Peptidergic sensory and parasympathetic fibre sprouting in the mucosa of the rat urinary bladder in a chronic model of cyclophosphamide-induced cystitis

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Abstract

Chronic bladder pain is becoming more and more prevalent and yet there is a lack of effective treatment because there is so little known about the mechanisms causing this type of pain. A possible etiology of chronic bladder pain is persistent inflammation. It has been proposed that inflammation initiates neurological changes in the bladder and CNS which could lead to sensitization of the bladder to cause chronic pain and frequency. Both autonomic and sensory innervation changes in the periphery can lead to hyperalgesia and allodynia, thus we have chosen to look for autonomic and sensory innervation changes that occur in the bladder in a model of chronic cystitis.

In this study, we used a well established animal model to investigate changes in the peptidergic and parasympathetic innervation of the bladder following chronic bladder inflammation. Adult female Sprague-Dawley rats were injected with either 70mg/kg cyclophosphamide diluted in saline, i.p., once every 3 days or saline. After 10 days, all animals were tested for urinary frequency and number of low volume voids, as well as symptoms of spontaneous pain. At the end of 12 days, all animals were perfused with histological fixatives and the urinary bladders processed for immunofluorescence using antibodies against calcitonin gene-related peptide (CGRP) and the vesicular acetylcholine transporter (VAChT) as markers, respectively, of peptidergic primary afferent fibres and parasympathetic efferent fibres. We show that animals treated with cyclophosphamide had inflamed bladders and displayed high urinary frequency as well as some indicators of spontaneous pain, such as piloerection and a rounded-back posture. Furthermore, they had a significant increase in the density of both parasympathetic and peptidergic sensory fibres in the bladder mucosa and an increase in peptidergic sensory fibres in the detrusor muscle.

Thus from our results, we propose that parasympathetic and sensory peptidergic innervation changes can occur in the bladder following chronic cystitis. Since we observed that the parasympathetic and peptidergic fibres often wrapped around one another or were in close proximity, these two fibre populations may be interacting with each other to lead to and maintain sensitization. These innervation changes may be

responsible for prolonged and exacerbated urinary symptoms and may play a role in diseases where there is chronic urinary pain, such as in interstitial cystitis.

RESUME

La douleur chronique de la vessie est de plus en plus commune et, à ce jour, il y a un manque de médicaments efficaces parce que les mécanismes responsables de ce type de douleur sont très peu connus. Une des causes possibles de douleur chronique de la vessie est l'inflammation persistante. Il a été proposé que l'inflammation pouvait déclencher des changements neurologiques dans la vessie et le SNC qui entraineraient une sensibilisation menant à une douleur chronique et une fréquence urinaire plus élevée. Les changements d'innervation des systèmes autonome ou sensoriel dans la périphérie peuvent entrainer à la fois hyperalgésie et de allodynie. C'est pour ces raisons que nous avons choisi d'observer les changements d'innervation autonomique et sensorielle se produisant dans la vessie dans un modèle de cystite chronique.

Dans cette étude, nous avons utilisé un modèle animal bien établi pour observer les changements de l'innervation peptidergique et parasympathique de la vessie en état d'inflammation chronique. Des rates Sprague-Dawley adultes ont été injectées soit avec 70mg/kg de cyclophosphamide diluée dans de la saline, i.p., une fois tous les trois jours , ou de la saline. Après 10 jours, la fréquence urinaire, le nombre de vidages de petit volume, ainsi que les symptômes de douleur spontanée ont été évalué pour tous les animaux. Après 12 jours, tous ont été perfusés avec des fixateurs histologiques et les vessies ont été traitées pour l'immunofluorescence utilisant des anticorps dirigés contre le peptide relié au gène de la calcitonine *(CGRP)* et le *vésiculaire transporteur acétylcholine (VAChT)* comme marqueurs respectifs des fibres primaires afférentes peptidergiques et des fibres efférentes parasympathiques. Nous démontrons que les animaux traités avec de la cyclophosphamide avaient des vessies inflammées et montraient une grande fréquence urinaire ainsi que certains indicateurs de douleur spontanée, telles que la piloérection et la courbure du dos. De plus, ces vessies montraient aussi une augmentation significative de la densité des fibres parasympathiques et peptidergiques sensorielles dans la muqueuse ainsi qu'une augmentation de la densité des fibres peptidergiques sensorielles dans le muscle detrusor.

Suite à nos résultats, nous proposons que des changements au niveau de l'innervation sensorielle peptidergique et de l'innervation parasympathique peuvent se

produire dans la vessie suite a l'état de cystite chronique. Puisque nous avons observé que les fibres parasympathiques et peptidergiques étaient très près les unes des autres ou même s'enroulaient, ces deux populations pourraient interagir ensemble pour créer et maintenir la sensibilité. Ces changements d'innervation pourraient être responsables de symptômes urinaires prolongés et exacerbés et pourraient jouer un rôle dans les maladies de douleur chronique urinaire, comme dans le cas de la cystite interstitielle par exemple.

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Abbreviations

ATP	Adenosine triphosphate
β2	Adrenergic beta-2 receptor
β3	Adrenergic beta-3 receptor
BDNF	Brain derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
Cyclo	Cyclophosphamide
DM	Detrusor muscle
GDNF	Glial derived neurotrophic factor
I.P	intraperitoneal
IC	Interstitial cystitis
IgG	Immuoglobin G
IR	Immunoreactivity
K+	potassium
L6	Lumbar segment six of spinal cord
LP	Lamina propria
LPS	lipopolysaccharide
M1	M1 muscarinic acetylcholine receptor
M2	M2 muscarinic acetylcholine receptor
M3	M3 muscarinic acetylcholine receptor
M3	Muscarinic M3 receptor
M4	Muscarinic M4 receptor
NGF	Nerve growth factor
NGS	Normal goat serum
NHS	Normal horse serum
P2X3	Pain-related cation-channel receptor
PBS	Phosphate buffered saline
PBS+T	Phosphate buffered saline with Triton-X-100
PFA	paraformaldehyde
S1	Sacral segment one of spinal cord
SP	Substance P
TrkA	Tyrosine receptor kinase
TrpV1	transient receptor potential cation channel, subfamily V, member 1
Ur	Urothelium
VAChT	Vesicular acetylcholine transporter

Contributions of Authors

This thesis is based on data obtained for the generation of the following manuscript:

Peptidergic sensory and parasympathetic fibre sprouting in the mucosa of the rat urinary bladder in a chronic model of cyclophosphamide-induced cystitis
A. Dickson, A. Avelino, F. Cruz and A. Ribeiro-da-Silva

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Responsibilities of authors and co-authors:

A. Ribeiro-da-Silva, MD, PhD: Principal investigator of project. Provided intellectual influence on planning the experiment and the manuscript. Edited and revised the manuscript and aided with the preparation of the final version of the figures. He edited the grant application to the Louise that funded this research, which was written by A. Dickson (see below).

Dr. F. Cruz, MD, PhD, Departments of Histology and Embryology and of Urology, Faculty of Medicine of Porto, Portugal: Investigator in the project. Provided a clinical perspective to the manuscript. Collaborated extensively in the writing of manuscript and its revision.

A. Avelino, PhD, Department of Histology and Embryology, Faculty of Medicine of Porto, Portugal: Investigator in the project. Provided advice on protocols and edited and revised the manuscript.

A. Dickson: Devised all experiments included in this thesis and executed them. She performed all the drug administrations to rats and the behaviour analyses, the animal perfusions with fixatives, the tissue processing for immunocytochemistry, and observed all bladder sections by fluorescence and confocal microscopy. She also obtained all the micrographs and performed all quantifications using image analysis. She wrote the initial version of the manuscript submitted to Neuroscience. Furthermore, she wrote a successful grant application to the Louise Edwards Award in Pain Research, which funded this project and wrote the animal protocols for approval by the Animal Care Committee.

The current thesis has been significantly altered from the manuscript prepared and submitted to Neuroscience.

CHAPTER ONE:

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1: The History of Pain

"The art of life is the avoiding of pain." (Thomas Jefferson, 1743-1826).

Pain in man has its origins at the genesis of humankind; it is one of the most basic and wretched of human experiences. Milton (1608-74) stated in Paradise Lost, "But pain is perfect miserie, the worst of evils..." In seeking to alleviate pain, there is a history of many drastic measures taken. Many ancient cultures believed pain was a punishment for human folly. Human sacrifices were made to placate the angry gods and it was believed that after a sacrifice was made, the gods would grant relief to the sufferer (Hyson, 2001). From early skeletal remains, anthropologists have demonstrated evidence of diseases and injuries (abscessed teeth and unhealed fractures) which undoubtedly caused pain to the individual. In some of these skeletal remains there are small, round holes drilled into the skulls, this is evidence of trepanation, an operation performed by the witch doctors to let out the evil spirits, the cause of the pain (Hyson, 2001). Since these ancient times, the experience and origin of pain has been discussed, debated, studied and researched. The link between disease and pain, emotion and pain, suffering and pain, the physical body and pain and the influence of the soul in pain are some topics covered over and over again in medical, religious and general literature. Although we have made advancements in the understanding and treatment of pain, pain is still the symptom of the majority of diseases that causes debilitation and that impedes a good quality of life (Ashburn and Staats, 1999).

1.2. General Overview of Pain

The best definition of pain is that endorsed by the International Association for the Study of Pain: "Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Merskey H and Bogduk N, 1994). Although unpleasant, pain is a protective mechanism that has evolved to prevent tissue damage or alert one of tissue damage so that it can be treated (Cousins, 1989; Millan, 1999). The ability to perceive pain is essential for survival, as is demonstrated in patients who were born without the capacity to feel pain. This happens in individuals with hereditary sensory autonomic abnormalities (Millan, 1999; Nagasako et al., 2003). These patients with insensitivity to pain often die as children from preventable injuries which lead to fatal illness (Nagasako et al., 2003). However, although acute pain is essential, pain can also be maladaptive and destructive if it goes on chronically. Chronic pain may be defined as "pain that has lasted 6 months or longer, is ongoing, is due to non-life threatening causes, has not responded to currently available treatment methods, and may continue for the remainder of the patient's life" (Dunajcik, 1999). Chronic pain is a world wide problem with a prevalence of between 2% and 46.5% (Elliott et al., 1999; Gureje et al., 1998; Verhaak et al., 1998). While acute pain is treatable, chronic pain is more difficult to control and thus those with chronic pain often have difficulty being optimistic about ever improving (Loeser and Melzack, 1999). Chronic pain patients are thus more likely to experience depression, anxiety, activity limitations and financial problems (Ashburn and Staats, 1999; Latham and Davis, 1994; Melzack, 1990; Rudy et al., 1988). Several studies have found that chronic pain patients have elevated rates of suicidal ideation (Breslau, 1992; Chaturvedi, 1989; Hitchcock et al., 1994), attempts (Magni et al., 1998; Stenager et al., 1994), and completions (Fishbain

et al., 1991; Fishbain, 1996; Magni et al., 1998; Penttinen, 1995). One survey reported that 50% of chronic pain patients have seriously considered committing suicide because of pain (Hitchcock et al., 1994). Thus learning more about chronic pain should be a priority due to the suffering it causes worldwide.

1.3 Causes of Chronic Pain

Chronic pain may be due to the persistent stimulation of nociceptors in areas of ongoing tissue damage, for example, in patients with osteoarthritis. Frequently, however, chronic pain presents without any identified ongoing tissue damage or injury (Ashburn and Staats, 1999; Bonica, 1990; Bonica, 1990). Many patients with chronic pain are currently diagnosed on the basis of clinical criteria alone, as there are no well-defined laboratory studies or tests to identify the disease. These common pain syndromes include uretheral syndrome, inflammatory bowel syndrome, myofacial pain syndrome, causalgia and fibromyalgia to name a few (Bonica, 1990; Goldenberg, 1987; Long and Kephart, 1998; Bonica, 1990). Chronic pain can be classified as somatic or visceral, based on its origin. Pain that affects the skin, muscles, joints, ligaments or bones is usually described as somatic pain. Pain arising from internal organs is defined as visceral pain (Cervero, 1988). Somatic and visceral pain can be caused by many factors. Three main sources of pain are injury and inflammation of tissue, direct injury to the peripheral nervous system (neuropathic pain), or changes in the central nervous system (Levine and Taiwo, 1994; Marchand et al., 2005; Nicholson, 2004; Woolf and Mannion, 1999). Knowledge about the underlying pathology that leads to chronic pain is limited and often there are many possible etiologies. It was

previously thought that pain was fairly homogeneous, no matter what the source, however, new data is revealing that somatic pain and visceral pain are quite different (Cervero and Laird, 1999). In fact, the subtypes within each origin (neuropathic, inflammatory and centrally mediated pain) also have very different mechanisms in causing pain. Traditionally, acute somatic inflammatory pain has been researched the most. However as there is an increasing incidence of visceral pain and as evidence points to mechanisms of visceral pain being different from somatic pain, current research is now starting to look specifically at visceral pain (Cervero and Laird, 1999).

Although all types of pain are important, this thesis will concentrate on visceral pain, specifically possible mechanisms of chronic bladder pain. A review of the literature will be presented for bladder function and dysfunction as well as bladder pain. The importance of this research will be highlighted by drawing clinical correlations to interstitial cystitis, to which our results are relevant. Finally a rationale for the current thesis will be briefly summarized

1.4 Visceral Pain

Visceral pain is the most common form of pain produced by disease and one of the most frequent reasons why patients seek medical attention (Bonica, 1990). Until recently, visceral pain was not considered to be a major problem by the clinical specialists that dealt with it. Obstetricians, gynaecologists, cardiologists, gastroenterologists and urologists considered pain to be a symptom of an underlying disease and were mainly concerned with the diagnosis and treatment of the disease. Their approach was to assume that if the disease went away so would the pain (Cervero and Laird, 1999). However, many visceral conditions

such as irritable bowel syndrome and functional dysplasia are characterized solely by chronic pain with undefined pathology to the organ (Cervero and Laird, 1999; Mayer and Raybould, 1990). Only recently, and mainly because of popular pressure, has pain become a subject that can be treated directly and independently of the accompanying disease as doctors realize that this 'symptom' is often the very centre of the problem and the reason for the debilitating effects of the disease (Cervero and Laird, 1999).

Visceral pain has some important clinical characteristics distinct from somatic pain. First, although somatic pain can be seen as protective, as it can alert one of external threats so that the organism can mount defensive behaviour, the role of visceral pain is less clear (Cervero and Laird, 2004). Some major forms of life threatening tissue destruction such as perforation of hollow organs or malignant growths in organs are frequently non-painful (McMahon et al., 1995). Some internal organs in fact are insensitive to pain and extensive damage to organs like the liver or the lungs can go unnoticed until it is too late to do anything about it (Cervero and Laird, 2004). Conversely, minor stimuli that do not cause tissue damage can be very painful such as distension of a hollow viscera. Thus it is not easy to find a close association between injury to internal organs and pain or to attribute a protective role to all forms of internal pain as it is to somatic pain (Cervero, 1994; Cervero and Laird, 2004). Second, pain of cutaneous origin is usually focal and well localized whereas visceral pain is either a diffuse "deep" pain or is referred to other locations. Visceral pain tends to be diffuse because of the higher central divergence of visceral input compared with that of somatic input (Cervero and Morrison, 1986) also, it has been shown that spinal neurones which receive visceral input present larger receptive fields than those receiving somatic input (Akeyson and Schramm, 1994; Cervero, 1983; Euchner-Wamser et al., 1993). Visceral pain is often felt in

somatic areas remote from the site of the originating cause; for example during a heart attack pain is often referred to the left shoulder and arm. The most widely accepted mechanism for referred pain is that visceral and somatic primary sensory neurones converge onto common spinal neurones. This theory proposes that the activity in ascending spinal pathways is misconstrued as originating from somatic structures and thus the feeling that the pain is coming from the skin or muscle instead of an internal organ (Cervero and Laird, 1999). Finally unlike somatic pain, visceral pain is often accompanied with motor and autonomic reflexes such as nausea (Cervero and Laird, 1999; Cortelli and Pierangeli, 2003). These differences from somatic pain are important in illustrating that visceral pain is distinct. In fact there is sufficient data to indicate that somatic and visceral pain differ at the molecular, cellular and systems level (Cervero and Laird, 2004). Due to this distinction, the treatment often used for somatic pain is often not sufficient for visceral pain. Although we now know that visceral pain is different from somatic pain, we still have very little understanding of the pathology of visceral pain.

