP expression in the lumbar spinal cord. d: NK1R-IR is present in superficial lamina I-II, in deeper laminae, area around the CC (lamina X). NK1 immunostaining also extends laterally to the lateral collateral pathway (LCP) and is distributed in the IML. e: CGRP-IR in L6: CGRP fibers in sacral cord are primarily distributed in the superficial dorsal horn in lamina I-II, extending from Lissauer’s tract in lamina I to the lateral collateral pathway of the Lissauer (LCP) and to the region of parasympathetic nucleus (IML). f: Partial co-expression of NK1 and CGRP in the sacral cord. Scale bar represents 150µm and applied to all panels.
This area of the sacral spinal cord contains interneurons, projection neurons and an intermediolateral column of neurons (IML) that contains cell bodies of parasympathetic preganglionic autonomic neurons. The IML is not present in the lumbar enlargement. NK1R-positive neurons and fibres were present in the IML region of the sacral cord. The NK1R-positive dendrites were seen to travel from the IML medially towards the central canal. It was noticed that the CGRP positive fibres showed a pattern of staining similar to NK1R immunoreactivity in the superficial dorsal horn. Intense CGRP immunostaining was observed in the superficial dorsal horn in both medial and lateral aspects (Fig. 2.1 e). CGRP fibres also travelled in the ventrolateral direction along the lateral collateral pathway and terminated in the IML region. The merged image of NK1R and CGRP in Figure 2.1 shows the close relationship between immunoreactivity of both markers. This is an important anatomical finding because it shows that the CGRP positive peptidergic fibres project to two regions of the sacral cord, the dorsal horn and the IML. The NK1R expression by IML neurons, together with the peptidergic axons in the IML indicates that IML is a second potential target of peptidergic nociceptors in the sacral cord.

To verify the peptidergic targets in the IML region we co-stained NK1R with SP or CGRP. Figure 2.2 shows the immunohistochemistry of SP and CGRP in relationship to the NK1R-positive IML neurons. SP staining was done to determine if there is a local source of SP to activate the NK1R-positive neurons in the IML. SP immunoreactivity was seen in the IML region (Fig. 2.2 a), where SP-positive axons and boutons could be seen in close proximity to the NK1R positive neurons (Fig. 2.2 b). The close relationship between immunoreactivity of SP and NK1R-positive neurons in the IML (Fig. 2.2 c) revealed that the preganglionic neurons are a potential functional target of
these peptidergic fibres. However, in the sacral spinal cord, peptidergic fibres are not the only source of SP. It is also released by the descending projections from supraspinal neurons (Hokfelt et al. 2001), hence immunoreactivity for SP alone in the IML does not distinguish the source of SP. On the other hand, CGRP is present only in sensory fibres (Keast and De Groat 1992), and can be used to identify the peptidergic afferents. CGRP immunoreactivity was seen (Fig. 2.2 d) in close relationship to the NK1R-positive neurons (Fig. 2.2 e) in the IML region. CGRP-positive axons and boutons were observed in close proximity to the NK1R-positive neurons (Fig. 2.2 f).
Figure 2.2. Representative images of the IML region in L6 showing the relationship between NK1R-IR and neuropeptide (CGRP and SP) IR. a & b represent images of NK1R positive neurons in the IML. Intensely labeled plasma membrane of NK1R positive cell bodies can be seen in the IML. Some of the neuronal processes (potentially axons or dendrites can also be seen passing through the IML. c: CGRP fibres can be seen extending into the region of the IML. CGRP positive boutons and dendrites are located near and around the NK1R positive neurons. d: SP releasing fibres can also be seen terminating in the IML. Although we did not quantify the number of SP fibres in this area, it appeared in some sections that there was a smaller number of SP fibres in the IML compared to the CGRP fibres. e-f Co-staining of CGRP and SP fibres with the NK1R respectively. Scale bar represents 20 µm and applies to all panels.
2.3.2 Neurokinin 1 receptor expression in parasympathetic preganglionic neurons

In the IML at the sacral spinal cord level, NK1R was co-expressed with both ChAT and the NOS immunoreactive populations of preganglionic neurons (Fig. 2.3 a-f). ChAT is an enzyme that catalyses the reaction to combine Acetyl-CoA with choline to synthesise acetylcholine. In the spinal cord, two major types of cells have been reported to use acetylcholine as their neurotransmitter. These are the preganglionic autonomic neurons and the somatic motor neurons (Barber et al. 1984). The preganglionic neurons of the sacral cord IML and motor neurons of the ventral horn showed immunoreactivity for ChAT, consistent with the previous findings (Fig. 2.3 b). Figures 2.3 g-i show high magnification confocal images of these neurons. We looked at the co-expression of ChAT with NK1R and found that many of the preganglionic neurons express both NK1R (Fig. 2.3g) and ChAT (Fig. 2.3 h). The co-expression of both these markers can be seen in the merged image (Fig. 2.3 i). The presence of receptor for substance P on ChAT positive neurons suggests that these neurons are a functional target of the substance P releasing peptidergic fibres.

Nitric oxide synthase (NOS) is an enzyme that catalyses the production of nitric oxide from L-arginine. Nitric oxide (NO) is a neurotransmitter in the central nervous system and is involved in the development of central sensitisation and synaptic plasticity after peripheral tissue damage or inflammation (Meller and Gebhart 1993). It is also involved in cyclophosphamide-induced acute and chronic cystitis (Souza-Fiho et al. 1997). The NOS positive terminals were seen in many regions of the sacral cord including lamina II of the dorsal horn, dorsal commissure and the area around the central canal (Fig. 2.3 d-f). The neurons expressing NOS were present around the central canal and in the IML region of the cord, consistent with the previously described immunolabeling of NOS in
Figure 2.3. NK1R, ChAT and NOS-IR in the sacral spinal cord. a-f: Low power (10x) confocal images of the L6 spinal cord showing NK1R-IR and its co-expression with choline acetyl-transferase (ChAT) and nitric oxide synthase (NOS) in many of the intermediolateral column (IML) neurons. a: NK1R positive neurons of the IML. b: ChAT positive preganglionic neurons in the IML. c: The co-expression of NK1R in ChAT neurons can be seen in the IML. d: NK1R positive neurons in the IML. e: NOS positive neurons in the IML. f: The co-expression of NK1R in NOS neurons is also seen in the IML. Arrowheads (a-f) point to the IML region. g-l: High power (40x), confocal images of the co-expression of NK1, ChAT and NOS in neurons of the IML. g: NK1R positive neurons of the IML region, NK1R is expressed on the plasma membrane of the neurons. h: Neurons showing ChAT immunoreactivity, ChAT is present in the cytosol of the neurons. i: At the cellular level, NK1R is partially co-expressed with the preganglionic neuronal marker ChAT. j: NK1R expression in the IML neurons. k: Neurons showing NOS immunoreactivity; NOS is present in the cytosol of the neurons. l: At the cellular level, NK1R is also partially co-expressed with the preganglionic neuronal marker NOS. The arrowheads in (i) and (l) represent ChAT and NOS neurons that express the NK1R. Not all of the IML preganglionic neurons express NK1R: broken arrows in (i) & (l). There are also some NK1 positive neurons in the IML that do not co-express ChAT or NOS and likely represent the local interneuron population. Scale bar in panel a represents 150µm (applies to a-f) and in panel g represents 10µm (applies to g-l).
the sacral cord (Vizzard et al. 1996). We next looked at the co-expression of NK1R with NOS in the preganglionic neurons. Figure 2.3 (j-l) shows high power confocal images of NK1R (j) and NOS (k) and co-expression (l) of both markers. Hence the qualitative analysis shows that the NK1R is co-expressed with both the preganglionic neuronal markers, ChAT and NOS. In the preganglionic neurons, ChAT and NOS immunolabeling were primarily confined to the soma. On the other hand, NK1R immunoreactivity was observed along the length of neuronal processes. It was generally not possible to determine which of these were axons or dendrites. A small number of NK1R-positive neurons were also seen (Fig. 2.3 i and l) that do not express ChAT or NOS. These are likely to be local interneurons. In summary, these immunohistochemical analyses confirmed the presence of receptor for substance P on the autonomic neurons.

We next made quantitative analysis to identify what proportion of these neurons express the NK1R. For this analysis, a minimum of 50 IML neurons were counted from each animal for each population. Neuronal counts in control (saline treated) animals showed a similar expression of NK1R in both populations, with approximately 80% of the neurons expressing the NK1R (78.7 ± 3.3, ChAT and 77.3 ± 2.8, NOS) in the IML. We next investigated if these proportions of receptor expression alter in either population of neurons after activation of capsaicin sensitive afferents using the TRPV1 agonist. Capsaicin is the active ingredient in chili peppers and is known to act as an agonist of the TRPV1 receptor commonly expressed by nociceptive afferents (Sun et al. 2010). Capsaicin was administered in the hindpaw of one group of animals to first reproduce the published studies of NK1R internalisation in somatosensory inflammation. As compared to the group treated with saline, animals treated with capsaicin showed no
significant difference in NK1R expression in ChAT (83.2 ± 2.2%) and NOS (69.7 ± 5.1%) neurons. The quantitative analysis confirmed that a majority of preganglionic neurons express the receptor for substance P. This suggests that these neurons may be a functional target of the bladder projecting peptidergic afferents.

2.3.3 NK1 receptor internalisation after noxious stimulation of the nociceptive afferents using a TRPV1 agonist

Capsaicin was also administered intravesically to stimulate the TRPV1 expressing population of nociceptive bladder afferents (visceral noxious stimulation). The activation of sacral spinal cord neurons was investigated in response to the capsaicin treatment. NK1R internalisation was used to identify neurons that were activated in response to peripheral noxious stimulation. The number of endosomes was counted in z-stacks of the imaged sections (see 2.2.5). The group of animals injected with capsaicin in the hindpaw showed NK1R internalisation (Fig. 2.4 a & b) in neurons only on the ipsilateral to the side of the injection (P < 0.05, Fig. 2.5 a & c). The group of animals treated with capsaicin (intravesical) also showed a significant increase in the number of NK1R endosomes/neuron (Fig. 2.4 c-f) in the dorsal horn compared to the saline treated group (9.34± 1.05 capsaicin, 0.37±0.02 saline; P < 0.01, Fig. 2.5 b & d), although this was a much smaller response than seen with the hindpaw injection. Endosomes were also counted in the ChAT/NK1R positive neurons of the IML. Again there was a small but significant increase in the number of endosomes/neurons in capsaicin treated compared to saline treated group (8.68±0.54 capsaicin, 0.82±0.28 saline, P < 0.01). These results suggest that the capsaicin treatment causes an increase in substance P
Figure 2.4. High power (63x) confocal images of NK1R internalisation after TRPV1 activation using capsaicin. **a:** L5 dorsal horn neuron ipsilateral to the capsaicin treatment in the hindpaw, arrowheads point to internalised endosomes. **b:** DH neurons contralateral to the unilateral intraplantar injection of capsaicin do not show NK1R internalisation. **c:** NK1R-IR remains confined to the membrane in DH neurons in the saline treated group. **d:** Neurons in the IML also do not show NK1R internalisation after intravesical injection of saline. **e:** In groups with intravesical capsaicin treatment NK1 receptor was internalised in DH neurons. **f:** Internalised endosomes were also observed in the IML neurons after capsaicin treatment. Arrowheads in **e** and **f** point to the internalised NK1R in the form of endosomes. Scale bar represents 15µm and apply to all panels (n=4).
Figure 2.5 Effect of noxious stimulation on somatic and visceral afferents using TRPV1 agonist (capsaicin). a: Effect of peripheral nociceptive stimulation (TRPV1 activation) of afferent terminals on NK1R internalisation after intraplantar injection of capsaicin. Paired Student’s t test was used to determine if there was a difference in mean number of endosomes between ipsilateral and contralateral groups of neurons. Capsaicin injection in the hindpaw produced significant NK1R internalization in the superficial dorsal horn neurons ipsilateral to the injection, (* P < 0.05). NK1R internalisation was not observed in neurons contralateral to capsaicin treatment. Data are expressed as mean number of endosomes ± SEM.

b: Effect of peripheral noxious stimulation (TRPV1 activation) of bladder afferent terminals on NK1R internalisation after intravesical injection of capsaicin. An unpaired Student’s t test was used to determine statistical significance between saline and capsaicin treated groups. No. of endosomes per neurons significantly increases in DH and IML neurons after capsaicin treatment compared to the saline group,(*** P < 0.01). Data are expressed as mean no of endosomes ± SEM.

c & d: Scatter plots show pooled data from ~ 30 neurons from each animal after the intraplantar and intravesical treatment.
release from the peptidergic bladder nociceptive afferents in both locations in the sacral cord. The increased levels of neuropeptide release causes more binding of substance P to its receptor. As a result, the receptor internalises and is seen in the form of the small endosomes in the cytoplasm of the neurons.

The scatter plots for each experiment show individual neurons plotted against the number of internalised endosomes. It can be seen that there was a large proportion of neurons in the saline group that did not have any endosomes (Fig. 2.5 c & d). However, in each of the treatment groups only some of the neurons showed minimal or no endosome internalization. That is, for each treatment, most of the dorsal horn and IML neurons appeared to be activated by SP, while some neurons could be considered “non-responders” (see 2.4).

The estrous cycle of each animal was monitored but not controlled for. For the intravesical capsaicin experiments, the stage of the estrous cycle for each animal is indicated in Figure 2.6 where each number refers to an individual animal in each treatment group plotted in the order of their appearance on the scatter plot. Figure 2.6 shows the individual internalisation response of each animal in saline and capsaicin treatment groups. The stage of the estrous cycle can be a confounding factor in measurements of nociceptive response to noxious bladder stimulation in female rats, however to specifically test if this was impacting on our analysis would require a much larger and differently designed study. We next analysed if a particular stage of the estrous cycle affected the NK1R internalisation response. However, in the saline treated groups, the majority of the neurons did not show internalisation. This response is very similar in all the animals. The number of non-responding neurons changed after the
capsaicin treatment but the distribution of number of endosomes per neuron in the saline and capsaicin treated groups looked very similar despite animals being in a different stage of the estrous cycle (Fig. 2.6 a & b). All animals in the capsaicin group had DH neurons with more 30 internalised endosomes, however this response was not exclusive to animals in a particular stage. Similarly some neurons in the IML also showed a high level of NK1R internalisation after capsaicin treatment (> than 30 endosomes), but this response was also not confined to a particular stage of the estrous cycle.
Figure 2.6. The scatter plot shows the estrous cycle stage of each animal in saline and capsaicin treated groups. The estrous cycle of the animals was monitored but not controlled for. Raw data is presented for each animal. Numbers on the x-axis refer to individual animals in each treatment group. LPE = late proestrous, EP = early proestrous, 3 = DE = diestrous, E = estrous. There does not seem to be a correlation between the stage of the estrous cycle and the treatment in either DH (a) or IML neurons (b).
2.4 Discussion:

In this study we have used CGRP-immunoreactivity to identify visceral nociceptive afferents that terminate near the IML preganglionic neurons. The peptidergic afferent axons have been reported to project to the lumbosacral parasympathetic nucleus (de Groat 2006). A majority of the CGRP positive fibres also release SP. However peptidergic visceral afferents are not the only source of SP in the sacral spinal cord. The sacral cord also receives its SP input from descending fibres originating in the brain stem (Hokfelt et al. 2001), so CGRP was used to identify regions of the sacral cord where visceral nociceptive afferents terminate. The CGRP immunoreactivity in the dorsal horn is very similar to previously published reports (Vizzard 2001; Hwang et al. 2005). Our results also showed CGRP positive terminals in the IML region where CGRP boutons could be seen surrounding the preganglionic ChAT/NK1R positive neurons. This suggests that autonomic neurons innervating the bladder maybe directly innervated by the visceral nociceptive peptidergic afferents.