1.5. An Overview of the Bladder

To understand bladder dysfunction and pain, the normal structure, innervation and function of the bladder itself must first be examined.

1.5.1. Anatomy of the Bladder

The bladder is hollow organ located in the lower abdomen. It is held in place by ligaments that are attached to other organs and the pelvic bones. The bladder is typically divided into three regions; the upper dome, the body and the trigone (Figure 1). The upper

dome is the most cranial region and is defined as the dome shaped section of the bladder. The region directly basal to the upper dome is the body region. The body and trigone regions are divided by the uretheral openings, the region above the uretheral openings is the body region, while, the triangular area beneath the urethral openings is the trigone region. It is the trigone region of the bladder that empties into the urethra (Gabella and Davis, 1998). The bladder can also be divided into three layers; the urothelium, the lamina propria and the detrusor muscle (figure 1). The regions and layers are important in the function of the bladder and will be discussed further.

1.5.2. Bladder Physiology and Function

The urinary bladder has two important functions: storage of a socially adequate volume of urine and voluntary excretion of urine at a convenient time (Chancellor and Yoshimura, 2002). The bladder receives urine from the kidney via the ureters and expels it through the urethra. Although this seems relatively simple, the bladder is a fairly complex organ that requires precise coordination of detrusor muscle relaxation and urethral sphincter contraction during the filling phase and the converse during micturition (Ferguson and Christopher, 1996). The bladder has a mucosal layer and a smooth muscle layer. The mucosa is made up of an urothelium to line the lumen of the bladder and a lamina propria. The smooth muscle layer consists of several layers of detrusor smooth muscle. Outside the muscular wall is connective tissue called adventitia. The bladder is innervated by an intricate network of parasympathetic, sympathetic and sensory afferent fibres which are responsible for controlling the function of the organ.

For the bladder to function adequately and efficiently, it must first be able to stretch and rearrange itself to allow an increase in bladder volume without a significant rise in pressure. First, if the bladder is not compliant, intraluminal pressure will become high with only a small volume of urine and the micturition reflex will be activated prematurely (Chancellor and Yoshimura, 2002). Second, the smooth muscle and intrinsic nerves have to be protected from exposure to urine by the urothelium, which itself must also expand during bladder filling. Third, bladder emptying requires synchronous activation of all the smooth muscle because if only part of the wall contracted, the uncontracted compliant areas would stretch and prevent the increase in pressure necessary for urine to be expelled (Chancellor and Yoshimura, 2002). To further examine the physiology and pathophysiology of the bladder, a closer look at the anatomy and innervation of the bladder is necessary.

1.6 Bladder Structure

1.6.1. The Urothelium

The urothelium is the epithelial lining of the urinary tract. It consists of three distinct layers: the basal cell layer, the intermediate cell layer and the umbrella cell layer. Umbrella cells form the luminal surface of the urothelium, are the largest epithelial cells in the body, measuring 100 to 200 μm in diameter and can flatten and increase their surface area with stretching (Chancellor and Yoshimura, 2002). The umbrella cell stratum contains tight junctions that prevents the passage of ions between the urine and the blood stream (De Groat, 2004). Traditionally the role of the urothelium was thought to be solely a passive barrier

between urine and plasma; however it is now thought to play more complex roles in regulating bladder sensation and activity.

It has been shown that urothelial cells can respond to mechanical as well as chemical stimuli and in turn release chemicals such as ATP (Ferguson et al., 1997; Knight et al., 2002) and nitric oxide (Birder et al., 1998). These agents are known to modulate the sensory input of afferent nerves which are located in or close to the urothelium (Bean, 1990; Dmitrieva et al., 1998; Yoshimura and De Groat, 1997). Thus, the urothelium plays a role in bladder sensation by responding to local chemical and mechanical stimuli to send chemical signals to afferent nerves which then convey information to the central nervous system (Ferguson et al., 1997).

The urothelium responds to stretch by releasing ATP which acts on submucosal nerves to sensitize primary afferent fibres and causes feelings of bladder fullness (Dmitrieva et al., 1998; Pandita and Andersson, 2002). In fact, it had been postulated that the release of ATP from the urothelium is the most important mechanism for sensing bladder distension (Namasivayam et al., 1999). There are two possible mechanisms that have been proposed to explain how ATP decreases the activation threshold of afferent nerves. First, ATP may directly sensitize sensory nerves in the bladder through the purinergic P2X3 receptor (Dmitrieva et al., 1998; Lee et al., 2000; Namasivayam et al., 1999). However, there is some controversy as to whether P2X3 receptors exist in submucosal nerves because there is evidence that the bladder only contains peptidergic sensory fibres which by definition do not have the P2X3 receptor (Avelino et al., 2002; Gabella and Davis, 1998). There is agreement in the literature that P2X3 receptors exist on urothelial cells (De Groat, 2004; Elneil et al.,

2001; Lee et al., 2000; Sun and Chai, 2004; Tempest et al., 2004). Thus, the second proposed action of ATP is by an indirect mechanism through the P2X3 receptors in the urothelium, which may then cause the urothelium to release a chemical that activates the sensory fibres.

The urothelium has receptors for various neurochemicals including the vanilloid receptor TRPV1, cholinergic nicotinic and muscarinic receptors, all of which contribute to the function of the bladder (De Groat, 2004). Recent evidence demonstrates that activation of muscarinic (M3) receptors in the urothelium causes the release of ATP. A putative mechanism for muscarinic receptor-mediated release of ATP from the urothelium is that calcium influx mediated by activation of M3 receptors in the urothelium facilitates exocytosis of ATP (Birder et al., 2003; De Groat, 2004). As previously discussed, ATP released from the urothelium can act on sensory nerves to cause the sensations of bladder fullness and pain (Cervero and Laird, 2004). ATP release from the urothelium during bladder filling appears to be upregulated in chronic bladder disorders such as interstitial cystitis (IC) and may be important in the urinary symptoms of IC (Sun and Chai, 2004). In fact, the upregulated ATP release observed in feline interstitial cystitis is mediated, at least in part, by muscarinic receptors in the urothelium (Birder et al., 2003). Thus, recent research has revealed the importance of the urothelium in bladder sensation and function as well as in bladder pathology and pain.

1.6.2. The Detrusor Muscle

1.6.2.1 Anatomy and General Function of the Detrusor Muscle

In the specialized literature, the smooth muscle layers of the bladder are normally called as a group the detrusor muscle. It consists of three rather poorly defined layers of smooth muscle. The cells of the outer and inner layers tend to be oriented longitudally and those in the middle layer circularly (Keane and O'Sullivan, 2000). In the human detrusor muscle, bundles of muscle cells of varying size are surrounded by connective tissue rich in collagen. The individual smooth muscle cells in the detrusor are typical smooth muscle cells, being long and spindle-shaped cells with a central nucleus (Chancellor and Yoshimura, 2002).

The detrusor muscle is important for relaxation and contraction of the bladder to allow adequate filling and then voiding, respectively. Normal detrusor function is controlled by a complex set of interactions between the voluntary and involuntary nervous systems (Chess-Williams, 2002). Innervation of the bladder will be discussed in further detail in another section; however the mechanisms by which innervation directly controls the function detrusor muscle will be discussed here briefly.

1.6.2.2 The Role of the Detrusor Muscle in Relaxation

The bladder normally fills with urine by a series of peristaltic contractions at a rate between 0.5 and 5mL/min (Keane and O'Sullivan, 2000). Intravesical pressure measurements during bladder filling in both humans and animals reveal low and relatively constant bladder pressures when bladder volume is below the threshold for inducing voiding

(De Groat, 1999; Frazier et al., 2005). The bladder undergoes an incredible change in size from empty to full. The percent change is truly unmatched by any other organ in the body (Chancellor and Yoshimura, 2002). The accommodation of the bladder to increasing volumes of urine is dependent on the intrinsic properties of bladder smooth muscle, the stimulation of sympathetic activity and quiescence of the parasympathetic system (De Groat, 1993; Torrens and Morrison, 1987; Yoshimura and De Groat, 1997).

Smooth muscle is essential for bladder function because it is much more adaptable than skeletal muscle and is able to adjust its length over a much wider range than skeletal muscle. It is a crucial adaptation because the volume contained in the bladder depends on the cube of the length of the individual muscle fibres (Chancellor and Yoshimura, 2002). A stretched striated muscle can shorten by about a third of its initial length whereas a smooth muscle can shorten by more than two thirds of its initial length. The ability of the detrusor smooth muscle to change its length to such a large degree permits the bladder to adjust to much wider variations in volume than would be possible for skeletal muscle (Brading et al., 1996; Brading, 1999; Gabella, 1995). Thus the bladder requires the unique properties of smooth muscle to accomplish its job.

During the filling or storage phase, distension of the bladder produces low-level bladder afferent firing, which in turn activates the sympathetic system (Chancellor and Yoshimura, 2002). Norepinephrine is released from the hypogastric nerve and primarily acts on β-adrenergic receptors in the detrusor muscle to promote relaxation during the storage phase (Frazier et al., 2005; Michel and Peters, 2004). β-Adrenergic receptor activation is considered to be the most important physiological mechanism mediating urinary bladder

relaxation during the filling/storage phase of the micturition cycle (Andersson, 2004; Yamaguchi, 2002). It is stimulation of the β_2 and β_3 adrenergic receptors that exist in the human detrusor that results in direct relaxation of its smooth muscle cells (Andersson, 1993; Chancellor and Yoshimura, 2002; Levin and Wein, 1995; Morita et al., 1993; Nishimoto et al., 1995). The smooth muscle relaxant effect of β-adrenergic receptors is possibly through two mechanisms, an increase in cellular cAMP and an activation of K^+ channels in the muscle (Frazier et al., 2005).

The low level afferent firing during filling inhibits parasympathetic activity to the bladder. Although individual muscle cells may contract spontaneously, contraction of the bladder as a whole requires stimulation by the parasympathetic nerves (Andersson, 1993).

The plasticity of the bladder smooth muscle allows a fairly large volume of urine to be stored while inhibition of the parasympathetic system and the activation of the sympathetic system maintain bladder relaxation until the desire or need to void.

1.6.2.3 The Role of the Detrusor Muscle in Voiding

The storage phase of the bladder can be switched to the voiding phase either involuntarily (as a reflex) or voluntarily. The former is readily demonstrated in the human infant or in patients with neuropathic bladder when the volume of urine exceeds the micturition threshold (Chancellor and Yoshimura, 2002). At this point, increased afferent firing from tension receptors in the bladder reverses the pattern of efferent outflow, the sympathetic system is inhibited and the parasympathetic system is activated.

Parasympathetic activity causes a release of acetylcholine into the detrusor muscle. The human urinary bladder smooth muscle contains a mixed population of M_2 and M_3 muscarinic receptor subtypes (Chapple, 2000). The M_2 receptor is the predominant subtype in the bladder comprising about 80% of the total muscarinic receptor population, but mediation of bladder contraction by the minor population of M_3 receptors is well documented (Wang et al., 1995). Stimulation of M_3 receptors by acetylcholine leads to phophoinositol hydrolysis, and ultimately to accumulation of intracellular calcium and smooth muscle contraction (Harriss et al., 1995). The functional involvement of the M_2 receptor is beginning to be understood. Activation of the M_2 receptor leads to the inhibition of adenylate cyclase and thus to a decrease in cAMP. This is thought to inhibit sympathetically mediated augmentation of cAMP levels and bladder relaxation (Chapple, 2000). Therefore it has been suggested that activation of M_3 receptors by acetylcholine evokes direct smooth muscle contraction while stimulation of the M_2 receptors reverses sympathetically mediated smooth muscle relaxation.

The sympathetic system is inhibited directly by the increased afferent firing, leading to a decrease in sympathetic efferent outflow. This, accompanied by the activation of the parasympathetic system, leads to a more efficient voiding of urine.

1.6.3. The Lamina Propria

The lamina propria is located between the urothelium and the detrusor muscle. It consists of loose fibroelastic connective tissue, nerves, fibroblasts, blood capillaries, scattered smooth muscle cells and in some regions glands (Chancellor and Yoshimura, 2002). Recently

the existence of another type of cell, the *myofibroblast*, has been noted in the lamina propria. This cell has some of the structural characteristics of both a smooth muscle cell and a fibroblast. Reportedly, it occurs in several normal tissues, including in the stomach, testicle, prostate, liver, spleen, kidney and lung. The role of the myofibroblast in the bladder has not yet been determined, however there is evidence that it may function as a bladder stretch receptor and may, therefore, play a role in the sensation of bladder fullness (Wiseman et al., 2003). In other tissues overactivity of the myofibroblast can lead to pathological conditions; these include burn contractures, lung fibrosis, liver cirrhosis and atherosclerotic plaques. Whether the myofibroblast is important in some pathological states in the bladder has yet to be known. Future research need to be done to determine the role of the myofibroblast in both normal and pathological bladder physiology.

1.7. Innervation of the Bladder

1.7.1. Distribution of Afferent Fibres in the Bladder

All components of the lower urinary tract have to be co-ordinated to allow normal storage and evacuation of urine from the bladder. This co-ordination is achieved by a complex neural control system, in which afferent activity from the bladder plays an important role. Adequate sensory input is the prerequisite for bladder control (Andersson and Hedlund, 2002). In fact, surgical or chemical destruction of the sensory nerve fibres results in the loss of the ability to void (FitzGerald and Mueller, 2004). In the urinary bladder of humans and animals, sensory nerves have been identified in the lamina propria as well as in the detrusor

muscle. In the lamina propria, these nerves form a plexus that lies immediately beneath the urothelial lining, with some terminals reaching into the basal parts of the urothelium. In the upper dome of the bladder, the mucosal plexus is relatively sparse, however it becomes progressively denser near the basal portion of the bladder and is particularly prominent in the trigone (Gabella and Davis, 1998). Nerve endings that connect to the hypogastric nerves are mainly located in the bladder base and urethra, whereas those connecting to the pelvic nerves are more evenly distributed throughout the bladder but with predominance in the bladder body (Andersson and Hedlund, 2002). The pudendal nerve receives sensory afferent nerve fibers from the striated muscle in the urethra (Andersson and Hedlund, 2002). The hypogastric and pelvic pathways can be implicated in the sensations associated with normal bladder filling, but also with bladder pain. The pelvic and pudendal pathways convey the sensation that micturition is imminent as well as thermal sensations from the urethra (Andersson and Hedlund, 2002). In addition to relaying bladder sensation, afferent influx controls the activity in the parasympathetic, sympathetic and somatic efferent nerves to the lower urinary tract (FitzGerald and Mueller, 2004).

1.7.2. Role of Bladder Afferents in Sensation and Pain

Unlike the skin, the majority of the nociceptive afferents in visceral organs are sensory peptidergic in nature (Cervero and Laird, 2004). There are few, if any, non-peptidergic sensory afferents in the bladder (Avelino et al., 2002; Gabella and Davis, 1998). The peptidergic population expresses substance P (SP), calcitonin gene related peptide (CGRP) and the NGF high affinity receptor, trkA (Alvarez and Fyffe, 2000). The peptidergic

sensory nerves supplying the bladder are either thinly myelinated Aδ fibres or unmyelinated C fibres. Both A delta and C fibres transmit sensory information from the lower urinary tract to the spinal cord via the pelvic, hypogastric and pudendal nerves (De Groat, 1986; Janig and Morrison, 1986; Yoshimura and De Groat, 1997).

Pelvic and hypogastric A delta fibres are likely all peptidergic (see above) and constitute the afferent part of the normal micturition reflex. They exhibit no ongoing resting activity and appear to be low threshold mechanoreceptors (Bahns et al., 1987; Habler et al., 1993b; Habler et al., 1993a; Janig and Morrison, 1986). These myelinated fibres are silent when the urinary bladder is empty and during the initial phase of slow filling, which corresponds well to the fact that humans do not notice bladder filling until a certain volume has been reached (Denny-Brown and Robertson, 1933). However, after exposure to intravesical irritants, such as occurs in inflammation, the properties of Aδ fibres may change and they exhibit ongoing activity and show a heightened response to mechanical stimulation. This may lead to symptoms of bladder fullness even when there is only a small volume of urine in the bladder (Habler et al., 1990).