The cellular targets of visceral nociceptive afferent projections in the superficial dorsal horn and IML region of the sacral spinal cord have been confirmed. Many studies have been conducted that identify preganglionic parasympathetic neurons in the IML across different species. Electrophysiological and horseradish peroxidase studies have identified preganglionic parasympathetic neurons in sacral cord of the cat (De Groat and Ryall 1968; Nadelhaft et al. 1980). HRP retrograde tracing studies in rats also show that the preganglionic parasympathetic axons travelling in the pelvic nerve originate in the autonomic neurons of the sacral spinal cord IML region (Hancock and Peveto 1979; Nadelhaft and Booth 1984). Our study confirmed these findings and quantified the
NK1R expression in the IML region. This is the first study to quantify expression of NK1R in the intermediolateral column (IML) of preganglionic parasympathetic neurons. The expression of SP receptor in the IML preganglionic neurons could indicate that these neurons (potentially including those involved in the micturition reflex) are a target of SP releasing nociceptive bladder afferents. For the purpose of this study, this finding is a step towards using the noxious stimulation to observe cellular activation of NK1R expressing preganglionic neurons.

Preganglionic autonomic neurons were identified using immunohistochemical techniques against chemical markers such as ChAT and NOS. We found a similar pattern of ChAT staining in the sacral cord L6/S1 segments as described previously (Barber et al. 1984; Hinrichs and Llewellyn-Smith 2009). Neuronal counts revealed that NK1R is expressed by the majority (~80%) of sacral preganglionic neurons. Substance P binds to the NK1R after it is released in response to noxious stimulation (Abbadie et al. 1996; Abbadie et al. 1997; Marvizon et al. 1997). As expected, our results showed that the number of neurons expressing NK1R in the IML preganglionic neurons is unaffected by acute capsaicin treatment to cause noxious stimulation of bladder nociceptive afferents. The results also highlighted that NK1R phenotype in both populations is the same, hence allowing for flexibility in using the antisera alternatively for further investigations in the future. Further analysis of internalisation was only conducted in neurons that co-express NK1R with ChAT. This co-expression is a reliable indicator that the quantification only included preganglionic autonomic neurons (ChAT positive) and not local interneurons.
In all groups treated with capsaicin, animals were perfused 10 minutes after the intravesical administration of stimulus. This time course has previously been shown to be sufficient for maximum internalisation of endosomes in dorsal horn neurons (Mantyh et al. 1995; Wang and Marvizon 2002; Huang et al. 2011).

The NK1R immunoreactivity and internalisation assay has been widely used in research involving various somatic and some visceral pain states (see Table 1.1). One such example is use of Complete Freund’s Adjuvant (chemical noxious stimulus) to induce inflammation in the hindpaw that results in an increase in the NK1R immunoreactivity in the lumbosacral cord (Abbadie, Brown et al. 1996). Capsaicin induced stimulation of spinal cord slices has been known to result in NK1R internalisation in the lumbar cord (Marvizon et al. 2003). Similarly colonic distension has been used as a visceral inflammation model to study NK1R internalisation (Honore et al. 2002). Our results further demonstrated the sensitivity and reliability of the NK1R internalisation assay in identifying cellular activation in a visceral inflammation model.

We used unilateral capsaicin injection in the hindpaw to confirm previous findings of activation of dorsal horn neurons in the lumbar cord in response to acute noxious stimulation. This is consistent with the findings of Mantyh (Mantyh et al. 1995) and Marvizon (Marvizon et al. 1997) who found NK1R to internalise in response to intraplantar capsaicin injection. Capsaicin was then used to activate the peripheral nociceptive bladder afferent endings of TRPV1 expressing fibres. Our results showed a small but significant increase in the NK1R internalisation in the superficial dorsal horn neurons after capsaicin-induced stimulation of bladder nociceptive afferent terminals. The internalisation response after visceral noxious stimulation was smaller than the somatosensory stimulation. However activation of neurons after visceral noxious
stimulation was consistently observed in all animals treated with capsaicin. There was very little or no response in the saline treated group. Hence we conclude that the NK1R internalisation observed in the treated group is induced by noxious stimulation.

The increase in internalisation was also observed in IML preganglionic neurons that express the NK1R. The expression of NK1R by ~80% of ChAT expressing autonomic neurons suggests that these neurons are a target of the peptidergic afferents. It is also possible that the autonomic neurons are a site of termination of these peptidergic afferents. Although we have not quantified individual substance P fibres terminating on or near the IML autonomic neurons, Vizzard (Vizzard 2001) has shown immunoreactivity of substance P in the sacral cord IML. Some neurotransmitters in the nervous system act at a distance well beyond their site of release (from a cell or synapse). This is a phenomenon known as volume transmission. It allows for diffusion of neurotransmitter over a distance larger than a synaptic cleft (Agnati et al. 1995). It is possible that substance P released from afferents terminating at a distance from the autonomic neurons is diffused through volume transmission to the IML neurons.

NK1R internalisation may have occurred in the proximal and distal dendrites as well, but only endosomes in the soma were quantified. In a histological section, all the dendrites cannot be traced to their cell bodies and hence any endosomes seen in the dendrites were excluded from quantification to maintain consistency in the analysis. This technical limitation may have led us to under-estimate the extent of NK1R internalisation in response to noxious stimulation that may be higher (in-vivo) than reflected by our counts. We only sampled every second section to quantify endosomes (to avoid double-counting) and this would also have led to under-estimation of the total
number. Therefore our results should be viewed as demonstrating relative rather than absolute numbers between treatment groups. The difference in NK1R internalisation response between the somatic and visceral noxious stimulation can be due to a number of reasons. It is possible that there is a difference in the amount of SP released from the visceral afferents compared to the somatic afferents. As mentioned in Section 1.1.2, although extensively arborized, the viscera are sparsely innervated compared to a large number of somatic afferents that innervate the skin, muscles and joints. The large number of somatic afferents innervating the hindpaw may account for a higher level of SP release, hence resulting in a bigger NK1R internalisation response after hindpaw capsaicin treatment. Capsaicin was dissolved in ethanol and administered intravesically and saline was used for the control group. A true vehicle control should have been used comprised of animals receiving intravesical ethanol injections. However due to time limitation, we could not perform an additional set of experiment using ethanol as a vehicle control group. Nevertheless, ethanol has previously been used as a vehicle control in other visceral inflammation studies such as colitis with no significant damage to the colon tissue (Rojas-Cartagena et al. 2005).

The NK1R internalisation was observed in many but not all of the dorsal horn and IML neurons. This is because not all neurons in the dorsal horn receive inputs from the bladder afferents and may be innervated by somatic afferents or afferents from other pelvic viscera. It is also known that only a small proportion of dorsal root ganglion neurons in the lumbosacral cord will project to the bladder (Robinson and Gebhart 2008). Due to the convergence of visceral and somatic afferents in the dorsal horn (see 1.1.2), it is possible that some of the neurons activated by intravesical capsaicin also receive somatic afferent inputs. There is also a chance that the random sampling of
cells did not include a majority of neurons that are innervated by TRPV1 expressing afferents. Similarly not all preganglionic IML neurons are involved in the bladder function and instead are responsible for mediating reflexes of the lower bowel or the reproductive organs. Therefore we expected only a minority of neurons to respond to intravesical capsaicin treatment. Our study did not show a relationship between the levels of internalisation in an animal to its particular stage of the cycle, a more extensive study would be required to determine this. Our estrous cycle data suggests that estrous cycle stages of the female rats were not a confounding factor in nociceptive response to a 10-minute intravesical noxious stimulation.

Previously studies have used immediate early gene “c fos” expression to identify regions of the spinal cord that are activated in response to electrical stimulation of the ureter in rats (Matsumoto et al. 1996). Studies have also shown c fos expression induced by bladder stimulation after spinal cord injury (Vizzard 2000) and following cystitis (Vizzard 2000). These studies have mapped the regions of the spinal cord that are activated in response to bladder stimulation. However, my study is the first to identify individual neurons in the sacral spinal cord (DH and IML) that are activated in response to noxious stimulation of peptidergic bladder projecting afferents. My study has also validated the use of NK1R internalisation assay in identifying neurons in a visceral model of noxious stimulation. This opens up a window of opportunity to use the assay to study physiology and plasticity of individual neurons within bladder regulatory pathways in bladder inflammation. This assay also makes it possible to further characterise the chemistry and projections of the activated neurons.
Chapter 3: Effects of bladder inflammation on nociceptive and micturition reflex pathways.