Most C fibres from both the hypogastric and pelvic nerves have no ongoing activity and are irresponsive to bladder distension even at high pressures (Habler et al., 1990; Morrison, 1999). These fibres are therefore called silent receptors and their role during normal bladder filling is questioned. However, after chemical irritation, the C-fibre afferents exhibit spontaneous firing when the bladder is empty and increased firing during bladder distension (Habler et al., 1990). This increased afferent input may cause hypersensitivity (urgency and pain at low bladder contents) seen in various inflammatory bladder diseases

(Habler et al., 1990; Janig and Koltzenburg, 1990). There is a small proportion of C fibres that do show mechanosensitivity. They do not respond to normal non-painful pressures in the bladder but react to pressures in the noxious range such as in over-distension and, therefore, lead to a painful sensation (Habler et al., 1990). C-fiber afferents are thus believed to have primarily nociceptive functions, although they do contribute to micturition in the fetus, in the neonatal period and when the bladder and/or the micturition reflex is damaged in adults (Chancellor and De Groat, 1999; Chancellor and Yoshimura, 2002; Cruz, 1998; Dinis et al., 2004; Fowler, 2002). In conclusion, based on the available evidence, it seems that both Aδ and C fibres in the pelvic and hypogastric nerves are able to signal noxious events and give rise to abnormal sensations such as urgency and pain.

Activity from both Aδ and C fibres is also noted during bladder contraction and is essential for the voiding reflex (FitzGerald and Mueller, 2004). In the normal bladder, activity from these fibres during contraction is not painful. The afferent firing is due to the motor activity initiated in the CNS and is recognised as such by the CNS (Wyndaele and De, 2003). This may explain why high pressures during voiding are not painful in contrast to high pressures during the passive distension of the bladder.

1.7.3. Transduction of afferent signalling from the bladder to the CNS

Normal micturition in both humans and animals occurs in response to afferent signals from the lower urinary tract, and is controlled by neural circuits in the brain and spinal cord. These circuits coordinate the activity of smooth muscle in the detrusor and urethra with that

of striated muscle in the urethral sphincter and pelvic floor (Andersson, 2004). They are believed to act as on/off switches to shift the lower urinary tract between two modes of operation: storage and voiding (De Groat, 1975). During storage, sympathetic activity to the bladder is activated while parasympathetic activity is inhibited, and during voiding the converse occurs. In adults, urine storage and voiding are subject to voluntary control mediated by the cerebral cortex. In infants, however, these switching mechanisms function in a reflex manner to produce involuntary voiding (De Groat and Booth, 1993; De Groat, 1999). Injuries or diseases of the nervous system in adults can disrupt voluntary control of micturition, and cause re-emergence of reflex micturition, resulting in over active bladder syndrome and incontinence.

1.7.3.1 CNS signalling during bladder storage for reflex voiding

During the storage of urine, distension of the bladder produces low-level bladder afferent firing. These afferent fibres synapse into the spinal cord. The primary afferent neurons of the pelvic and pudendal nerves are contained within the sacral dorsal root ganglia whereas afferent innervation in the hypogastric nerves arises in the rostral thoracolumbar dorsal root ganglia (De Groat, 1993; De Groat et al., 1997; FitzGerald and Mueller, 2004). Spinal reflex pathways cause sympathetic outflow to the bladder outlet and body via the hypogastric nerve and somatic outflow to the external urethral sphincter via the pudendal nerve, meanwhile there is an inhibition of parasympathetic activity to the bladder (Andersson, 2004; Chancellor and Yoshimura, 2002; De Groat and Theobald, 1976; De Groat, 1993). This causes bladder body relaxation and the closure of the urethral sphincter allowing bladder filling and storage. In neonates and in patients with neuropathic bladder, storage and voiding

of urine is involuntary. Once afferent firing reaches a threshold, efferent outflow is reversed so that sympathetic activity is inhibited and parasympathetic activity is activated causing bladder wall contraction and urethral sphincter relaxation so that voiding is initiated (De Groat, 1999). However as we mature voiding comes under the control of higher brain centers and becomes voluntary (FitzGerald and Mueller, 2004).

The signals of bladder filling reach into the brain stem and brain. As the bladder is filling, afferent fibres relay information about bladder fullness to the dorsal horn, which relays information to the pontine continence center, the cerulean nucleus, the ventral posterior nucleus and the periaqueductal grey matter (FitzGerald and Mueller, 2004). The pontine continence center and cerulean nucleus reflexively maintain the storage phase while the ventral posterior nucleus and the periaqueductal grey matter are important for the conscious feeling of fullness and the initiation of voiding respectively.

The pontine continence center responds to increased afferent signalling by stimulating the pudendal nerve to cause contraction of the external urethral sphincter (Athwal et al., 2001; FitzGerald and Mueller, 2004; Holstege et al., 1986; Mallory et al., 1991). Meanwhile, the locus coeruleus responds to afferent signals of bladder filling by stimulating sympathetic activity to the bladder and urethra via the hypogastric nerve which reduces detrusor tone, while heightening protective tone in urethral smooth muscle (FitzGerald and Mueller, 2004; Mallory et al., 1991). These actions are reflexively done to maintain bladder filling until voiding is stimulated. Other ascending afferent branches synapse onto the ventral posterior nucleus of the thalamus which relays the signals to the cingulate cortex; these signals are responsible for the conscious feeling of bladder filling or fullness (De Groat et al., 1998; De

Groat, 2002; FitzGerald and Mueller, 2004). Finally increased afferent activity from the bladder activates the periaqueductal grey matter which responds by lowering the threshold of the pontine micturition center (M region in the pons). As the bladder fills, there is more afferent activity from the bladder, thus more activity is relayed to the pontine micturition center, eventually leading to the activation of the voiding mechanism (Blok et al., 1997; Blok et al., 1998). If there were no suprapontine regions to control voiding, the increased afferent activity would eventually activate voiding via the pontine micturition center (this the mechanism for involuntary voiding).

1.7.3.2 CNS signalling during bladder storage for voluntary continence

Continence is maintained until a socially acceptable time by various suprapontine regions, there is limited information on these regions in the literature but there is evidence that the basal region of the right prefrontal cortex and the motor cortex play a role in inhibiting voiding. The basal region of the prefrontal cortex inhibits the cingulated cortex, the preoptic area and the periaqueductal grey matter by exciting local inhibitory internuncial neurons. Inhibition of the right anterior cingulated cortex diminishes the sense of urgency (FitzGerald and Mueller, 2004). Inhibition of the preoptic area of the hypothalamus reduces parasympathetic activity to the detrusor muscle (Athwal et al., 2001). Inhibition of the periaqueductal grey matter reduces activity in the pontine micturition center (M region of the pons), which in turn inhibits parasympathetic activity to the bladder (Barrington, 1925). In support of the effort to maintain continence, the relevant region of the motor cortex is recruited to produce mass contraction of pelvic floor muscles (Blok et al., 1998). All these actions prevent the switch from the storage phase to the elimination phase by ultimately

maintaining activity of the sympathetic system to the bladder while inhibiting parasympathetic activity to the bladder. Thus voiding is inhibited until a socially acceptable time.

1.7.3.3 CNS Signalling to Induce of Voiding

Once micturition is appropriate, the inhibitory action of the prefrontal cortex ceases, the activity of the periaqueductal grey matter is thus disinhibited and the preoptic area joins the periaqueductal grey matter in activating the pontine continence center (M region). These regions also inactivate the pontine continence center via inhibitory interneurons. Parasympathetic activity to the bladder is initiated while sympathetic activity is inhibited causing bladder wall contraction and urethral relaxation, allowing voiding. Maintaining the voiding reflex is through ascending afferent input from the bladder (Athwal et al., 2001; Barrington, 1925; Holstege et al., 1986; Mallory et al., 1991).

1.7.4. The Parasympathetic System

1.7.4.1 The Functional Role of the Parasympathetic System

As already mentioned above, activity of the parasympathetic system is essential for micturition. Parasympathetic preganglionic neurons innervating the lower urinary tract are located in the lateral part of the sacral intermediate grey matter in a region termed the sacral parasympathetic nucleus (Chancellor and Yoshimura, 2002; Morgan et al., 1981; Nadelhaft et al., 1980). Parasympathetic preganglionic axons travel in the pelvic nerve to ganglion cells in

the pelvic plexus; parasympathetic postganglionic axons are sent from the pelvic plexus to all regions of the bladder (De Groat and Booth, 1993). The detrusor muscle has a dense parasympathetic innervation throughout the bladder, while the distribution of parasympathetic fibres in the mucosa had not been examined (Andersson, 1993) until the current study. As has previously been discussed, when the parasympathetic system is active, the post-ganglionic parasympathetic fibres release acetylcholine which acts on the M2 and M3 receptors in the detrusor muscle to mediate contraction. However, there are also muscarinic receptors on the parasympathetic nerves themselves which are important for normal bladder function. The muscarinic receptors M1 and M2/M4 are located prejunctionally on cholinergic nerve endings in the bladder (D'Agostino et al., 2000; Somogyi et al., 1996). Activation of M1 prejunctional receptors facilitates acetylcholine release whereas activation of the M2/M4 receptors inhibits it. It has been proposed that inhibitory M2/M4 receptors are preferentially activated by autofeedback mechanisms during short periods of low frequency nerve activity and thereby suppress cholinergic transmission during urine storage (Braverman et al., 1998; D'Agostino et al., 1997; Somogyi and De Groat, 1992). Conversely, M1 receptors are activated during more prolonged high frequency nerve firing that would occur during voiding and thus participate in an amplification mechanism to promote complete bladder emptying (Somogyi et al., 1996; Somogyi et al., 1997). Thus, the release of acetylcholine not only acts on receptors in the bladder smooth muscle but also on the presynaptic receptors of cholinergic fibres to facilitate contraction. In fact, contractile responses can be completely abolished by atropine, thus identifying cholinergic activity to be the important mechanism responsible for bladder contraction (Sibley, 1984). Anticholinergics are the main drugs given for urinary incontinence and overactive bladder, to suppress

detrusor muscle hyperactivity and stop the involuntary bladder contractions (Chapple, 2000; Herbison et al., 2003).

1.7.4.2 The Parasympathetic System and Pain in the Bladder

Recent evidence indicates that the parasympathetic system is not only imperative in controlling bladder contraction but may also be important in bladder sensation and pain. As was discussed in the section on the urothelium, there are muscarinic receptors in the urothelium. When these cholinergic receptors are activated, ATP is released. This molecule is known to be important in the sensations of bladder fullness and pain by either directly or indirectly sensitizing primary afferent fibres (Dmitrieva et al., 1998; Pandita and Andersson, 2002). Acetylcholine may also directly activate peptidergic sensory fibres, as these sensory fibres express cholinergic receptors (Coggeshall and Carlton, 1997). For this sensitization to occur, there must be a close proximity between the varicosities from the parasympathetic fibres and the sensory fibres as acetylcholine is promptly destroyed by cholinesterases, as has been pointed out in a publication from our laboratory (Ramien et al., 2004). Thus there is evidence that, along with being essential for causing bladder contraction, the parasympathetic system may be important in sensations of bladder fullness and pain.

1.7.5. The Sympathetic System

1.7.5.1 The Functional Role of the Sympathetic System

The sympathetic system is essential for urine storage in the bladder. Activation of sympathetic nerves induces relaxation of the bladder body and contraction of the urethra (Andersson, 1993; Chancellor and Yoshimura, 2002). Sympathetic preganglionic pathways

emerge from the lumbar spinal cord and pass to the sympathetic chain ganglia and then, through the inferior splanchnic nerves, to the inferior mesenteric ganglia. Preganglionic and postganglionic sympathetic axons then travel in the hypogastric nerve to the pelvic plexus and then to the bladder body and to the urethra (Kihara and De Groat, 1997). Most sympathetic fibres are found in blood vessel wall and in the smooth muscle of the bladder and the urethra. In the normal bladder, the sympathetic system activates β adrenergic receptors in the detrusor and α adrenergic receptors in the urethra, causing relaxation of the bladder body and contraction of the urethra, respectively, and allowing for urine storage. Under normal conditions, α adrenergic stimulation is not very prominent in the bladder wall. However, under pathological conditions, the α adrenergic receptor density can increase to such an extent that the norepinephrine-induced responses in the bladder are converted from relaxation to contraction. It has been hypothesized that this shift in response may contribute to the bladder hyperactivity observed in incontinence and obstructive uropathy (Andersson, 1997). It has been demonstrated that unstable human bladders have significantly lower levels of muscarinic receptors and significantly higher levels of α adrenergic receptors compared to normal human bladders (Lepor et al., 1989).

1.7.5.2 The Sympathetic System and Pain in the Bladder

The sympathetic system has been shown to play a role in pathological pain in many systems including the skin (Grelik et al., 2005). It has been suggested that sensory fibres express α_2 receptors and that activation of these receptors causes sensitization of the afferent fibres (Sato and Perl, 1991). Although the sympathetic system in the bladder has also been implicated in painful disorders of the bladder (Peeker et al., 2000), we did not find any

evidence that the sympathetic system plays a role in cyclophosphamide-induced cystitis, as we did not observe any morphological changes in the sympathetic innervation (unpublished observations).

1.8. Painful Conditions of the Bladder

The most common chronic pain disorder of the bladder is interstitial cystitis (IC). In the USA the prevalence has been estimated to be between 30 and 67 per 100,000 human beings (Curhan et al., 1999; Tincello and Walker, 2005). The prevalence may indeed be much higher. One study (Hanno and Sant, 2001)theorizes that, after correcting for misdiagnosis, closer to 10.8 million patients have IC in the United States. IC is a term given to a chronic relapsing syndrome of bladder pain associated with urgency, frequency and nocturia in the absence of any other identifiable pathology (urinary tract infection, bladder carcinoma or cystitis induced by medication or radiation) (Butrick, 2003). A set of inclusion and exclusion criteria for the diagnoses of interstitial cystitis have been established by the National Institutes of Arthritis, Diabetes, Digestive and Kidney diseases (Gillenwater and Wein, 1988). Although mostly a diagnosis of exclusion, one of the criteria is that patients must present either glomerulations (pinprick haemorrhaging) or Hunner's ulcers, identified by cystoscopy with hydrodistension. Although IC is not a rare disease, it is not widely known in the medical field. Typically IC is misdiagnosed as something else or ignored altogether for 4-7 years before finally being identified as IC (Butrick, 2003). In fact, medical providers frequently trivialize the illness experiences that IC patients report, thus delaying further referral for further evaluation and treatment (Azevedo and Payne, 2001). The severity of this disease can vary from being merely annoying to being disabling. An ethnographic study

indicated that interstitial cystitis symptoms substantially impact the patient's quality of life, affecting mood, social relationships, employment and education opportunities as well as family relationships (Azevedo and Payne, 2001). In one study, 46% of patients with IC reported moderate or serious depression (Tincello and Walker, 2005). More than half of the IC patients report daily or constant pain (Koziol, 1994) and there are several cases where the pain was so unbearable that the bladder was removed from the patient (Baskin and Tanagho, 1992). Interestingly, in a majority of the cases, this did not lead to a resolution of pain postulating that once the cause leading to the onset of IC has resolved, there are nervous changes that leave behind a chronic visceral pain syndrome (Baskin and Tanagho, 1992). The pain presenting in these patients is most often described as "pressure" or "aching" (Wesselmann, 2001). Other types of pain reported are "shooting pains" and "burning pain". Patients with interstitial cystitis may feel pain in their lower back, urethra, vaginal or penis as well as in their bladder (Wesselmann, 2001) Once identified, IC is difficult to treat: the effectiveness of drugs for the disease are only slightly better than placebo (Chancellor and Yoshimura, 2004; Tincello and Walker, 2005). The lack of effective treatment is due to the lack of knowledge about the disease. Scientists have debated the etiology of IC ever since it was first identified by AJC Skene in 1897. Leading theories of pathogenesis include 1) change in urothelial permeability; 2) increased mast cell activity; 3) neuron-immune abnormalities; 4) neuroplasticity of the nervous system; 5) infectious etiologies. One reason why this debate continues may be because there is no single etiology of IC and any one of the proposed etiologies may in fact be the trigger for an individual patient. However, the key to understanding IC is to understand the neuropathology that develops as a result of prolonged inflammatory or noxious stimuli (Butrick, 2003; Driscoll and Teichman, 2001). It is

hypothesized that the neural changes following bladder insult result in a self-perpetuating chronic pain syndrome.

1.9. Rationale for Study

As has previously been discussed in this thesis, the peptidergic sensory, parasympathetic and sympathetic systems can all contribute to pain and other urinary symptoms in the bladder. Thus, our objective in this thesis was to determine whether a change in peptidergic and autonomic innervation in the bladder could be a possible mechanism contributing to prolonged bladder pain. A possible trigger for interstitial cystitis is bladder infection or inflammation (Butrick, 2003; Driscoll and Teichman, 2001; Sadhukhan et al., 2002), thus we set out to determine whether autonomic and afferent innervation changes occur following bladder inflammation. It has been established that sprouting of autonomic and sensory fibres is correlated to pain in the skin (Grelik et al., 2005). Although there have been no studies done to correlate pain with peripheral sprouting in visceral organs, there is evidence that there is increased autonomic and peptidergic sensory innervation in the bladders of patients with interstitial cystitis (Christmas et al., 1990; Clemens et al., 2005; Pang et al., 1995; Peeker et al., 2000) . Also, the treatments offered for interstitial cystitis implicate that neurological changes in the autonomic and sensory fibres play a role in causing the symptoms of IC (Gosling et al., 1977; Lasanen et al., 1992a; Lasanen et al., 1992b). Previous studies have shown an increase in neurotrophic factors in the bladder following inflammation in both animal models and humans (Vizzard, 2000). With an increased expression of neurotrophic factors there is a possible mechanism for the sprouting of autonomic and peptidergic sensory fibres. Therefore due to the evidence implicating a

neurological component to bladder pain and other urinary symptoms we generated the following hypothesis:

HYPOTHESIS: Sprouting of autonomic and sensory fibres in the bladder mucosa and muscle following chronic inflammation may contribute to long-term pain and other urinary symptoms.

We used a well established model of chronic bladder inflammation to look at changes in the parasympathetic and peptidergic sensory fibres following bladder inflammation as preliminary results suggested that there are no significant changes in the sympathetic fibre density in the bladder following inflammation. By evaluating autonomic and sensory innervation changes following inflammation, we hoped to uncover possible mechanisms that lead chronic bladder pain.

CHAPTER TWO:

MATERIALS AND METHODS

Experimental Procedures

2.1 Animals

Female Sprague-Dawley rats (200-250g), obtained from Charles River (Saint-Constant, QC, Canada), were used in all experiments. Female rats were used due to anatomical reasons; the bladders are much easier to extract.

2.2 Induction of Cystitis

Eight rats were treated with 70mg/kg cyclophosphamide diluted in saline, i.p., once every 3 days until their 12 day experimental endpoint. Control animals (n=7) were given saline only.

2.3 Preparation of Tissue

At the experimental endpoint of 12 days, all animals were deeply anaesthetized with Equithesin (6.5mg chloral hydrate and 3 mg sodium pentobarbital in a volume of 0.3mL, i.p./ 100g body weight). The abdomen was opened up and the bladders manually emptied. The rats were then perfused intracardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The bladders were then excised, weighed and post-fixed for 2 hours in the same fixative at 4°C. Subsequently, the bladders were cryoprotected by infiltration with 30% sucrose in phosphate buffer for 48 h at 4°C. Under a dissecting microscope, each bladder was cut into 4 specific regions: upper dome, body, lower body and trigone. Tissue from each region of the bladder was then embedded in an Optimal Cutting Temperature compound (OCT; Tissue-Tek), sectioned into 12 μm-thick cross sections on a cryostat (Leica Canada),

and collected onto gelatine-subbed slides. To avoid any confusion, tissue from each region of the bladder was placed on a separate slide.

2.4 Histological Staining

To qualitatively assess inflammatory changes in the bladder, sections from each bladder region of the cyclophosphamide treated rats and the control rats were processed for haematoxylin and eosin staining as described in detail elsewhere (Miao et al., 2004). The haematoxylin and eosin used were purchased from Surgipath Canada.

2.5 Immunofluorescence

Bladder sections processed for immunofluorescence were attached to slides. All steps were carried out at room temperature, unless stated otherwise

2.5.1 *Single Labelling for Quantification.*

Sections were treated with 50% ethanol for 30 minutes and then rinsed for 30 min in 3 changes of phosphate-buffered saline (PBS) with 0.2% Triton X-100 (+T). To block non-specific staining, tissue was placed in 10% normal horse serum (NHS) for 60 minutes and then incubated for 48 hours, at 4°C, with one of two primary antibodies, in a humid chamber. The antibody used to stain for parasympathetic fibres was a rabbit polyclonal serum against the vesicular acetylcholine transporter (VAChT; 1:5000; Gilmor et al., 1996). The anti-VAChT antibody was generated against a fusion protein containing the amino acid sequence from 478-530 of the C terminus of the VAChT molecule; its specificity has been studied by Western blot analysis and shown to be highly specific for VAChT (Gilmor et al., 1996). The

ability of this antibody to detect specifically cholinergic fibres in the central and peripheral nervous systems has been well established (for recent review, see Ramien et al., 2004). The antibody used to label sensory peptidergic fibres was a rabbit polyclonal against calcitonin gene related peptide (CGRP; 1:2000; Sigma). This antibody was raised against rat CGRP and does not cross-react with any other peptide except human CGRP and rat and human βCGRP (data supplied by Sigma). Primary antibodies were diluted in a solution containing 10% NHS in PBS+T. After 48 hours of incubation with either the anti-CGRP or anti-VAChT antibodies, the sections were thoroughly rinsed with PBS+T (3 washes of ten minutes each). The sections were then incubated in a solution of donkey anti-rabbit IgG conjugated to Rhodamine Red-X (1:200; Jackson Immunoresearch) in 5% NHS diluted in PBS+T for two hours. Sections were then washed in PBS for 40 minutes (4 washes, ten minutes each). The slides with the sections were then let to dry overnight, coverslipped with Aqua Polymount (Polysciences) and kept at 4°C until examined. Sections from control and experimental groups which were used for quantification were processed simultaneously, applying the same batches of antibodies.

2.5.2 Double Labelling for VAChT and CGRP.

Sections were treated with 50% ethanol for 30 minutes and then rinsed in PBS+T for 30 minutes (3 washes of ten minutes each). For blocking non-specific staining, tissue was placed in 10% normal horse serum (NHS) and 10% normal goat serum (NGS) in PBS+T for 60 minutes. Sections were incubated in a mixture of the VAChT antibody generated in rabbit and a guinea-pig anti-CGRP primary antibody (1:800; Peninsula), diluted in a solution containing 5% NHS and 5% NGS in PBS+T and placed, for 48 hours, at 4°C in a humid chamber. After washing with PBS+T, sections were incubated with a biotinylated goat anti-

rabbit IgG (1:200; Vector laboratories) in PBS+T for two hours. Subsequently, sections were washed with PBS+T and incubated in a mixture of the secondary antibodies for 2 hours. The secondary antibodies used were Alexa Fluor 488 conjugated to streptavidin (1:200; Molecular Probes) and donkey anti-guinea pig IgG conjugated to Rhodamine Red-X (1:200; Jackson Immunoresearch). The sections were then washed with PBS, air-dried, coverslipped and placed in the refrigerator until observed under the microscope. The material was examined with both the Zeiss Axioplan 2 Imaging fluorescence microscope, using a X40 objective, and Zeiss LSM 510 confocal microscope equipped with Argon and Helium Neon lasers and a X63 oil-immersion plan-apochromatic objective. The confocal microscope was used to rule out any possibility of co-localisation of CGRP and VAChT immunoreactivities within the same fibres. For this purpose, a multitrack approach was used together with a very small pinhole to obtain optical sections of less than 0.5 μm.

2.5.3 Immunocytochemical controls.

To ensure that the immunostaining was specific, some sections of bladder were immunostained using primary antibodies pre-absorbed with the corresponding blocking peptides for the anti-VAChT antibody or for the anti-CGRP antibodies (both the rabbit and guinea-pig). Other sections were incubated with pre-immune serum of the same species in which the antibodies were generated instead of the primary antibody. No staining was observed in any of these experiments.

2.6 Quantitative Analysis of Fibre Density.

For the quantification of both the parasympathetic and sensory fibres, only material processed for single labelling was used. A total of ten animals were used, 5 of which were from the control group and 5 from the cyclophosphamide-treated group. From each animal ten sections per immunostaining were chosen at random per region (upper dome, body, lower body and trigone) to be quantified. Four pictures per section were then randomly taken from each of the above 4 regions of the bladder. Therefore, per immunostaining, we obtained a total of 40 pictures per region of bladder per animal. These images were captured with a Zeiss Axioplan 2 Imaging fluorescence microscope, equipped with a high resolution colour digital camera and connected to a computer with Zeiss Axiovision 4.1 Software (Zeiss, Canada). Micrographs were obtained with a X20 objective and were stored in the Zeiss Axiovision format. To avoid variation that could affect the quantification, the exposure settings of the digital camera were kept constant for all micrographs.

The calibrated images were then exported to the TIFF format and analyzed with an image analysis system (MCID Elite version 7, Imaging Research Inc., St. Catharines, ON, Canada). Fibre length was assessed using a tool in the program configured to measure total fibre length per unit area in a given area of the image. This approach involved skeletonization of the fibres, meaning that each fibre was reduced to a thickness of 1 pixel. In each section, the mucosa and the detrusor muscle were quantified separately. The area of measurement corresponded to the portion of either the mucosa or muscle present in each image and was drawn with computer mouse. When quantifying the muscle, the connective tissue between the smooth muscle was excluded from the measured area.

Values were expressed as total fibre length per unit area per region ± SEM, in the mucosa or muscle. To obtain these values, we calculated the average density of fibres per region in each rat (based on the measurement of 40 pictures) and used these numbers to find the means and SEM for each treatment group per region. To analyse whether there was an overall change in the innervation of the mucosa or muscle of the bladder, we calculated the average length of fibres per unit area after pooling the data from all 4 regions.

For statistical comparisons, we used either an unpaired t-test or a Mann-Whitney test. The objective was to determine whether the treatment group was significantly different from the control group. Since assumptions of t-tests are that the standard deviations are similar and that there is a Gaussian distribution, we determined whether the treatment groups followed these assumptions. If there was a failure to meet one or both of these assumptions, the non-parametric test Mann-Whitney was used. Values of $P < 0.05$ were considered as statistically significant.

2.7 Behaviour

On day 10, all rats were tested for frequency and for low volume voids. The short term voluntary urination in freely moving rats was assessed using a simple behaviour test. Water was taken away from the rats two hours before the test. The rats were placed individually in cages lined with filter paper (with no bedding). After a twenty-minute period, the numbers of small and large diameter urine spots were counted. A UV light source was used to visualize and trace the urine spots on the filter paper. The total number of spots was used to determine urinary frequency. The diameter of the spots was used to roughly determine the volume of each voiding (Birder et al., 2002). Spots with a diameter larger than

0.5 cm were considered to be normal volume voids. The total number of voids and the number of low volume voids were compared between the control and treatment group. Statistical comparisons we carried out using the unpaired Student's t-test or the non-parametric Mann Whitney test. Values of $P < 0.05$ were considered as statistically significant.

Furthermore, all rats were also qualitatively assessed for known signs of pain such as piloerection and a rounded-back posture.

CHAPTER THREE:

RESULTS

3.1 Pattern of innervation in the normal bladder

3.1.1 Mucosa

Using CGRP immunoreactivity as a marker of the distribution of peptidergic afferent axons, we have found that the innervation of the mucosa in the upper dome and body was rather sparse (Figs.1A, 1C and 2A), with extensive areas devoid of any fibres, particularly in the upper dome. This afferent innervation became progressively denser as we approached the neck of the bladder, and was particularly prominent in the trigone (Fig. 2A). These data concurs with what was previously found (Gabella and Davis, 1998).

We also studied the distribution of cholinergic (VAChT-immunoreactive) fibres in the mucosa (Figs. 2B, 3A, 3C and 3E) and found that these fibres had a similar distribution to the peptidergic sensory fibres, being the sparsest in the upper dome (Figs. 2B, 3A) and becoming progressively denser approaching the trigone (Fig. 2B).

The double-labelling study showed that, in the mucosa, peptidergic and cholinergic fibres were close in proximity and often wrapped around each other (Fig. 4A). In the regions where they wrapped around each other, the varicosities of VAChT- and CGRP-IR fibres were sometimes in close apposition. However, the two fibre types represented entirely separate populations, as no double-labelled fibres were found.

3.1.2 Muscular layer

We also studied (Fig. 5) and quantified (Figs. 6 and 7) the distribution of both CGRP- and VAChT-immunoreactive (IR) fibres in the detrusor muscle of the different regions of the normal bladder and did not find any significant differences in the density of these fibres between regions. (Figs. 6 and 7). Like in the mucosa, the parasympathetic and sensory

afferent fibres in the muscle formed a close association with one another, but always represented completely independent populations (data not shown).

3.2 Cyclophosphamide-induced cystitis

3.2.1 Histopathology

All cyclophosphamide-treated rats displayed oedema, red blood cell extravasation and leukocyte infiltration in the lamina propria of the mucosa. The inflamed bladders also had urothelial tears in some regions and, occasionally, thickening of the urothelium (Fig. 8B). None of these signs was present in vehicle treated animals (Fig. 8A). The weight of the bladders from cyclophosphamide-treated rats was on average about 120 mg, whereas, in saline-treated animals, bladders were around 80 mg.

3.2.2 Behaviour

After ten days we found that that the rats with cystitis had lower volume voids than the control rats ($p<0.01$) and had increased urinary frequency ($p<0.05$) (Fig. 9). We also qualitatively observed that, on day 10, all rats with bladder inflammation showed piloerection while none of the normal rats showed this behaviour. Six out of eight rats with cystitis showed a hunched back position while none of the control rats displayed this position ($n=7$). These behaviours were interpreted to be pain related (Boucher et al., 2000)

3.3 Innervation changes following cyclophosphamide administration

3.3.1 CGRP-IR fibres in the mucosa

We noticed a striking increase in the density of CGRP-IR fibres in the mucosa of the upper dome and body of the bladders from animals with cystitis when compared to vehicle-treated rats (Figs. 1 and 10A). Although the differences were not significant in the lower body and trigone, there was a trend towards an increase in peptidergic innervation in these two regions of the inflamed bladders (Fig. 10A). When we pooled together the data from all regions of the bladder, however, we detected an overall increase in the density of CGRP-IR fibres in the mucosa of the inflamed bladders (Fig. 10B).

3.3.2 VAChT-IR fibres in the mucosa

There were prominent changes in the density of VAChT-IR fibres in the mucosa following inflammation. There were very significant increases in the innervation by VAChT-IR fibres in the upper dome, body and lower body (Figs. 3 and 11A), but not in the trigone (Fig. 11A). Overall, the inflamed bladders had a much higher density of VAChT-IR fibres in the mucosa than controls (Fig 11B). As shown in Figures 4B and 4C, these VAChT-IR fibres wrapped around CGRP-IR fibres and the varicosities of the two fibre types were often closely apposed (Fig. 4C). Although no direct quantification was attempted, it was very clear that overall the number of apposed VAChT-IR and CGRP-IR varicosities was much higher in the inflamed than in the normal bladders.

3.3.3 Peptidergic fibres in the muscular layer

There was a trend towards an increase in the density of CGRP-IR fibers in the muscle layer in all regions of the inflamed bladders compared to controls (Fig. 6A). Such increase, however, only reached significant levels in the body region (Figs. 5A, 5B and 6A). Overall throughout the bladder there was an increase in density of CGRP-IR fibres in the muscular layer of the inflamed bladders when compared to controls (Fig. 6B).

3.3.4 VAChT-IR fibres in the muscular layer

There was a significant increase in density of VAChT-IR fibres in the upper dome muscle of the inflamed bladders (Figs. 5C, 5D and 7A). No significant differences in the density of VAChT-IR fibres in the muscular layer occurred in the body, lower body and trigone regions of the inflamed bladders when compared to controls (Fig. 7A). Overall, there was no significant difference in the density of VAChT-IR fibres in the muscle of inflamed bladders compared to controls when all regions were pooled together (Figure 7B).