3.1 Introduction

The main purpose of this study was to understand plasticity of sacral spinal circuits in inflammation. The first part of the study has validated the use of NK1R internalisation assay in identifying neurons that are activated in response to noxious stimulation of peripheral nociceptive afferent terminals. We showed that the internalisation assay could be reliably applied to a visceral noxious stimulation model. Hence we used this assay to address the next set of aims. 1) We wanted to determine whether intravesical activation of TRPV1 and TRPA1 activate similar populations of spinal neurons. To address this aim we used a TRPA1 receptor agonist mustard oil, to induce noxious stimulation of the bladder afferents. TRPA1 is expressed in a subpopulation of TRPV1 expressing afferents ([Streng et al. 2008; Sun et al. 2010]. We wished to investigate if activation of TRPA1 channels will result in response in a similar population as TRPV1 activation. We also wished to investigate if spinal neurons in both nociceptive and autonomic pathways will again be activated with mustard oil induced noxious stimulation. 2) To define the level of basal activation of peptidergic bladder afferent neurons after acute and chronic bladder inflammation. This was achieved by establishing acute and chronic cyclophosphamide (CYP) induced bladder inflammation. We wished to determine if acute and chronic inflammation could stimulate the NK1R in the sacral cord. We wished to investigate if this activation of NK1R will occur both in the nociceptive (DH) and autonomic (IML) neurons. Finally we compared the response of levels of neuronal activation between acute and chronic
inflammation models. Effects of intravesical MO administration in animals with chronic inflammation were also studied. 3) **We wanted to determine if bladder voiding (continuous cystometry) activates NK1R internalisation in dorsal horn and IML neurons and if this internalisation is influenced by prior bladder inflammation.** We used cystometry to record bladder activity during normal voiding behavior. This was compared to cystometric recordings in animals with acute and chronic CYP induced bladder inflammation. NK1R internalisation was quantified in control and inflammation groups after cystometric recordings to identify any increase in levels of neuronal activation due to inflammation during normal voiding behavior.

### 3.2 Methods:

All procedures were performed under ketamine (80mg/ml) and xylazine (60mg/ml) anaesthesia administered intraperitoneally. In a group of animals (n=5), mustard oil (100µl of 2.5%MO, 7.5% DMSO in saline, intravesically) was injected using an insulin syringe with a 30G needle. A lower midline abdominal incision was made to expose the urinary bladder. The bladder was emptied manually by applying gentle pressure before injection of mustard oil. Animals were perfused intracardially 10 minutes after the injection. A control group of animals (n=3) were injected with vehicle (100µl, 7.5% DMSO in saline) and perfused intracardially 10 minutes after the injection.
3.2.1 Cyclophosphamide (CYP) induced cystitis

An animal model of inflammation was established by using cyclophosphamide (CYP) to induce inflammation of the lower urinary tract (Vizzard 2001). All injections of CYP were performed under isoflurane (3% in O2) anaesthesia. For acute inflammation, CYP (75mg/kg in sterile 0.9% saline i.p.) was injected and animals were kept for 48 hours before spinal cords were harvested (see 2.2.2 for perfusion details). For chronic inflammation animals were injected every third day for ten days (n=6). Animals receiving injections of sterile saline were used as controls in these experiments. In a separate group of animals, acute and chronic inflammation was induced as described above before cystometry was performed to assess bladder function. All animals were intracardially perfused after treatment to harvest spinal cords for NK1R immunoreactivity in the sacral dorsal horn and IML neurons. In a third group of animals with chronic inflammation, mustard oil was intravesically administered before animals were intracardially perfused.

3.2.2 Cystometry

Animals were deeply anaesthetised with urethane (1.2g/kg of 30% urethane solution) before a lower midline abdominal incision was made to expose the urinary bladder. A catheter was inserted in the dome of the bladder and connected via a three-way stopcock to a pressure transducer for recording intravesical pressure and a syringe pump for saline infusion in the bladder. Saline (room temperature) was infused at a rate of 40µl/min. Rhythmic contractions of the bladder were allowed to stabilise for atleast 30 minutes before a recording of 90-120 minutes was made for analysis. The parameters
measured include intercontractile interval, peak pressure, voiding pressure threshold, and baseline pressure after micturition. After cystometric recordings animals were intracardially perfused with fixative and spinal cords were harvested and processed for immunohistochemistry.

Please see chapter 2 methods, sections “2.2.2” and “2.2.4” for details of antibodies used, tissue processing and analysis criteria for endosomal counts.

3.2.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0. Results are given as Mean ± SEM. A parametric test (unpaired Student’s t-test) was used to test the effect of MO treatment on NK1R expression in preganglionic autonomic neurons (proportion of NK1R-positive neurons). A one-way analysis of variance was used to determine any statistically significant differences between saline, acute and chronically CYP treated groups followed by a post hoc test (Bonferroni). For all statistical tests, a $P$-value of less than 0.05 was considered significant.
3.3 Results:

3.3.1 Acute noxious stimulation of TRPA1 bladder afferents

Mustard oil (MO) is a known ligand of the TRPA1 channel (Story et al. 2003). TRPA1 is expressed on a subpopulation of TRPV1 expressing sensory neurons (Bautista et al. 2006). In this study, we used MO to activate the TRPA1 expressing bladder afferents to determine if TRPA1 and TRPV1 expressing afferents activate similar populations of neurons in the sacral cord. We quantified the number of endosomes in both the dorsal horn and IML neurons.

As seen in Chapter 2, (section 2.3.3), neurons in both DH and IML show NK1R internalisation in response to TRPV1 activation (Fig. 3.1 a). On the other hand, we found that noxious stimulation of bladder afferents by MO produced different effects in the dorsal horn and IML neurons. Neurons in the dorsal horn did not show a significant increase in levels of NK1R internalisation compared to the vehicle control group (Fig. 3.1 b). On the other hand, a significant increase in the number of endosomes was observed in preganglionic neurons of the IML. Neurons in the IML showed NK1R internalisation (6.8 ± 1.3 MO, 1.82 ± 0.3 DMSO; $P < 0.05$, Fig. 3.1 b) similar to that seen in the capsaicin treated group (~ 9 endosomes/neuron).

The NK1R internalisation response after TRPA1 activation was different to TRPV1 activation. Neurons in both the nociceptive (dorsal horn) and autonomic (IML) spinal regions were activated in response to TRPV1 activation. The pooled data of number of endosomes in DH and IML neurons was plotted for each treatment. We found that as seen earlier after TRPV1 activation (Fig. 3.1 c), there were neurons both in the dorsal horn and IML regions that did not show NK1R internalisation after MO treatment (Fig. 3.1 d). This is likely because not all the neurons in the dorsal horn and IML project to
the bladder or are innervated by afferents expressing the appropriate TRP, hence it is expected that not all the neurons will show NK1R internalisation in response to a particular bladder stimulus.
Figure 3.1. Effect of TRPV1 (red, n=4) and TRPA1 (blue, n=5) activation on NK1R internalisation. a & b: Data are expressed as mean no. of endosomes ± SEM. An unpaired Student’s t test was used to determine statistical significance between vehicle and TRP agonist treatment groups. a: A significant increase in number of endosomes was observed in both the dorsal horn and IML neurons in the capsaicin treated group, (*** P < 0.01) b: Similar to TRPV1 activation, there was a significant increase in NK1R internalisation in IML but not dorsal horn neurons after MO treatment, (* P < 0.05). c & d: represent pooled data from ~ 30 neurons from each animal after capsaicin and MO treatment respectively.
3.3.2 NK1R expression in preganglionic neurons after CYP induced cystitis

CYP was used to elicit acute and chronic inflammation of the bladder. We investigated if the proportion of NK1R expression in autonomic neurons changes after bladder inflammation. In the control group (acute saline treatment) 70.0 ± 2.2% of ChAT and 71.5 ± 1.6% of NOS neurons expressed NK1R. This proportion of NK1R expression did not significantly alter after acute bladder inflammation (72.8 ± 3.2% ChAT, 73.8 ±3.2% NOS). In the group of animals treated with saline over a ten day time period, the NK1R expression in the ChAT and NOS neurons was 80.7 ±1.1% and 65±1.9% respectively. In the chronically inflamed group of animals the expression in either of the population remained unaltered. (77.5 ±2.6% ChAT, 70 ± 2.9% NOS).

3.3.3 NK1R internalisation was significantly increased after acute and chronic inflammation of the bladder

Acute and chronic bladder inflammation was induced to analyse the activation of NK1R in the dorsal horn and IML neurons. Figures 3.2 a-d are examples of neurons in the dorsal horn and IML. The NK1R internalisation can be seen in the dorsal horn and IML neurons after the acute (Fig 3.2 a & b) and chronic bladder inflammation (Fig 3.2 e & d). The arrowheads in b, c and d indicate the internalised endosomes. In the acutely treated group the average number of endosomes/neuron were ~ 5 in DH neurons (0.4 ± 0.1 saline, 5.4 ± 0.1 CYP; P < 0.01, Fig. 3.2 e ) and 7 in the IML neurons (0.3 ± 0.1 saline, 6.9 ± 0.3 CYP; P < 0.01, Fig. 3.2 f). In the chronic group, an increase was observed in number of endosomes both in DH (0.4 ± 0.1saline, 5.2±1.3 CYP; P < 0.01 Fig. 3.2 e) and IML neurons (0.6 ± 0.1 saline, 8.6 ± 1.5 CYP; P < 0.001,Fig. 3.2 f).
There appeared to be a similar levels of internalisation in the in dorsal horn and autonomic neurons in animals with acute and chronic inflammation. Figures 3.2 g and h represent pooled data of number of endosomes in DH (g) and IML (h) neurons after acute and chronic bladder inflammation.
Figure 3.2. Effect of acute (orange, n=4) and chronic (red, n=6) cyclophosphamide treatment on NK1R internalization in sacral cord. a-d represent high power (63x) confocal images of NK1R positive neurons in dorsal horn and IML regions from CYP treated groups. a & b: acute CYP treatment, c & d: chronic CYP treatment. Arrowheads in a-d point to internalized endosomes. Scale bar represents 10µm and applied to a-d. One-way analysis of variance was used to test for statistical significance followed by Bonferroni’s multiple comparison test. e: The number of endosomes in the DH neurons significantly increased after cyclophosphamide induced acute and chronic inflammation compared to the saline controls (**) $P < 0.01$). f: NK1 internalisation was also significantly higher in the IML neurons after both acute, (**) $P < 0.01$) and chronic inflammation, (***) $P < 0.001$). Data are expressed as mean no of endosomes ± SEM. g & h represent pooled data from ~ 30 neurons in DH and IML respectively. Data is presented as raw counts from each animal in all treatment groups.
3.3.4 NK1R internalisation after MO administration in CYP treated animals

significantly increases in the IML neurons

As described above, after noxious stimulation of the TRPV1 and TRPA1 expressing bladder afferents, there was a significant number of endosomes in the IML neurons. However the TRPA1 stimulation did not result in a significant increase in the number of endosomes in the DH neurons. To further investigate the difference in neuronal activation in DH and IML neurons we established CYP induced chronic inflammation in a group of animals (see 3.2.1). On day 10 of the inflammation, animals were anaesthetised and mustard oil was injected in the bladder; animals were perfused 10 minutes after the injection. The number of endosomes quantified in the DH neurons in the group with chronic inflammation and intravesical mustard oil application was not statistically different to the numbers observed in animals with chronic inflammation alone or mustard oil only treatment. (Fig. 3.3 a). However the level of internalisation in the IML neurons was higher in the group CYP + MO group (13.5 ± 0.9; P < 0.05, Fig. 3.3 b) compared to the group with only mustard oil treatment (Fig.3.3 b). Figures 3c & d show the spread of the endosomes after each treatment in both dorsal horn (Fig. 3.3 c) and IML neurons (Fig. 3.3 d). In each treatment there was a small number of neurons that did not show internalisation.
Figure 3.3. Effect of mustard oil (MO) treatment on NK1R internalisation in animals with CYP induced chronic inflammation. One way analysis of variance was used to test for statistical significance followed by Bonferroni’s multiple comparison test. a: Number of endosomes in the DH neurons in the MO + CYP (n=4) treated group was not significantly different compared to group with MO (n=4) treatment or chronic inflammation (n=4). b: Significant increase in number of endosomes is observed in the IML neurons after the MO + CYP treated group compared to the mustard oil only treatment, (* P < 0.05). Data are expressed as mean no of endosomes ± SEM. c & d represent pooled data from ~ 30 neurons in DH and IML neurons, respectively. Data is presented as raw counts from each animal in all treatment groups.
3.3.5 Cystometry

Cystometry is a method that allows measurement of bladder function using continuous bladder infusion. Continuous infusion of the bladder results in activation of bladder sensory afferents that detect a change in intravesical pressure and trigger a voiding reflex. This change in various pressure parameters and frequency of voiding cycles can be measured from the cystometrograms. We have measured voiding frequency and changes in pressure parameters in control, acute and chronic inflammation groups. Figure 3.4a is an annotated trace of a typical cystometrogram recorded during continuous saline infusion. Threshold pressure (TP) refers to the intravesical pressure immediately before the voiding starts. Peak pressure (PP) refers to the maximum pressure during a voiding cycle. BP denotes the base line pressure that is the pressure at the end of each voiding cycle. The cystometrograms can also indicate the interval between each voiding cycle. This is known as the inter-contractile interval or ICI. A short ICI indicates increase in the voiding frequency (Andersson et al. 2011).

Table 3.1 shows the ICI and pressure parameters measured during cystometry conducted in saline, acute and chronic inflammation groups. During continuous saline infusion, the control group of animals underwent regular voiding cycles after every 3 minutes (Fig. 3.4 a, Table 3.1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ICI (min)</th>
<th>TP (mmHg)</th>
<th>PP (mmHg)</th>
<th>BP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.5±0.76</td>
<td>8.12±0.46</td>
<td>20.03±0.70</td>
<td>3.39±0.42</td>
</tr>
<tr>
<td>CYP acute (48hrs)</td>
<td>3.48±0.63</td>
<td>9.11±2.10</td>
<td>18.74±1.44</td>
<td>3.42±1.13</td>
</tr>
<tr>
<td>CYP chronic (10days)</td>
<td>2.92±0.52</td>
<td>10.75±2.02</td>
<td>22.96±2.65</td>
<td>5.02±0.76</td>
</tr>
</tbody>
</table>

Table 3.1 Cystometric measurements
There was no change in voiding frequency after acute inflammation compared to the control group (Fig 3.4 b). While in some CYP-treated animals there was a shorter ICI (e.g. Fig 3.4 c), when the group statistics were compared, there was no statistically significant effect of acute or chronic CYP on voiding frequency. We did not detect a significant change in threshold pressure or peak pressure in either of the inflammation groups. A small change in baseline pressure was detected in the chronic inflammation group indicating incomplete emptying of the bladder, however this was not found to be significantly different to the control group.