Figure 1

Figure revised from a website illustration of the anatomy of the human male urinary bladder. The regions and layers of the bladder are depicted in this image. Figure was modified from http://www.malecare.org/bladder.jpg

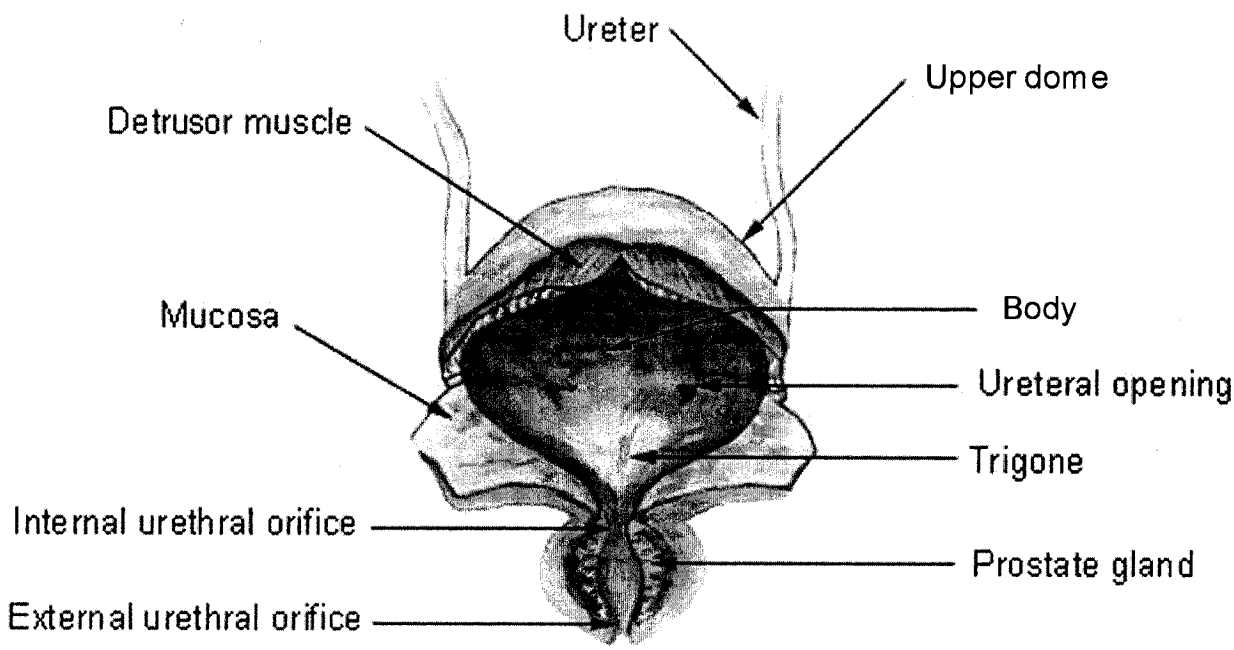

Ureter
Upper dome
Detrusor muscle
Mucosa
Body
Ureteral opening
Trigone
Internal urethral orifice
Prostate gland
External urethral orifice

Figure 2

Micrographs showing the normal innervation by CGRP-IR fibres of the mucosa of urinary bladder in the upper dome (A) and body (C), and changes in cyclophosphamide cystitis (B and D). Note that in vehicle-treated animals (**A** and **C**), CGRP-IR fibres were sparse in the upper dome and body (and not detected in the two micrographs show here). In contrast, cyclophosphamide-treated animals (B and D) displayed a novel innervation by CGRP-IR fibres. These new fibres penetrated into the urothelium (arrows) and were also found in the lamina propria (arrowheads) close to the urothelium. Ur, urothelium; LP, lamina propria; DM, detrusor muscle; cyclo, cyclophosphamide. Scale bar applies to all micro micrographs.

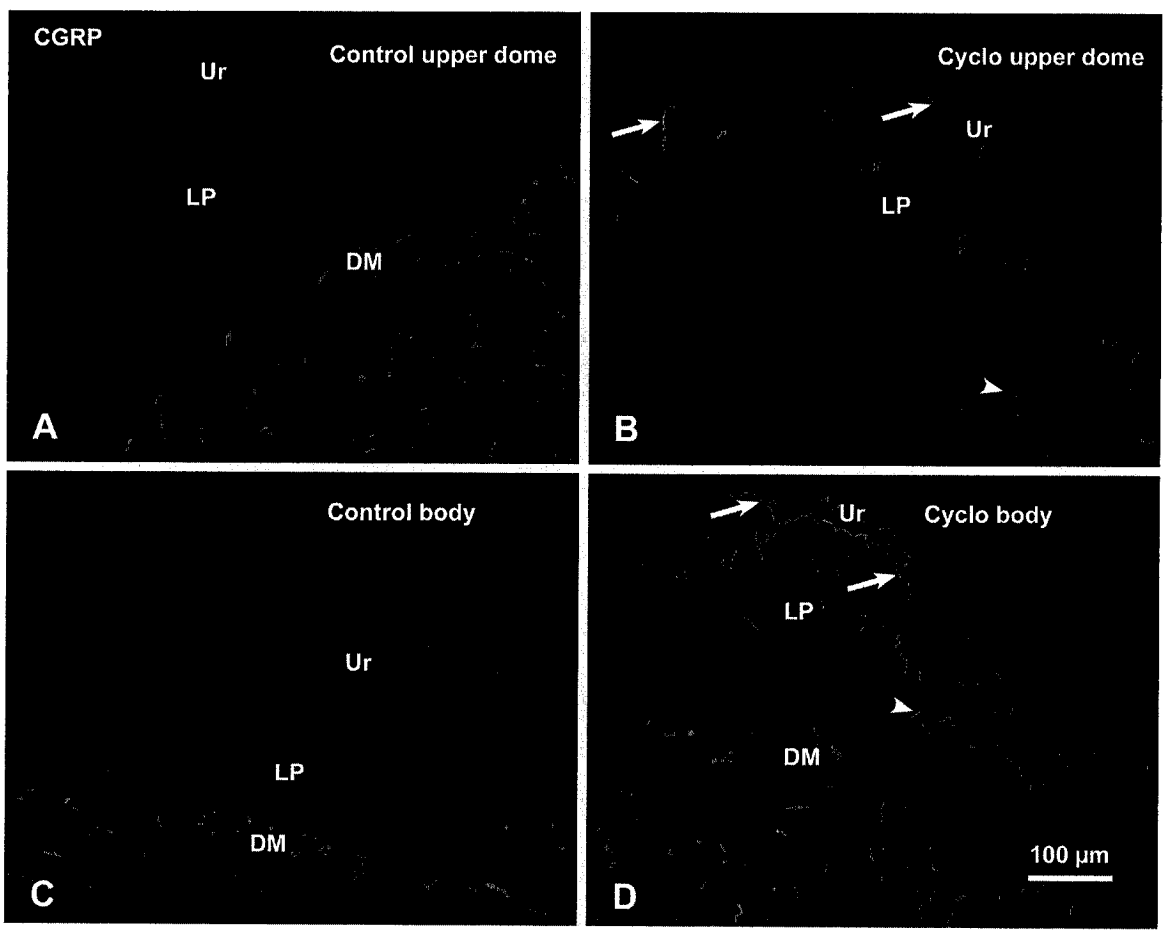
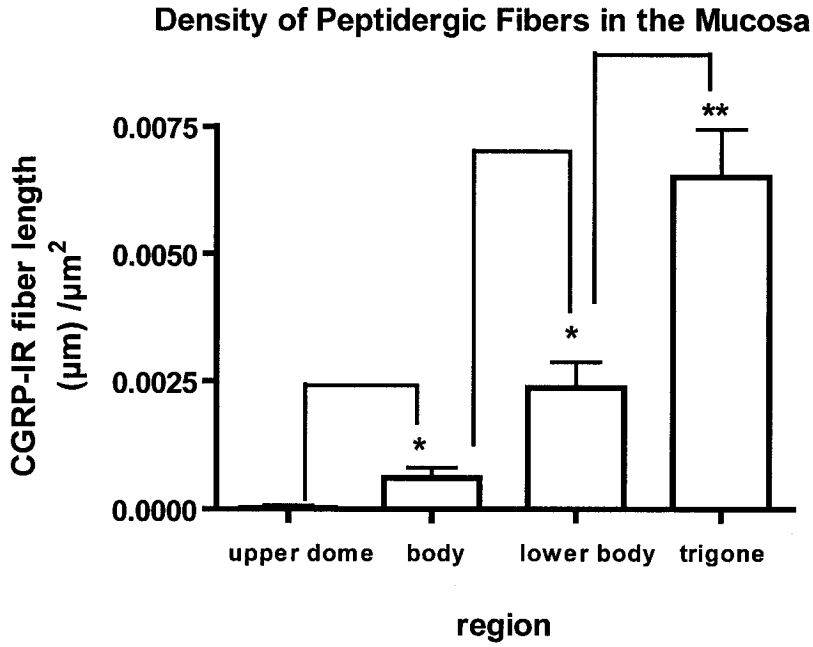

CGRP
Control upper dome
Ur
LP
DM
A
Cyclo upper dome
Ur
LP
B
Control body
Ur
LP
DM
C
Ur
Cyclo body
LP
DM
100 µm
D

Figure 3

Normal distribution of peptidergic and parasympathetic fibres in the mucosa in the various regions of the bladder. Both populations of fibres became progressively denser from the upper dome to the trigone. These differences with the region were statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

A


Density of Peptidergic Fibers in the Mucosa
CGRP-IR fiber length (µm) /µm2
0.0075
0.0050
0.0025
0.0000
*
*
**
upper dome
body
lower body
trigone
region

B

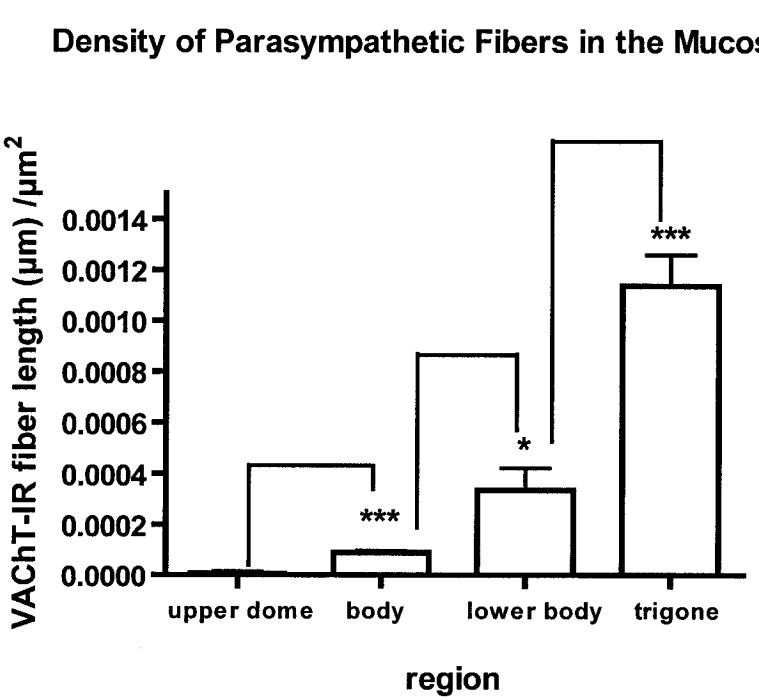

Density of Parasympathetic Fibers in the Mucosa
VAChT-IR fiber length (µm) /µm2
0.0014
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
***
*
***
upper dome
body
lower body
trigone
region

Figure 4

Micrographs showing the normal innervation by VAChT-IR fibres of the mucosa of urinary bladder in the upper dome (A), body (C) and lower body (E), and changes in cyclophosphamide cystitis (B, D and F). In vehicle-treated animals (**A** and **C**), VAChT-IR fibres were sparse in the upper dome and body (and not detected in the two micrographs show here), and slightly more abundant in the lower body (arrowhead in F). In contrast, cyclophosphamide-treated animals (B and D) displayed a novel innervation by CGRP-IR fibres in the upper dome and body and a more abundant innervation than vehicle-treated animals in the lower body (F). Like for the CGRP-IR, the sprouted VAChT-IR fibres were particularly abundant close to the urothelium (arrowheads). Ur, urothelium; LP, lamina propria; DM, detrusor muscle; cyclo, cyclophosphamide. Scale bar applies to all micrographs.

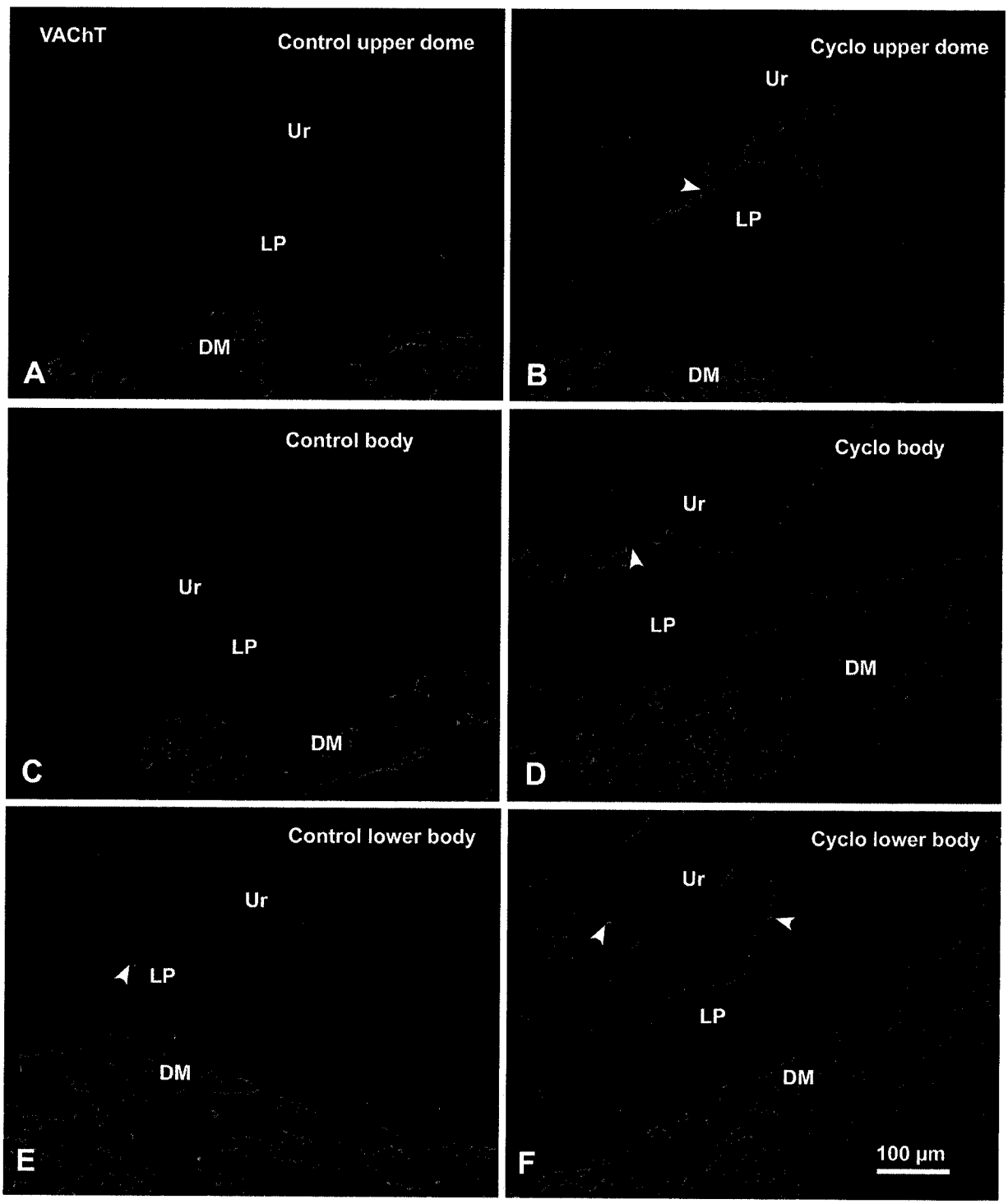

VAChT
Control upper dome
Ur
LP
DM
A
Cyclo upper dome
Ur
LP
B
DM
Control body
Ur
LP
DM
C
Cyclo body
Ur
LP
DM
D
Control lower body
Ur
LP
DM
E
Cyclo lower body
Ur
LP
DM
F
100 µm

Figure 5

Micrographs illustrating the complete absence of co-localization of CGRP and VAChT immunoreactivities in the bladder of vehicle-treated and cyclophosphamide-treated rats. In **A**, note the close proximity of VAChT-IR and CGRP-IR fibres in the mucosa of from a vehicle-treated rat, which wrapped around each other where indicated with an arrowhead. In **B**, note that in cyclophosphamide-treated rats there was an increased number of CGRP-IR and VAChT-IR fibres in the mucosa, which frequently wrapped around each other (arrowhead). In **C,** the complete absence of co-localization of the two markers within the same fibres was confirmed using confocal microscopy; arrowheads indicate areas of extensive wrapping around each other of the two fibre types. Note also that the varicosities of the fibres of the two types were in close proximity. Ur, urothelium; LP, lamina propria; DM, detrusor muscle; cyclo, cyclophosphamide.

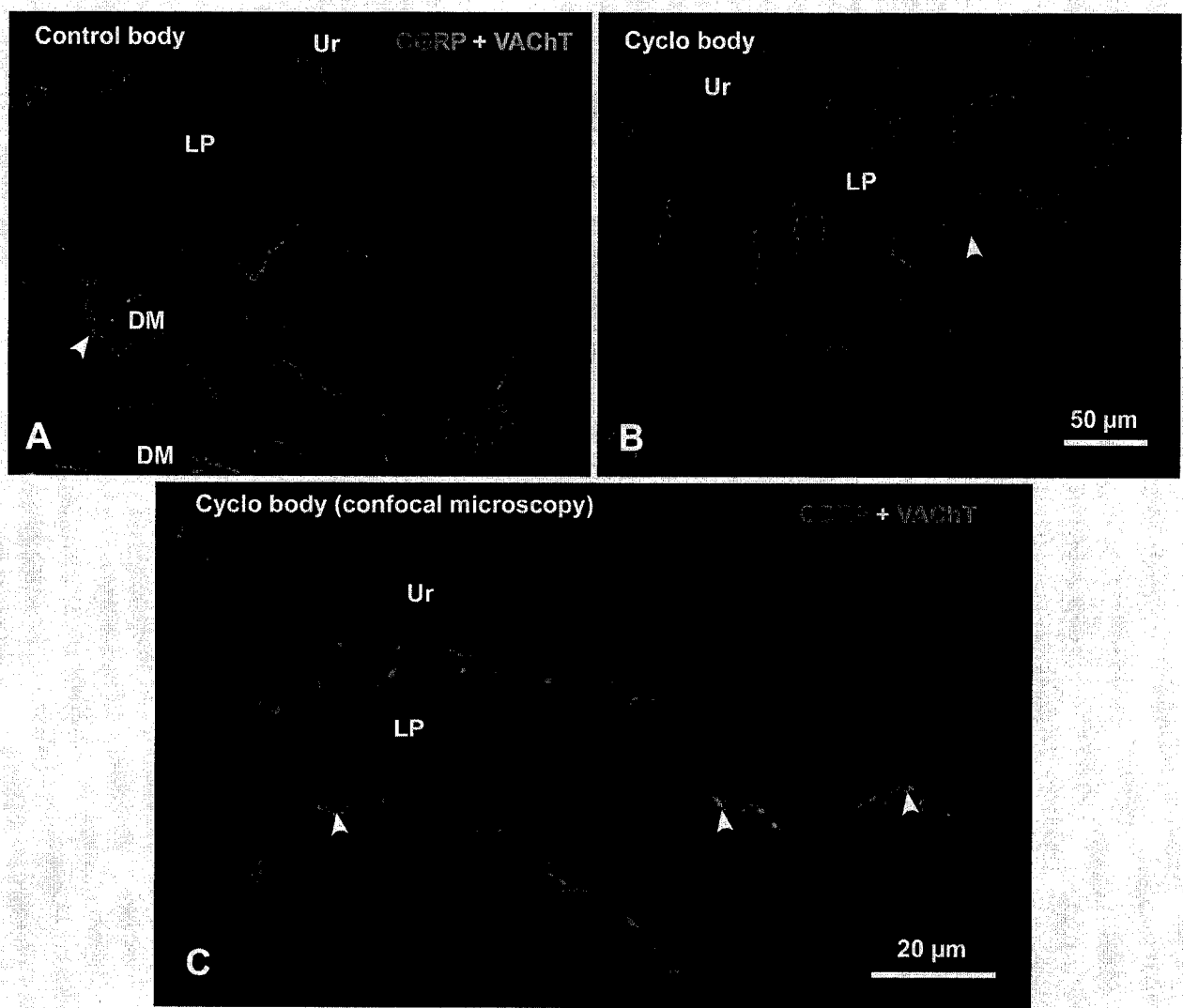

Control body
Ur
CGRP + VAChT
LP
DM
A
DM
Cyclo body
Ur
LP
B
50 µm
Cyclo body (confocal microscopy)
+ VAChT
Ur
LP
C
20 µm

Figure 6

Micrographs of the innervation of the detrusor muscle by CGRP-IR (A and B) and VAChT-IR (C and D) fibres in vehicle-treated and cyclophosphamide-treated rats. In vehicle-treated animals, the density of CGRP-IR fibres in the detrusor muscle was much lower than that of the VACT-IR in all regions of the bladder (compare **A** and **C**). **B** and **D**, from cyclophosphamide-treated rats, show representative examples of increased densities relative to vehicle-treated animals (**A** and **C**) of CGRP-IR and VAChT-IR fibres. It should be noted, however, that the overall density of VAChT-IT fibres in muscle was not significantly higher in cyclophosphamide-treated rats. DM, detrusor muscle; cyclo, cyclophosphamide.

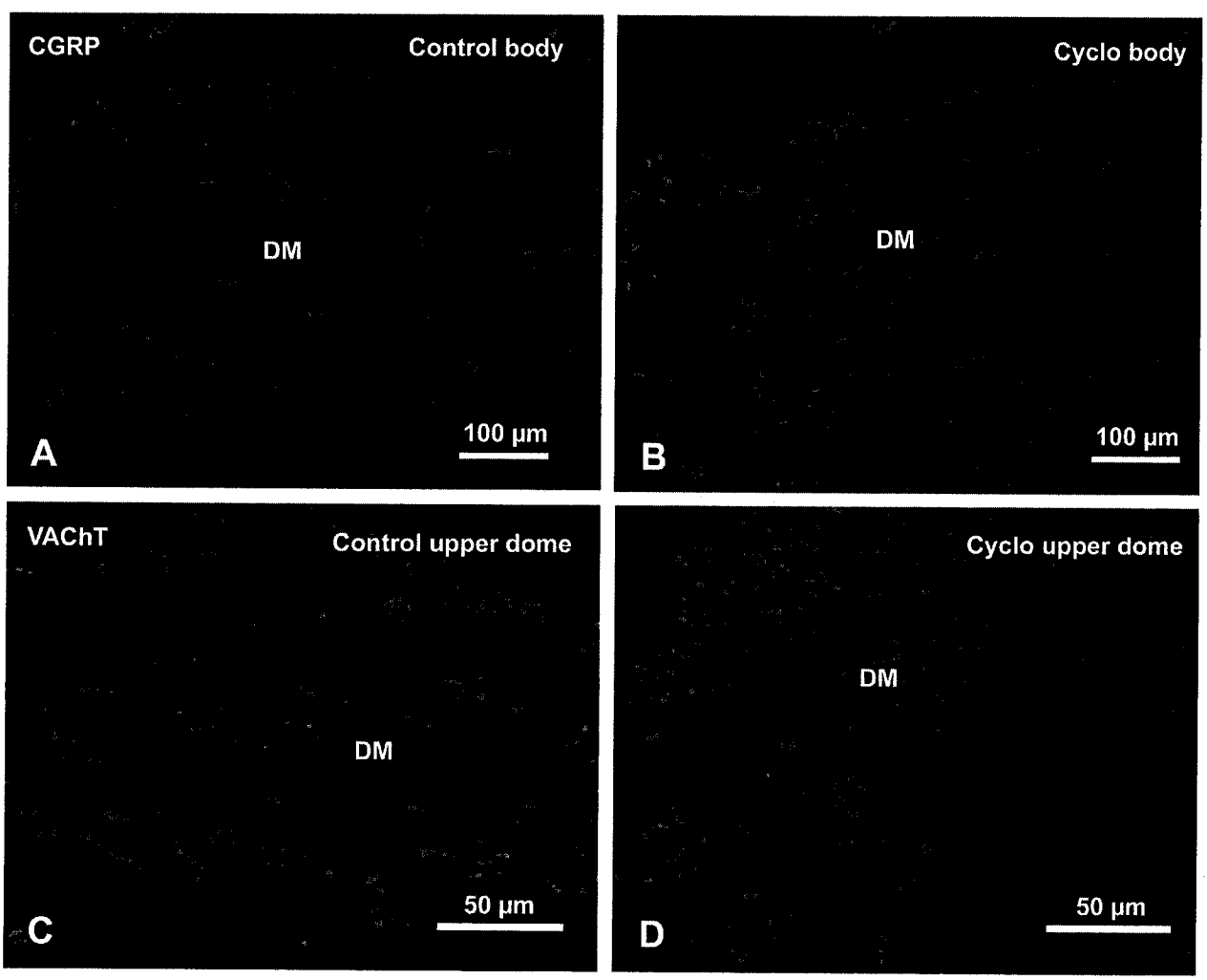
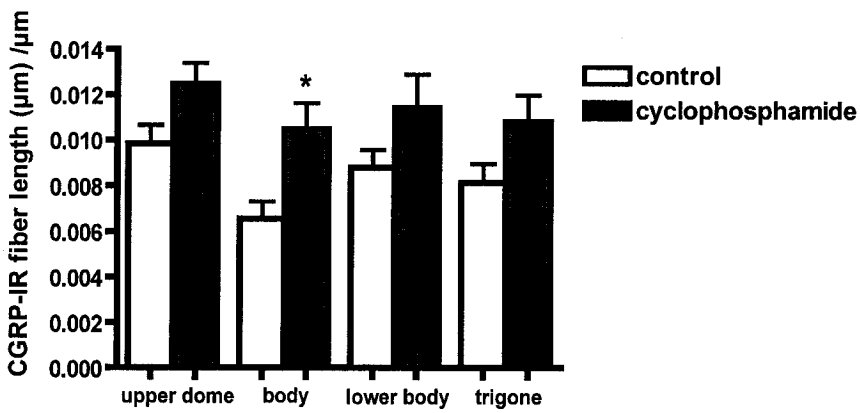

CGRP
Control body
DM
100 µm
A
Cyclo body
DM
100 µm
B
VAChT
Control upper dome
DM
50 µm
C
Cyclo upper dome
DM
50 µm
D

Figure 7

Changes in the density of CGRP-IR fibres in the detrusor muscle following cyclophosphamide cystitis. A - There was only a significant difference in the body muscle between the treatment groups, the inflamed bladder having increased CGRP-IR fibres density ($p<0.05$). **B** - Overall, throughout the bladder, the muscle from cyclophosphamide-treated animals had a higher density of CGRP-IR fibres than in vehicle-treated rats ($p<0.05$).

A Density of Peptidergic Fibers in the Muscle



B Overall Density of Peptidergic Fibers in the Muscle

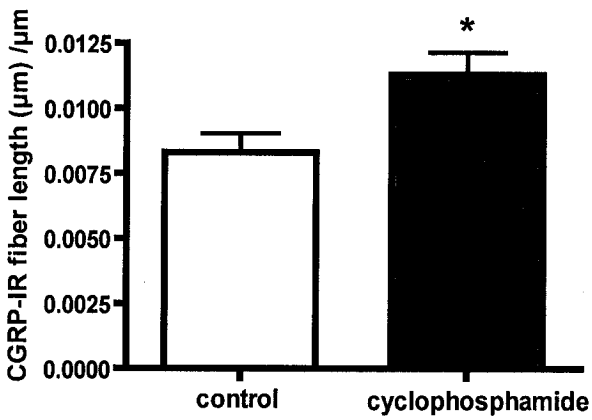
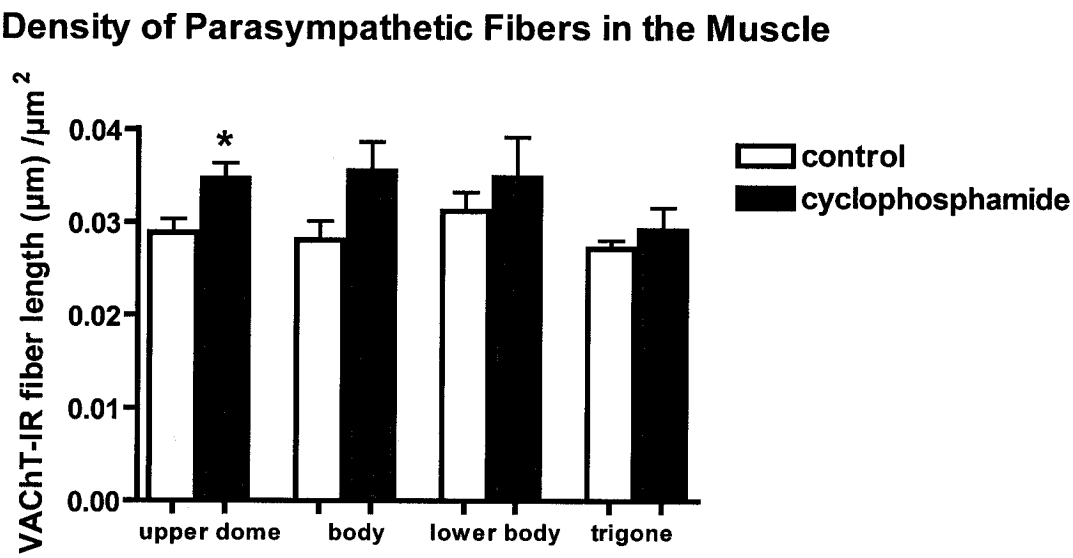


Figure 8

Changes in the density of VAChT-IR fibres in the detrusor muscle following cyclophosphamide cystitis. A – Concerning changes per region, there was only a significant increase in density of fibres in the muscle of the upper dome while in the other regions there is no significant difference from the controls. **B** - Overall there were no significant differences in VAChT-IR fibre density between treatment groups in the muscle of the bladder.

A


Density of Parasympathetic Fibers in the Muscle
VAChT-IR fiber length (µm) /µm 2
0.04
0.03
0.02
0.01
0.00
*
upper dome
body
lower body
trigone
control
cyclophosphamide

B

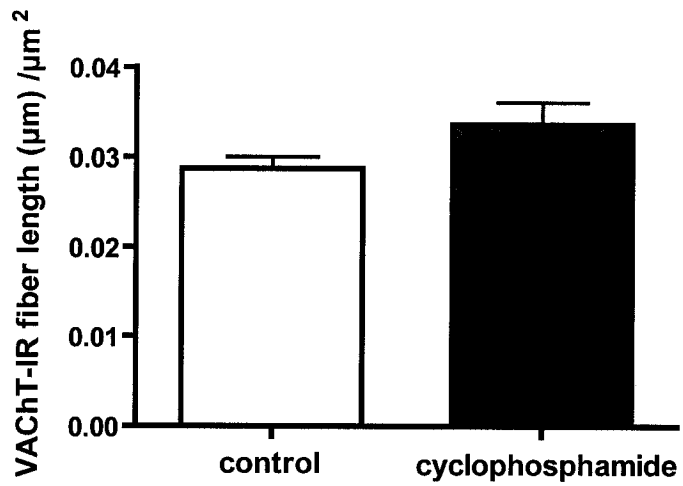

Overall Parasympathetic Fiber Density in the Muscle
VAChT-IR fiber length (µm) /µm 2
0.04
0.03
0.02
0.01
0.00
control
cyclophosphamide

Figure 9

Haematoxylin and eosin staining in sections of the body of the bladder from saline (A) and cyclophosphamide-treated (B) rats. In **A**, note the normal morphology of the mucosa, which comprises the urothelium (Ur) and the lamina propria (LP). Observe also the normal morphology of the smooth muscle of the bladder (DM). In **B**, note the edema, red blood cell extravasation (arrows), thickening of the urothelium, with a discontinuity of the epithelial lining (arrowhead).