At the end of the cystometric recordings, each animal was intracardially perfused and processed for NK1R immunohistochemistry in the sacral cord. Figure 3.5 shows quantification of NK1R internalisation in the dorsal horn and IML neurons. Overall there was an increase in internalisation within neurons in the acute and chronic inflammation groups after physiological stimulation of bladder afferents. In the dorsal horn neurons from the chronic inflammation group, there was a significant increase in NK1R internalisation compared to the saline group. Surprisingly, in the acute inflammation group, the increase in number of endosomes/neuron did not reach statistical significance compared to the control group (Fig. 3.5 a & c). In the IML neurons, the NK1R internalisation was significantly increased compared to the control group but not to the same extent as observed in the earlier described chronic inflammation group (Fig. 3.5 b & d). For each group of neurons (dorsal horn and IML neurons), there was no difference in the levels of neuronal activation between the acute and chronic groups, just as observed earlier.
Figure 3.4. Representative cystometrograms during infusion of saline into bladders of urethane anaesthetised rats using an infusion rate of 40μl/ml. One way analysis of variance was used to test for statistical significance followed by Bonferroni’s multiple comparision test. a: represents recording from a control animal while b and c show recordings from CYP treated animals (acute and chronic respectively). TP = Threshold pressure, PP = Peak Pressure, ICI = Intercontractile Interval, BP = Baseline Pressure. The red bars in a represent the baseline pressure. There was no significant difference among the groups (n=4 for all treatment groups).
Figure 3.5. Effects of physiological stimulation on NK1R internalisation in control and acute (orange) and chronic (red) inflammation groups. One way analysis of variance was used to test for statistical significance followed by Bonferroni’s multiple comparison test. a & b: There was a significant increase in the number of endosomes in DH (a, * P < 0.05) and IML neurons (b, **P < 0.01) in the chronic inflammation group after continuous infusion of bladder with saline but not after acute inflammation. Data are expressed as mean no of endosomes ± SEM. c & d represent pooled data from ~30 neurons from each animal in all treatment groups and data is presented as raw counts (n=4 for all treatment groups).
3.3.6 Analysis of estrous cycle stages of female rats in different treatment groups

It is important to identify any changes seen in the experimental outcome in relation to the stage of the estrous cycle. Figure 3.6, 3.7 and 3.8 show the estrous cycle of animal used in different treatment groups. We plotted the pooled data of number of endosomes against the cycle stage of each animal to identify any trends in the cycle stage and correlate to the treatment group.

As mentioned earlier in (section 2.3.3), the estrous cycles of the animals were monitored but not controlled for. This means that the number of animals in each stage of the cycle is not the same and in most cases there are only small numbers of each animal in each stage of the cycle for a given treatment group. Therefore this does not give us the flexibility to perform statistical analysis on the data to test the effect of estrous cycle in each treatment group. However, the monitoring of the estrous cycle could allow us to identify any potential confounding effects of the estrogens on specific treatment groups. This is most likely if animals in a specific treatment group all belonged to one stage of the cycle and controls had been from another stage. Our results indicate that this was not the case as the animals in a treatment or control group did not all belong to a particular stage of the cycle. Hence the increase in NK1R internalisation was most likely to be an effect of the treatment and not confounded by the stage of the estrous cycle. Secondly the animals in a particular stage of the cycle did not always show the same response to treatment. For example after MO treatment (Fig. 3.6 b), two animals in the estrous stage of the cycle showed different numbers of neurons with a high number of endosomes. Similarly, animals that showed a very similar response (spread of the endosomes) to acute inflammation (animals 3 and 4 in acute CYP
treatment group, Fig. 3.7a) belonged to different stages of the cycle. Hence we conclude that our results are not significantly confounded by the stage of the estrous cycle.
Figure 3.6. Estrous cycle of animals in the MO and vehicle (DMSO) treated groups. a: dorsal horn, b: IML. Numbers on the x-axis refer to individual animals in each treatment group LPE=late proestrous, EPE= early proestrous, DE= diestrous, E= estrous.
Figure 3.7. Estrous cycle of animals in the saline, acute and chronic inflammation groups a: dorsal horn, CYP (acute), b: IML, CYP (acute), c: dorsal horn, CYP (chronic), d: IML, CYP (chronic). Numbers on the x-axis refer to individual animals in each treatment group LPE=late proestrous, EPE= early proestrous, DE= diestrous, E= estrous.
Figure 3.8. Estrous cycle of animals in the control, acute and chronic inflammation cystometry groups. **a**: dorsal horn, **b**: IML. Numbers on the x-axis refer to individual animals in each treatment group LPE=late proestrous, EPE= early proestrous, DE= diestrous, E= estrous.
3.4 Discussion:

3.4.1 Mustard oil induced activation of the TRPA1 channel

TRPA1 is expressed by a subset of TRPV1 expressing afferents (Story et al. 2003; Kobayashi et al. 2005; Nilius et al. 2007). This means that a proportion of TRPV1 expressing fibres do not express TRPA1 and are exclusively activated by capsaicin. TRPA1 immunoreactivity is found on unmyelinated nerve fibres throughout the bladder, in urothelium, suburothelium space, muscle layer and blood vessels (Streng et al. 2008; Skryma et al. 2011). Almost 50% of bladder projecting dorsal root ganglion neurons show TRPA1 immunoreactivity, co-localising with CGRP immunoreactivity, indicating that these are nociceptive neurons (Du et al. 2007). This abundance of TRPA1 in the bladder afferents and the urothelium implies that it may play an essential role in bladder sensory processing. In previous reports, MO application during cystometry has resulted in an increase in voiding frequency and a decrease in voided volume in rodents (Everaerts et al. 2011). The parasympathetic IML neurons are involved in the micturition reflex. Consistent with these studies, we found that the NK1R internalisation response elicited after intravesical mustard oil (MO) administration was significantly elevated in IML neurons.

As mentioned earlier, TRPA1 expression is expressed to be on temperature sensitive, TRPV1 expressing small diameter nerve fibres (Du et al. 2007; Skryma et al. 2011). Like TRPV1, TRAP1 channel modulates synaptic transmission on to dorsal horn nociceptive neurons of the lumbo-sacral spinal cord (Wrigley et al. 2009). TRPA1 expressing activation of dorsal roots is known to increase the excitatory postsynaptic synaptic currents in the lumbo-sacral dorsal horn neurons, and this excitation increases in the presence of TRPA1 agonists (Kim et al. 2010; Uta et al. 2010). Hence, it was
surprising we did not observe NK1R internalisation in the nociceptive neurons in the dorsal horn region after intravesical MO treatment. Although TRPA1 is reported to be involved in mediating nociception, very little is known about how the nociceptive information is processed in the spinal cord.

Studies conducted in the rodent lumbar spinal cord (L4 level) indicate that dense TRPA1 positive axons are distributed in the superficial lamina (Uta et al. 2010). However there are no autonomic preganglionic neurons in this spinal level and it is possible that in the L6/SI spinal segments, where many preganglionic neurons exist, there is a difference in distribution of TRPA1 expressing bladder projecting terminals. Our results suggest that TRPA1 is only expressed on the peripheral projections of neurons that project exclusively to the IML region (including the neurons involved in micturition reflex) but not to the dorsal horn. In contrast, capsaicin activates NK1R internalisation in both IML and dorsal horn, consistent with TRPV1 being expressed by both sets of neurons. This also suggests that populations of neurons expressing only TRPV1 (not TRPA1) are involved in transmitting nociceptive signals. To further investigate this, experiments using antibodies against the TRPA1 channels should be conducted to conduct qualitative and quantitative analysis of the expression of TRPA1 positive terminals in the L6/S1 spinal dorsal horn.

3.4.2 CYP induced bladder inflammation

Once cellular targets of nociceptive signal transmission were identified in both regions (DH and IML), we established rodent models of Cyclophosphamide (CYP) induced acute and chronic lower urinary tract (LUT) inflammation. CYP induced inflammation
is a well-established model to investigate the underlying mechanisms for some of the symptoms that mimic the clinical condition of interstitial cystitis (IC).

A significant level of NK1R internalisation was observed in the dorsal horn neurons after both acute and chronic inflammation. This indicates activation of projection and interneurons of the ascending pain pathway involved in processing nociceptive information and responsible for eliciting the painful symptoms of inflammation. The observed increase in NK1R internalisation in IML neurons may contribute to the bladder hyperactivity that is observed after chemical induced cystitis in rats (de Groat and Yoshimura 2001) reported in LUT inflammation (Berkley 2005; Bogart et al. 2007; French et al. 2009; Grover et al. 2011). The NK1R internalisation response in both acute and chronic models suggests that there is an increase in tonic activity of peptidergic afferents in the DH (putative nociceptive) and IML (autonomic) regions of the cord. There was no difference in the levels of NK1R internalisation between acute and chronic treatment groups. Hence no additive affect of multiple doses of CYP is seen after 10 days of treatment.