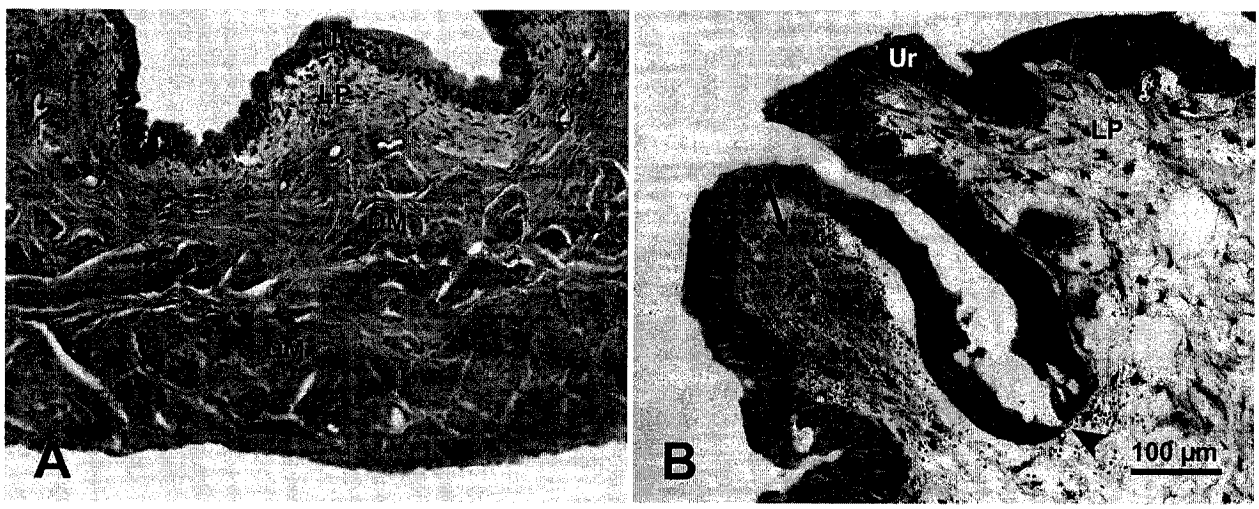
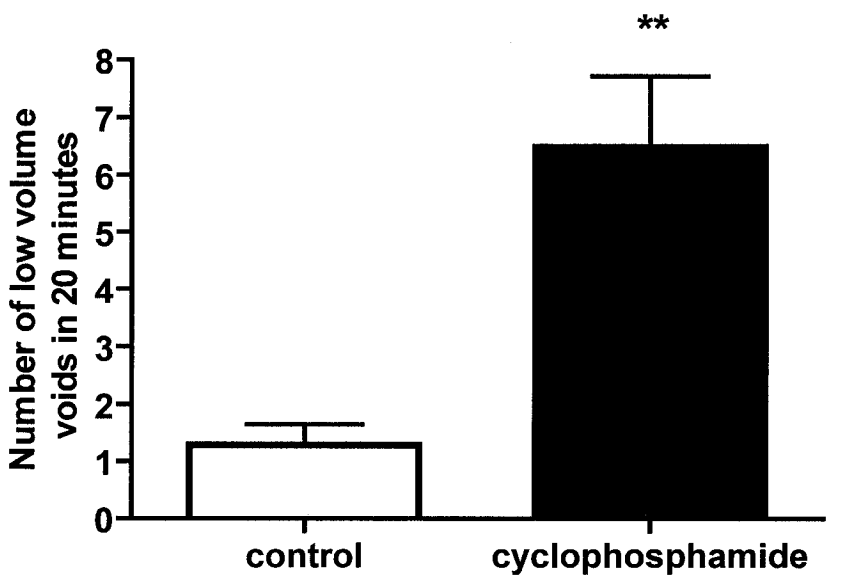

LP
A
Ur
LP
B
100 µm

Figure 10

Behaviour on day 10 following repeated i.p. injections of saline or cyclophosphamide. Cyclophosphamide-treated rats had increased number of low volume voids (**A**; **$p<0.01$) as well as an increase in urinary frequency (**B**; *$p<0.05$) compared to saline-treated rats.

A


Frequency of Low Volume Voids
**
Number of low volume voids in 20 minutes
0
1
2
3
4
5
6
7
8
control
cyclophosphamide

B

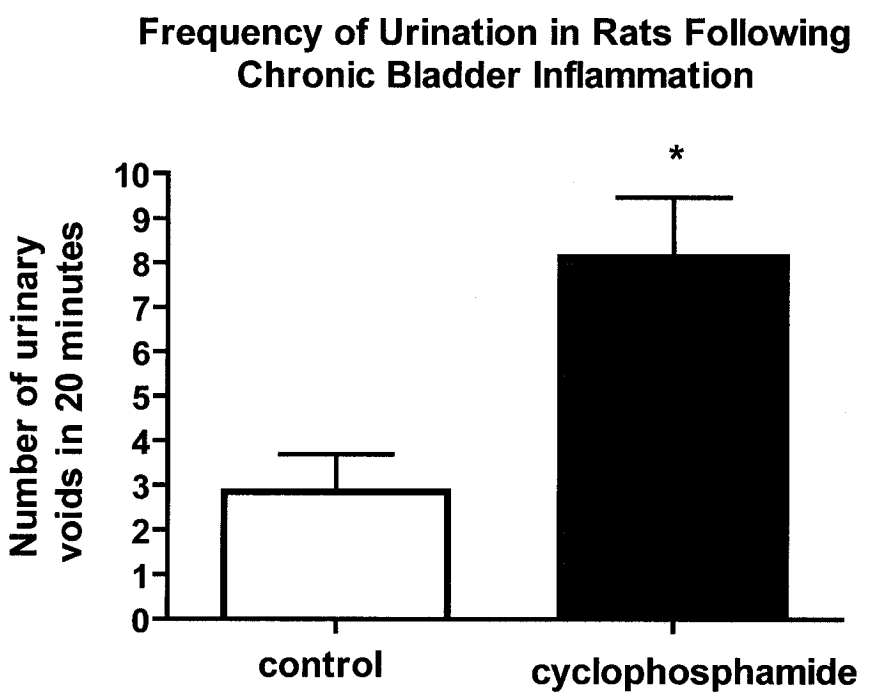
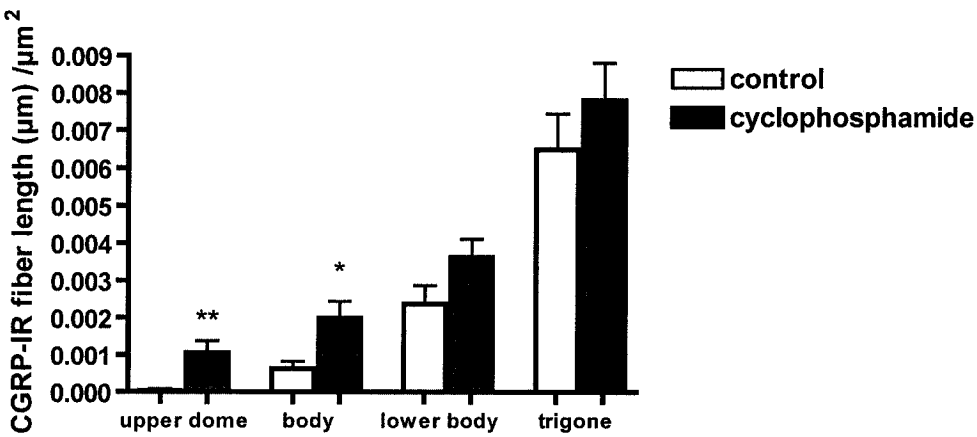

Frequency of Urination in Rats Following Chronic Bladder Inflammation
*
Number of urinary voids in 20 minutes
0
1
2
3
4
5
6
7
8
9
10
control
cyclophosphamide

Figure 11

Changes in CGRP-IR fibre density in the mucosa following cyclophosphamide administration. A – Changes per region; **B** – overall changes. In A, observe that following cyclophosphamide, the mucosa of upper dome had a very significant increase (**$p<0.01$) while the body had a significant increase (*$p<0.05$) in innervation density. No significant changes were observed in the lower body and trigone. In B, note that overall there is a significant increase in density of CGRP-IR fibres in the mucosa throughout the bladder following cyclophosphamide administration.

A Density of Peptidergic Fibers in the Mucosa



B Overall Density of Peptidergic Fibers in the Mucosa

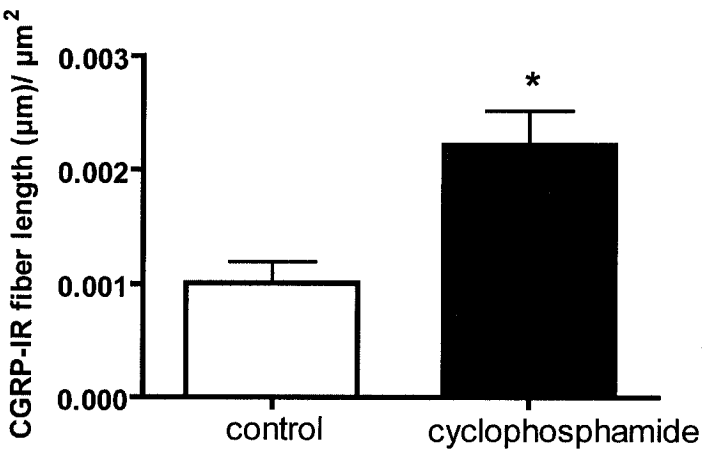
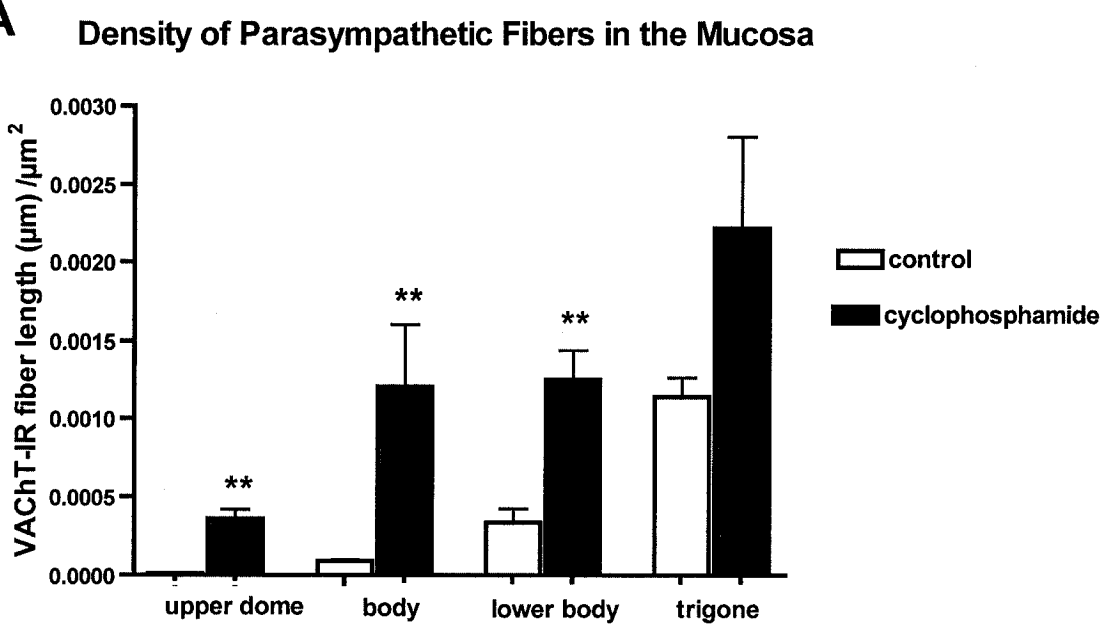


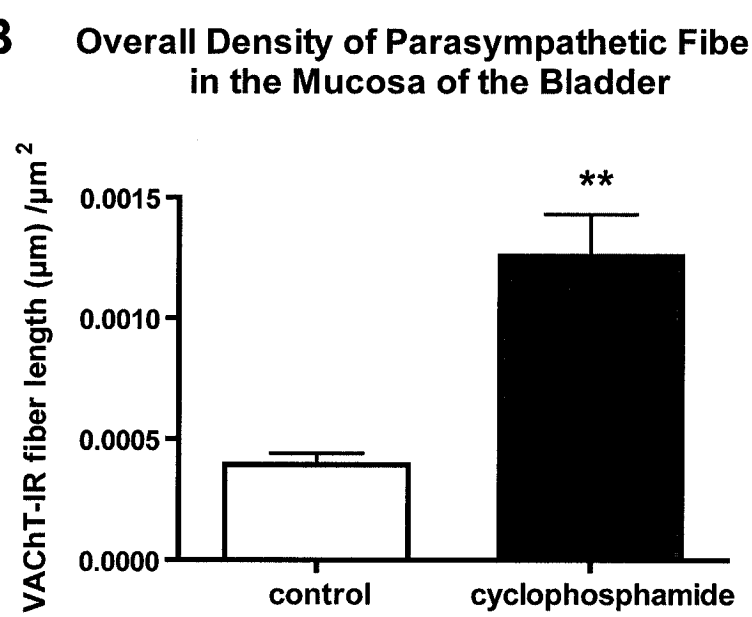
Figure 12

Changes in VAChT-IR fibre density in the mucosa following cyclophosphamide administration. A – Changes per region; **B** – overall changes. In **A**, note that the upper dome, body and lower body had very significant changes (**$p<0.01$), but not the trigone, where density values did not differ among treatment groups In **B**, note that overall there was a very significant increase in density of VAChT-IR fibres in the mucosa throughout the bladder following cyclophosphamide administration.

A


Density of Parasympathetic Fibers in the Mucosa
VAChT-IR fiber length (µm) /µm 2
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
**
**
**
upper dome
body
lower body
trigone
control
cyclophosphamide

B


Overall Density of Parasympathetic Fibers in the Mucosa of the Bladder
VAChT-IR fiber length (µm) /µm 2
0.0015
0.0010
0.0005
0.0000
**
control
cyclophosphamide

CHAPTER FOUR:

DISCUSSION

In this study, we show that following chronic bladder cystitis induced by cyclophosphamide there was a significant increase in the density of both parasympathetic and peptidergic sensory fibres in the bladder mucosa and an increase in density of peptidergic sensory fibres in the muscular layer. Furthermore, we provide evidence that these rats displayed urinary symptoms (frequency) as well as some indicators of spontaneous pain, such as piloerection and a rounded-back posture.

4.1 Choice of Animal Model

We chose to use cyclophosphamide-induced cystitis as a model of chronic bladder inflammation. This model has been well characterized in the literature, and its mechanism is well understood. Cyclophosphamide is known to induce bladder irritation in humans and rodents through the action of the toxic metabolite acrolein which undergoes urinary excretion (Brock and Pohl, 1983; Cox, 1979). The urinary bladder is the organ most affected by cyclophosphamide because of its reservoir function which leads to relatively long exposure of the mucosa to acrolein. This exposure results in a painful hemorrhagic cystitis in humans (Foad and Hess, 1976) and a pain-related behaviour, frequency and low volume voiding in the rat (Boucher et al., 2000; Lanteri-Minet et al., 1995). It has been previously reported in the literature that, following cyclophosphamide treatment, the urinary symptoms persisted even after the bladder had apparently recovered the normal morphology (Wood et al., 2001; Edrees et al., 1988). In addition, previous studies in the rat have shown an important involvement of sensory fibres in micturition changes (Dinis et al., 2004b; Vizzard, 2000a). Based on the above, we decided to use a cyclophosphamide-induced cystitis model to try to

establish whether it induced innervation changes that could be responsible for the bladder symptoms.

4.2 Choice of Fibre Systems to Study and of Markers to Label Them

We chose to focus on changes in sensory and parasympathetic fibres because of their known importance in bladder function and symptoms in disease. As a marker of sensory fibres, we used CGRP. Bladder afferents are either thinly myelinated (Aδ) or unmyelinated (C fibres) and CGRP is known to be expressed in over 80% of them (Cervero and Laird, 2004; Gabella and Davis, 1998). Since both A delta and C fibres are known to be able to signal bladder noxious events (Habler et al., 1990; Janig and Koltzenburg, 1990; Wyndaele and De, 2003; Habler et al., 1990; Janig and Koltzenburg, 1990; Wyndaele and De, 2003), this peptide seems to be the ideal marker for such afferents. Furthermore, co-localization studies have shown an almost 100% overlap of CGRP and TRPV1 immunoreactivities in nerve fibres coursing the rat bladder wall (Avelino et al., 2002). TRPV1 confers to nerve fibres sensitivity to capsaicin, heat and protons (Caterina et al., 1997) and its activation gives rise to pain-related behaviour and increased reflexogenic activity of the organ (Dinis et al., 2004a; Ishizuka et al., 1994; Ishizuka et al., 1995). In addition, the TRPV1 receptor seems essential to bladder hyperactivity associated with acute and chronic inflammation (Dinis et al. 2005). Therefore, by choosing CGRP as a marker we are confident that we are labelling the great majority of bladder afferents involved in nociception.

In what concerns the parasympathetic innervation of the bladder, as parasympathetic postganglionic fibres are all cholinergic and VAChT is the most reliable marker of

cholinergic fibres in the peripheral nervous system (for recent review see Ramien et al., 2004), we used a highly specific and well characterized anti-VAChT antibody.

4.3 Innervation Changes Following Cyclophosphamide Treatment

Twelve days after the start of the cyclophosphamide administration regime, we detected increases in both peptidergic sensory and parasympathetic innervation of the bladder. As we did not take into account the increase in weight of the bladder, which is caused by the edema of inflammation, we certainly underestimated the increase in bladder innervation following inflammation. Indeed, the area of the mucosa in particular increased substantially because of the edema. The changes in the innervation were particularly significant in the mucosa, but also occurred in the muscular layer. The increase in density of the peptidergic and parasympathetic fibres in the mucosa were most prominent in the upper regions of the bladder where there was little innervation in control animals. We also detected a significantly elevated innervation of the muscle by peptidergic fibres. However, the parasympathetic innervation in the muscle was not significantly changed, although there was a trend towards an increase. We have shown that in both the treated and the control animals there was no colocalization of VAChT and CGRP immunoreactivities in the bladder.

4.4 Increase in Sensory Afferent Density

Previous studies have shown that inflammation can alter the threshold of bladder afferents which could be responsible for the urinary symptoms of frequency, urgency and pain during inflammation (Yoshimura and De Groat, 1999). However, until now, there were no studies investigating whether peripheral sprouting could be a mechanism for maintaining

and exacerbating urinary pain and reflex bladder activity, in spite of the evidence obtained in the skeletal muscle showing that peripheral sprouting can occur in peptidergic fibres following inflammation (Reinert et al., 1998). We suggest that this increased density of afferent fibres that we observed in the inflamed bladders may increase the sensation of bladder filling and create bladder symptoms such as frequency and pain. We did not investigate in the present work the mechanisms by which peptidergic sprouting occurs during chronic inflammation. However, as peptidergic fibres are mostly NGF sensitive and an increase in the mRNA of this neurotrophic factor was observed in the bladder following chronic cyclophosphamide cystitis (Vizzard, 2000b), increased local synthesis of NGF could be a mechanism for the increased density of CGRP-IR fibres throughout the bladder.

4.5 Increase in Parasympathetic Fibre Density

We have shown an increase in parasympathetic fibre density in the mucosa following persistent inflammation. It is interesting that we found an increase in parasympathetic fibres in the mucosa and not in the muscular layer. If the sprouting had been shown in the muscle then we could infer that the increased parasympathetic tone could cause increased contraction of the bladder and lead to symptoms such as frequency and urgency. The level of parasympathetic sprouting in the upper dome, body and lower body mucosa following inflammation is quite striking. The occurrence of parasympathetic fibres in the mucosa is intriguing, as they serve no known function, except for those in the wall of vessels which are known to participate in vasodilatation. However, in this study, we found that the majority of the parasympathetic fibres in the mucosa were very close to the peptidergic fibres. It has previously been reported that there are both muscarinic and nicotinic receptors on sensory

fibres (Coggeshall and Carlton, 1997). A distinct possibility is that acetylcholine release from the parasympathetic fibres directly sensitize peptidergic fibres. Recent studies support this hypothesis. In fact, the blockade of muscarinic receptors present in somatic (Bernardini et al., 2002; Kim et al., 2005) sensory fibres has antinociceptive properties. The close proximity of the varicosities from the parasympathetic fibres and the sensory would be required as acetylcholine is promptly destroyed by cholinesterases and cannot act at a remote site. This was exactly the arrangement found in the cyclophosphamide-treated animals, with parasympathetic and sensory fibre wrapping around each other. We noticed that with the increase of both types of fibres in the bladders of the cyclophosphamide treated group there was also an obvious increase in the number of these two types of fibres wrapping around each other. This resulted in a marked increase in the number of varicosities of VAChT-IR which were apparently in close contact with varicosities of CGRP-IR fibres. How close the proximity of the two types of varicosities was could not be accurately determined using confocal microscopy, even though we used an oil-immersion, plan-apochromatic objective and scanned at very high magnification with small pinholes. Electron microscopy would be required to clarify this point. It should be noted that inflammation did increase the number of varicosities that were in apparent apposition, but very close proximities were observed also in non-inflamed bladders. Interestingly, a similar parasympathetic fibre sprouting was observed previously in our lab in the skin of the rat lower lip following nerve injury and was suggested as a mechanism for hyperalgesia occurring in this model (Ramien et al., 2004). Thus we speculate that the increased density of parasympathetic fibres in the mucosa following inflammation may cause sensitization of the sensory afferents in the mucosa leading to a decreased threshold and increased frequency and pain.

Again, we did not investigate the mechanisms for parasympathetic nerve fibre sprouting in the bladder mucosa. However, neurotrophic factors looks like ideal candidates. Chronic inflammation induced by cyclophosphamide increases NGF and BDNF protein in the major pelvic ganglia in the rat probably due to their increased retrograde transport following an augment of TrkA and TrkB receptors in the bladder post-ganglionic parasympathetic efferents (Murray et al., 2004).

4.6 The Role of the Sympathetic System

From preliminary data using an antibody against Dopamine-Beta-Hydroxylase (DBH) to mark sympathetic fibers, we did not see any sprouting of sympathetic fibers in any of the regions of the bladder. The focus of our study was not on changes in the sympathetic system thus we did not do quantification of this system. Since sensory afferent fibers and sympathetic fibers are usually supported by the same trophic factors it is strange that there was sprouting of the sensory afferent fibers without sprouting of the sympathetic fibers. Furthur studies would be necessary to resolve this problem and to analyze more thoroughly the role of the sympathetic system in cyclophosphamide cystitis.

4.7 Interaction Between Afferent and Efferent Pathways

Afferent activity controls parasympathetic, sympathetic and somatic efferent nerves to the lower urinary tract. An increased afferent activity eventually initiates an activation of the micturition reflexes leading to an increased response of the parasympathetic system (Andersson and Hedlund, 2002). Thus we can infer from what we found that following

chronic inflammation there are more sensory afferent fibres that can be activated, increasing the input to the spinal cord. It is well established that peptidergic afferents from the bladder terminate, among other locations, around the parasympathetic preganglionic neurons in the spinal cord (the parasympathetic nucleus) (Chancellor and Yoshimura, 2002). A previous study has shown, by densitometry, an increase of CGRP immunoreactivity in the parasympathetic nucleus of the L6-S1 segments following chronic cyclophosphamide treatment (Vizzard, 2001). Therefore, it is very likely that the increased input through the small diameter fibres could lead to an increased activation of the parasympathetic system. In fact, previous work has shown that there is an increase in activation in the parasympathetic nucleus following cyclophosphamide cystitis by using c-fos as a marker (Vizzard, 2000a). Thus we propose that after chronic cystitis there is an increase in afferent activity to the parasympathetic nucleus which would cause an increase in parasympathetic activity. This increase in parasympathetic activity accompanied by a higher number of ectopic cholinergic fibres in the bladder mucosa would lead to activation of sensory fibres, creating a vicious circle of increased activity leading to bladder symptoms.

4.8 Implications and Relevance to Human Studies

Bladder inflammation, whatever its origin, has been proposed as an important pathophysiological factor in interstitial cystitis (Chancellor and Yoshimura, 2004). The mechanisms by which inflammation could lead to persistent urgency, frequency and pain in interstitial cystitis are, however, unknown. This study proposes that inflammation can lead to neuronal changes such as nerve sprouting, which could explain the association of bladder inflammation with long-term urinary symptoms. In agreement with this suggestion, patients

with IC have more nerve fibres, including SP positive sensory fibres, within the mucosa and detrusor muscle than asymptomatic controls (Christmas et al., 1990; Pang et al., 1995).

In accordance with the concept of sensory and parasympathetic hyperinnervation some of the treatments often effective in reducing bladder pain in patients with IC patients may act by decreasing bladder innervation. Hydrodistension was shown to lead to the destruction of sensory and parasympathetic fibres in the nerve plexus of the bladder (Gosling et al., 1977; Lasanen et al., 1992a; Lasanen et al., 1992b). Intravesical resiniferatoxin, a capsaicin analogue (Cruz, 2004; Lazzeri et al., 2000), was shown to reduce bladder peptidergic innervation. Thus there is clinical evidence that hyperinnervation may be causing bladder symptoms in interstitial cystitis. Our results demonstrate that inflammation can cause an increase in sensory and parasympathetic innervation. Whether, as we suggest, such changes in the innervation may lead to an increase in bladder sensation, thus providing a mechanism of long-term bladder symptoms such as pain and frequency, needs to be clarified in future studies.

4.8. Future Directions

We used a simple test for investigating urinary symptoms. Although this test was sufficient for examining urinary behaviour and pain in our experiment, there are other tests that are more accurate but required techniques and/or equipment that we did not possess. These techniques could provide more detailed information about the urinary symptoms following inflammation of the bladder. Cystometrograms have been used to measure bladder activity. A cystometrogram is the measurement of intravesical pressure during the course of bladder filling (Chancellor and Yoshimura, 2002). Previous experiments have shown that

cyclophosphamide administration induces a marked reduction of bladder capacity (hyperreflexia). This is interpreted to mean that the bladder is painful at a lower volume as well (Maggi et al., 1992). It would be advantageous to use this test for our behaviour tests as this is a well accepted method of recording bladder activity and is more precise than the simple frequency test that we used. Another accurate method for measuring frequency has applied in studies in mice: changes in voided volume and frequency have been measured non-invasively using computerized digital balances. When using this test, urine falls directly from the cages onto electronic balance pans and the computer records the time and weight of each void (Wood et al., 2001). This method gives very accurate information about frequency and volume at which the animal voids. Thus, in future research on this project, one of these more accurate tests would be more appropriate to evaluate the urinary symptoms in the animals.

Although we have shown that there is an increase in parasympathetic and sensory peptidergic innervation following bladder inflammation, we have not shown that urinary symptoms persist in hyperinnervated animals once the inflammation has subsided. Thus, a future experiment would be to induce chronic inflammation in the bladder of a number of rats, then allow the rats sufficient time for the inflammation to subside. We could do the behaviour tests mentioned above to monitor urinary symptoms and then correlate the urinary symptoms to the innervation density. This would directly inform us as to whether the hyperinnervation induced by inflammation can cause long-term bladder pain, urgency and frequency.

It would also be interesting to investigate the mechanism of the fibre sprouting. Previous work using molecular techniques has shown that there is an increase in nerve growth factor in the bladders of both interstitial cystitis patients and rats with cystitis (Lowe et al., 1997; Vizzard, 2000). It has been found in many studies that an overexpression of NGF in the skin causes sprouting of peripheral sensory fibres (Albers et al., 1994; Davis et al., 1993) and it may be that this neurotrophic factor may be responsible for the sensory fibre sprouting in the bladder. A recombinant NGF sequestering protein, tyrosine receptor kinase A Ig2 (REN1820) has previously been used in an acute model of cyclophosphamide cystitis model. REN1820 was found to decrease bladder overactivity (Hu et al., 2005). It would be interesting to use REN1820 in a chronic model of cystitis and determine whether it blocks the sprouting of sensory fibres in the bladder. If the sensory sprouting is blocked then NGF must be the neurotrophin responsible for sensory afferent sprouting in the bladder. This finding would have possible clinical implications, as a trkA antagonist (the major NGF receptor on peptidergic fibres) or a NGF sequestering drug may be useful in the early treatment of interstitial cystitis.

As for parasympathetic sprouting, the mechanism is unknown in the bladder. Previous studies in the skin have indicated that GDNF is the most important neurotrophin responsible for parasympathetic neuron migration (Enomoto et al., 2000). However, one study done in the rat cystitis model showed that after chronic bladder inflammation there was not a significant increase in GDNF mRNA in the bladder (Vizzard, 2000). Further studies should be done to research whether this factor is indeed not significant in parasympathetic sprouting in the bladder and which other factors may be involved.

Another interesting experiment would be to determine the receptors that are important for the signalling of pain in the bladder. It has been suggested in this thesis that both the parasympathetic system and the sensory peptidergic system are important for pain signalling in the bladder. Ultimately, however, it is the activation of certain receptors on the sensory fibres that leads to pain signalling and increased sensitivity of the bladder. Therefore, the changes in receptors on sensory fibres are important to characterize. Muscarinic receptors are likely candidates to play a major role in increased sensitivity of the bladder (Birder et al., 2003; Coggeshall and Carlton, 1997; De Groat, 2004). Antagonists for the muscarinic receptors found on peptidergic fibres and in the urothelium may prevent the parasympathetic system from sensitizing the primary afferents, thus inhibiting bladder symptoms of pain and frequency. Therefore, it would be interesting to use selective muscarinic receptor antagonists to determine what muscarinic subtype is important in bladder symptoms. Selective muscarinic antagonists for the M2 and M3 receptors could be given to rats with chronic cystitis to see if the drug could ameliorate the urinary symptoms. Finding a selective antagonist is important as unselective muscarinic antagonists affect a large number of body systems and can lead to many adverse effects which are intolerable. None of the currently available drugs selectively targets the M3 or M2 receptor. If a selective antagonist is found to be effective in the rats with cystitis, the drug could possibly be tested in humans for efficacy in treating interstitial cystitis. Since almost all, if not all, of the sensory afferent in the bladder are peptidergic (Avelino et al., 2002; Gabella and Davis, 1998) it is very likely that tachykinins released from these fibres play a significant role in creating bladder pain (Cervero and Laird, 2004).

Finally although, we used the model of cyclophosphamide cystitis, it is important to develop a new model for chronic bladder pain, as this anti-cancer agent has many side effects. A major weakness of the chronic cyclophosphamide cystitis model is that many behavioural changes can not be specifically attributed to the bladder as cyclophosphamide is toxic to many body systems (Frasier LH et al., 1991). Intravesical instillation of LPS (lipopolysaccharide) could be a good model as it specifically targets the bladder, where it produces inflammation (Dupont et al., 2001; Luber-Narod et al., 1997). Therefore, behavioural changes could all be accredited to be due to the bladder symptoms. Another benefit of the LPS model is that the bladder can be kept inflamed for a longer period of time. Indeed, when using the cyclophosphamide model the animals cannot be kept for more than two weeks, whereas LPS can be used to cause bladder inflammation for over one month (Kawai et al., 1993).

CHAPTER FIVE:

CONCLUSION

Interstitial cystitis is a chronic visceral pain syndrome, characterized by debilitating pelvic/perineal pain and urinary frequency and urgency. The mechanisms by which bladder pain persists in this disease are unknown and treatments for the disease remain ineffective. The purpose of the present study was to provide a possible mechanism for persistent bladder pain, with the expectation that the clarification of such mechanism might lead to better treatment of diseases such as interstitital cystitis. As bladder inflammation has been proposed as an etiological component of interstitial cystitis, we used an inflammation model to investigate changes in the innervation of the bladder. In this thesis, we provide evidence that persistent urinary bladder inflammation can lead to peripheral sprouting of paraysympathetic and peptidergic sensory fibres. We should point out that there is evidence that sprouting of these fibres can contribute to hypersensitivity in the skin. There is also evidence from the literature that there is an hyperinnervation in interstitial cystitis. Thus we propose that this neuroplasticity observed in the bladder may contribute significantly to long-lasting urinary symptoms. We further suggest in this thesis that sensory and parasympathetic fibres may interact to create an ongoing feedback loop that may maintain and exacerbate chronic allodynia and hyperalgesia. Further evidence is needed to determine the importance of peripheral nerve fibre sprouting in the bladder but it is well possible that such fibre changes may be be a crucial factor required for chronic bladder pain to develop. If this proves to be true, therapies aimed at preventing fibre sprouting in the bladder could be developed. Such therapeutical approaches might be extremely valuable for chronic bladder diseases such as interstitial cystitis.

CHAPTER SIX: LITERATURE CITED

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