In an earlier study of colonic inflammation induced by zymosan administration and colorectal distension, an increase in NK1R internalisation was observed in thoracic and sacral cord lamina 1 neurons (Honore et al. 2002). In this study only neurons containing 20 or more endosomes were considered as having undergone NK1R internalisation. In contrast, in our study neurons were chosen randomly and a minimum number of endosomes was not set as a criterion for a neuron to be counted as internalised. This allowed us to unmask any subtle changes in the magnitude of internalisation in response to inflammation that enabled us to identify neurons activated in bladder inflammation. The sparse distribution of the visceral afferents may also account for the low internalisation response. As was mentioned in Chapter 1 section 1.1.2, although there is
an extensive branching of the visceral afferent terminals, there are comparatively fewer afferents innervating the viscera compared to afferents innervating the skin, muscles or joints (Robinson and Gebhart 2008). Termination of visceral afferents on multiple and rostro-caudal segments also causes the nociceptive signals to be diffused and poorly localised. The diffusion of nociceptive signals could be one of the reasons that the NK1R internalisation response after acute and chronic inflammation are smaller than observed after a somatic noxious stimulation. It may also mean that less substance P is released from visceral compared with somatic afferents.

We wished to further investigate if noxious stimulation using TRPA1 agonist has an additive effect on a group of animals with chronic inflammation. We found that the restricted effect of MO on IML neurons also occurred after inflammation. Once again sensitisation of bladder afferents after chronic bladder inflammation did not reveal any effects of MO on the dorsal horn neurons. This implies that there may be a difference in the neurotransmitter release in both regions of the cord (see Chapter 4, General Discussion).

**3.4.3 Cystometry control vs acute and chronic CYP treated groups**

Functional analysis of the bladder was conducted by taking cystometric recordings from the bladders of control and CYP treated animals. Saline was infused at 40µl/min as per previously established protocol for cystometric recordings in anaesthetised animals (Du et al. 2007). The purpose of activating bladder function by cystometry was to determine if a more physiological stimulus (i.e. regular bladder filling) was able to stimulate NK1R internalisation in neurons involved in the micturition pathway. Furthermore, we wished to determine if this physiological stimulus would also result in NK1R
internalisation in the nociceptive neurons in groups with acute and chronic inflammation.

Unlike some previous reports (Hu et al. 2003; Buyuknacar et al. 2008; Klinger and Vizzard 2008) we did not observe a significant increase in bladder activity in animals with chronic CYP treatment compared to the control group. We observed very low values for the baseline pressure and peak pressure across all the groups. Du and colleagues have also reported baseline pressure and peak pressure values in control animals that are very similar to that observed by us (Du et al. 2007). In this study cystometric recordings were also performed under urethane anaesthesia. On the other hand, Klinger and Vizzard (Klinger and Vizzard 2008) and Hu and colleagues (Hu et al. 2003) reported much higher levels of baseline pressure, threshold pressure and peak pressure measured for their control groups, here cystometry was performed in un-anaesthetised, catheterised animals. In our experiments, the lack of change in cystometric parameters after bladder inflammation is surprising. Another difference from previous studies is that these cystometric recordings were performed over a few hours whereas ours were performed over a shorter (90 minutes after the initial 30 minute stabilisation period). It is possible that this shorter period did not provide a representative measurement of bladder function. We do not consider that the CYP treatment was inadequate in our study because macroscopic analysis of the bladder revealed characteristic morphological differences in bladders of animals treated with CYP compared to the saline control group. The bladders of animals with inflammation were heavier and showed signs of haemorrhage. During animal monitoring, animals exhibited peculiar rounded back posture immediately after injection that is a characteristic feature of development of CYP induced cystitis (Bon et al. 1997).
The NK1R internalisation was significantly increased in animals with chronic inflammation both in the DH and IML neurons for animals that had undergone cystometry for 90 minutes. However this level of internalisation was no different to the chronic CYP treated group without having gone continuous cystometry. This indicates that the added stimulation by continuous infusion of saline into the inflamed bladder had no additional affects on cellular targets in the spinal cord.

3.4.4 Estrous cycle and bladder activity

Functions of the lower urinary tract in women are reported to fluctuate in association with their menstrual cycle and there is a direct link between the gonadal hormones and pain sensitivity (Pang et al. 1995; Johnson and Berkley 2002; Powell-Boone et al. 2005). Previously published animal studies indicate that the symptoms of interstitial cystitis fluctuate and their severity increases during different menstrual cycle stages (Sant and Theoharides 1999; Bennett et al. 2003; Cheng and Keast 2009). Phases of the estrous cycle are known to impact on the levels of CGRP and SP in the bladder (Shaffer et al. 2011). Literature also suggests that the bladder projecting neurons express both the TRPV1 and estrogen receptors α & β (Bennett et al. 2003; Cheng and Keast 2009). This means that the bladder projecting nociceptive afferents are a potential target of the circulating estrogens. Hence, it is possible that a change in the estrogen levels during different stages of the menstrual cycle have an effect on bladder pain. Johnson and Berkley examined the effect of menstrual cycle on micturition threshold in control and inflamed group of female rats (Johnson and Berkley 2002) and found an increase in vulnerability of inflamed bladders to become hyperreflexic during different stages of the cycle.
Based on the previous findings, we considered that changes in the estrous cycle in inflammation groups may have an effect on bladder activity that could be reflected in an altered level of NK1R internalisation and confound our analyses. From our first assessment of the stages of estrous cycles in rats in different treatments, there did not seem to be a correlation between the stages of the cycle with the levels of NK1R internalisation. However limited numbers of samples at each stage of the cycle limited our ability to statistically test the effects of the estrous cycle on bladder activity and NK1R internalisation. In future, experiments with replicates at each stage of the estrous cycle could specifically evaluate any effects of the menstrual cycle on activation of peptidergic afferents, evidenced by NK1R1 internalisation.

3.5 Conclusions

By employing the NK1R internalisation method, we have investigated neuronal activation in two different regions of the sacral spinal cord in a visceral inflammation model. Both noxious stimulation of peripheral terminals of visceral afferents and chronic inflammation leads to activation of neurons in the superficial dorsal horn and in the IML. An activation of DH and IML neurons in inflammation suggests a contributing role in bladder hyperactivity and discomfort that characterises chronic bladder inflammation. Our results have also demonstrated a difference in TRPV1 and TRPA1 expressing afferent projections to the DH and IML neurons. Cystometric recordings reveal that the non-noxious stimulation of bladder afferents results in an increase in internalisation after acute and chronic inflammation. Our results provide valuable tools to further probe the mechanisms and effects of neuronal activation in a visceral model of pain.
Chapter 4: General Discussion

This study has attempted to understand mechanisms underlying some of the characteristic symptoms of cystitis such as pain and bladder hyperactivity. We have mimicked some of the symptoms of cystitis in rodents using noxious stimulation of the bladder afferents and chemically CYP induced cystitis. We chose to perform this study in female rats because interstitial cystitis (IC) is a problem that is more prevalent in women (Cheng and Keast 2009).

We have used a previously well-established technique (visualization of internalised NK1R) to identify regions of neuronal activation in response to bladder noxious stimulation and bladder inflammation. SP releasing peptidergic nociceptive bladder afferents were stimulated using an agonist for the TRPV1 channel, capsaicin. The neurons in the sacral spinal cord dorsal horn and IML region that express the NK1R were analysed to quantify NK1R internalisation. The first set of experiments verified the efficacy of using the NK1R internalisation method in a visceral model of noxious stimulation. The activation of neurons in the nociceptive region and in neurons responsible for micturition reflex led us to conduct further experiments using CYP induced bladder inflammation. This helped us identify the areas of the sacral cord that are activated in response to inflammation.

We have looked at the response of inflammation on neuronal activation in two regions of the sacral spinal cord that are known to be involved in nociception (DH) and micturition reflex (IML). This study has enabled us to identify targets of visceral nociceptive bladder afferents in the sacral spinal cord. Our study indicates that the
preganglionic autonomic region (IML) is a direct target of the SP releasing bladder afferents. It is also known that in the sacral cord, the peptidergic afferents are not the only source of SP. SP is also released from supraspinal sources (Hokfelt et al. 2001). To acquire an indication of whether the internalisation response is due to SP released in the IML region by peptidergic afferents alone, studies of CGRP and NK1 co-expression should be performed. The removal of supraspinal source of SP by means of a spinal cord transection (Llewellyn-Smith and Weaver 2001) will allow us to quantify the SP inputs exclusively from the bladder afferents. This could potentially confirm bladder afferents as the source of observed SP immunoreactivity in the IML, although remodeling of spinal circuitry after the injury could confound the results.

In the IML, a lot of the SP positive terminals were not in close proximity to the NK1R positive preganglionic neurons yet these NK1R positive neurons showed receptor internalisation. It is unclear how SP mediates its action on the NK1R in the IML when not all the SP fibres terminate on the preganglionic neurons. One possibility may be that SP acts on these neurons via volume transmission. SP is also one of the neurotransmitters that may diffuse over relatively long distances (Duggan et al. 1992; Baude and Shigemoto 1998). It is known that a mode of inter-neuronal communication other than synaptic transmission also exists, known as volume transmission (Agnati et al. 1995). Volume transmission is characterised by signal diffusion in extracellular fluid at points that may be remote from the target cells (Castaneda-Hernandez and Bach-y-Rita 2003). This means that structures that do not exhibit conventional synapses receive signals over a distance. Several studies have suggested that in the central nervous system, volume transmission of neurotransmitters is used as a source of communication. Dopamine, serotonin, noradrenaline and neuropeptides have all been shown to use
volume transmission as a mode of non-synaptic communication (Agnati et al. 1995; Zoli and Agnati 1996). Some peptidergic fibres are observed in the region dorsal to the IML, where no NK1R positive cell bodies are seen. It is possible that no SP is released from these synapses, where the major neurotransmitter is likely to be glutamate, as for other afferents. The upper lumbar spinal cord also receives inputs from the bladder afferents (Blok 2002; de Groat 2006). NK1R internalisation should also be assessed in this segment of the spinal cord. Neurons in the upper lumbar cord are not involved in the micturition reflex but may still be involved in pain and be activated in response to bladder inflammation.

Activation of TRPV1 and TRPA1 channels elicited different responses of NK1R internalisation. As seen in section 3.3.1, the activation of TRPA1 channel led to neuronal activation only in the IML neurons. These results indicated that the amount of SP released by the bladder afferents that project to one region of the sacral cord (DH) may be different to another region (IML) and this may be due to a difference in the electrical activity of the afferents projecting to both regions. The literature indicates that in animal models and in people, cystitis is often accompanied by bladder hyper-reflexia and inflammatory pain (Sant and Theoharides 1999). Our results suggest that the activation of DH and IML neurons does not always occur in parallel. This means that bladder hyperactivity alone cannot be used as an indication of bladder pain. Many studies have been conducted to date that only use cystometric parameters and observed bladder hyperactivity, aiming to also understand the nociceptive symptoms caused by the inflammation (Du et al. 2007; Andersson et al. 2011). However they have not directly enhanced our understanding of circuitry actually involved in bladder pain. In this context, additional valuable studies have been performed that included
measurements of referred hyperalgesia (Guerios et al. 2009) in addition to cystometric measurements. Further, insight into mechanisms of dorsal horn and IML neurons independently of each other may lead to therapeutic developments that can target each symptom separately (pain and bladder hyperactivity).

One of the main findings of the study is that the response to TRPA1 activation in the dorsal horn and IML regions is different. TRPV1 activation resulted in eliciting a response of neuronal activation in the nociceptive and micturition reflex pathways. In previous cystometric studies, mustard oil has been used to test the effect of TRPA1 activation in bladder hyper-reflexia (Du et al. 2007). As mentioned earlier (Chapter 1, section 1.4) the properties of bladder nociceptive afferents change after inflammation and the bladder is known to become hyperreflexic (Vizzard 2001; Westropp and Buffington 2002; Bon et al. 2003). In future experiments, a TRPA1 agonist should also be infused in the bladders of animals with chronic inflammation to assess the effect of mustard oil on NK1R internalisation. Continuous stimulation of the bladder afferents during cystometry may reveal a different effect to what we have observed on the dorsal horn neurons after agonist-induced TRPA1 activation. Bladder noxious stimulation experiments should also be performed using other TRPA1 agonists such as allicin and diallyl disulfide to confirm this unique difference in activation of neurons in DH and IML neurons (Bautista et al. 2005).

Although we have seen that the IML neurons are a functional target of the bladder afferents, electrophysiological studies suggest that some afferent termination also occurs on local interneurons (Araki and De Groat 1996; de Groat and Yoshimura 2010). There is also a population of autonomic interneurons dorsal to the central canal. A lot of
NK1R positive cell bodies were observed in this region of the cord which could also be included in future quantification to analyse the extent of inflammatory response on a bigger population of neurons in the sacral cord that receive inputs from the peptidergic bladder afferents. The present study has focused on the NK1R internalisation assay that has limited our analysis to only one neuropeptide involved in nociception. Electrophysiological studies could also be used to analyse other synaptic communication in the sacral cord that uses neuropeptides other than SP or the impact of bladder inflammation on glutamate transmission.

We quantified NK1R internalisation in ChAT positive preganglionic autonomic neurons. As mentioned earlier, the preganglionic neurons of the IML control not only the bladder, but some instead regulate the colon and reproductive organs (Keast and de Groat 1989; Keast 2006). Therefore we would not expect that all IML neurons respond to bladder stimulation by noxious agents or inflammation. In the future, we would like to confirm that the IML response we observed is specific to bladder regulating neurons. Previous studies have used injections of retrograde tracer dyes such as Fluorogold and Fast Blue into the bladder, colon and penis to label and characterise postganglionic neurons in the major pelvic ganglion (Keast and de Groat 1989). Another study using the retrograde labeling technique has been conducted to study the immunohistochemical properties of bladder projecting neurons in the major pelvic ganglion (Callsen-Cencic and Mense 1997). However, these dyes do not cross synapses so will not label the preganglionic spinal neurons innervating (e.g.) bladder-projecting pelvic ganglion neurons. For identification of preganglionic neurons in the sacral cord, one can inject a retrograde labeling dye directly into the pelvic ganglion to label all preganglionic neurons in the sacral cord. However, there are currently no structural or chemical
markers to distinguish the function of these preganglionic neurons. Another approach could be to use viral tracing from the bladder to label these neurons. The literature shows that neurotropic viruses can be transported in a transneuronal manner (Strack et al. 1989). Pseudo-rabies virus (PRV) has been shown to transport in a retrograde fashion in studies of the central nervous system (Strack et al. 1989). Previously, neurons in the central nervous that innervate the bladder have been identified using the PRV injections in the external urethral sphincter and the bladder (Marson 1997), and into the urethra (Vizzard et. al.; 1995). A PRV study would enable us to label the bladder projecting neurons in the sacral spinal segment. However, labeling studies label only a proportion of the neurons and there maybe some unlabeled neurons that are bladder-projecting neurons. Irrespective, we could use the virus to conduct NK1R internalisation analysis in all the labeled neurons, and conduct parallel studies to specifically label those projecting to the reproductive organs and colon. We hypothesize that only the bladder projecting labeled neurons will show internalised endosomes in response to a bladder stimulus.

Our experiments also faced some technical limitations. The microscopy technique itself is limited in its use to show the entire neuron intact with a dendrite. Hence the data we collected was only from the soma of neurons. Secondly, the sample size was small. Acquisition of more data would require more assessment of sections, for a large number of neuronal counts, and counts of endosomes in the DH and IML. The confocal microscopy and counting of the endosomes in each individual cell is a laborious process. If the number of neurons counted was bigger and we had characterised more than one neurotransmitter, we may have seen the effect of inflammation on a particular
subpopulations of neurons. This would also give a better idea of the features contributing to the plasticity in the spinal cord.

This study has provided an insight into the underlying features of characteristic symptoms of cystitis. Our study showed that despite the differences between somatic and visceral afferents, the previously established assay for measuring neuronal activity in somatic inflammation can be employed in a model of visceral inflammation. Our study also highlights that both symptoms of cystitis, bladder pain and hyperactivity should be studied because the nociceptive and micturition reflex pathways can be activated independently. Despite some limitations, this study has provided us with a powerful tool to identify individual neurons involved in nociception and the micturition reflex, which are activated in response to bladder inflammation.
References:


