Neuroplasticity of Micturition Reflex Pathways with Cyclophosphamide-Induced Cystitis

Mary Beth Klinger

University of Vermont

Follow this and additional works at: http://scholarworks.uvm.edu/graddis

Recommended Citation
Klinger, Mary Beth, "Neuroplasticity of Micturition Reflex Pathways with Cyclophosphamide-Induced Cystitis" (2008). Graduate College Dissertations and Theses. Paper 123.
NEUROPLASTICITY OF MICTURITION REFLEX PATHWAYS WITH CYCLOPHOSPHAMIDE-INDUCED CYSTITIS

A Dissertation Presented

by

Mary Beth Klinger

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Anatomy and Neurobiology

October, 2008
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Anatomy and Neurobiology.

Dissertation Examination Committee:

Advisor
Margaret A. Vizzard, Ph.D.

Gary M. Mawe, Ph.D.

Cynthia J. Forehand, Ph.D.

Chairperson
Mark T. Nelson, Ph.D.

Vice President for Research and Dean of Graduate Studies
Frances E. Carr, Ph.D.

Date: July 14, 2008
Micturition requires the precise reciprocal function of the urinary bladder and urethral outlet. Perhaps due to this degree of precision, micturition is prone to dysfunction with injury or disease. One such disease, interstitial cystitis (IC)/painful bladder syndrome (PBS), is characterized by urinary urgency, frequency and pelvic pain. Inflammation has been implicated as a factor in IC/PBS. The overall hypothesis of this project is that urinary bladder inflammation induces expression of inflammatory mediators and changes neurotrophin receptor expression that contribute to functional changes in the urinary bladder. Using a well-characterized rat model of cyclophosphamide (CYP)-induced urinary bladder inflammation, the expression and function of cyclooxygenase-2 (COX-2), a known inflammatory enzyme, and the p75 neurotrophin receptor (p75NTR), involved in neurotrophin signaling, were examined in micturition reflex pathways using neuroanatomical, biochemical, molecular and physiological techniques.

Although COX-2 expression is increased in urinary bladder and involved in bladder hyperreflexia after CYP-induced cystitis, the localization and time course of upregulation was not known. We hypothesized increased COX-2 expression in specific tissue compartments (urothelium or smooth muscle) of the urinary bladder with CYP-induced inflammation. Western blotting for COX-2 showed a significant increase in COX-2 expression in both detrusor and urothelium/suburothelium, with the greatest increase in the urothelium/suburothelium. Immunostaining showed increased COX-2 staining in suburothelium with cystitis, co-localized with CD86, a marker for dendritic cells and macrophages.

Nerve growth factor (NGF) has been implicated in inflammation, increased voiding frequency and altered sensation in urinary bladder. The specific NGF tyrosine kinase receptor, TrkA, is increased in bladder afferent cells with CYP. NGF also binds p75NTR. The second goals were to examine the expression and functional role of p75NTR in urinary bladder pathways in control and CYP-treated rats. We hypothesized that p75NTR is constitutively expressed in micturition pathways and upregulated with cystitis. With cystitis, p75NTR expression was increased in lumbosacral spinal cord and in bladder afferent cells in dorsal root ganglia. Western blotting for p75NTR showed increased expression in whole urinary bladder with cystitis. Based on bladder function effects of TrkA blockade with cystitis, we hypothesized that p75NTR blockade in the urinary bladder would also decrease bladder hyperreflexia with cystitis. The functional role of p75NTR was studied by intravesical blockade by immunoneutralization with a monoclonal antibody to p75NTR and by PD90780, known to block NGF-p75NTR binding. Both forms of p75NTR blockade significantly decreased bladder capacity in control and CYP-treated rats. Changes in micturition and threshold pressure, and non-voiding contractions were also demonstrated.

In conclusion, these dissertation studies demonstrate that CYP-induced bladder inflammation alters expression of inflammatory mediators and neurotrophin receptors in micturition pathways. This altered expression can affect overall urinary bladder function.
Citations

Material from this dissertation has been published in the following form:


Dedication

A dedication to my parents,
my foundation and inspiration.
Acknowledgements

The past five years have been filled with an incredible amount of learning and wonderful experiences, and I have many people to thank. I would like to first thank Dr. Margaret Vizzard, for all of the guidance, support and time that she has put into my graduate training; she is a fantastic role model and mentor. The experience of being a part of the Vizzard lab is one that I will never forget. I would like to thank all past and present members of the Vizzard laboratory, especially Susan Malley, Abbey Dattilio, and Kimberly Corrow for all of the laboratory techniques they have taught me over the years and for always being there to provide a helping hand.

I would also like to thank my dissertation committee, Dr. Mark Nelson, Dr. Cynthia Forehand, and Dr. Gary Mawe for offering so much of their time and helpful suggestions. The University of Vermont Department of Anatomy and Neurobiology has been a fantastic environment in which to do my graduate work, with innumerable admirable people always ready with advice and support. I hope to always be among such brilliant and thoughtful people throughout my career.

I have made many friends while here in Vermont who have helped to make this journey that much easier and more fun. There are too many to name individually, although I would like to and I thank you all. A special thank you to Dr. Julie Simpson, Dr. Martin Hruska, and Dr. Eric Krauter, who inspired me as I watched them finish graduate school and who were always there to help me throughout my own graduate school journey. Also a special thank you to Ryan Lawrence, who has shown me constant support, patience, and understanding throughout the past four years.

Finally, I am forever grateful to my parents Mark and Mary Klinger and to my brother John for always knowing what to say to me when I felt down, and who have always made me feel that I can accomplish absolutely anything.
Table of Contents

CITATIONS .................................................................................................................................................. ii
DEDICATION .................................................................................................................................................. iii
ACKNOWLEDGEMENTS .............................................................................................................................. iv
LIST OF FIGURES ......................................................................................................................................... viii
LIST OF TABLES ............................................................................................................................................ x

CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW ................................................................. 1

I. INTRODUCTION ........................................................................................................................................ 1

II. ANATOMY AND FUNCTION OF THE LOWER URINARY TRACT ................................................... 3

   A. Lower Urinary Tract Anatomy ........................................................................................................... 3
   B. Micturition Reflex .............................................................................................................................. 6
   C. Urinary Bladder Innervation ............................................................................................................ 11

III. BLADDER INFLAMMATION AND ASSOCIATED CHANGES ..................................................... 16

   A. Cyclophosphamide (CYP) Cystitis .................................................................................................. 16
   B. CYP-induced cystitis: Changes in the micturition reflex pathway ................................................ 19
   C. How CYP cystitis can relate to human conditions ......................................................................... 22

IV. CYCLOOXYGENASE-2 (COX-2) AND INFLAMMATION ............................................................. 25

   A. General Background ....................................................................................................................... 25
   B. COX-2 and urinary bladder inflammation ..................................................................................... 29

V. NERVE GROWTH FACTOR (NGF) AND THE P75 NEUROTROPHIN RECEPTOR (P75NTR) .............. 31

   A. NGF ................................................................................................................................................. 31
   B. Receptor tyrosine kinase A (TrkA) ................................................................................................. 36
   C. p75NTR ......................................................................................................................................... 39

VI. PROJECT AIMS AND HYPOTHESES ............................................................................................ 47

REFERENCES FOR COMPREHENSIVE LITERATURE REVIEW .................................................... 50

CHAPTER 2: EXPRESSION OF CYCLOOXYGENASE-2 IN URINARY BLADDER IN RATS WITH CYCLOPHOSPHAMIDE-INDUCED CYSTITIS ................................................................. 68

ABSTRACT ................................................................................................................................................. 69

INTRODUCTION ......................................................................................................................................... 70

MATERIALS AND METHODS .................................................................................................................. 73
APPENDIX A: FUNCTIONAL AND SENSORY CHANGES IN P75<sup>NTR</sup><sup>−/−</sup> MICE

INTRODUCTION............................................................................................................................... 246

MATERIALS AND METHODS ........................................................................................................... 247

RESULTS....................................................................................................................................... 249

FIGURES ....................................................................................................................................... 251

DISCUSSION................................................................................................................................. 256

REFERENCES ............................................................................................................................... 258
List of Figures

CHAPTER 1

FIGURE 1: BASIC BLADDER ANATOMY AND THE SUBUROTHELIAL PLEXUS ................................................................. 4
FIGURE 2: IMPORTANT PARAMETERS OF CYSTOMETRY ................................................................................................. 10
FIGURE 3: NEURAL CIRCUITRY INVOLVED IN THE MICTURITION REFLEX ............................................................... 14
FIGURE 4: THE PRODUCTION OF PROSTAGLANDINS FROM ARACHIDONIC ACID ....................................................... 26
FIGURE 5: THE CYCLOOXYGENASE CONCEPT. ........................................................................................................... 27
FIGURE 6: NEUROTROPHINS AND THEIR RECEPTORS. ............................................................................................... 36

Chapter 2

FIGURE 1: WESTERN BLOT IN DETRUSOR AND UROTHELIUM/SUBUROTHELIUM ...................................................... 91
FIGURE 2: COX-2 EXPRESSION IN URINARY BLADDER SECTIONS ........................................................................... 92
FIGURE 3: COX-2-IR NERVE FIBERS IN SUBUROTHELIAL PLEXUS ........................................................................ 93
FIGURE 4: COX-2 AND PGP9.5 EXPRESSION IN URINARY BLADDER WHOLE MOUNTS ............................................ 94
FIGURE 5: WHOLE MOUNT PREPARATION OF URINARY BLADDER ........................................................................ 95

Chapter 3

FIGURE 1: P75NTR<sup>exonIII-/-</sup> MICE ...................................................................................................................... 133
FIGURE 2: P75<sup>NTR</sup> -IR IN LUMBOSACRAL SPINAL CORD ......................................................................................... 134
FIGURE 3: P75<sup>NTR</sup> -IR CELLS IN THE LUMBOSACRAL DORSAL ROOT GANGLIA ................................................. 135
FIGURE 4: NUMBERS OF P75<sup>NTR</sup> -IR CELLS IN THE LUMBOSACRAL DORSAL ROOT GANGLIA .......................... 136
FIGURE 5: PERICELLULAR P75<sup>NTR</sup> -IR IN DRG FROM CONTROL AND CYP-TREATED RATS. .......................... 137
FIGURE 6: P75<sup>NTR</sup> –IR IN BLADDER AFFERENT CELLS IN LUMBOSACRAL DORSAL ROOT GANGLION .............. 138
FIGURE 7: P75<sup>NTR</sup> MRNA IN L6-S1 DRG ........................................................................................................... 139
Chapter 4

Figure 1: Urinary Bladder Whole-Mount Preparations ................................................................. 183

Figure 2: Western Blot for p75NTR Expression in Whole Urinary Bladder .......................... 184

Figure 3: Intravesical Infusion of Anti-p75NTR Monoclonal Antibody .................................. 185

Figure 4: Voiding Frequency and Inter-contraction Interval ...................................................... 186

Figure 5: Intravesical Infusion of Anti-p75NTR Monoclonal Antibody (100 µg/ml) .............. 186

Figure 6: Intravesical Infusion of PD90780 .............................................................................. 188

Figure 7: Voiding Frequency and Inter-contraction Interval in Control Rats ....................... 189

Figure 8: Voiding Frequency and Inter-contraction Interval in CYP-Treated Rats ............. 190

Chapter 5

Figure 1: p75NTR Blockade Leads to Increased Bladder Activity ........................................... 208

Appendix A

Figure 1: Genotyping of p75NTRexonIII Null Mice, Wildtype and Heterozygous p75NTRexonIII Mice .... 251

Figure 2: Cystometry in p75NTRexonIII Null Mice ................................................................. 252

Figure 3: Tyrosine Hydroxylase (TH) Expression in p75NTRexonIII Null Mice ................. 253

Figure 4: Vasoactive Intestinal Polypeptide (VIP) Expression in p75NTRexonIII Null Mice .... 254

Figure 5: Calcitonin Gene-related Peptide (CGRP) Expression in p75NTRexonIII Null Mice .... 255
List of Tables

CHAPTER 2

TABLE 1: PRIMARY AND SECONDARY ANTIBODIES USED, SOURCES AND APPLICATIONS ................................96
TABLE 2: EXPERIMENTS OF URINARY BLADDER SECTIONS OR WHOLE MOUNT PREPARATIONS .......................96

CHAPTER 3

TABLE 1: OLIGONUCLEOTIDE PRIMER SEQUENCES FOR P75NTR AND THE HOUSEKEEPING GENE, L32........140

Chapter 4

TABLE 1: EFFECTS OF INTRAVESICAL INFUSION OF AN ANTI-P75NTR MONOCLONAL ANTIBODY ON
CYSTOMETRIC PARAMETERS...............................................................................................................181
TABLE 2: EFFECTS OF INTRAVESICAL INFUSION OF PD90780 ON CYSTOMETRIC PARAMETERS.................182
Chapter 1: Comprehensive Literature Review

I. Introduction

Micturition is an important process requiring complex neuronal organization that involves the storage and periodic elimination of urine. Micturition is highly regulated and involves peripheral and central innervation and pathways that are prone to injury and disease. While much is now understood of normal micturition reflex circuitry, there is much less information available about how the micturition reflex changes with injury or disease. Bladder outlet obstruction, multiple sclerosis, Parkinson’s disease, overactive bladder, spinal cord injury, and interstitial cystitis/painful bladder syndrome (IC/PBS) are all conditions that affect the organized processes of bladder storage and efficient elimination. Thus, neural injury or disease can affect the lower urinary tract and result in pain, hyperreflexia, incontinence, or even bladder underactivity, which can severely affect the quality of life of those affected (Michael et al., 2000).

IC/PBS is a syndrome characterized by urgency, frequency and pelvic pain including pain at low to moderate bladder pressure. The cause of IC/PBS is unknown, although many theories of etiology exist, including toxic agents in the urine, autoimmune disorder, deficiency in the bladder wall, urothelial cell deficiency, pelvic floor injury, and neurogenic causes (Erickson, 1999). Whatever the cause, inflammation of the bladder has been implicated as a factor in IC/PBS; most patients with IC/PBS often show some degree of inflammation, as evidence by the infiltration of inflammatory cells and mediators in the bladder (Erickson, 1999). Chronic tissue inflammation can lead to
neuroplasticity, involving the sensitization of primary afferents or changes in central synapses, resulting in hyperalgesia and allodynia (Cheppudira, 2006; Thompson et al., 1995; Yoshimura and de Groat, 1999). In bladder, this could translate to uncomfortable sensation/pain and increased frequency of elimination. It has already been shown that bladder inflammation results in a multitude of effects centrally and peripherally, including changes in neurochemistry, neurotrophin expression, prostaglandin expression, electrophysiology of bladder afferents, and the re-organization of micturition reflex pathways, among many others (Hu et al., 2003; Vizzard, 2000b; 2001; Yoshimura and de Groat, 1999). The overall goal of this project was to further understand and characterize the changes associated with bladder inflammation and specifically, the role that both cyclooxygenase-2 (COX-2) and the neurotrophin receptor p75 Neurotrophin receptor (NTR) play. This project focused on COX-2 and p75 NTR in micturition reflex pathways as well as the role of p75 NTR in bladder function.
II. Anatomy and Function of the Lower Urinary Tract

A. Lower Urinary Tract Anatomy

The lower urinary tract is made up of the urinary bladder and urethra (Figure 1). The urinary bladder is divided into two main parts. Most superiorly, the body of the bladder is located above the ureteral orifices. The bladder base consists of the trigone, urethrovessical junction (bladder neck) and anterior bladder wall (Andersson and Arner, 2004; Elbadawi, 1996). The trigone is the area of bladder bounded by the ureteral orifices and the urethrovessical junction.

The wall of the urinary bladder is organized in layers (Figure 1), with the urothelium as the closest layer to the bladder lumen. Below the urothelium is the lamina propria layer, which contains the suburothelial plexus of nerves (containing mostly afferent nerves) and blood vessels (Andersson and Arner, 2004). The outermost layer of the bladder is the detrusor smooth muscle, which consists of circular as well as longitudinal smooth muscle layers. The urethra is also surrounded by smooth muscle, although this is structurally and functionally different than bladder smooth muscle (Andersson and Arner, 2004). There is an internal urethral sphincter that is under involuntary control at the inferior end of the bladder and the superior end of the urethra (Elbadawi, 1996). The external urethral sphincter is made of striated muscle to allow for conscious control of bladder function and this is located inferior to the prostate in males and at the inferior end of the urethra in females (Andersson and Arner, 2004).
Figure 1: Basic bladder anatomy and the suburothelial plexus.

(A) Basic bladder anatomy includes the dome as the most superior part of the bladder. The body is the part of the bladder located above the ureteral orifices. The neck is the base of the bladder. The urethral outlet allows for the release of urine. (B) The bladder wall consists of urothelial cells closest to the bladder lumen. The lamina propria layer is underneath the urothelium and it contains the suburothelial plexus of nerves and blood vessels. The smooth muscle layer is the furthest from the bladder lumen and contains longitudinal as well as circular muscle. (C) Example of a urinary bladder taken from a female rat. The bladder is pinned out on Sylgard, and detrusor (yellow arrows) is carefully separated from the inner urothelial layer (red arrows). The suburothelial plexus comes off attached to the urothelial layer (Zvarova et al., 2004). (D) Staining in the suburothelial plexus for PGP9.5, a neuronal marker. The suburothelial plexus contains most of the afferent nerves in the urinary bladder.
The urothelium has three basic layers of cells: a basal cell layer with a basement membrane underneath, an intermediate layer of cells, and a layer of transitional epithelium made of hexagonal cells (also known as “umbrella cells”) that provide the luminal lining of the urinary bladder (Birder, 2005; Cohen, 1995). A band of tight junctions connects the cells to act as a barrier from the contents of the urine (Cohen, 1995).

Urothelial cells were once thought to simply provide a barrier between urine and the underlying tissues. However, many recent studies have shown that urothelial cells express receptors and ion channels associated with neuronal function, specifically afferent neuronal function, which include bradykinin receptors, neurotrophin receptors TrkA, TrkB and p75NTR, purinergic receptors P2X and P2Y, α- and β-adrenergic receptors, and transient receptor potential (TRP) channels such as TRPV1 (Kullmann et al., 2008; Murray et al., 2004). Urothelial cells also release neuroactive compounds (i.e. acetylcholine, ATP, nitric oxide) (Birder, 2005). Bladder afferent nerves are located in close proximity to urothelial cells, suggesting that signaling likely occurs between afferents and urothelium (Birder, 2005; Kullmann et al., 2008).

The urine itself is made of multiple organic and inorganic substances, including urea (formed from ammonia and CO₂), uric acid (the product of the oxidation of purines), creatinine (hydrated creatine), sodium, potassium, phosphates, sulfates, bicarbonate, and ammonia (Cohen, 1995). Levels of each constituent as well as urinary pH vary widely with food consumption and time of day (Cohen, 1995). It has been suggested that some constituents of urine could impact the bladder by way of the urothelium (Cohen, 1995).
B. Micturition Reflex

The urinary bladder always exists in one of two modes: storage mode and elimination mode. The bladder smooth muscle and the urethral outlet must function reciprocally for the efficient elimination of urine (Fowler et al., 2008). During storage mode, the bladder smooth muscle must remain relaxed to allow for filling, while the urethral outlet is contracted (Fowler et al., 2008). Elimination mode requires contraction of the bladder smooth muscle with relaxation of the urethral outlet, to allow urine flow (Andersson and Arner, 2004). Precise organization of the reciprocal functions of the urinary bladder and urethra and complex neural organization are required (Fowler et al., 2008).

Storage mode

During storage mode, the detrusor smooth muscle must remain relaxed while the urethral outlet remains contracted to ensure the retention of urine and continence. Storage mode involves the slow filling of the urinary bladder, signaled by low-level afferent firing of myelinated Aδ fibers (Figure 3) (Holstege, 2005). The sympathetic pathway has a role in inhibition of parasympathetic efferent input to the detrusor smooth muscle and in inhibition of parasympathetic activity in the autonomic ganglia (Fowler et al., 2008). Sympathetic outflow and reflex pathways keep the urethra and urethral sphincters contracted (Fowler et al., 2008). Conscious control of continence is provided by cholinergic motor neurons to the striated muscle in the external urethral sphincter...
(Fowler et al., 2008) (Elbadawi, 1996; Holstege, 2005). Studies have also shown that there is activation of the periaqueductal gray (PAG) during bladder filling in humans, which in turn influences the pontine micturition center (PMC), a part of the brainstem involved in the switch between filling and voiding (Figure 3) (Blok and Holstege, 2000; Fowler et al., 2008) (Holstege, 2005).

The PMC, located in the brainstem, is thought to be involved in coordinating the switch between storage and elimination activity (Holstege, 2005). Drugs applied to the PMC can change the bladder volume set point (i.e. threshold) to induce bladder voiding activation (Fowler et al., 2008). The PMC receives bladder afferent projections and in turn sends excitatory projections to the bladder motoneurons in the preganglionic parasympathetic nucleus of the spinal cord (Holstege, 2005). The PMC also excites GABA-ergic and glycine-ergic inhibitory interneurons of urethral sphincter (Holstege, 2005). In humans and cats, bladder filling afferent information most likely is relayed first to the PAG, which then has projections to the PMC (Blok and Holstege, 2000; Fowler et al., 2008). In rats, there is likely direct access between lumbosacral afferent neurons and the PMC, possibly making rat voiding behavior less easily extrapolated to human conditions with respect to the supraspinal elements of the micturition reflex (Blok and Holstege, 2000).

Elimination mode

The elimination of urine involves the coordinated contraction of the detrusor and relaxation and dilation of the urethral outlet. This involves inhibition of the sympathetic output to the bladder and urethral outlet, and activation of the parasympathetic pathway.
Relaxation of the urethral smooth muscle is achieved by removal of adrenergic and cholinergic excitatory inputs and the release of nitric oxide (NO) to elicit smooth muscle relaxation (Fowler et al., 2008). Contraction of the detrusor smooth muscle is activated by parasympathetic cholinergic efferent input to this muscle. The PMC has also been shown to exhibit firing during bladder voiding activity (Fowler et al., 2008; Holstege, 2005).

**Components of the micturition reflex as seen during cystometry**

Cystometry is a technique used to measure bladder function *in vivo*. Cystometry involves the constant infusion of saline into the urinary bladder of an experimental animal through tubing that is secured in the dome of the bladder. The tubing is connected to a pressure transducer, which then measures the changes in pressures associated with micturition cycles.

Both conscious cystometry and cystometry in anesthetized animals are used experimentally. There are some differences between methods, not the least of which is a difference in bladder capacity (Cannon and Damaser, 2001; Ghoniem et al., 1996). Animals under anesthesia have a significantly larger bladder capacity than conscious rats (Cannon and Damaser, 2001; Ghoniem et al., 1996). It is also said that anesthesia may compromise urodynamic parameters and may have profound effects on bladder function, perhaps because general anesthesia may block glutamate transmission in the brain (Cannon and Damaser, 2001; Ghoniem et al., 1996; Yokoyama et al., 1997). Careful interpretation must be exercised when interpreting data from experiments using anesthetized animals (Ghoniem et al., 1996). It is also true that conscious cystometry
might more closely reflect normal bladder functions. However, in both models, the implantation of the tubing into the bladder may affect urothelial sensation or bladder capacity. This is why a period of several days is recommended for the recovery of the animal from surgery.

Figure 2 illustrates an example of one micturition cycle during a typical cystometrogram recording in a conscious rat. Important parameters of cystometry include filling pressure (the pressure during the filling phase of the micturition cycle), threshold pressure (pressure at the beginning of the void event), micturition pressure (the peak in pressure during the void event), inter-micturition interval (the amount of time between the end of one void event and the end of the next), the frequency of non-voiding contractions, and void volume (Maggi et al., 1986).

Changes that are detected in certain urodynamic parameters with experimentation can be attributed to changes in afferent or efferent function. Experiments involving blockade of nicotinic receptors in the pelvic ganglia in order to impair the efferent branch of the micturition reflex (Maggi et al., 1986) demonstrated that threshold pressure, void volume and inter-micturition interval are associated with the afferent limb of the micturition reflex. Changes in micturition pressure are associated with changes in efficiency of neurotransmission in the efferent branch of the micturition reflex (Maggi et al., 1986).

Multiple possibilities exist for changes in the number of non-voiding contractions with cystometry, including myogenic or neurogenic activities. Non-void contractions during the filling phase could mean an increase in detrusor activity, including
Figure 2: Important parameters of cystometry

ICI: inter-contraction interval, FP: filling pressure, TP: threshold pressure, MP: micturition pressure.

spontaneous contractions of the detrusor smooth muscle. This is often seen in spinal cord-injured rats (Brading, 1997; Herrera et al., 2003; Yoshimura et al., 2006). Other explanations include changes in the response of the urothelium to bladder distension, possibly affecting the bladder afferents located in the suburothelial plexus (Herrera et al., 2003). Experimental effects on afferent or efferent nerves are also possible sources of neurogenic changes in the number of non-void contractions (Herrera et al., 2003).
C. Urinary Bladder Innervation

Sympathetic Innervation

Preganglionic sympathetic neurons are located in the intermediolateral (IML) region (laminae V-VII) of the thoracolumbar spinal cord (L1-L2 in rats; T11-L2 in humans) (Fowler et al., 2008). Sympathetic efferents travel in the hypogastric nerve to the inferior mesenteric ganglia, then traveling in the hypogastric nerve to the pelvic ganglia and lower urinary tract, where they activate α-adrenergic excitatory receptors in the bladder base and urethral smooth muscle and β-adrenergic inhibitory receptors on the detrusor smooth muscle (Figure 3)(Fowler et al., 2008).

Parasympathetic Innervation

Parasympathetic preganglionics are located in the sacral parasympathetic nucleus (SPN) of the IML region (laminae V-VII) of sacral spinal cord (L6-S1 in rats; S2-S4 in humans). Parasympathetic efferents travel in the pelvic nerve, with postganglionics located in the major pelvic ganglion of rats (Keast et al., 1989). Postganglionic parasympathetic nerves have an excitatory effect on detrusor smooth muscle and an inhibitory effect on bladder base and urethra (Fowler et al., 2008). Post-junctional M₃ muscarinic receptors expressed on the bladder smooth muscle are the receptors thought to be most involved in excitatory transmission in the detrusor (Fowler et al., 2008). However, a recent experiment showed that activation or inhibition of muscarinic acetylcholine receptors (mACHRs) at the luminal surface of the bladder can affect bladder function. This is presumably through interactions between the urothelium and the C-fiber
sensory nerves located within close proximity, demonstrating that parasympathetic muscarinic transmission could be important not only in the detrusor smooth muscle, but in the urothelium as well (Kullmann et al., 2008). Non-cholinergic parasympathetic innervation involves adenosine triphosphate (ATP) activation of P2X receptors and NO transmission, which is inhibitory to urethral smooth muscle (Fowler et al., 2008).

**Somatic Innervation**

Cholinergic neurons also innervate the striated muscle of the external urethral sphincter, keeping the sphincter under conscious control (Fowler et al., 2008). These neurons originate in the ventral horn of lumbosacral spinal cord (lamina IX) in the dorsolateral nucleus and travel via the pudendal nerve to synapse on external urethral sphincter muscle (Figure 3).

**Bladder Afferent Pathways**

Bladder afferent nerve fibers travel in both the hypogastric and pelvic nerves, with their cell bodies in dorsal root ganglia (DRG) at spinal segment levels S2-S4 and T11-L2 in humans and L6-S1 and L1-L2 in rats (Fowler et al., 2008). Bladder afferents consist of lightly myelinated Aδ fibers and unmyelinated C-fibers. As stated above, sensations of bladder filling are conveyed by Aδ fibers, the most important mechanoreceptors of the bladder. C-fibers are normally “silent” but they do respond to chemical or noxious stimuli (Figure 3) (Fowler et al., 2008). Aδ and C-fibers terminate in the urothelium, suburothelium, and smooth muscle layers of the bladder (Kullmann et al., 2008). Most of the bladder afferents project to lumbosacral spinal cord levels, as this
is the most important region for signaling the micturition reflex (Holstege, 2005). In the bladder, most of the sensory nerves are located in a dense suburothelial plexus just beneath the urothelium (Andersson and Arner, 2004)(Figure 1).

Bladder primary afferent fibers in the pelvic nerve project to the lumbosacral spinal cord via Lissauer’s tract. Afferents from urinary bladder, uterus, and colon project along the lateral side of the dorsal horn, forming the lateral collateral pathway (LCP) (Kawatani et al., 1990). Afferent projections from the pudendal nerve and genital structures project along the medial edge of the dorsal horn into the dorsal commissure region, forming the medial collateral pathway (MCP) (Kawatani et al., 1990).

Many bladder afferents further project to the SPN, synapsing with preganglionic parasympathetic neurons as well as interneurons (Morgan et al., 1981) (Fowler et al., 2008). Primary bladder afferents from the pelvic and hypogastric nerves also project to dorsal commissure and the superficial dorsal horn (Fowler et al., 2008). Lumbosacral dorsal commissure, superficial dorsal horn and the parasympathetic nucleus all contain interneurons important to urinary bladder function (Fowler et al., 2008). These interneurons project locally in the spinal cord or to the brain (Fowler et al., 2008). Some bladder afferents synapse with ascending pathways in the spinal cord which project to neuronal populations in the brain involved in micturition control, including the PMC (Fowler et al., 2008). Neuronal tracing studies using horseradish peroxidase showed that the dorsal commissure and sacral parasympathetic nucleus are located periodically at approximately 200 μm intervals (Morgan et al., 1981).
During filling, there is low-level A\(\delta\) afferent firing, stimulating sympathetics in the hypogastric nerve to keep the detrusor smooth muscle relaxed and the urethra contracted. The pudendal nerve provides conscious control to keep the external urethral sphincter contracted. C-fiber unmyelinated afferents do not respond to normal bladder filling, but are “awakened” and activated after spinal cord injury or by noxious stimulants, such as cold. Intense afferent firing during bladder filling activates spinobulbospinal pathways, with afferent input to the periaqueductal grey (PAG) and pontine micturition center (PMC), which in turn activate preganglionics in the sacral parasympathetic nucleus (SPN) of lumbosacral spinal cord. Postganglionics in the pelvic nerve then excite the detrusor smooth muscle, whereas sympathetic and pudendal input to the urinary bladder and urethra are inhibited. The cerebral cortex also provides conscious control over micturition.
The use of the rat model in studying micturition reflex pathways is very common, as the comparison of rat anatomy to human anatomy is well known. Analogous spinal cord segments involved in micturition reflex circuitry exist in rats, including the L1-L2 spinal cord segment that is analogous to the human T11-L2 spinal cord region which contains the sympathetic outflow and some of the bladder afferent input. Analogous to the human S2-S4 region is the L6-S1 region in rat, which contains the parasympathetic outflow and receives most of the bladder afferent input (Shefchyk, 2002). It is also true that rats, like humans and cats, have supraspinal control over the micturition reflex, with a distinct region in the pons analogous to the PMC; the destruction of this region leads to total urinary retention (Blok and Holstege, 2000; Maggi et al., 1986). However, rats appear to have direct lumbosacral input to their PMC, perhaps the reason for a more reflexic voiding behavior than “guarded” reflex behavior as seen in humans and cats (Blok and Holstege, 2000). Guarded reflex behavior in humans and cats involves voiding in a safe location.
III. Bladder Inflammation and Associated Changes

Bladder inflammation is often experimentally induced to study the effects of acute and chronic bladder inflammation on bladder function and micturition reflex pathways in experimental animals (Westropp and Buffington, 2002). Multiple models of cystitis are in use, including cystitis that results from the intraperitoneal injection of cyclophosphamide (CYP). The CYP model has been widely studied in order to characterize the changes associated with this purely visceral inflammation model (Lanteri-Minet et al., 1995). The results of experiments done in the CYP-treated animals may have relevance to human conditions that involve bladder inflammation, including IC/PBS (Westropp and Buffington, 2002).

A. Cyclophosphamide (CYP) Cystitis

CYP was originally used as an anti-tumor chemotherapeutic agent that had an unfortunate side effect of painful hemorrhagic cystitis (Bautista et al., 2006; Cox, 1979b; Maggi et al., 1992). Side effects of CYP therapy also include painful voiding, bladder hyperreflexia and gross hematuria (Stillwell and Benson, 1988; Watson and Notley, 1973). Symptoms of CYP cystitis can also include increased somatic sensitivity (Guerios et al., 2006). CYP is often used to model bladder inflammation because of its ease of observation behaviorally and histologically, and because the inflammation is confined specifically to the bladder (Bon et al., 2003; Lanteri-Minet et al., 1995). Experimental CYP cystitis in animals results in histological changes in the urinary bladder that include
edema, vasodilation, infiltration of inflammatory cells such as mast cells, and proliferation of nerve fibers (Yoshimura and de Groat, 1999). Bladder hyperreflexia is seen as an increase in voiding frequency with CYP-induced cystitis, resulting in a shorter inter-micturition interval and a decreased void volume; the presence of non-voiding contractions during the filling phase is also seen (Hu et al., 2003; Maggi et al., 1992). The causative agent of this effect of CYP was proven to be acrolein, a metabolite of CYP that accumulates in the urine and contacts the bladder wall while being stored before elimination (Cox, 1979b).

The activation of capsaicin-sensitive primary afferents by acrolein in urinary bladder plays a significant role in the hyperreflexia associated with CYP-induced cystitis, leading to neurogenic inflammation and nociception (Ahluwalia et al., 1994; Geppetti et al., 2008; Maggi et al., 1992). Capsaicin is the pungent component of spicy chili peppers. Pretreatment with capsaicin abolished the hyperreflexia seen with CYP (Maggi et al., 1992). The TRPA1 channel was found to underlie responsiveness to acrolein toxicity (Bautista et al., 2006; Dinis et al., 2004; Geppetti et al., 2008). Activation of this channel in capsaicin-sensitive primary afferents by acrolein in the bladder leads to depolarization of sensory neurons (Bautista et al., 2006; Dinis et al., 2004). This leads to numerous effects, including the release of neuropeptides, vasodilation and other neurogenic inflammatory effects, which in turn lead to hypersensitivity to mechanical stimuli and bladder hyperreflexia (Ahluwalia et al., 1994; Bautista et al., 2006; Maggi et al., 1992). This accounts for the initial steps of acrolein toxicity seen in urinary bladder (Bautista et al., 2006). Neurochemical and electrophysiological changes in afferents also occur in
response to activation of primary afferents by acrolein exposure (Maggi et al., 1992; Vizzard, 2000c; 2001; Yoshimura and de Groat, 1999).

Both TRPV1 and TRPA1 receptors are expressed by a subset of capsaicin-sensitive afferents and both have been examined for their potential role in responding to acrolein exposure (Bautista et al., 2006). The TRPV1 channel is activated by heat as well as capsaicin (Bautista et al., 2006). While the TRPV1 channel is involved in the progress of bladder hyperreflexia and peripheral mechanical hypersensitivity associated with CYP cystitis, it is not as a direct result of acrolein exposure (Ahluwalia et al., 1994; Dinis et al., 2004; Wang et al., 2008). It has been shown that TRPA1 is the presumptive channel required for the inflammatory effects of acrolein exposure in the urinary bladder (Geppetti et al., 2008).

TRPA1 is known to be activated by isothiocyanate or thiosulfinate compounds, the pungent components of mustard oil and garlic (Bautista et al., 2006). Recent in vitro studies have shown that cells expressing TRPA1 were responsive to acrolein application, while cells expressing only TRPV1 were not (Bautista et al., 2006; Dinis et al., 2004). Also, TRPV1-expressing neurons did not produce any current in response to acrolein exposure, while TRPA1-expressing neurons did produce current (Bautista et al., 2006). This demonstrated that TRPA1 is the receptor responsible for the depolarization of afferents in response to acrolein, thus eliciting inflammatory pain (Dinis et al., 2004).

Other experimental approaches to induce cystitis include intravesical instillation of lipopolysaccharide (LPS), acetic acid, mustard oil, hydrochloric acid (HCl), or turpentine. Instillation of all of these compounds leads to bladder irritation and
inflammation by various methods. While there is no perfect model of human bladder inflammation or of the human condition of IC/PBS, these models do provide the opportunity to examine the effects of acute and chronic bladder inflammation and the mechanisms of inflammation including structural, neurochemical and functional changes in micturition reflexes (Westropp and Buffington, 2002). These models also often mimic the inflammation-related histological changes seen in patients with IC/PBS, and many also lead to bladder hyperreflexia, also a part of the constellation of symptoms seen with IC (Kirimoto et al., 2007; Wheeler et al., 2001). However, some of these models induce damage or effects that are not present in the human condition of IC/PBS, including detrusor damage (Westropp and Buffington, 2002). Also, some models do not mimic all of the symptoms of IC/PBS (Westropp and Buffington, 2002). However, IC/PBS does occur naturally in cats. This feline IC model reproduces more features of human IC/PBS than any other animal model previously described, including a waxing and waning course (Westropp and Buffington, 2002). Limitations of the feline model include limited availability of cats diagnosed with the disease and the fact that it is unknown how widespread feline IC really is. These cats also do not provide the ability to examine acute aspects of inflammation (Westropp and Buffington, 2002).

B. CYP-induced cystitis: Changes in the micturition reflex pathway

The inflammation or irritation of tissue can result in chronic pathological conditions that induce changes in somatic sensory pathways, leading to hyperalgesia and allodynia. This increased sensation can be caused by sensitization of peripheral afferents (Dmitrieva and McMahon, 1996; Woolf et al., 1997) or changes in central synaptic
connections that process nociception (Lewin et al., 1992; Yoshimura and de Groat, 1999). A multitude of changes in the micturition reflex pathway have been documented with CYP-induced cystitis. These include bladder hyperreflexia, as mentioned above, and also organizational changes, electrophysiological changes, neurochemical changes as well as changes in somatic sensation (Hu et al., 2003; Vizzard, 2000b; 2001; Yoshimura and de Groat, 1999).

The protein product of the immediate early gene c-fos (Fos) has been used to mark the postsynaptic activation of spinal cord neurons receiving afferent input from the lower urinary tract (Birder and de Groat, 1992; 1993). Growth-associated protein-43 (GAP-43) is a calmodulin-binding protein associated with synapse formation and an ongoing capability for synaptic plasticity in adults (Vizzard and Boyle, 1999). With CYP treatment in rats, changes in the distribution of the Fos protein in lumbosacral spinal cord segments and expansion of spinal cord segments involved in micturition reflexes have been demonstrated (Lanteri-Minet et al., 1995; Vizzard, 2000a). This suggests a change in the organization of micturition reflex pathways, resulting in changes in synaptic connections, afferent excitability or pain modulation systems (Vizzard, 2000a). Chronic CYP treatment in rats also leads to increased expression of GAP-43 in micturition pathways in the dorsal commissure, dorsal horn and IML cell column of the spinal cord, suggesting the possibility of synaptic plasticity with CYP cystitis (Vizzard and Boyle, 1999).

Electrophysiological changes documented in bladder afferent cells with chronic CYP-induced cystitis demonstrate an enhanced electrical excitability of C-fiber bladder
afferents, as well as significant hypertrophy of afferent cell bodies in rats in the L6-S1 DRG (Yoshimura and de Groat, 1999). CYP treatment in rats also leads to the increased afferent firing in the pelvic nerve in response to distension, decreased the firing threshold and increased the area of evoked action potentials (Yu and de Groat, 2008). Increased excitability of bladder afferent neurons to stimulation could certainly contribute to pain and hyperactivity of the urinary bladder as seen with CYP cystitis.

Neurochemical changes with CYP cystitis include an increase in the expression of the neuropeptides calcitonin gene-related peptide (CGRP) and substance P as well pituitary adenylate cyclase-activating polypeptide (PACAP) in lumbosacral DRG and spinal cord regions involved in micturition afferent pathways (in rats: L1-L2, L6-S1)(Vizzard, 2000c; 2001). The expression of the neurotrophin tropomyosin receptor kinases A and B (TrkA and TrkB) and phosphorylated Trk (pTrk) also change in bladder afferent neurons with CYP cystitis in rats, and this could occur in response to the changed expression of neurotrophic factors in the inflamed urinary bladder (Qiao and Vizzard, 2002; Vizzard, 2000b). Bladder afferents in lumbosacral DRG exhibit an increased expression of the transcription factor cAMP responsive-element binding protein (CREB) with CYP-induced cystitis (Qiao and Vizzard, 2004). Phosphorylated CREB (p-CREB) is co-localized with phosphorylated Trk receptors in lumbosacral DRG cells, suggesting that p-CREB activity may be related to a neurotrophin/Trk signaling pathway in bladder afferents (Qiao and Vizzard, 2004). Enhanced expression and function of the ATP receptor purinergic 2X (P2X) in pelvic and lumbar splanchnic pathways has been
reported, suggesting a role for ATP signaling through this receptor in hyperreflexia and hypersensitivity with CYP (Dang et al., 2008).

Sprouting of CGRP and vesicular acetylcholine transporter-immunoreactive (IR) fibers were reported in the bladder urothelium and peptidergic sensory fibers were seen in the detrusor smooth muscle with CYP cystitis, (Dickson et al., 2006) suggesting the possibility that an increase in these fibers could result in bladder hyperreflexia and hypersensitivity.

Symptoms of CYP cystitis can also include increased somatic sensitivity, as evidenced by mechanical hypersensitivity of the hind limbs induced by application of von Frey filaments in experimental animals (Bielefeldt et al., 2006; Guerios et al., 2006; Guerios et al., 2008; Lamb et al., 2006)(Cheppudirah and Vizzard, unpublished observations). Sensitization of pain pathways by inflammation can result in the hypersensitization of neighboring organs and referred somatic cutaneous sites (Bielefeldt et al., 2006).

These changes may play a role in the altered visceral sensation and bladder hyperreflexia involved in human clinical syndromes, such as IC/PBS.

C. How CYP cystitis can relate to human conditions

The CYP-induced experimental model of urinary bladder inflammation could be useful in modeling such syndromes as IC/PBS, which involves a persistent inflammatory state in some patients. IC/PBS is more recently being called painful bladder syndrome (PBS), so as to use a more symptom-oriented description (FitzGerald et al., 2006). IC/PBS is a painful chronic disorder of the urinary bladder that exhibits urinary
frequency, urgency, and suprapubic pressure and pain with only small amounts of bladder filling (Erickson, 1999).

IC/PBS affects anywhere from 44,000 to 1 million people in the United States (Erickson and Davies, 1998) and bladder dysfunction in general affects an estimated 17 million Americans. The cost of treating urinary incontinence was estimated at $25 million in 1995.

Patients with IC/PBS show hypersensitivity to somatic stimuli, including sensitivity to deep tissue pressure, the ischemic forearm test of pain tolerance, and bladder distension (Ness et al., 2005). Some patients even report discomfort to sites other than suprapubic, including lower back, buttocks, and upper thighs (FitzGerald et al., 2005). Patients with IC/PBS also have more non-bladder related symptoms, such as tingling of the fingers and toes and headaches, than age-matched controls (Westropp and Buffington, 2002).

Most biopsies of bladders from patients with IC/PBS show some degree of inflammation, as evidence by the infiltration of inflammatory cells and inflammatory mediators such as lymphocytes, T cells, B cells, mast cells, interleukin-6 and interleukin-2 (Erickson, 1999). The etiology of IC/PBS is unknown, although various theories have been proposed, including neurogenic causes, infection, toxic agents in the urine, deficiency in bladder wall lining, urothelial disorder, disorder of the pelvic floor musculature, underlying endocrine abnormality, and autoimmune disorder (Buffington, 2004; Erickson, 1999). IC/PBS often shows comorbidity with arthritis, allergies, endometriosis, fibromyalgia, migraine, irritable bowel syndrome and panic syndrome, all
of which become worsened by stress (Buffington, 2004; Theoharides, 2007; Westropp and Buffington, 2002).

A link between chronic stress and the onset of symptoms of IC/PBS has been reported (Million et al., 2007; Rothrock et al., 2001). Corticotropin-releasing factor (CRF)-over-expressing mice were examined in order to investigate this link. CRF initiates the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which leads to acute behavioral and visceral stress responses; an overactive HPA axis has also been implicated in the pathogenesis of PBS/IC (Buffington, 2004; Million et al., 2007). CRF-over-expressing mice show a significant increase in frequency of elimination in a novel environment and an overall enhanced pelvic response to stressors (Million et al., 2007). The CRF receptor type 1 has been implicated in visceral hyperalgesia seen in stressed rats (Larauche et al., 2008). CRF has also been implicated in overactive bladder and the stimulation of bladder activity, and a link has been shown between bladder disorders and anxiety (Klausner and Steers, 2004).
IV. Cyclooxygenase-2 (COX-2) and inflammation

A. General Background

The cyclooxygenase enzymes are responsible for the synthesis of prostaglandins from arachidonic acid. Prostaglandins have many roles in physiological functions, including physiological “housekeeping” as well as inducible inflammatory functions. Arachidonic acid is found in cell membrane phospholipids and must be liberated from the membrane-bound phospholipids by phospholipase enzymes before COX enzymes can form prostaglandins (Figure 4) (Mitchell and Warner, 1999). Two isoforms of COX have been identified: COX-1 and COX-2. While both COX enzymes are involved in beneficial, homeostatic functions as well as inflammatory and pain effects, a distinction is often drawn between the two. In general, COX-1 is thought of as the constitutive isoform, involved with many of the physiological functions of gastrointestinal, renal, immune and cardiovascular systems (Parente and Perretti, 2003). COX-2 is thought of as the inducible isoform, with its metabolites being released locally in sites of inflammation or systemically after infection (Figure 5) (Mitchell and Warner, 1999). Cytokines and growth factors have been implicated in the induction of COX-2 expression (Mitchell and Warner, 1999). However, more research has shown that the story is not really as simple as this.

Both COX-1 and COX-2 are responsible for the production of prostaglandin G2 (PGG2) and prostaglandin H2 (PGH2) (Mitchell and Warner, 1999). The presence of different downstream enzymes then determines the formation of further prostaglandins
Arachidonic acid is produced from membrane-bound phospholipids by the enzyme phospholipase A$_2$. Cyclooxygenase (COX) enzymes then produce the prostaglandins PGG$_2$ and PGH$_2$ from arachidonic acid. PGH$_2$ is then formed into multiple other prostaglandins, some of which are involved in inflammatory processes.
Figure 5: The cyclooxygenase concept.

The COX enzymes are involved in homeostatic functions (COX-1) as well as inducible inflammatory functions (COX-2). The presence of inflammatory cytokines and growth factors contributes to the induction of COX-2, which then produces prostaglandins, which sensitize nociceptors and lead to increased pain sensation. However, recent studies have also shown an inducible role for COX-1 in pain.
from PGG₂ and PGH₂ (Figure 4) (Mitchell and Warner, 1999). PGH₂ can be further transformed into PGI₂, thromboxane A₂ (TXA₂), PGE₂, PGD₂ and PGF₂α (Parente and Perretti, 2003). Important for inflammation, PGE₂ is involved in pain sensitization centrally and at nerve terminals in the periphery, and acts as a hyperalgesic and vasodilator (Ahmadi et al., 2002; Mitchell and Warner, 1999; Parente and Perretti, 2003). PGE₂ also could function in a positive feedback loop, inducing the expression of more COX-2 at inflammatory sites (Nantel et al., 1999; Parente and Perretti, 2003). PGI₂ acts as a vasodilator and both PGE₂ and PGI₂ are known to be hyperalgesic, potentiating nociception induced by other factors via the cAMP pathway and activation of protein kinase A (PKA) (Parente and Perretti, 2003; Richardson and Vasko, 2002). Among other functions, TXA₂ and PGD₂ are involved in platelet aggregation, and PGF₂ is involved in vasoconstriction and bronchoconstriction (Parente and Perretti, 2003).

While a clear distinction between the roles of COX-1 and COX-2 remains to be established, it is known that selective inhibition of COX-2 does reduce inflammatory pain in animal models and in humans (Morrison et al., 1999; Parente and Perretti, 2003; Riendeau et al., 1997). In most mammalian tissues, COX-2 is undetectable in normal conditions. COX-2 is then rapidly induced in inflammatory cells such as fibroblasts, monocytes, and vascular endothelium in response to inflammatory mediators such as growth factors and cytokines (Figure 5) (Parente and Perretti, 2003). Still, a role for COX-1 in nociception and inflammation has also been reported and it is likely that inflammation and inflammatory hyperalgesia result from cooperation between prostaglandins produced by both COX enzymes (Parente and Perretti, 2003).
biological functions of the two COX enzymes are much more complex and interrelated than initially thought (Parente and Perretti, 2003).

**B. COX-2 and urinary bladder inflammation**

COX pathways and prostaglandins have a cytoprotective role in urinary bladder and also are involved in micturition reflex (Wheeler et al., 2001). Prostaglandins modulate the basal tone of the detrusor and influence detrusor contraction as well as target primary bladder afferents, thus influencing the micturition reflex (Angelico et al., 2006; Maggi, 1992). Endogenous prostaglandins are produced in the bladder wall during distension and these may have an influence on afferent input, leading to alterations in void contractions (Maggi, 1992; Wheeler et al., 2001). Application of prostaglandins to the detrusor stimulates the micturition reflex (Maggi et al., 1984). PGE$_2$ and PGI$_2$ are the predominant prostaglandins found in the rat and human urinary bladder, although PGD$_2$ is also expressed (Hu et al., 2003; Wheeler et al., 2001). Treatment with systemic non-steroidal anti-inflammatory drug (NSAID), which specifically targets the COX enzymes, increases bladder capacity in experimental rats and NSAID application in vitro leads to detrusor muscle relaxation (Abrams et al., 1979; Maggi et al., 1984; Maggi et al., 1988b). Prostaglandins TXA$_2$, PGE$_2$, PGD$_2$ and PGF$_{2\alpha}$ influence contractile activity of detrusor smooth muscle in vitro (Angelico et al., 2006). COX inhibitors have been shown to attenuate detrusor instability in humans (Lecci et al., 2000). Furthermore, intravenous administration of non-selective COX inhibitors reduces bladder activity in both control rats and rats with bladders inflamed by intravesical administration of acetic acid (Angelico et al., 2006). It has been postulated that COX-1 could be involved in
modulating the contraction threshold, while COX-2 could have more involvement in the inflammatory response (Lecci et al., 2000).

In addition to the roles of COX pathways and prostaglandins in normal bladder function, numerous studies have suggested a role for COX-2 in bladder inflammation and even in the associated bladder hyperreflexia. We know that LPS bladder inflammation in rats leads to the elevation of COX-2 protein in urinary bladders, but does not elevate levels of COX-2 mRNA or COX-1 protein or mRNA (Wheeler et al., 2001). Urinary levels of PGE$_2$ and synthesis of PGE$_2$ in bladder are also increased with LPS, suggesting the importance of this molecule in the LPS inflammatory process (Wheeler et al., 2001). Acute CYP-induced cystitis in female rats increased the expression of COX-2 immunoreactive cell profiles throughout the urinary bladder wall as well as increased protein expression of PGD$_2$ and PGE$_2$ in the whole bladder (Hu et al., 2003). Functionally, it has been shown that a COX-2 specific inhibitor, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl2(5H)-furanone (DFU) attenuates the bladder hyperreflexia associated with CYP-induced cystitis in rats and rescues bladder capacity (Hu et al., 2003). Prostaglandins and COX-2 may play a prominent role in bladder function as well as a pathophysiological role in bladder inflammation and the associated hyperreflexia.
V. Nerve Growth Factor (NGF) and the p75 neurotrophin receptor (p75NTR)

NGF was first discovered by Rita Levi-Montalcini in the 1950s as a target-derived factor vital to the survival and outgrowth of sensory neurons (Aloe, 2004). Since then, the specific receptor for NGF, TrkA, has been discovered and attributed as the receptor required for the important neuronal survival functions of NGF. More recently, the discovery of a pan-neurotrophin receptor, p75NTR, has made the study of neurotrophins and NGF more interesting. p75NTR was initially discovered as a receptor for proNGF, which led to apoptosis (Barker, 2004). Since then, p75NTR has been implicated in myriad neuronal processes, including apoptosis and regeneration. For our purposes, p75NTR has also been implicated as a co-receptor for TrkA, possibly enhancing TrkA binding and specificity for NGF. Both NGF and TrkA are implicated in the processes of bladder inflammation, NGF inducing bladder hyperreflexia and TrkA upregulated after CYP-induced cystitis. The next step is to determine what role p75NTR may play, if any, in bladder inflammation and hyperreflexia.

A. NGF

NGF is one of a family of four mammalian neurotrophins that also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. Neurotrophins are often target-derived and their retrograde transport to the perikaryon is thought to be required for the neurotrophin effect on the neuron (Zweifel et al., 2005). NGF was the first neurotrophin identified and it is involved in the development and survival of peripheral sympathetic and sensory neurons (Chao et al., 2006; Frossard et al., 2004).
Centrally, NGF is involved in survival and maintenance of cholinergic neurons in the basal forebrain (Chao et al., 2006). It has been shown that in the adult, NGF maintains the normal properties of C-fiber afferents as well as all small size sensory neurons (Chuang et al., 2001a). Other NGF functions include neuronal differentiation, neurite outgrowth and sprouting, modulation of synaptic activity, gene regulation, survival maintenance in the adult, and inflammatory pain (Friedman and Greene, 1999).

NGF has a prominent role in inflammation, central and peripheral pain processing, and in the hyperalgesic effects of inflammation. Nociceptors continue to express NGF receptors into adult life in humans and animals and NGF is now additionally thought of as a “peripheral pain mediator” (Pezet and McMahon, 2006); NGF is upregulated in many chronic pain conditions and inflamed tissues. Upregulation of NGF is seen with peripheral inflammation and tissue injury (Lindholm et al., 1987; Micera et al., 2007; Woolf et al., 1997). The induction of NGF expression is caused in part by the actions of pro-inflammatory cytokines, including interleukin-1 and tumor necrosis factor (Hefti et al., 2006). NGF levels can remain increased with chronic inflammation (Hefti et al., 2006). Mast cells are an important inflammatory cell type; mast cells stimulate the release of NGF, and NGF in turn stimulates mast cells under conditions of tissue injury or inflammation, possibly creating a positive feedback loop for inflammatory responses (Hefti et al., 2006). NGF also sensitizes TRPV1 receptors, by initiating post-translational changes in the receptor, resulting in greater excitability (Hefti et al., 2006). TRPV1 is a non-selective cation channel originally identified as the capsaicin receptor (Hefti et al., 2006). Retrograde transport of NGF to the perikaryon
also increases the transcription of several nociception-related molecules, including substance P, BDNF, the Na⁺,1.8 ion channel and TRPV1 (Hefti et al., 2006). Substance P and BDNF are involved in central sensitization to increase the excitability of second-order spinal neurons; the Na⁺,1.8 Na⁺ channel is expressed solely in nociceptors (Hefti et al., 2006).

Injection of NGF into rat hindpaw creates allodynia and heat hyperalgesia due to a wind-up response in the spinal cord (Thompson et al., 1995). Injection of carrageenan or complete Freund’s adjuvant into the hindpaw of mice leads to a rise in endogenous levels of NGF as well as the development of mechanical and heat hyperalgesia (Guerios et al., 2006). Administration of NGF to DRG neurons or afferent terminals leads to sensitization of these primary afferents by increasing the transcription levels of TRPV1, upregulating neuropeptides (CGRP, substance P) and BDNF (Allen and Dawbarn, 2006; Pezet and McMahon, 2006). The neuropeptides substance P and CGRP are released in the periphery and centrally from activation of small-diameter sensory afferents and through actions on endothelial cells, mast cells, immune cells and arterioles, they initiate such inflammatory effects as vasodilation, plasma extravasation, and sensory neuron hyperexcitability, resulting in redness, swelling, and hypersensitivity (Richardson and Vasko, 2002). Exogenously applied NGF can also lead to the rapid sensitization of Na⁺,1.8 sodium channel that is expressed only on nociceptors, contributing to the sensitization of primary afferent neurons after injury or inflammation (Allen and Dawbarn, 2006). NGF protein levels are also increased on colonic epithelium after
trinitrobenzene sulphonic acid (TNBS)-induced colitis (Stanzel et al., 2008). NGF likely serves as a link between inflammation and hyperalgesia (Lewin and Mendell, 1993).

There is a large body of evidence that supports the concept that NGF also has a role in mediating the micturition reflex. NGF is expressed under normal conditions in the urinary bladder, and part of its function is likely in the maintenance of sensory afferents there (Chao and Hempstead, 1995; Chuang et al., 2001b). It also can interact with afferents and contribute to pathological conditions including inflammation and bladder hyperreflexia. Sources of NGF in the bladder include urothelium, detrusor smooth muscle and inflammatory infiltrates, especially mast cells (Steers and Tuttle, 2006; Vizzard, 2000b; Zvara and Vizzard, 2007). Mechanical stretch of the detrusor smooth muscle results in the release of NGF mRNA and protein from the muscle (Vaidyanathan et al., 1998).

There is also a wide range of evidence supporting the role of NGF in lower urinary tract disorders: NGF levels are increased in the urine of patients with IC (Okragly et al., 1999) and increased levels of NGF are seen in the bladders of patients with sensory urgency (Lowe et al., 1997). NGF mRNA levels are increased in rat bladders with chemically-induced cystitis (Oddiah et al., 1998; Vizzard, 2000b) and with bladder outlet obstruction (Steers and Tuttle, 2006). While NGF levels in urinary bladder are reduced with CYP cystitis, this could be attributed to retrograde transport of NGF to cell bodies in the major pelvic ganglion or DRG (Birder et al., 2007; Murray et al., 2004; Vizzard, 2000b).
There is much functional evidence to support the role of NGF in bladder reflexes as well as sensation. It has been shown that intravesical instillation of NGF sensitizes bladder afferents, increasing their response to stimuli and making some responsive that were not responsive before the instillation of NGF (Dmitrieva and McMahon, 1996). Over-expression of NGF in detrusor in rats leads to hyperreflexia (Clemow et al., 1998; Zvara and Vizzard, 2007). Sequestration of NGF reduces inflammation-induced hyperreflexia in a rat model (Dmitrieva et al., 1997). Blockade of NGF reduces the peripheral hypersensitivity seen with CYP cystitis also (Guerios et al., 2008). Systemic administration of REN1820, an NGF scavenging agent and a recombinant protein of the D5 extracellular domain of the NGF receptor tyrosine kinase TrkA, also reduces bladder hyperreflexia in a CYP-induced inflammation model in rats (Hu et al., 2005). Intrathecal administration of NGF increases NGF expression in bladder afferents, sensitizes bladder afferents as well as increases bladder hyperreflexia, demonstrating that increased NGF levels in micturition afferent pathways can contribute to bladder afferent plasticity, contributing to changes in the micturition reflex (Yoshimura et al., 2006). Exogenous NGF application to the rat bladder detrusor through an osmotic pump reduced bladder capacity as well as increased expression of CGRP and Fos in lumbosacral spinal cord, suggesting re-organization of micturition reflex pathways (Zvara and Vizzard, 2007). It has also been suggested that Aδ fibers play a more important role in NGF-induced bladder hyperreflexia than C-fibers (Chuang et al., 2001b).
Figure 6: Neurotrophins and their receptors.

Each neurotrophin binds specifically to a Trk receptor. They all also bind p75NTR, which is known as the pan-neurotrophin receptor. p75NTR is a member of the tumor necrosis factor-α family of receptors. Trk receptors have five extracellular domains. The D5 domain is important for binding ligands (Allen and Dawbarn, 2006).

B. Receptor tyrosine kinase A (TrkA)

Neurotrophins bind with greater affinity to certain receptors. NGF specifically binds TrkA, BDNF and NT-4/5 specifically bind TrkB, and NT-3 binds TrkC (Figure 6). The Trk receptors have an intracellular structure consisting of a transmembrane domain, a kinase domain and a 15-residue C-terminal tail. Extracellularly, Trk receptors can be divided into five distinct domains D1-D5, where D5 is the closest to the cell membrane and also serves as the binding domain (Allen and Dawbarn, 2006). Neurotrophin binding to Trk receptors results in receptor dimerization and transphosphorylation of the
intracellular kinase domains (Allen and Dawbarn, 2006). Phosphorylated tyrosines then can become docking sites for many signaling molecules, including those for the Ras, phosphoinositide 3-kinase (PI3K) and phospholipase C-γ (PLC-γ) pathways (Allen and Dawbarn, 2006; Chao and Hempstead, 1995; Kalb, 2005). Induction of p38 MAPK, ERK and c-JUN N-terminal kinase are also achieved by NGF and possibly involved in NGF-mediated hyperalgesia (Hefti et al., 2006). This signaling through MAP kinases alters and influences post-translational processes as well as transcription (Richardson and Vasko, 2002).

Neurotrophin signaling also often requires retrograde transport of neurotrophins from the periphery to the perikaryon, resulting in retrograde effects on the cell body from what are often great distances from the nerve terminals (Allen and Dawbarn, 2006; Miller and Kaplan, 2001; Zweifel et al., 2005). It is unclear if internalization of a complete ligand-receptor complex is necessary or if other modes of signal propagation are used for the neurotrophin signal (Zweifel et al., 2005). However, there is considerable evidence for a clathrin/dynamin dependent process of NGF-TrkA ligand-receptor internalization and transport, although pincher-mediated endocytosis as well as caveolin-mediated endocytosis have also been implicated (Zweifel et al., 2005).

As explained above, a role for NGF in sensory and sympathetic cell development, maintenance, and inflammatory pain states has been described extensively. All of these functions of NGF are mediated through the TrkA receptor. Perhaps underlining the importance of NGF-TrkA signaling to pain sensation, the condition known as congenital insensitivity to pain with andhidrosis (CIPA) results from a rare autosomal-recessive
genetic mutation in the TrkA gene (Allen and Dawbarn, 2006). NGF is involved in regulating the gene expression of nociception-related molecules in TrkA-expressing neurons (Allen and Dawbarn, 2006; Pezet and McMahon, 2006). Many have been described above. These include substance P, CGRP, sodium channels and TRPV1 as well as ion channels, and the P2X3 purinergic receptor (Allen and Dawbarn, 2006; Pezet and McMahon, 2006). Post-translationally, increased neuronal sensitivity is achieved through increased expression of CGRP and substance P release and the phosphorylation of TRPV1 seen after NGF treatment of TrkA-expressing cells (Pezet and McMahon, 2006). It has also been demonstrated that TrkA-mediated activation of the MEK1-ERK1/2 and PI3K pathways is involved in local axonal sprouting (Miller and Kaplan, 2001).

In urinary bladder afferents, TrkA immunoreactivity was upregulated in bladder-projecting afferent cells in DRG neurons at spinal cord levels L1 and L6 after acute and chronic CYP-induced cystitis (Qiao and Vizzard, 2002). TrkA is also upregulated in the major pelvic ganglion after CYP-induced cystitis (Murray et al., 2004). Phosphorylated Trk expression is also increased in CYP cystitis, suggesting that the increased phosphorylation of TrkA may be increasing the response of neurons to target-derived NGF (Qiao and Vizzard, 2002). Also, the systemic administration of k252a, a Trk specific antagonist, decreased the bladder activity associated with CYP-induced cystitis, suggesting a role for NGF-TrkA signaling in CYP-induced bladder hyperreflexia (Hu and Vizzard, unpublished observations).
C. p75NTR

While all neurotrophins NGF, BDNF, NT-3 and NT-4/5 bind to their specific Trk receptors, they also all bind to p75NTR with comparable affinity (Allen and Dawbarn, 2006). As well as binding all neurotrophins and functioning independently, p75NTR also regulates the affinity of Trk receptors for their specific ligands, thus leading to enhanced Trk function and ligand specificity (Chao and Hempstead, 1995; Chao et al., 2006). p75NTR has an extracellular domain that contains 4 cysteine-rich domains that are required for ligand binding (Allen and Dawbarn, 2006; Barker, 2004). The intracellular portion of p75NTR contains a type II death domain that is implicated in apoptosis. This domain is common in members of the tumor necrosis factor-α (TNF-α) family of receptors, although the structure of the death domain of p75NTR is distinct from other receptors in the TNF-α family (Barker, 2004). p75NTR often acts as a co-receptor for activating signaling cascades (especially with Trk receptors), but also functions on its own (Chao and Hempstead, 1995). The results of the multiple interactions with co-receptors and ligands of p75NTR often depend on the developmental stage and cell type where signaling is occurring.

While p75NTR functions in the presence of Trk receptors are very important, p75NTR has many other interesting binding partners and functions, including apoptosis and neuronal growth regulation. For example, sortilin is a co-receptor for p75NTR binding the NGF precursor molecule proNGF, and this signaling partnership results in apoptosis (Barker, 2004; Chao and Hempstead, 1995). The affinity of proNGF for p75NTR is higher than that of mature NGF (Barker, 2004). This cell death function of p75NTR may also be
important in regulating the development of central nervous system cell populations (Friedman and Greene, 1999). It has also been shown that p75\textsuperscript{NTR} stimulation activates the transcription factor NF\textkappa B in Schwann cells and oligodendrocytes and the c-Jun N-terminal kinase (c-JNK) pathway in oligodendrocytes, facilitating cell survival (Chao and Hempstead, 1995; Freund-Michel and Frossard, 2008; Friedman and Greene, 1999). The consequences of this signaling are not yet fully understood (Friedman and Greene, 1999). p75\textsuperscript{NTR} also may form a three-receptor complex with the Nogo receptor and with Lingo-1 which results in neuronal growth inhibition (Barker, 2004). This p75\textsuperscript{NTR} complex can bind myelin-based growth inhibitors including Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgP) and result in the regulation of RhoA, thus affecting neuronal growth (Barker, 2004; Dechant and Barde, 2002; Wong et al., 2002).

The mechanisms of the internalization and movement of the p75\textsuperscript{NTR} signal are as yet poorly understood and many possibilities exist (Bronfman and Fainzilber, 2004). Internalization of the receptor could result in lysosomal degradation or recycling of the ligand-receptor complex or adaptor proteins could form a retrograde transport complex with or without a signaling endosome (Bronfman and Fainzilber, 2004). A clathrin-dependent internalization of p75\textsuperscript{NTR} has been demonstrated in PC12 cells, with internalization occurring 3-fold slower than TrkA internalization in PC12 cells (Freund-Michel and Frossard, 2008). This possibly leads to recycling of p75\textsuperscript{NTR} at the cell membrane (Freund-Michel and Frossard, 2008). p75\textsuperscript{NTR} can also be subjected to
regulated intramembrane proteolysis, which means that the intracellular cleavage of \( p75^{\text{NTR}} \) could have its own signaling capabilities (Bronfman and Fainzilber, 2004).

In neurons expressing Trk receptors, \( p75^{\text{NTR}} \) is typically thought to function as an assistant to NGF signaling through TrkA. In studies in thoracic rat DRG, \( p75^{\text{NTR}} \) was found exclusively expressed in cells that also expressed TrkA (Wright and Snider, 1995). Co-expression of \( p75^{\text{NTR}} \) with members of the Trk family is thought to enhance binding, increase sensitivity and to sharpen the discrimination of Trks for their specific neurotrophin ligand (Barker, 2004; Chao and Hempstead, 1995; Lee et al., 2001; Teng and Hempstead, 2004). It is also possible that \( p75^{\text{NTR}} \) forms a complex with TrkA and NGF, allowing for increased ligand selectivity of TrkA (Chao et al., 2006; Kahle et al., 1994; Verdi et al., 1994). However, through analysis of the crystal structures of the extracellular domains of TrkA and NGF, a recent study has determined that the formation of an actual trimeric NGF- \( p75^{\text{NTR}} \)-TrkA structure cannot occur (Wehrman et al., 2007). However, a ligand-passing model has been suggested that involves the rapid binding of NGF to the extracellular domain of \( p75^{\text{NTR}} \), and then NGF binding to TrkA. NGF can then bind a second TrkA molecule, allowing for dimerization of TrkA upon NGF binding (Barker, 2007; Wehrman et al., 2007). It is possible that TrkA-\( p75^{\text{NTR}} \) signaling pathways are different from TrkA signaling alone (Nykjaer et al., 2005). Convergence of downstream signaling pathways of TrkA and \( p75^{\text{NTR}} \) is also a likely possibility for the communication between TrkA and \( p75^{\text{NTR}} \) (Freund-Michel and Frossard, 2008).

While in some systems, \( p75^{\text{NTR}} \) increases TrkA binding affinity for NGF, in other systems, \( p75^{\text{NTR}} \) also could function to reduce NGF-TrkA signaling by sequestering NGF,
leaving less available to bind TrkA (Coome et al., 1998; Dhanoa et al., 2006; Hannila et al., 2004; Nykjaer et al., 2005). This scenario is possible in pathophysiological conditions such as tissue injury or inflammation that involve the increased expression of NGF; it is possible that TrkA and p75NTR no longer work synergistically in these situations (Dhanoa et al., 2006). Sprouting is an NGF-mediated process of peripheral axons. Mice that do not express p75NTR show increased sprouting in cerebellar and spinal cord sensory neurons (Coome et al., 1998; Scott et al., 2005). p75NTR expression in sympathetic neurons is important to ensure normal target sympathetic innervation and to limit axonal arborization in tissues where NGF is elevated, such as in inflammation or injury (Dhanoa et al., 2006). Increased sprouting in the p75NTR knockout mice has also been shown for postganglionic sympathetic projections into cerebellum and trigeminal ganglia (Dhanoa et al., 2006). Mice that over-express NGF and do not express p75NTR show an increase in sympathetic fiber sprouting in target peripheral tissues, and the authors suggest that NGF-TrkA signaling is enhanced in situations without p75NTR and with the over-expression of NGF (Dhanoa et al., 2006). Enhanced sprouting of central sensory processes is also seen in p75NTR knockout mice after dorsal rhizotomy, again suggesting that the TrkA-NGF-dependent process of sensory neuron sprouting is enhanced in the absence of p75NTR. It has been suggested that the binding of NGF to p75NTR leaves less NGF available to signal via TrkA, thus permitting more NGF-TrkA-mediated sprouting (Coome et al., 1998; Hannila and Kawaja, 2005; Miller et al., 1994). The ratio of p75NTR to TrkA may determine neuronal responsiveness and survival (Chao et al., 2006).
In a knockout mouse model involving the deletion of exon III from the \( p75^{\text{NTR}} \) gene, distinct changes were seen in the sensory innervation phenotype (Lee et al., 1992). The mice had decreased sensory innervation of CGRP and substance P-expressing neurons in their footpads, which led to loss of heat sensitivity and ulceration of distal limbs (Lee et al., 1992). This effect was rescued by crossing a human transgene encoding \( p75^{\text{NTR}} \) into mutant mice, indicating that \( p75^{\text{NTR}} \) has an important role in sensory innervation (Lee et al., 1992). A second line of transgenic mice involves deletion of exon IV from the \( p75^{\text{NTR}} \) gene. This line demonstrated a similar decrease in sensory innervation and decreased temperature sensation as the exon III knockout mice did (Dhanoa et al., 2006). Changes in levels of sympathetic axonal sprouting have since been demonstrated in both lines of \( p75^{\text{NTR}} \) transgenic mice (Coome et al., 1998; Dhanoa et al., 2006).

It is possible that \( p75^{\text{NTR}} \) functions in the sensation of pain as well as in inflammatory plasticity, including long-term changes in sensory pathways after inflammation. \( p75^{\text{NTR}} \) is increased in DRG after nerve injury and may be associated with the neuropathic pain seen after nerve injury (Obata et al., 2006). The neuropeptide substance P is upregulated and released from sensory neurons after exposure to high levels of NGF. An \textit{in vitro} experiment using sensory neurons showed that blockade of TrkA and \( p75^{\text{NTR}} \) independently inhibited the release of substance P from these neurons, suggesting that both TrkA and \( p75^{\text{NTR}} \) are required for the upregulation and release of substance P in this system (Skoff and Adler, 2006). Freund’s adjuvant-induced arthritis is a chronic inflammatory pain model that results from injection of complete Freund’s
adjuvant (CFA) into the hindpaw of an experimental animal. This treatment increases TrkA and p75NTR protein expression in DRG cells and in the dorsal horn of lumbar spinal cord levels L3-L5, which receive afferents from hind paws (Pezet et al., 2001). This suggests that this chronic inflammatory pain model involves NGF, which may signal via TrkA and p75NTR receptors, thus contributing to central neuroplasticity (Pezet et al., 2001). The mRNA expression of p75NTR is also increased in DRG with peripheral inflammation resulting from the injection of CFA into the hindpaws of rats (Cho et al., 1996). The same technique was used in neonatal rats and mRNA expression of p75NTR and TrkA were upregulated in the DRGs of rat pups, implicating the signaling pathways of p75NTR and TrkA in the development of sensory and sympathetic neurons and in the neuroplasticity associated with inflammation in newborn pups (Chien et al., 2007).

The roles of p75NTR have not been completely resolved, but there are lines of evidence indicating that it is an active player in neuronal plasticity. Research examining p75NTR expression in the bladder includes experiments in the streptozotocin (STZ)-induced diabetes model, neuropathic bladder in human patients, in experimental nerve regeneration and in bladder inflammation models (Cho et al., 1996; Elsasser-Beile et al., 2000; Tong and Cheng, 2005; Vaidyanathan et al., 1998; Wakabayashi et al., 1998; Wakabayashi et al., 1996; Wakabayashi et al., 1995). STZ is used to model diabetes in experimental rats and diabetes is often associated with bladder dysfunction (Tong and Cheng, 2005). Both protein and mRNA expression of NGF were down-regulated in the bladder of rats with STZ-induced diabetes, and p75NTR mRNA expression is also down-regulated (Delcroix et al., 1998; Tong and Cheng, 2005). This reduction in neurotrophic
factor as well as neurotrophin receptor could indicate the importance of NGF and the p75\textsuperscript{NTR} receptor in the maintenance of bladder afferents, and may indicate that the loss of these in the bladder could lead to the bladder pathology seen with diabetes (Delcroix et al., 1998; Tong and Cheng, 2005). It is also possible that target-derived changes in NGF levels drive changes in p75\textsuperscript{NTR} expression (Delcroix et al., 1998). Biopsies taken from patients with neuropathic bladder were examined with immunohistochemistry for p75\textsuperscript{NTR}-immunoreactivity (IR) and IR was seen in urothelium as well as in nerve fibers located near to the urothelium (Vaidyanathan et al., 1998). Nerve crush injury was performed at the major pelvic ganglion of experimental male rats and regeneration occurred after about 60 days (Wakabayashi et al., 1998). After this time, bladders were examined for p75\textsuperscript{NTR}–IR and GAP-43-IR. The density of both p75\textsuperscript{NTR}–IR and GAP-43-IR nerve fibers was increased in the bladders 30 days after the crush, implicating both GAP-43 and p75\textsuperscript{NTR} in nerve regeneration and neuroplasticity in the urinary bladder after nerve injury (Wakabayashi et al., 1998). A few other studies have examined p75\textsuperscript{NTR}–IR in the rat urinary bladder with or without CYP-induced cystitis (Wakabayashi et al., 1996; Wakabayashi et al., 1995). Most of the p75\textsuperscript{NTR} staining found in control bladders was in nerve fibers running freely in the lamina propria, on the surface of Schwann cells, and in the vicinity of blood vessels (Wakabayashi et al., 1996; Wakabayashi et al., 1995). A few nerve fibers were also labeled in the control detrusor and some p75\textsuperscript{NTR}-IR fibers also co-labeled with tyrosine hydroxylase (Wakabayashi et al., 1995). No staining was seen in the urothelium (Wakabayashi et al., 1995). Staining after 2 or 3-day CYP treatment was increased in thick nerve fiber bundles in the smooth muscle. The authors
suggested that $p75^{\text{NTR}}$ expression in the muscle could be involved in the hyperalgesia associated with CYP cystitis due to the increased expression of NGF in the urinary bladder smooth muscle (Wakabayashi et al., 1996).

While it is clear that $p75^{\text{NTR}}$ is expressed in urinary bladder reflex pathways and that its expression is plastic with some pathological conditions, much remains to be determined. It is likely that the ratio of expression of TrkA to $p75^{\text{NTR}}$ receptors influences the overall NGF binding affinity and signaling effects (Chao and Hempstead, 1995).

While the importance of $p75^{\text{NTR}}$ is becoming more clear in many tissue types and in NGF signaling, virtually nothing is known about $p75^{\text{NTR}}$ signaling in the bladder, even though NGF signaling appears to have importance in bladder activity with and without bladder inflammation. This dissertation focused on determining the expression and functional role of $p75^{\text{NTR}}$ in the urinary bladder.
VI. Project aims and hypotheses

The goal of this project was to examine changes in micturition reflex pathways, including the urinary bladder, lumbosacral spinal cord and DRG and bladder function with bladder inflammation using the CYP model of cystitis. The overall hypothesis of this project is that urinary bladder inflammation induces expression of inflammatory enzymes and changes in neurotrophin receptor expression that contribute to functional changes in the urinary bladder. This project focused specifically on the expression of COX-2 (Aim 1) and p75NTR (Aim 2) in the urinary bladder and micturition reflex pathways, and on the functional role of p75NTR (Aim 3) in the urinary bladder of experimental animals.

COX-2 has been implicated in normal bladder function as well as in bladder hyperreflexia seen with bladder inflammation (Wheeler et al., 2001). Systemic administration of a COX-2 inhibitor resulted in the attenuation of bladder hyperreflexia seen with CYP cystitis (Hu et al., 2003). Inflammatory stimuli increases COX-2 expression in inflamed tissues, and COX-2 protein and mRNA expression in the urinary bladder are increased with CYP treatment (Hu et al., 2003), but the localization and cellular sources of the COX-2 upregulation remained to be shown. We hypothesized increased COX-2 expression in specific tissue compartments of the urinary bladder (urothelium or detrusor) with CYP-induced inflammation. This was the goal of Aim 1, described in Chapter 2.
NGF is also implicated in normal bladder function as well as in the plasticity of bladder function with cystitis (Chao and Hempstead, 1995; Chuang et al., 2001b). NGF is increased in the urine of patients with IC/PBS and in experimentally induced inflammation, and exogenous application of NGF results in bladder hyperreflexia and increased afferent sensitivity (Clemow et al., 1998; Dmitrieva and McMahon, 1996; Dmitrieva et al., 1997; Okragly et al., 1999; Zvara and Vizzard, 2007). The specific NGF tyrosine kinase receptor, TrkA, is increased in bladder afferent cells with CYP. While there is evidence for a role of NGF and NGF-TrkA signaling in inflammatory responses as well as in bladder inflammation, thus far very little attention has been paid to the role that the pan-neurotrophin receptor p75NTR might play in normal micturition reflexes and how this might change in response to bladder inflammation. Thus, aims 2 and 3 of this project focused on the expression and function of p75NTR in bladder and micturition reflex pathways in control and CYP-treated rats. We hypothesized that p75NTR is constitutively expressed in micturition pathways and upregulated with cystitis. Based on bladder function effects of TrkA blockade with cystitis, we also hypothesized that p75NTR blockade in the urinary bladder would also decrease bladder hyperreflexia with cystitis. The functional role of p75NTR was studied by intravesical blockade by immunoneutralization with a monoclonal antibody to p75NTR and by PD90780, known to block NGF-p75NTR binding. A summary of aims and hypotheses for this dissertation follows below.
**Aim 1:** To determine the expression of COX-2 in urinary bladder urothelium, suburothelium and detrusor smooth muscle in control animals and after acute and chronic CYP-induced cystitis.

**Hypothesis:** COX-2 expression is increased in specific tissue compartments of the urinary bladder with the bladder inflammation with CYP-induced cystitis.

**Aim 2:** To determine the expression p75\textsuperscript{NTR} in micturition reflex pathways in control animals and after CYP-induced cystitis.

**Hypothesis:** p75\textsuperscript{NTR} is constitutively expressed in micturition reflex pathways and expression is upregulated with urinary bladder inflammation.

**Aim 3:** To determine the effect of intravesical p75\textsuperscript{NTR} blockade on bladder function in both control and CYP treated animals.

**Hypothesis:** Blockade of p75\textsuperscript{NTR} in urinary bladder decreases voiding frequency in control rats and decreases bladder hyperreflexia in CYP-treated rats.
References for Comprehensive Literature Review


neurotrophin-3 and glial cell line-derived neurotrophic factor levels in the urine of interstitial cystitis and bladder cancer patients. J Urol 161(2):438-441; discussion 441-432.


drugs (NSAIDs) and selective COX-2 inhibitors, using sensitive microsomal and platelet assays. Can J Physiol Pharmacol 75(9):1088-1095.


64


Chapter 2: Expression of Cyclooxygenase-2 in Urinary Bladder in Rats with Cyclophosphamide-induced Cystitis

Abstract

These studies examined the expression of cyclooxygenase-2 (COX-2) expression in the urothelium and suburothelial space and detrusor from rats treated with cyclophosphamide (CYP) to induce acute (4 hr), intermediate (48 hr) or chronic (10 day) cystitis. Western blotting and immunohistochemistry were used to demonstrate COX-2 expression. In whole mount preparations of urinary bladder, nerve fibers in the suburothelial plexus and inflammatory cell infiltrates were characterized for COX-2 expression after CYP-induced cystitis. COX-2 expression significantly ($p \leq 0.01$) increased in the urothelium + suburothelium and detrusor smooth muscle with acute, intermediate and chronic (10 day) CYP-induced cystitis but expression in urothelium + suburothelium was significantly greater. CYP-induced upregulation of COX-2 showed by immunostaining in the urothelium + suburothelium was similar to that observed with western blotting and also demonstrated COX-2 inflammatory cell infiltrates (CD86+) and nerve fibers (PGP+) in the suburothelial plexus. Although COX-2 expression was significantly ($p \leq 0.01$) increased in detrusor smooth muscle, immunohistochemistry failed to demonstrate an obvious change in COX-2-IR in detrusor muscle but COX-2 inflammatory infiltrates were present throughout the detrusor. COX-2-IR nerve fibers exhibited increased density in the suburothelial plexus with acute or chronic CYP-induced cystitis. COX-2-IR macrophages (CD86+) were present throughout the urinary bladder with acute and chronic CYP-induced cystitis. These studies demonstrate cellular targets in the urinary bladder where COX-2 inhibitors may act.
Introduction

Experiments involving a chemically (cyclophosphamide, CYP)-induced urinary bladder inflammation have demonstrated alterations in neurochemical (Braas et al., 2006; Dattilio and Vizzard, 2005; Vizzard, 2001), organizational (Vizzard, 2000b; Vizzard and Boyle, 1999) and electrophysiological (Yoshimura and de Groat, 1999) properties of micturition reflex pathways. Possible mechanisms underlying the neural plasticity following CYP-induced cystitis may involve altered expression of neurotrophic factors (Murray et al., 2004; Vizzard, 2000b), cytokines (Malley and Vizzard, 2002) and/or neural activity in the urinary bladder. The presence of proinflammatory cytokines and growth factors can induce cyclooxygenase-2 (COX-2), an inflammatory response gene (Mitchell and Warner, 1999). CYP-induced cystitis induces a number of neurotrophic factors (Murray et al., 2004; Vizzard, 2000b), including nerve growth factor, in the urinary bladder and pelvic ganglia.

In addition to COX-2 being induced in response to inflammatory stimuli (Fujisawa et al., 2005; Hu et al., 2003; Linden et al., 2004; Mitchell and Warner, 1999; Schafers et al., 2004; Yiangou et al., 2006), studies have demonstrated upregulation of COX-2 in the urinary bladder as a result of urinary bladder outlet obstruction (Park et al., 1999; Park et al., 1997) and postnatal development (Park et al., 1997). Complete bladder outlet obstruction in mice significantly upregulated COX-2 expression in detrusor smooth muscle cells and this has been suggested to be a result of mechanical stretch (Park et al., 1999). During development, embryonic expression of COX-2 transcript in bladder is
100-fold higher compared to postnatal or adult bladder (Park et al., 1997). In our previous studies, COX-2 mRNA was increased in the whole urinary bladder after acute and chronic CYP treatment (Hu et al., 2003). COX-2 protein expression in inflamed bladders paralleled that of COX-2 mRNA (Hu et al., 2003). Prostaglandins generated by the COX-2 enzyme are also mediators of altered neuronal activity in inflamed tissues and have been demonstrated to stimulate the micturition reflex, possibly through activation of capsaicin sensitive bladder afferents (Andersson and Wein, 2004; Maggi, 1992; Maggi et al., 1988a). Prostaglandins have been suggested to play a physiological role in contributing to the basal tone of the detrusor and modulating activity of bladder nerves (Andersson and Wein, 2004; Maggi, 1992; Maggi et al., 1988a). A number of COX-2 inhibitors have also been shown to increase bladder capacity in experimental cystitis induced by CYP (Hu et al., 2003; Lecci et al., 2000).

An increase in COX-2 expression induced by complete bladder outlet obstruction has been specifically localized to the detrusor smooth muscle cells (Park et al., 1999). With respect to COX-2 upregulation induced by CYP-induced cystitis (Hu et al., 2003), there is currently no information that addresses the cellular sources in the urinary bladder that express COX-2 with bladder inflammation of varying duration. The purpose of this study was to determine: (1) COX-2 protein expression in the urothelium + suburothelium compared to detrusor smooth muscle by western blotting after CYP-induced cystitis of varying duration; (2) cellular location of COX-2 in urinary bladder of control rats or after CYP-induced cystitis using immunohistochemistry with an emphasis on urothelial cell, nerve fiber and inflammatory cell infiltrate expression; (3) intensity of COX-2
immunoreactivity in the urothelium after CYP-induced cystitis using semi-quantitative image analysis. In contrast to COX-2 expression in detrusor smooth muscle cells with outlet obstruction (Park et al., 1999), the present study demonstrates robust expression of COX-2 protein in the urothelium, inflammatory cell infiltrates and to a lesser extent, detrusor smooth muscle with CYP-induced cystitis.
Materials and Methods

Adult female Wistar rats (150 - 250g) were purchased from Charles River Canada (St. Constant, Canada). Chemicals used in these studies were purchased from Sigma ImmunoChemicals (St. Louis, MO). Primary antibodies for immunohistochemistry were purchased from commercial sources (Tables 1). Secondary antibodies were purchased from Jackson ImmunoResearch, West Grove, PA (Table 1).

Cyclophosphamide (CYP)-Induced Cystitis-Acute, Intermediate or Chronic

Chemical cystitis was induced in adult female Wistar rats by CYP treatment. CYP is metabolized to acrolein, an irritant eliminated in the urine (Cox, 1979a). CYP was administered in one of the following ways (Corrow and Vizzard, 2007a): (1) 4 hr (150 mg/kg; i.p.) prior to euthanasia of the animals to elicit acute inflammation (n = 21); (2) 48 hr (150 mg/kg; i.p.) prior to euthanasia to examine an intermediate inflammation (n = 21) or (3) administered every third day for 10 days to elicit chronic inflammation (n = 21; 75 mg/kg; i.p.). All injections of CYP were performed under isoflurane (2%) anesthesia. Control animals (n= 21) were gender matched to the experimental groups and received a corresponding volume of saline (0.9%) or distilled water injected (i.p.) under isoflurane (2%) anesthesia. Animals were euthanized by isoflurane anesthesia (4%) plus thoracotomy at the indicated time points and the urinary bladder was harvested and weighed. The University of Vermont IACUC approved all experimental procedures (#06-014) involving animal use. Animal care was under the supervision of UVM’s Office of Animal Care in accordance with AAALAC and NIH guidelines. All efforts
were made to minimize animal stress/distress and suffering and to use the minimum number of animals.

**Western Blotting for COX-2**

The urothelium + suburothelium was dissected from the detrusor smooth muscle using fine forceps under a dissecting microscope (Dattilio and Vizzard, 2005). Urothelium + suburothelium or detrusor were homogenized separately in tissue protein extraction agent with protease inhibitors (Roche, Indianapolis, IN) and aliquots were removed for protein assay. Samples (20 µg) were suspended in sample buffer for fractionation on gels and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes and efficiency of transfer was evaluated. Membranes were blocked overnight in a solution of 5% milk, 3% bovine serum albumin in Tris-Buffered Saline with 0.1% Tween. Membranes were incubated in goat anti-COX-2 (Table 1) overnight at 4 °C. Washed membranes were incubated in a species-specific secondary antibody (Table 1) for 2 hr at room temperature for enhanced chemiluminescence detection (Pierce, Rockford, IL). Blots were exposed to Biomax film (Kodak, Rochester, NY), and developed. The intensity of each band was analyzed and background intensities subtracted using Un-Scan It software (Silk Scientific, Orem, UT). Human recombinant COX-2 (1:1000; Cayman Chemical Co., Ann Arbor, MI) was used as a positive control and Western blotting of erk1 and erk2 (1:1000; Cell Signaling Technology, Danvers, MA) in samples was used as a loading control. Additional loading controls including GAPDH and L32 revealed identical results to those obtained with
erk1/2. Preabsorption of COX-2 antisera with appropriate immunogen (1 µg ml⁻¹) reduced staining in blots to background levels. To confirm the specificity of our split bladder preparations, urothelium + suburothelium and detrusor samples were examined for the presence of α-smooth muscle actin and uroplakin II by western blotting. In urothelium + suburothelium samples, only uroplakin II was present. Conversely, in detrusor samples, only α-smooth muscle actin was present.

**Immunohistochemistry**

Cryostat sections of the bladder (10 µm) from control (n = 7) and experimental treatments (acute, intermediate, chronic CYP-induced cystitis; n = 7 each) were examined for COX-2 immunoreactivity (IR). The urinary bladder was post-fixed in 4% paraformaldehyde, placed in ascending concentrations of sucrose (10-30%) in 0.1 M PBS for cryoprotection, sectioned on a freezing cryostat and directly mounted on gelled (0.5%) microscope slides for on-slide processing (Vizzard, 1997). Briefly, sections were incubated overnight at room temperature with mouse anti-COX-2 (Table 1) in 1% donkey serum and 0.1 M PBS, and then washed (3 × 10 min) with 0.1 M PBS, pH 7.4. The tissues were then incubated with secondary antibody (Table 1) for 2 hr at room temperature. Following washing, the slides were coverslipped with Citifluor (Citifluor Ltd., London). Control sections incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining was observed.

**Whole Mount Bladder Preparation**
The urinary bladder from control (n = 5) and experimental treatments (n = 5 each) was dissected and placed in Krebs solution. The bladder was cut open along the midline and pinned to a sylgard-coated dish. The bladder was incubated for 1.5 hr at room temperature in cold fixative (2% paraformaldehyde + 0.2% picric acid) and urothelium removed as previously described (Zvarova and Vizard, 2005). Urothelium and bladder musculature were processed for COX-2-IR (as described above). Whole mounts stained for COX-2 were also stained with antiserum to the pan-neuronal marker, protein gene product (PGP)9.5. COX-2-immunoreactive cells and associated processes in the suburothelial space were obvious with CYP treatment but were not PGP positive. To determine the cell types expressing COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (GFAP, vimentin), glial and Schwann cells (S-100), interstitial cells (c-kit), macrophages, dendritic cell populations and monocytes (CD86), and activated B lymphocytes (CD80) was performed (Table 1). For single staining for COX-2-IR, we used a mouse anti-COX-2 antibody (Table 1). For double staining procedures, either a mouse anti-COX-2 or rabbit anti-COX-2 antibody was used depending upon the host of the other primary antibody (Table 1). COX-2-IR in the urinary bladder was equivalent with either the mouse anti-COX-2 or rabbit anti-COX-2 antibody. Control (n = 4) and CYP-treated tissues (n = 4 each) were incubated overnight at room temperature in a cocktail of COX-2 antiserum (as above) plus PGP, vimentin, GFAP, S-100, c-kit, CD80 or CD86 (Table 1). After washing, the tissues were incubated in a cocktail of species-
specific secondary antibodies (Table 1) for 2 h at room temperature, followed by washing
and coverslipping.

**Assessment of Positively Stained Urinary Bladder Regions**

Immunohistochemistry on bladder sections or whole mounts was performed on
control and experimental tissues simultaneously to reduce the incidence of staining
variation that can occur between tissues processed on different days. Staining observed
in experimental tissue was compared to that observed from experiment-matched negative
controls. Urinary bladder sections or whole mounts exhibiting immunoreactivity that was
greater than the background level observed in experiment-matched negative controls
were considered positively stained.

**Visualization and quantitative analysis of COX-2 –IR in urothelium**

Six to 10 urinary bladder sections from control and experimental groups were
examined under an Olympus fluorescence photomicroscope with a multiband filter set for
simultaneous visualization of the Cy3 and Cy2 fluorophores. Cy3 was visualized with a
filter with an excitation range of 560-596 nm and an emission range from 610-655 nm.
Cy2 was viewed by using a filter with an excitation range of 447-501 nm and an emission
range from 510-540 nm. Quantification of COX-2-IR in the urothelium was performed as
previously described (LaBerge et al., 2006a; Yuridullah et al., 2006). Grayscale images
acquired in tiff format were imported into Meta Morph image analysis software (version
4.5r4; Universal Imaging, Downingtown, PA). The opened image was first calibrated for
pixel size by applying a previously created calibration file. The free hand drawing tool
was selected and the urothelium was drawn and measured in total pixels area. A
threshold encompassing an intensity range of 100-250 grayscale values was applied to the region of interest in the least brightly stained condition first. The same threshold was subsequently used for all images. Percent COX-2 expression above threshold in the total area selected was then calculated. Quantification of COX-2 expression in nerve fibers in the suburothelial plexus was performed as previously described (Corrow and Vizzard, 2007a; LaBerge et al., 2006a; Yuridullah et al., 2006) and modified from Brady et al. (Brady et al., 2004). Grayscale images acquired in tiff format were imported into Image J (Abramoff et al., 2004) and images were thresholded. Images were acquired from the trigone region of the suburothelial plexus in control and treated rats. A rectangle of fixed dimension (500 x 500 pixels) was placed on the section according to a random selection of x and y coordinates. This process was repeated 7 times for each image. The average density of COX-2-IR nerve fibers was then calculated.

**Statistics**

All values are means ± S.E.M. Comparisons of COX-2 densitometry values from western blots of urinary bladder samples were made using ANOVA. Percentage data from image analysis were arcsin transformed to meet the requirements of this statistical test. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. When F ratios exceeded the critical value (p ≤ 0.05), the Dunnett’s post-hoc test was used to compare the control means with each experimental mean.

**Figure Preparation**

Digital images were obtained using a CCD camera (MagnaFire SP; Optronics; Optical Analysis Corp., Nashua, NH) and LG-3 frame grabber attached to an Olympus
microscope (Optical Analysis Corp.). Exposure times were held constant when acquiring images from control and experimental animals processed and analyzed on the same day. Images were imported into Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA) where groups of images were assembled and labeled.
Results

COX-2 protein expression in detrusor smooth muscle, urothelium + suburothelium with CYP-induced cystitis

COX-2 protein expression in detrusor as determined by western blotting significantly (p ≤ 0.01) increased (6-fold) with acute (4 h), intermediate (48 h; 4.2-fold) or chronic CYP-treatment (6.3-fold)(Fig. 1A, B). COX-2 protein expression in urothelium + suburothelium also significantly (p ≤ 0.01) increased with acute (15-fold), intermediate (16-fold) or chronic (17-fold) CYP treatment (Fig. 1C, D). Basal COX-2 expression in detrusor was significantly (p ≤ 0.01) greater than that in urothelium + suburothelium. The fold-increase in COX-2 expression in the urothelium + suburothelium induced by CYP-induced cystitis was significantly (p ≤ 0.01) greater at all time points examined compared to detrusor (Fig. 1E).

COX-2-Immunoreactivity (IR) in Urinary Bladder with CYP-Induced Cystitis

The expression of COX-2-IR was virtually absent in the urothelium of urinary bladder sections from control rats (Fig. 2A). However, some COX-2-IR was present in the detrusor smooth muscle of control rats (Fig. 2A). With acute (4 hr; Fig. 2B, C), intermediate (48 hr) and chronic CYP-treatment (Fig. 2D, E), COX-2-IR was increased primarily in the urothelium and in the suburothelial region. COX-2-IR in the urothelium and suburothelial regions induced by cystitis was non-homogenous throughout the urothelium; patchy in appearance and intensity. COX-2-IR was present in detrusor smooth muscle with CYP-treatment but changes in COX-2 staining intensity were not
obvious. We chose to focus the quantitation of COX-2 expression on the urothelium because expression was dramatically and obviously upregulated in the urothelium with CYP-treatment. COX-2 expression in the urothelium significantly ($p \leq 0.001$) increased in the urothelium with acute (4 hr; 2.4-fold), and chronic CYP-treatment (3.5-fold).

**COX-2 expression in urinary bladder nerve fibers and inflammatory infiltrates after CYP treatment**

To determine if COX-2-IR was present in the suburothelial nerve plexus, whole mount preparations of urinary bladder were prepared to aid in the visualization of the nerve plexus. COX-2-IR nerve fibers were observed in the suburothelial plexus from control rats (Fig. 3A) and the density significantly ($p \leq 0.01$) increased with CYP-induced cystitis (4 hr; Fig. 3B and chronic; Fig. 3D, Fig. 3E). COX-2-IR nerve fibers were most obvious in the trigone region. COX-2-IR nerve fibers in suburothelial nerve plexus exhibited colocalization with the pan-neuronal marker, PGP9.5 (Fig. 4). In whole mount and sections of the urinary bladder after CYP treatment of all durations, fusiform cells with short processes in the suburothelial region were observed to exhibit COX-2-IR (Fig. 2C-F). In whole mounts, these cells and processes were located in a different focal plane compared to the suburothelial plexus (Fig. 3, Fig. 5B). COX-2-IR cells were abundant within the suburothelial region (Fig. 2B-E) and scattered throughout the detrusor smooth muscle in bladder sections. These cells displayed a similar morphology to macrophages previously described in the urinary bladder after CYP-induced cystitis (Corrow and Vizzard, 2007a) but we pursued additional double-labeling studies with markers of other cellular candidates based upon morphology to determine the identity of
these cells (Table 2). Double-labeling experiments of urinary bladder sections or whole mount preparations demonstrated that these COX-2-IR cells expressed the CD86 antigen (e.g., macrophages, dendritic cells) (Fig. 2F; Fig. 5B) (Table 2) but did not express immunoreactivity for vimentin, GFAP, c-kit, S-100 or CD80 (Table 2).
Discussion

These studies demonstrate several novel findings with respect to COX-2 expression in micturition reflexes with CYP-induced cystitis. COX-2 expression is significantly increased in the urothelium + suburothelium and detrusor smooth muscle with acute (4 hr), intermediate (48 hr) and chronic (10 day) CYP-induced cystitis as determined by western blotting but expression in urothelium + suburothelium is significantly greater. COX-2 immunostaining in the urothelium + suburothelium generally mirrored that observed by western blotting and also demonstrated COX-2 inflammatory cell infiltrates and nerve fibers in the suburothelial plexus. Although COX-2 expression was significantly increased in detrusor smooth muscle as determined by western blotting, immunostaining failed to demonstrated an obvious change in COX-2-IR in detrusor muscle but COX-2 inflammatory infiltrates were present throughout the detrusor. COX-2-IR nerve fibers exhibited increased density in the suburothelial plexus in whole mount preparations with acute or chronic CYP-induced cystitis. Migration of macrophages (CD86+) in the suburothelial space and detrusor with CYP-induced cystitis is dramatic and expression of COX-2-IR is present in both acute and chronic inflammation of the urinary bladder. Inhibitors of COX-2 that have been shown to reduce bladder overactivity (Hu et al., 2003; Jang et al., 2006; Lecci et al., 2000) with bladder inflammation are likely to exert effects on the urothelium, inflammatory cell infiltrates and suburothelial nerve plexus among other targets.
A number of previous studies have demonstrated roles for COX-2 and prostaglandins in bladder overactivity induced by bladder inflammation (Angelico et al., 2006; Hu et al., 2003; Jang et al., 2006; Lecci et al., 2000; Maggi, 1992; Maggi et al., 1988a). It has previously been suggested that prostanoids are key mediators following the induction of CYP-induced cystitis (48 hr) (Hu et al., 2003; Lecci et al., 2000). Our previous studies (Hu et al., 2003) have also demonstrated mRNA and protein for COX-2 upregulation in whole urinary bladder with CYP-induced cystitis. PGE$_2$ and PGD$_2$ expression in the urinary bladder with acute, intermediate and chronic CYP-induced cystitis is also increased (Hu et al., 2003). Previous studies using selective or non-selective inhibitor of COX-2 demonstrated an increase in bladder capacity in rodents treated with CYP (Angelico et al., 2006; Hu et al., 2003; Lecci et al., 2000). In these studies (Angelico et al., 2006; Hu et al., 2003; Lecci et al., 2000), COX-2 inhibitors were delivered systemically and any understanding of potential sites of action was limited. In addition, our previous study (Hu et al., 2003) used whole urinary bladder with no emphasis on cell types in the urinary bladder that may express COX-2 with CYP-induced cystitis. In the present study, we demonstrate several potential sites of action of such inhibitors in the urinary bladder including the urothelium, macrophages and suburothelial nerve plexus. The functional studies are consistent with an effect of COX-2 inhibitors on bladder afferents or urothelium (increased bladder capacity, reduced micturition frequency) (Hu et al., 2003; Lecci et al., 2000). The present studies also provide anatomical data to support the use of an intravesical route of administration of COX-2 inhibitors (Jang et al., 2006) in reducing bladder overactivity induced by CYP.
A number of reports have demonstrated upregulation of COX-2 in response to inflammatory stimuli (Fujisawa et al., 2005; Hu et al., 2003; Linden et al., 2004; Mitchell and Warner, 1999; Schafers et al., 2004; Yiangou et al., 2006). A recent report (Liu et al., 2006) emphasizes the inclusion of a number of controls to be certain that a signal obtained by western blotting, is, indeed, reflective of COX-2. We have performed each of these suggested controls. [1] We have included a purified, recombinant COX-2 protein as a positive control in our western blots that is located at 74-kD. A review of the literature reveals that the mature, enzymatically active form of COX-2 is located in the 70- to 74-kD range on denaturing acrylamide gel electrophoresis. This is consistent with the COX-2 protein identified from urinary bladders in the present and previous (16) study. The difference in band location for the positive control and the samples may be attributed to several issues: species differences as the positive control is from human, nonglycosylated forms of COX-2 and/or incomplete proteolysis. [2] We have identified (16) increased COX-2 mRNA in urinary bladder induced by CYP treatment to further support our western blot results. [3] We have demonstrated upregulation of two prostaglandins (PG), PGE\textsubscript{2} and PGD\textsubscript{2}, in urinary bladder with CYP treatment as further evidence that COX-2 may affect bladder function through downstream activation of PG (16).

Immunohistochemical studies demonstrated that increases in COX-2 protein determined by western blotting are a reflection of increased urothelial cell expression of COX-2, COX-2-IR macrophages (CD86\textsuperscript{+}), and COX-2-IR nerve fibers in the suburothelial plexus. A number of studies have demonstrated COX-2-IR in nerves (i.e.,
endoneurium) in response inflammatory stimuli alone or as a result of neural injury (Durrenberger et al., 2006; Pesce et al., 2006). In the present study, we have confirmed that COX-2-IR is present in nerve in the suburothelial plexus and that COX-2-IR nerve fibers increase in density in the trigone region of the urinary bladder with acute and chronic CYP-induced cystitis. One limitation to this study is the method used to determine density of innervation. All COX-2-IR structures in the whole mount within the same plane of focus are captured in the frame and density determination is performed on all COX-2-IR structures. Therefore, COX-2-IR macrophages or other COX-2-IR inflammatory cell infiltrates of unknown cellular phenotype may have increased the density determinations that we have attributed to nerve fibers. However, a large proportion of the COX-2-IR nerve fibers were not located in the same focal plane as the macrophages so we feel that this possibility is limited in the present study. Afferent nerve fibers make a large contribution to this neural plexus although some contribution from efferent sources cannot be ruled out (Dixon et al., 2000). Previous studies have shown COX-2-IR in primary afferent cells in dorsal root ganglia (Vizzard et al., preliminary data) (Dou et al., 2004; Durrenberger et al., 2006) and therefore there is precedent for COX-2 expression in afferent nerves.

Urothelial cells share a number of similarities with sensory neurons and the urothelium has been suggested (Birder, 2005; 2006) to have ‘neuronal-like’ properties. Urothelial cells express a number of receptors and ion channels similar to those found in sensory neurons (Birder, 2005; 2006; Corrow and Vizzard, 2007a; LaBerge et al., 2006a; Murray et al., 2004) and it was therefore not surprising to observe COX-2 expression in
the urothelium after CYP-induced cystitis. COX-2-IR in the urothelium was observed in apical, intermediate and basal cells. Immortalized human urothelial cells express COX-2 and inducible nitric oxide synthase (iNOS) after stimulation of β-adrenergic receptors (Harmon et al., 2005). The present study adds to the growing list of similarities between urothelial cells and sensory neurons and may also suggest that urothelial cells participate in the transduction of inflammatory signals to the central nervous system (Birder, 2005; 2006).

A number of previous studies have also demonstrated COX-2 expression in macrophages (Tsai et al., 2007; Yamashita et al., 2007; Yiangou et al., 2006). Surprisingly, in our previous study (Hu et al., 2003), we failed to demonstrate COX-2-IR in macrophages, although present in the bladder after CYP-induced cystitis, but demonstrated COX-2-IR in mast cells in the inflamed bladder. In the present study, the immunostaining confirms that a proportion of COX-2-IR in the suburothelial space and detrusor express the antigen CD86. Although our present study does not rule out a contribution from mast cells, the prominent, cellular staining in the suburothelial region largely represents macrophages. The reason for this difference is not known but likely reflects our choice of COX-2 antiserum as has been suggested in the COX-2 literature (Yiangou et al., 2006). We used a monoclonal COX-2 antibody in the present study because the polyclonal antibody used in our previous study was inconsistent in its staining for this study.

Previous studies have demonstrated robust COX-2 expression in the detrusor smooth muscle after complete bladder outlet obstruction in mice and this increase has
been attributed to mechanical stretch (Park et al., 1999; Park et al., 1997). In contrast, no COX-2 expression was present in the urothelium or suburothelial region in control tissues or after outlet obstruction (Park et al., 1999; Park et al., 1997). The present study clearly demonstrates a larger COX-2 contribution from the urothelium and suburothelial region in response to CYP-induced cystitis. This difference probably is a reflection of COX-2 protein induced by an inflammatory stimulus versus a mechanical stimulus. In both bladder inflammation and outlet obstruction, COX-2 is either demonstrated or hypothesized to contribute to bladder overactivity/hyperactivity (Hu et al., 2003; Lecci et al., 2000; Park et al., 1999; Park et al., 1997) although the source of COX-2 and resultant production of prostaglandins is likely to be different. It is interesting that COX-2 is highly expressed in the urinary bladder during development and that bladder outlet obstruction represents reactivation of this gene (Park et al., 1997). The urinary bladder during early postnatal development exhibits spontaneous bladder contractions (Ng et al., 2007; Park et al., 1997) but the contribution of COX-2 to this function is presently unknown.

COX-2 expression can be stimulated by growth factors, proinflammatory cytokines and chemokines (Khanapure et al., 2007; Yermakova and O'Banion, 2000). Changes in neurotrophic factor expression in the urinary bladder with cystitis including β-nerve growth factor, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, neurotrophin (NT)-3 and NT-4 (Murray et al., 2004; Vizzard, 2000b) have been demonstrated. Robust (Malley and Vizzard, 2002) changes in a number of urinary bladder cytokines including IL-1-β, IL-2, IL-4, IL-6 and more modest changes in
TNF-α/ or TNF-β with CYP-induced cystitis have also been demonstrated. Most recently, we have demonstrated upregulation of the chemokine, fractalkine, and fractalkine receptor in the urinary bladder and specifically in the urothelium with CYP-induced cystitis (Yuridullah et al., 2006). Separately or in combination, neurotrophic factors, proinflammatory cytokines and chemokines expressed in the inflamed urinary bladder may also contribute to COX-2 upregulation.

In summary, these studies have demonstrated significant changes in COX-2 expression in the urinary bladder after CYP-induced cystitis examined at three time points (acute, intermediate and chronic). Specifically, COX-2 expression is significantly increased in the urothelium, in nerve fibers in the suburothelial plexus and in macrophages in the suburothelial space. Although western blotting demonstrates COX-2 expression in detrusor smooth muscle, this change likely reflects COX-2 expression in inflammatory cell infiltrates as immunostaining for COX-2 in detrusor smooth muscle did not exhibit robust changes in COX-2 expression. A number of cellular sources in the urinary bladder express COX-2 after CYP-induced cystitis that may be induced by neurotrophic factors, cytokines and chemokines in the inflamed bladder. The present study defines some bladder cellular sources that are likely targets of COX-2 inhibitors.
Grants

This work was funded by NIH grants DK051369, DK060481, DK065989.
Figures

Figure 1: Western blot in detrusor and urothelium/suburothelium

A. Representative example of a western blot of detrusor smooth muscle (20 µg) for COX-2 expression in control rats and those treated with cyclophosphamide (CYP) for varying duration. Total ERK staining was used as a loading control. Total ERK staining was used as a loading control. COX-2 expression in detrusor smooth muscle is significantly increased with acute (4 hr), intermediate (48 hr) and chronic (10 day) CYP-treatment. *, p ≤ 0.01. n = 5-7. C. Representative example of a western blot of urothelium + suburothelium (20 µg) for COX-2 expression in control rats and those treated with cyclophosphamide (CYP) for varying duration. Total ERK staining was used as a loading control. D. Histogram of relative COX-2 band density in all groups examined normalized to total ERK in the same samples. COX-2 expression in urothelium + suburothelium is significantly increased with acute (4 hr), intermediate (48 hr) and chronic (10 day) CYP-treatment. *, p ≤ 0.01. n = 5-7. E. The fold change in COX-2 expression in the urothelium (U) and suburothelium (SubU; white bars) is greater compared to that in detrusor (black bars) with CYP-induced cystitis. *, p ≤ 0.01 with statistical analyses performed on raw data.
Figure 2: COX-2 expression in urinary bladder sections

Fluorescence images of COX-2 expression in urinary bladder sections of control (A), 4 hr (B, C), and chronic (D, E) CYP treated rats. For all images, exposure times were held constant and all tissues were processed simultaneously. In control rats, little if any COX-2 expression was visible in the urothelium (U; A). CYP treatment (4 hr, B, C; chronic, D, E) upregulated expression of COX-2 in the urothelium in all layers (apical, intermediate and basal) of the urothelium. CYP-induced cystitis (all time points) also increased COX-2-IR in the suburothelium (Sub U; C, D, E). In normal urinary bladder, the detrusor smooth muscle expressed basal COX-2 but increases with cystitis were not obvious. F. COX-2-IR inflammatory cell infiltrates were present in the urinary bladder of rats with CYP treatment (4 hr (C), chronic (D, E). Merged image of double-label immunostaining revealed that COX-2-IR (red) was present in cells that expressed the cytoplasmic antigen, CD86 (e.g., macrophages, dendritic cells) (F, arrows). L, lumen; Sub U, suburothelium; sm, smooth muscle. Calibration bar represents 50 µm.
Figure 3: COX-2-IR nerve fibers in suburothelial plexus

Fluorescence photographs of COX-2-IR nerve fibers in the suburothelial plexus in whole mount preparations of the urothelium/suburothelium in control (A), acute (B), intermediate (C) and chronic CYP treated rats (D). COX-2-IR nerve fibers in the suburothelial plexus in the urothelium/suburothelium whole mount preparation in the trigone region increased in density with CYP-induced cystitis (4 hr (B) and chronic (D)). Single COX-2-IR nerve fibers (arrows) as well as COX-2-IR nerve bundles (asterisks) were observed in the suburothelial plexus. E. Summary histogram of increase in COX-2-IR nerve fiber density with CYP-induced cystitis. *, p ≤ 0.01. Calibration bar represents 80 µm.
Figure 4: COX-2 and PGP9.5 expression in urinary bladder whole mounts

COX-IR fibers (A, C) expressed immunoreactivity for the pan neuronal marker, protein gene product (PGP9.5; B, D). Fluorescence images of COX-2-IR (A, C) processes in urinary bladder whole mounts with CYP-induced cystitis and PGP9.5 staining (B, D) in the same whole mounts to demonstrate colocalization of COX-2-IR and PGP9.5 staining. COX-2-IR was present in single nerve fibers (A, B, arrowheads) and nerve bundles (A-D, arrows). Some COX-2-IR nerve fibers exhibited a winding trajectory (A-D, arrows) whereas other COX-2-IR nerve fibers coursed linearly and lacked obvious varicosities (A, B, arrowheads). Numerous COX-2-IR macrophages were also present (C). Calibration bar represents 40 µm.
Figure 5: Whole mount preparation of urinary bladder.
A. Dissection of urinary bladder into urothelium + suburothelium and detrusor components. The urothelium and suburothelium layer (red arrows) is reflected back from the detrusor (yellow arrows). B. Numerous CD86+ macrophages were present in whole mount preparations of the urinary bladder with CYP-induced cystitis (4 hr). Calibration bar represents 80 µm in B.
Table 1: Primary and secondary antibodies used, sources and applications

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Primary Dilution</th>
<th>Secondary Antibody</th>
<th>Secondary Dilution</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-COX-2</td>
<td>Santa Cruz, Santa Cruz, CA</td>
<td>1:200</td>
<td>HRP donkey anti-goat</td>
<td>1:15K</td>
<td>WB</td>
</tr>
<tr>
<td>Mouse anti-COX-2</td>
<td>Cayman, Ann Arbor, MI</td>
<td>1:50</td>
<td>Cy3 donkey anti-mouse</td>
<td>1:100</td>
<td>IHC</td>
</tr>
<tr>
<td>Rabbit anti-COX-2</td>
<td>Santa Cruz</td>
<td>1:500</td>
<td>Cy3 donkey anti-mouse</td>
<td>1:100</td>
<td>IHC</td>
</tr>
<tr>
<td>Mouse anti-GFAP</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
<td>1:5000</td>
<td>Cy2 donkey anti-mouse</td>
<td>1:100</td>
<td>IHC</td>
</tr>
<tr>
<td>Rabbit anti-S-100</td>
<td>Cayman, Ann Arbor, MI</td>
<td>1:200</td>
<td>Cy2 goat anti-mouse</td>
<td>1:500</td>
<td>IHC</td>
</tr>
<tr>
<td>Rabbit anti-c-kit</td>
<td>Santa Cruz</td>
<td>1:1000</td>
<td>Cy2 goat anti-rabbit</td>
<td>1:500</td>
<td>IHC</td>
</tr>
<tr>
<td>Mouse anti-CD86</td>
<td>Biolegend, San Diego, CA</td>
<td>1:25</td>
<td>Cy2 donkey anti-mouse</td>
<td>1:100</td>
<td>IHC</td>
</tr>
<tr>
<td>Mouse anti-CD80</td>
<td>Biolegend</td>
<td>1:25</td>
<td>Cy2 donkey anti-mouse</td>
<td>1:100</td>
<td>IHC</td>
</tr>
<tr>
<td>Mouse anti-PGP</td>
<td>Abcam, Inc., Cambridge, MA</td>
<td>1:15</td>
<td>Cy2 donkey anti-mouse</td>
<td>1:100</td>
<td>IHC</td>
</tr>
</tbody>
</table>

Table 2: Experiments of urinary bladder sections or whole mount preparations

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Staining of cryostat bladder sections</th>
<th>Staining of bladder whole-mounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>vimentin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GFAP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S-100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>c-kit</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD86</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD80</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

The phenotype of COX-2-IR cells in the suburothelial space and detrusor in bladder sections and whole mounts induced by CYP-induced cystitis was determined using a number of antisera. COX-2-IR cell bodies in the suburothelial space and throughout the detrusor only expressed immunoreactivity to the CD86 antigen. +, presence of staining; - absence of staining; ND, not determined.
References


14. **Fujisawa H, Nakagawa S, Ohkubo Y, Matsu M, Yamaguchi S, Kawamura M, Hatanaka K, Kawakubo Y, Hiramoto Y, Kobayashi H, and Harada Y.** Local and


Chapter 3: $p75^{NTR}$ Expression in Rat Urinary Bladder Sensory Neurons and Spinal Cord with Cyclophosphamide-Induced Cystitis

Abstract

A role for nerve growth factor (NGF) in contributing to increased voiding frequency and altered sensation from the urinary bladder has been suggested. Previous studies have examined the expression and regulation of tyrosine kinase receptors (Trks) in micturition reflexes with urinary bladder inflammation. The present studies examine the expression and regulation of another receptor known to bind NGF, p75NTR, after various durations of bladder inflammation induced by cyclophosphamide (CYP). CYP-induced cystitis increased (p ≤ 0.001) p75NTR expression in the superficial lateral and medial dorsal horn in L1-L2 and L6-S1 spinal segments. The number of p75NTR-IR cells in the lumbosacral dorsal root ganglia (DRG) also increased (p ≤ 0.05) with CYP-induced cystitis (acute, intermediate and chronic). Quantitative, real-time polymerase chain reaction also demonstrated significant increases (p ≤ 0.01) in p75NTR mRNA in DRG with intermediate and chronic CYP-induced cystitis. Retrograde dye-tracing techniques with Fastblue were used to identify presumptive bladder afferent cells in the lumbosacral DRG. In bladder afferent cells in DRG, p75NTR-IR was also increased (p ≤ 0.01) with cystitis. In addition to increases in p75NTR-IR in DRG cell bodies, increases (p ≤ 0.001) in pericellular (encircling DRG cells) p75NTR-IR in DRG also increased. Confocal analyses demonstrated that pericellular p75NTR-IR was not colocalized with the glial marker, glial fibrillary acidic protein (GFAP). These studies demonstrate that p75NTR expression in micturition reflexes is present constitutively and modified by bladder inflammation. The functional significance of p75NTR expression in micturition reflexes remains to be determined.
Introduction

Neurotrophic factors are important in the maintenance and survival of peripheral neurons as well as in neuronal plasticity (Allen and Dawbarn, 2006; Dmitrieva et al., 1997). Nerve growth factor (NGF) binds to two receptors with high-affinity, the $p75^{\text{NTR}}$ receptor, a member of the tumor necrosis factor family of receptors and TrkA, a receptor tyrosine kinase. The majority of NGF-responsive cells express both $p75^{\text{NTR}}$ and TrkA (Chao and Hempstead, 1995) and TrkA is exclusively expressed with $p75^{\text{NTR}}$ in DRG (Wright and Snider, 1995). NGF selectively binds TrkA whereas $p75^{\text{NTR}}$ can bind all neurotrophins and precursor molecules with the same affinity (Huang et al., 1999). The ability of NGF to bind two very distinct receptors and the resulting signaling via this receptor complex has puzzled researchers (Barker, 2007). The original function attributed to $p75^{\text{NTR}}$ was as an accessory protein that modulates Trk receptors to regulate their affinity for their appropriate ligand (Barker, 2007; Chao et al., 2006). It has become a prevailing concept that $p75^{\text{NTR}}$ and TrkA combine to form a receptor complex that results in high-affinity NGF binding (Chao et al., 2006; Kahle et al., 1994; Verdi et al., 1994). However, a recent study (Wehrman et al., 2007) provides structural and mechanistic evidence for a ligand-passing model in which NGF rapidly associates with $p75^{\text{NTR}}$ and then is presented to TrkA (Wehrman et al., 2007).

At sites of tissue injury, inflammation or target organ hypertrophy, growth factors including NGF are upregulated (Lewin and Mendell, 1993; Lindholm et al., 1987; Meller et al., 1994; Thompson et al., 1995; Woolf et al., 1997). An increase in NGF and brain-derived neurotrophic factor (BDNF) in the urine and/or bladder are among previously
documented changes with bladder inflammation (Lowe et al., 1997; Oddiah et al., 1998; Okragly et al., 1999; Steers et al., 1994; Steers and Tuttle, 2006). Excess NGF in the urinary bladder may increase voiding frequency (Chuang et al., 2001b) secondary to neurochemical and other neurophenotypic responses. Intravesical administration of exogenous NGF in animals facilitates afferent firing and increases voiding frequency that can be blocked by anti-NGF (Dmitrieva et al., 1997). Exogenous administration of NGF into the detrusor smooth muscle also increased voiding frequency, augmented central responses to bladder distention and increased neuropeptide expression in the urinary bladder and lumbosacral spinal cord (Zvara and Vizzard, 2007). We have demonstrated (Hu et al., 2005) that the NGF scavenging agent (REN1820) reduces voiding frequency and pain behaviors in rats with cyclophosphamide (CYP)-induced bladder inflammation. Our previous studies (Qiao and Vizzare, 2002) have demonstrated significant upregulation of TrkA and TrkB in bladder afferent cells in lumbosacral DRG with CYP-induced bladder inflammation. The potential interactions between TrkA and p75NTR in micturition reflex pathways under control (non-inflamed) or inflamed conditions have not been investigated.

Few studies have examined the role of p75NTR in LUT pathways (Elsasser-Beile et al., 2000; Jahed and Kawaja, 2005) and only one (Wakabayashi et al., 1996) has examined expression in urinary bladder after inflammation. p75NTR expression in bladder afferent cells in the dorsal root ganglia (DRG) and central micturition pathways mediating micturition have not been examined. The present study was designed to determine p75NTR expression in bladder afferent pathways, including lumbosacral spinal
cord, and bladder afferent cells in DRG. We examined changes in $p75^{NTR}$ expression in micturition pathways and $p75^{NTR}$ mRNA in L6-S1 DRG with varying durations (4 hours (hr; acute), 48 hr (intermediate), and 10 days (chronic) of bladder inflammation induced by CYP.

Materials and Methods

Cyclophosphamide (CYP)-Induced Cystitis

Acute, intermediate and chronic CYP-induced cystitis rat models were examined in these studies (Corrow and Vizzard, 2007b; Klinger et al., 2007; Qiao and Vizzard, 2002; Vizzard, 2000b). Chronic CYP (Sigma)-induced cystitis: rats received drug injection (75 mg/kg, intraperitoneal, i.p.) every third day for 10 days. Intermediate CYP-induced cystitis: rats received a single injection (150 mg/kg, i.p.) and tissues were examined 48 hours (hr) later. Acute CYP-induced cystitis: rats received a single injection (150 mg/kg, i.p.) and survived for 4 hours (hr). Control rats received volume-matched injections of saline (0.9%; i.p.) or no treatment and no difference among the control groups was observed. All injections were performed under isoflurane (2%) anesthesia. All experimental protocols involving animal use were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC # 06-014). Animal care was under the supervision of the University of Vermont's Office of Animal Care Management in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress or distress.
Retrograde labeling of bladder afferent neurons

Four to six days prior to CYP injection, saline or no treatment, Fast-blue (FB; 4%, weight/volume; Polyol, Gross-Umstadt, Germany) was injected into the bladder to retrogradely label bladder afferent neurons in control (n=4) and CYP-treated (n=4 for each group) rats. As previously described (Qiao and Vizzard, 2002; Vizzard, 2000c), a total volume of 40 µl divided into six to eight injections was injected into the dorsal surface of the bladder wall with particular care to avoid injections into the bladder lumen, major blood vessels, or overlying fascial layers. At each injection site, the needle was kept in place for several seconds after injection, and the site was washed with saline to minimize contamination of adjacent organs with FB.

Tissue processing

After CYP treatment (4 hr, 48 hr, or chronic), animals were deeply anesthetized with isoflurane (3–4%) and then euthanized via thoracotomy. The spinal cord and DRG were quickly removed and postfixed in 4% paraformaldehyde for 3 hr or 1 hr, respectively. Tissue was then placed overnight in sucrose (30%) in 0.1 M PBS for cryoprotection. In pilot studies, intracardiac perfusion combined with post-fixation was also performed and p75NTR-IR was compared between different fixation protocols. p75NTR-IR was most consistent and robust with the post-fixation protocol. Thus, this method was used for all subsequent studies. Spinal cord segments were identified based upon at least two criteria: (1) the T13 DRG was present after the last rib, and (2) the L6 vertebra was the last moveable vertebra followed by the fused sacral vertebrae. Another
less precise criterion is the observation that the L6 DRG are the smallest ganglia following the largest DRG, L5. DRG and spinal cord segments (L1, L2, L5–S1) were sectioned parasagittally at a thickness of 20 or 30 µm, respectively, on a cryostat. Some DRG (L1, L2, L6, S1) were specifically chosen for analysis based upon the previously determined segmental representation of urinary bladder circuitry (Donovan et al., 1983; Keast and De Groat, 1992; Nadelhaft and Vera, 1995). Bladder afferents are not distributed within the L4–L5 DRG (Donovan et al., 1983; Keast and De Groat, 1992) that contain only somatic afferents nor are neurons that are involved in urinary bladder function observed in the L4–L5 spinal segments (Nadelhaft and Vera, 1995). Thus, the L5 DRG served as an internal control for these studies. Tissues from control animals were handled in an identical manner to that described above.

**p75NTR immunohistochemistry**

Spinal cord and DRG sections for both control and CYP-treated rats were processed for p75NTR immunoreactivity (IR) using an on-slide processing technique (Vizzard, 1997). Groups of control animals and experimental animals were processed simultaneously to decrease the possible incidence of variation in staining and background between tissues and between animals. Spinal cord and DRG sections were incubated overnight at room temperature with rabbit anti–p75NTR antibody (1:15,000, Advanced Targeting Systems, San Diego, CA) in 1% goat serum and 0.1 M KPBS (Phosphate Buffer Solution with potassium), and then washed (3×15 min) with 0.1 M KPBS, pH 7.4. Tissue was then incubated with Cy3-conjugated goat anti–rabbit IgG (1:500; Jackson
ImmunoResearch) for 2 hr at room temperature. After several rinses with 0.1 M KPBS, tissues were mounted with Citifluor (Citifluor, London, UK) on slides and coverslipped. Control tissues incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining was observed. Although the rabbit polyclonal antibody (1:15,000, Advanced Targeting Systems, San Diego, CA) was used in this study, staining with a mouse monoclonal antibody (1:500, Advanced Targeting Systems, San Diego, CA) was also performed and showed similar staining patterns in DRG and spinal cord but with reduced staining intensity.

**p75<sup>NTR</sup>exonIII** deficient mice

Breeding pairs of p75<sup>NTR</sup>exonIII<sup>+/−</sup> mice were generously provided by Dr. Hermes Yeh (Dept. of Physiology, Dartmouth Medical School). Mice were bred locally and DNA genotyping performed on tail snips. Wildtype and p75<sup>NTR</sup>exonIII deficient mice from the same litters were used to characterize the specificity of the antiserum to p75<sup>NTR</sup>. DRG and spinal cord were harvested and immunohistochemical processing was performed as described above. Tissues from littermate wildtype and p75<sup>NTR</sup>exonIII deficient mice were processed simultaneously using identical antibodies from start to finish.

**Antibody characterization**

Polyclonal anti-p75<sup>NTR</sup> is a purified rabbit polyclonal antibody. The antisera was developed in rabbit against an extracellular fragment from the mouse p75<sup>NTR</sup> (amino
This is a well-characterized antibody that was originally developed and the specificity demonstrated by Huber and Chao (Huber and Chao, 1995a; b) using a variety of approaches including the demonstration of p75NTR expression in transgenic mice expressing p75NTR gene sequences (Huber and Chao, 1995a; b). Specificity has also been demonstrated according to the manufacturer. This antibody also produces a similar pattern of immunoreactivity in rodent spinal dorsal horn and DRG as demonstrated in numerous previous publications (Delcroix et al., 1998; King et al., 2000; Hu and McLachlan, 2003; Obata et al., 2006) and in our pilot studies using a mouse monoclonal antibody to p75NTR. Mice with different splice variants of p75NTR are available (Dhanoa et al., 2006) and carry null mutations for p75NTRexonIII or p75NTRexonIV. These strains lack full-length p75NTR expression and exhibit loss of p75NTR function. In wildtype littermates and in mice with a null mutation for p75NTRexonIII, we examined p75NTR expression in DRG and spinal cord. Staining in the wildtype DRG was robust (Fig. 1A) whereas staining in DRG from p75NTRexonIII deficient mice revealed extremely faint and sparse p75NTR-IR (Fig. 1B). To obtain images that were not completely black, the exposure time for p75NTRexonIII/- DRG images was increased to 4 times longer than that used to acquire wildtype images. These results are similar to previous studies where reduced intensity and appearance of p75NTR-IR was observed in superior cervical ganglia in p75NTRexonIII deficient mice (Dhanoa et al., 2006).

Monoclonal anti-glial fibrillary acidic protein (GFAP) is a purified mouse monoclonal antibody. The antibody is derived from the hybridoma produced by the
fusion of mouse myeloma cells and splenocytes from an immunized mouse. The immunogen was purified GFAP from pig spinal cord (manufacturer’s technical information). The antibody recognizes the 50 kDa intermediate filament protein, GFAP, but not other intermediate filaments (manufacturer’s technical information). The epitope recognized by the monoclonal anti-GFAP is localized on the carboxy terminal Cys II fragment of GFAP and the N-terminal part of the tail sequence of the molecule (Debus et al., 1983; Shaw and Hawkins, 1992). This antibody produced the same pattern of immunoreactivity in rodent DRG as demonstrated in numerous previous publications (Hu and McLachlan, 2003; Karchewski et al., 2004; Obata et al., 2006; Xie et al., 2006).

**Assessment of positive staining in lumbosacral spinal cord and DRG**

Staining observed in experimental tissue was compared with that observed from experiment-matched negative controls. Tissues exhibiting immunoreactivity that was greater than the background level observed in experiment-matched negative controls were considered positively stained.

**Data analysis of p75NTR and FB-labeled DRG**

Tissues were examined under an Olympus fluorescence photomicroscope for visualization of Cy3 and FB. Cy3 was visualized with a filter with an excitation range of 560–596 nm and an emission range of 610–655 nm. In DRG from control and CYP-treated rats, p75NTR–immunoreactive (IR) cell profiles were counted in 5-8 sections of each selected DRG (L1, L2, L5–S1). Only cell profiles with a nucleus were quantified.
DRG sections with FB-labeled cells were viewed with a filter with an excitation wavelength of 340–380 nm and an emission wavelength of 420 nm. Cells colabeled with FB and p75\textsuperscript{NTR} –IR were similarly counted. Numbers of p75\textsuperscript{NTR} –IR cell profiles per DRG section are presented (mean ± SEM). The percentage of presumptive bladder afferent cells (FB-labeled) expressing p75\textsuperscript{NTR} –IR in each DRG examined is also presented (mean ± SEM). The results were not corrected for double-counting. p75\textsuperscript{NTR}-IR cells were quantified in a blinded fashion and p75\textsuperscript{NTR}-IR and FB-labeled cells were quantified by two individuals. Comparisons between control and CYP-treated groups were made by using analysis of variance (ANOVA). Percentage data were arcsin-transformed to meet the requirements of this statistical test. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. Thus, day was treated as a blocking effect in the model. Two variables were tested in the analysis: (1) duration of inflammation versus none (control), and (2) the effect of day (i.e., tissue from experimental and control groups of animals were processed on different days). When F ratios exceeded the critical value (p ≤ 0.05), the Newman–Keuls test was used for multiple comparisons among means.

**Quantitative analysis of pericellular p75\textsuperscript{NTR}–IR in DRG**

Six to 10 DRG sections from each of L1, L2, L5-S1 in control and experimental groups were examined under an Olympus fluorescence photomicroscope (Optical Analysis, Nashua, NH) with a multiband filter set for visualization of the Cy3 fluorophore. Grayscale images acquired in tiff format were imported into Meta Morph
image analysis software (version 4.5r4; Universal Imaging, Downingtown, PA) as previously described (Laberge et al., 2006b; Yuridullah et al., 2006). Four boxes of equal size were drawn and placed randomly over the DRG images. A threshold within an intensity range of 100–250 grayscale values was applied to the region of interest in the least brightly stained condition first. The same threshold was subsequently used for all images. Average intensity was calculated within the selected areas. The average intensity represents the average value of all of the pixels above the threshold value. Percent $p75^{NTR}$ expression above threshold in the total area selected was then calculated and averaged for all samples from control ($n = 4$) and CYP-treated ($n = 4$ for each experimental condition) rats. The percentage of $p75^{NTR}$–IR expression in CYP-treated rats was therefore expressed as a percentage of control.

$p75^{NTR}$-IR and glial fibrillary acidic protein (GFAP)-IR in lumbosacral DRG

To more fully characterize the pericellular $p75^{NTR}$-IR seen in lumbosacral DRG, double-labeling was performed with anti-$p75^{NTR}$ (as described previously) as well as mouse monoclonal anti-GFAP (1:5,000; Sigma-Aldrich, Inc., St. Louis, MO). Primary and subsequently, secondary antibodies (Cy3-conjugated goat anti-rabbit (1:500) and Cy2-conjugated goat anti-mouse (1:50; Jackson ImmunoResearch), were applied as a cocktail to tissue sections for on-slide processing. Tissue was examined and optical sections were acquired using a Zeiss LSM 510 confocal scanning system attached to a Zeiss LSM 510 microscope using a plan Fluor 40x oil objective. Excitation wavelengths
of 488 nm and 543 nm were used. Control experiments demonstrated that the Cy2 and Cy3 fluorochromes had no overlap in emission when sequential scanning was performed. To determine the degree of colocalization of GFAP with p75<sup>NTR</sup>, release 3.2 of the Carl Zeiss LSM software was used in optical sections. For these analyses, we demonstrated absence of bleed-through and background staining and autofluorescence was removed from all images, and analyses were performed only on optical sections. Changes in pericellular staining were most dramatic in L5-S1 DRG and thus, the colocalization analyses from control and CYP-treated rats were restricted to these DRG.

**Data analyses of p75<sup>NTR</sup>–IR in lumbosacral spinal cord**

Tissues were examined under an Olympus fluorescence photomicroscope for visualization of Cy3. Grayscale images were obtained in 8-bit monochrome tiff format files. No adjustments were made to the grayscale images. The density of p75<sup>NTR</sup>–IR in specific regions of spinal cord was determined by densitometry analysis (ImageJ, v. 1.34s). Spinal cord segments (L1-L2, L5-S1) were sectioned throughout the rostral-caudal extent of the segment. All tissue sections were then processed for p75<sup>NTR</sup>–IR as previously described. A 1:4 series of spinal cord sections was then used for semi-quantitative analysis of p75<sup>NTR</sup>–IR. Spinal cord sections were not selected based upon staining intensity, and no sections were discarded from the analysis because of low staining. The following regions of spinal cord from both CYP-treated and control animals were analyzed: superficial, lateral dorsal horn (LDH), medial dorsal horn (MDH), and dorsal commissure (DCM) regions, the region of the sacral parasympathetic
nucleus (SPN; L6, S1), the region of the intermediolateral nucleus (IML; L1, L2), and the region of the lateral collateral pathway (LCP; L6, S1). Spinal cord sections were viewed with a 20× objective and captured through a video camera attachment to the microscope with the exposure time, brightness, and contrast being held constant. The image was converted into grayscale. The spinal cord section was centered in the field, and four standard size squares were overlaid on the areas of interest (LDH, MDH, DCM, SPN, IML, and LCP regions). The labeled area within the square was measured. Transmittance \( t \) was calculated as \( t = (\text{gray level} + 1/256) \). Optical density (OD) was derived from \( \text{OD} = -\log t \). The background staining for each section was subtracted from the images. Differences in p75NTR expression in L1-L2 and L6-S1 spinal segments with cystitis were similar; these data (L1-L2 and L6-S1) were pooled for data presentation. Comparisons among control and experimental groups were made by ANOVA. When \( F \) ratios exceeded the critical value (\( p \leq 0.05 \)), Newman-Keuls test was used for multiple comparisons among means.

**Real-time quantitative reverse transcription-polymerase chain reaction (Q RT-PCR)**

L6 and S1 DRG were dissected under RNase-free conditions and total RNA was extracted using the STAT-60 total RNA/mRNA isolation reagent (Tel-Test ‘B’, Friendswood, TX, USA). Complementary DNA was synthesized using SuperScript II reverse transcriptase and random hexamer primers with the SuperScript II Preamplification System (Invitrogen, Carlsbad, CA, USA) in a 20 µl final reaction volume. The quantitative PCR standard for p75 and L32 transcripts was prepared with
the amplified p75 cDNA products ligated directly into pCR2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen). The nucleotide sequences of the inserts were verified by automated fluorescent dideoxy dye terminator sequencing (Vermont Cancer Center DNA Analysis Facility). To estimate the relative expression of the receptor transcripts, 10-fold serial dilutions of stock plasmids were prepared as quantitative standards. The range of standard concentrations was determined empirically.

Real-time quantitative PCR was performed using SYBR Green I detection as previously described (Girard et al., 2006). Complementary DNA templates, diluted 5-fold to minimize the inhibitory effects of the reverse transcription reaction components, were assayed using SYBR Green I JumpStartTM. Taq ReadyMix (Sigma, St. Louis, MO, USA) containing 3.5 mM MgCl₂, 200 mM dATP, dGTP, dCTP and dTTP, 0.64 U Taq DNA polymerase and 300 nM of each primer in a final 25 µl reaction volume. The real-time quantitative PCR was performed on an Applied Biosystems 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the following standard conditions: (i) initial heating 94 °C for 2 min; (ii) amplification over 40 cycles at 94 °C for 15 seconds (s) and 65 °C or 60 °C for 30 s. The amplified product from these amplification parameters was subjected to SYBR Green I melting analysis by ramping the temperature of the reaction samples from 60 °C to 95 °C. A single DNA melting profile was observed under these dissociation assay conditions demonstrating amplification of a single unique product, free of primer dimers or other anomalous products. Oligonucleotide primer sequences for p75NTR and the housekeeping gene, L32, used in these studies are described in Table 1.
For data analyses, a standard curve was constructed by amplification of serially
diluted plasmids containing the receptor target sequence. Data were analyzed at the
termination of each assay using the Sequence Detection Software version 1.3.1 (Applied
Biosystems, Norwalk, CT). In standard assays, default baseline settings were selected.
The increase in SYBR Green I fluorescence intensity (DRn) was plotted as a function of
cycle number and the threshold cycle was determined by the software as the
amplification cycle at which the DRn first intersects the established baseline.
Results

p75<sup>NTR</sup>-IR in spinal cord in control rats and after CYP-induced cystitis

In spinal cord levels examined (L1-L2, L5-S1), p75<sup>NTR</sup> was expressed in certain regions of the spinal cord in both control rats and after CYP-induced cystitis. In the spinal cord of control animals (L1-L2, L5-S1), p75<sup>NTR</sup>-IR was present in superficial dorsal horn (medial and lateral laminae I-II; Fig. 2). Faint p75<sup>NTR</sup>-IR was expressed in the dorsal commissure (DCM), located dorsal to the central canal and in the lateral collateral pathway of Lissauer (LCP) in L6-S1 spinal cord segments in control animals. p75<sup>NTR</sup>–IR was also observed in the SPN (L6-S1) or IML (L1-L2) regions in control rats. p75<sup>NTR</sup> staining in all regions was punctate in appearance and probably represented staining in nerve terminals because p75<sup>NTR</sup>-IR was not observed in any neuronal cell bodies in the dorsal or lateral horn. On rare occasions, p75<sup>NTR</sup>-IR ventral horn motoneurons were observed.

Following CYP treatment of varying duration, the intensity of p75<sup>NTR</sup> staining significantly increased in both medial (MDH) and lateral superficial dorsal horn (LDH) (Fig. 2B, C). In spinal cord segments L1-L2, p75<sup>NTR</sup> staining intensity increased significantly in both MDH and LDH for all CYP treatment durations examined (4 hr, 48 hr, or chronic; p ≤ 0.001)(Fig. 2E). p75<sup>NTR</sup> staining intensity in the LDH of L6-S1 also increased significantly (p ≤ 0.001) at all durations of CYP treatment (Fig. 2D). In addition to changes in the density of p75<sup>NTR</sup>–IR in the superficial laminae of the LDH and MDH, p75<sup>NTR</sup>–IR also extended into deeper laminae of the DH with CYP-induced cystitis (Fig. 2B, C). Although spinal cords demonstrated p75<sup>NTR</sup>-IR in DCM and LCP,
this staining was not different (Fig. 2) among control and experimental groups. \( p75^{\text{NTR}} \) –IR in the SPN region in the L6-S1 spinal cord or IML region of the L1-L2 spinal segments was also not changed with CYP-induced cystitis of any duration examined. No changes in \( p75^{\text{NTR}} \)-IR in the L5 spinal cord were demonstrated with CYP-induced cystitis of any duration examined.

**\( p75^{\text{NTR}} \)-IR is increased in lumbosacral dorsal root ganglion (DRG) cells after CYP-induced cystitis**

\( p75^{\text{NTR}} \) expression was observed in the cytoplasm of DRG cells in all levels examined (L1-L2; L5-S1). The number of cells that showed positive staining for \( p75^{\text{NTR}} \) (Fig. 3A-D; Fig. 4) increased significantly (\( p \leq 0.05 \)) in all levels examined after chronic CYP treatment. This increase in numbers of \( p75^{\text{NTR}} \)-IR DRG cells was also significant in L1 and S1 DRG after 48 hr CYP treatment and in L5-S1 DRG with acute (4 hr) CYP treatment (\( p \leq 0.05 \)) (Fig. 4).

**Pericellular \( p75^{\text{NTR}} \)-IR in lumbosacral DRG**

In addition to the \( p75^{\text{NTR}} \) expression seen in the cytoplasm, \( p75^{\text{NTR}} \) –IR was also observed surrounding individual DRG cells (Fig. 5B, D, F). This pericellular staining was observed in control animals as well as after acute (4 hr and 48 hr) and chronic CYP-induced cystitis in lumbosacral DRG (L5-S1)(Fig. 5B, D, F). The intensity of the staining was significantly (\( p \leq 0.001 \)) increased after chronic in L5-S1 DRG, after 48 hr CYP in L5 and L6 DRG and after 4 hr CYP treatment in L6 DRG (Fig. 5A, C, E, H).
To determine whether the pericellular staining was associated with glial cells in the DRG, DRG were stained with the antibodies to the glial cell marker, glial fibrillary acidic protein (GFAP), as well as with anti-p75\textsuperscript{NTR} and optical sections were used to determine colocalization. Upon examination with confocal microscopy (Fig. 5G; Fig. 6G), co-localization with these antibodies was not observed. Separate, non-overlapping GFAP-IR and p75\textsuperscript{NTR}-IR was observed in all DRG examined. Using colocalization software on the metaconfocal system, we determined that the greatest coefficient of colocalization for GFAP and p75\textsuperscript{NTR} in DRG examined from control or CYP-treated rats was 0.3 (range 0.05-0.3) indicating very little colocalization of GFAP with p75\textsuperscript{NTR}. As a comparison, a coefficient of colocalization of 1 is indicative of complete colocalization whereas 0 indicates no colocalization. Thus, we conclude that pericellular p75\textsuperscript{NTR} represents DRG cell membrane staining.

\textbf{p75\textsuperscript{NTR}-IR in bladder afferents}

To determine whether p75\textsuperscript{NTR}-IR is expressed in bladder afferent cells in DRG from control or after bladder inflammation, Fastblue (FB) was injected into the urinary bladder to retrogradely label bladder afferent cells in the L1, L2, L6 and S1 DRG (Fig. 6B, E). In control animals (Fig. 6A-C), 25-43% of bladder afferent cells expressed p75\textsuperscript{NTR}-IR in L1, L2 and L5-S1 DRG (Fig. 6G). After 4 hr CYP (Fig. 6H), there was a significant (p ≤ 0.01) increase in the percentage of bladder afferent cells that expressed p75\textsuperscript{NTR}-IR in L1 (58.4% ± 3.5), L2 (42.3% ± 1.6), L6 (53.3% ± 3.8), and S1 (71.3% ± 3.8) DRG. After 48 hr CYP treatment (Fig. 6D-F), a significant (p ≤ 0.01) increase in the
percentage of bladder afferent cells expressing p75\textsuperscript{NTR}-IR was demonstrated in L1 (63.2% ± 2.4), L6 (57.7% ± 4.8) and S1 (66.5% ± 3.0) DRG. After chronic CYP treatment (Fig. 6G, H), a significant (p ≤ 0.01) increase in the percentage of bladder afferent cells expressing p75\textsuperscript{NTR}-IR was demonstrated in L1 (67.6% ± 1.5), L2 (71.4% ± 4.5), L6 (69.4% ± 4.3), and S1 (67.5% ± 7.6). No changes were observed in the percentage of bladder afferents expressing p75\textsuperscript{NTR}-IR in the L2 DRG with 48 hr CYP treatment (Fig. 6H).

\textbf{p75\textsuperscript{NTR} mRNA expression in DRG with CYP-induced cystitis}

Acute (4 hr) CYP-induced cystitis produced a significant (p ≤ 0.01) reduction in p75NTR mRNA in the L6 DRG (Fig. 7). In contrast, a significant (p ≤ 0.01) increase in p75NTR mRNA was observed 48 hr after CYP-treatment (Fig. 7). With chronic CYP-treatment, no additional change in transcript expression was observed compared to control (Fig. 7). There was a significant (p ≤ 0.01) increase in p75NTR transcript with 48 hr and chronic CYP treatment compared to acute (4 hr) treatment (Fig. 7). A similar biphasic change in p75NTR mRNA expression in the S1 DRG with CYP treatment was also observed but these changes did not reach significance (Fig. 7).
Discussion

These studies demonstrate several novel findings: (1) constitutive $p75^{NTR}$ expression in spinal pathways involved in micturition reflexes in control rats is significantly increased after acute, intermediate or chronic CYP-induced cystitis; (2) constitutive $p75^{NTR}$-immunoreactivity (IR) in lumbosacral dorsal root ganglia (DRG) is significantly upregulated with CYP-induced cystitis of varying duration; (3) bladder afferent cells retrogradely labeled with Fastblue (FB) express $p75^{NTR}$-IR in control rats and the percentage of bladder afferents expressing $p75^{NTR}$ is significantly increased with CYP-induced cystitis; (4) CYP-induced cystitis also increased pericellular $p75^{NTR}$-IR in DRG and this staining did not exhibit colocalization with the glial marker, GFAP; (5) $p75^{NTR}$ transcript in DRG exhibited a biphasic response to CYP-induced cystitis with acute cystitis decreasing $p75^{NTR}$ mRNA whereas intermediate and chronic CYP-induced cystitis increased $p75^{NTR}$ transcript with respect to the acute condition. These studies demonstrate $p75^{NTR}$ expression in micturition pathways that is modifiable with varying degrees of bladder inflammation.

Although numerous studies have suggested a central or peripheral role for $p75^{NTR}$ after axotomy (Hannila and Kawaja, 2005; Keast and Kepper, 2001; Obata et al., 2006; Scott et al., 2005; Wakabayashi et al., 1998) or peripheral somatic inflammation (Chien et al., 2007; Cho et al., 1996; Pezet et al., 2001) few studies have examined the role of $p75^{NTR}$ in normal or inflamed lower urinary tract pathways (Elsasser-Beile et al., 2000; Jahed and Kawaja, 2005; Tong and Cheng, 2005; Vaidyanathan et al., 1998; Wakabayashi et al., 2002; Wakabayashi et al., 1998) (Wakabayashi et al., 1996). To our
knowledge, no studies have examined changes in p75NTR expression in central or peripheral micturition reflex pathways after bladder inflammation. In contrast, p75NTR expression in the urinary bladder has been examined in rodent and human conditions of neuropathic bladder inflammation and diabetes. Acute cystitis resulted in increased p75NTR-IR in nerve bundles in the detrusor smooth muscle (Wakabayashi et al., 1996). In patients with neuropathic bladder, increased expression of p75NTR has been shown in the umbrella and basal layers of the urothelium in addition to being expressed in bladder nerves (Vaidyanathan et al., 1998). In contrast, p75NTR mRNA is decreased in urinary bladder or sensory neurons in the DRG (Delcroix et al., 1998) of streptozotocin-induced diabetic rats but restoration of normal blood glucose level or exogenous, systemic treatment with NGF returned transcript expression to normal in urinary bladder (Tong and Cheng, 2005) or DRG (Delcroix et al., 1998). Thus, a number of studies have demonstrated that p75NTR expression is modifiable in the urinary bladder.

The current studies demonstrate significant increases in p75NTR in the superficial laminae of the dorsal horn; however, changes in p75NTR expression in other regions of the lumbosacral spinal cord involved in bladder reflex function (i.e., SPN, LCP, DCM) were not observed. Previous studies have suggested a role for p75NTR in both restraining and facilitating axonal growth in the dorsal horn after dorsal rhizotomy, spinal cord injury and during development (Hannila and Kawaja, 2005; Scott et al., 2005). p75NTR expression and upregulation with CYP-induced cystitis in the lumbosacral DH may represent central projections of sensory neurons, terminals of projection neurons and/or descending p75NTR systems (Pioro and Cuello, 1990; Sobreviela et al., 1994) exhibiting p75NTR–IR whereas
interneuronal expression of p75NTR is less likely (Bothwell, 1995). Most recently it has been demonstrated that p75NTR is anterogradely transported by the dorsal roots to central terminals in the spinal cord DH (Delcroix et al., 1998; Zweifel et al., 2005). Increased expression of p75NTR was observed in the superficial laminae of the DH in rostral lumbar (L1-L2) and caudal lumbosacral (L6-S1) spinal segments known to innervate the urinary bladder. Previous studies in this laboratory (Vizzard, 2001; Zvarova et al., 2004) have also demonstrated changes in calcitonin gene-related peptide, substance P, pituitary adenylate cyclase activating polypeptide and galanin expression in superficial laminae of the DH in the lumbosacral spinal cord after CYP-induced cystitis.

In support of p75NTR-IR nerve terminals in the DH of the spinal cord representing, in part, the central projections of sensory neurons in the lumbosacral DRG, the present study demonstrated p75NTR expression in DRG cells. Similar to what we observed in the DH of the spinal cord, CYP-induced cystitis increased p75NTR expression in lumbosacral DRG examined. Previous studies have demonstrated p75NTR-IR in DRG cells with increased expression after nerve injury (Obata et al., 2006). Previous studies have demonstrated p75NTR expression in both cell bodies in DRG (Bennett et al., 1996; Hu and McLachlan, 2000; Obata et al., 2006) and encircling (pericellular staining) (Hu and McLachlan, 2000; Obata et al., 2006) DRG cells. These two types of staining patterns are consistent with that observed in the present study. Previous studies have demonstrated co-labeling of the glial marker, GFAP and pericellular p75NTR expression in DRG (Hu and McLachlan, 2000; Obata et al., 2006). It was concluded that the
pericellular $p75^{\text{NTR}}$ staining was expressed in satellite glial cells after peripheral nerve lesion. In contrast, the present studies did not demonstrate significant overlap of $p75^{\text{NTR}}$ pericellular staining in lumbosacral DRG in control rats or after CYP-induced cystitis. It should be noted that our conclusions were based upon confocal imaging of DRG sections and determination of a coefficient of colocalization using optical sections. Previous studies have concluded colabeling of GFAP and pericellular $p75^{\text{NTR}}$ staining in DRG based upon the use of traditional epifluorescence microscopy (Hu and McLachlan, 2000; Obata et al., 2006). In addition, when DRG sections in the present study are imaged with epifluorescence microscopy and merged images are created, we would also conclude, incorrectly, that the pericellular $p75^{\text{NTR}}$ -IR was colocalized with GFAP. In addition to changes in $p75^{\text{NTR}}$ protein expression in DRG cells, we also demonstrated a biphasic response in $p75^{\text{NTR}}$ mRNA with CYP-induced cystitis. Increases in $p75^{\text{NTR}}$ mRNA were demonstrated with intermediate and chronic CYP-induced cystitis. In contrast, decreases in $p75^{\text{NTR}}$ mRNA were observed with acute CYP treatment. This decrease in $p75^{\text{NTR}}$ mRNA is at odds with the observed increase in $p75^{\text{NTR}}$ –IR in DRG with acute cystitis. Differences in transcript and protein expression are not uncommon and likely reflect an increase in translation efficiency, additional changes in posttranslational mechanisms, or increased stability of mRNA transcript (Kruttgen et al., 1998; Sherer et al., 1998a; Sherer et al., 1998b; Vizzard, 2000b).

Increased $p75^{\text{NTR}}$ expression was also observed in the L5 DRG known not to be involved in micturition reflexes but to receive sensory information from the hindpaw. It is interesting to note that the CYP-induced cystitis model in the rodent produces somatic
sensitization in the hindpaw (Guerios et al., 2006)(Vizzard and Cheppudirah, unpublished observations). Mechanical sensitivity as assessed with von Frey monofilaments is increased although thermal sensitivity remains unchanged (Guerios et al., 2006)(Vizzard and Cheppudirah, unpublished observations). Thus, increased p75NTR expression in the L5 DRG may be related to increased sensitivity in this referred site (Guerios et al., 2006)(Vizzard and Cheppudirah, unpublished observations). No changes in p75NTR expression in the L5 spinal segment were present with any duration of CYP treatment used in the present study. This may reflect a lack of anterograde transport of p75NTR in L5 DRG to central terminals in the spinal cord (Delcroix et al., 1998; Zweifel et al., 2005).

Although p75NTR expression has been demonstrated in sensory neurons in the DRG, the innervation targets of the DRG cells expressing p75NTR-IR have not been previously identified. In the present study, presumptive bladder afferent cells in the DRG were identified by injection of Fastblue into the urinary bladder and subsequent retrograde transport to lumbosacral DRG innervating the urinary bladder. These studies demonstrate that approximately 25-43% of bladder afferent cells express p75NTR-IR under control conditions. This percentage is in very close agreement with previous studies demonstrating a similar percentage of bladder afferent cells expressing the tropomyosin related tyrosine kinase receptors (Trk) TrkA or TrkB (Qiao and Vizzard, 2002). Previous studies have demonstrated almost complete colocalization of p75NTR and Trk receptors in DRG (Chao and Hempstead, 1995; Wright and Snider, 1995). CYP-
induced cystitis increased p75NTR expression in bladder afferent cells in all lumbosacral DRG examined (L1, L2, L6, S1). Increased expression occurred with acute, intermediate and chronic CYP-induced cystitis. The increased p75NTR expression observed in bladder afferent cells with cystitis was different than that observed for either TrkA or TrkB (Qiao and Vizzard, 2002). Increased TrkA expression was restricted to L1 and L6 DRG with varying durations of cystitis and increased TrkB expression was broadly distributed among L1, L2, L6 and S1 but only demonstrated an increase with acute CYP-induced cystitis (Qiao and Vizzard, 2002). Thus, although basal expression of p75NTR in bladder afferent cells resembled the percentage of bladder afferent cells expressing TrkA or TrkB, the response to CYP-induced cystitis differed. This difference may reflect the numerous, potential independent roles that p75NTR may play with neurotrophins and other receptors including, Trk, Nogo, LINGO-1 and sortilin (Allen and Dawbarn, 2006; Bronfman and Fainzilber, 2004; Nykjaer et al., 2005).

Target-derived changes in NGF with bladder inflammation may drive changes in p75NTR expression in central and peripheral micturition reflexes. Previous studies have demonstrated that exogenous or elevated endogenous NGF can alter p75NTR expression (Delcroix et al., 1998; King et al., 2000; Mousa et al., 2007). The exact contribution of NGF to bladder function is not known but a correlation between increased voiding frequency and elevated urinary bladder NGF has been suggested. Experimentally-induced cystitis increases voiding frequency and alters bladder NGF protein and transcript. Intrathecal (Yoshimura et al., 2006), intravesical delivery of NGF (Dmitrieva et al., 1997) or adenovirus-mediated NGF overexpression (Lamb et al., 2004) in the bladder increase
voiding frequency and bladder afferent cell hyperexcitability (Yoshimura et al., 2006; Yoshimura et al., 2002) in control rats. NGF scavenging methods (Dmitrieva et al., 1997; Hu et al., 2005) reduce voiding frequency in rats with experimentally induced inflammation. Elevated levels of neurotrophins are detected in the urine of women with painful bladder syndrome (Okragly et al., 1999) (PBS)/interstitial cystitis (IC) or in the urothelium of individuals with neuropathic bladder (Lowe et al., 1997). However, a recent study failed to demonstrate an association between increased urothelium/suburothelium NGF with detrusor overactivity or bladder sensation (Birder et al., 2007).

Neurotrophins, through interactions with Trk and/or p75NTR receptors, may contribute to neurochemical (Vizzard, 2000a; 2001), electrophysiological (Yoshimura et al., 2002) and organizational (Steers and Tuttle, 2006; Vizzard, 2000c) plasticity of lower urinary tract pathways after CYP-induced cystitis. Neurotrophin/Trk/p75NTR interactions could induce long-term changes in cells (Steers and Tuttle, 2006), including: (1) mediating neurotransmitter phenotype; (2) influencing dendrite size and synaptic reorganization; (3) increasing synaptic efficacy; and (4) controlling innervation density and target organ function. Additional p75NTR functions independent of Trk may also include myelination, axonal growth and regeneration (Nykjaer et al., 2005). Although p75NTR has been shown to increase binding affinity of TrkA for NGF and increase the activities of the TrkA receptor in some systems (Chao and Hempstead, 1995), p75NTR may also hinder TrkA binding to NGF by sequestering increased amounts of NGF, leaving less available to bind TrkA (Dhanoa et al., 2006; Hannila and Kawaja, 2005;
Nykjaer et al., 2005). Previous studies have demonstrated that expression of p75\textsuperscript{NTR} and TrkA can result in synergistic and antagonistic functions (Schweigreiter, 2006). It is likely that the role of p75\textsuperscript{NTR} in micturition reflexes is dependent on the balance between NGF/p75\textsuperscript{NTR} and NGF/TrkA signaling in bladder afferents and the extent to which this balance is altered with bladder inflammation. The function of p75\textsuperscript{NTR} in micturition reflex function in control rats and after CYP-induced cystitis is the subject of ongoing physiological studies.
Other Acknowledgements:

We gratefully acknowledge the generous gift of $p75^{NTRexonIII+/−}$ mice from Dr. Hermes Yeh, Dartmouth Medical School.
Figures

Figure 1: p75<sub>NTR</sub><sup>ExonIII<sup>-/-</sup> mice

p75<sub>NTR</sub><sup>ExonIII<sup>-/-</sup> mice exhibit minimal p75<sub>NTR</sub>-IR when tested with antisera used in the present study. Lumbosacral (L6-S1) dorsal root ganglion (DRG) sections from littermate wildtype and p75<sub>NTR</sub><sup>ExonIII<sup>-/-</sup> mice were used to determine the specificity of p75<sub>NTR</sub> antisera used in the present study. All DRG sections were reacted simultaneously using identical reagents. A. In wildtype S1 DRG, robust p75<sub>NTR</sub>-IR was present in neuronal cell bodies. B. In contrast, S1 DRG sections from p75<sub>NTR</sub><sup>ExonIII<sup>-/-</sup> mice only exhibited the faintest p75<sub>NTR</sub>-IR. Panels A and B represent images captured at different exposure times. To see any p75<sub>NTR</sub>-IR in the null mice, exposure times were increased to 4X that used to capture images from wildtype sections further demonstrating the faint p75<sub>NTR</sub>-IR. Calibration bar represents 80 µm.
p75^{NTR}-IR is increased in specific regions of the lumbosacral (L1, L2, L6, S1) spinal cord with cyclophosphamide (CYP)-induced cystitis. Fluorescence images of p75^{NTR} expression in the dorsolateral quadrant of lumbosacral (L6) spinal cord from control (A), 4 hour (B) and chronic (C) CYP treated rats. A-C. p75^{NTR}-IR is constitutively expressed in the superficial lateral (LDH) and medial dorsal horn (MDH); faint p75^{NTR}-IR was expressed in lateral collateral pathway (LCP) and dorsal commissure (DCM). With CYP-induced cystitis, p75^{NTR}-IR significantly (p ≤ 0.001) increased in the LDH and MDH of L6-S1 and L1-L2 spinal segments. D. Summary histogram of p75^{NTR} staining in L1-L2 spinal segments induced in LDH and MDH with varying duration of CYP-induced cystitis presented as a percentage of p75^{NTR} staining intensity in control tissues. E. Summary histogram of p75^{NTR} staining in L6-S1 spinal segments induced in LDH and MDH with varying duration of CYP-induced cystitis presented as a percentage of p75^{NTR} staining intensity in control tissues. Statistical analyses were performed on raw data. F. Drawing of a hemisection of lumbosacral spinal cord section for orientation purposes. * p ≤ 0.001. n=4. SPN, sacral parasympathetic nucleus; CC, central canal. Calibration bar represents 100 µm.
Figure 3: p75<sup>NTR</sup>-IR cells in the lumbosacral dorsal root ganglia

CYP-induced cystitis increases the number of p75<sup>NTR</sup>-IR cells in the lumbosacral dorsal root ganglia (DRG). Fluorescence images of p75<sup>NTR</sup>-IR in L1 DRG in control (A), 4 hour (B), 48 hour (C) and chronic (D) CYP treated rats. White arrows indicate cells demonstrate some examples of p75<sup>NTR</sup>-IR cells. Yellow arrowheads demonstrate some examples of DRG cells that did not exhibit p75<sup>NTR</sup>-IR. Calibration bar represents 60 µm.
Figure 4: numbers of p75<sup>NTR</sup>-IR cells in the lumbosacral dorsal root ganglia

Summary histogram of numbers of dorsal root ganglion (DRG) cells that exhibit p75<sup>NTR</sup>-IR under control conditions and after induction of CYP-induced cystitis (4 hour, 48 hour, chronic). (n = 5 for each; *, p ≤ 0.05)
Figure 5: Pericellular p75NTR-IR in DRG from control and CYP-treated rats.

Pericellular p75NTR-IR increased in lumbosacral dorsal root ganglia (DRG) with CYP treatment. A threshold encompassing an intensity range of 100-250 grayscale values was applied to the region of interest in control DRG (A). The same threshold was subsequently used for all images (C, E). Four boxes of fixed dimension were randomly placed in DRG images. Percent p75NTR expression above threshold was then calculated. Grayscale versions of p75NTR-IR in control DRG (A), or with 48 hour or chronic CYP-treatment (E). Images are thresholded and little p75NTR-IR (absence of purple within the outlined region) is above threshold in control DRG compared to significant p75NTR-IR that is above threshold after 48 hr CYP treatment (C, presence of purple) or chronic treatment (E). Fluorescence images of p75NTR-IR in S1 DRG from control (B), 48 hour (D), and chronic (F) CYP treatments. H. Summary histogram of p75NTR expression above threshold in selected regions was calculated and presented as a percentage of control (n=4; *, p ≤ 0.01). Calibration bar represents 40 µm (A-F), 20 µm (G).
Figure 6: p75NR-IR in bladder afferent cells in lumbosacral dorsal root ganglion

CYP-induced cystitis increases p75NR-IR in bladder afferent (Fastblue, FB-labeled) cells in the lumbosacral DRG. A-C. L6 DRG section from control rat demonstrating p75NR-IR (A), bladder afferent cells (FB-labeled) in same section (B) and merged image (C) demonstrating bladder afferent cells with p75NR-IR (pinkish-purple/magenta). D-F. L6 DRG section from rat with 48 hour induced cystitis (D), bladder afferent cells (FB-labeled) in same section (E) and merged image (F) demonstrating bladder afferent cells with p75NR-IR (pinkish-purple/magenta). White arrows indicate some examples of bladder afferent cells that exhibit p75NR-IR. Not all bladder afferent cells express p75NR-IR (yellow arrowheads) and not all cells expressing p75NR-IR are bladder afferent cells (green arrows). G. Triple-labeled image demonstrating p75NR-IR (red) in bladder afferent cells (blue) in L6 DRG section from a rat treated chronically with CYP. The DRG section was also stained for glial fibrillary acidic protein (GFAP; green). Bladder afferent cells exhibiting both FB and p75NR-IR appear pinkish-purple/magenta). (H) Summary histogram of the percentage of bladder afferent cells expressing p75NR-IR in control and CYP-treated rats. (n=4 for each group, #, p ≤ 0.01; *, p ≤ 0.001). Calibration bar represents 35 μm.
Figure 7: p75NTR mRNA in L6-S1 DRG

CYP-induced cystitis induces a biphasic response in p75NTR mRNA in L6-S1 DRG. For each experimental time period, total RNA from individual L6 or S1 DRG was reverse transcribed, and the cDNA templates synthesized from control and CYP-treated DRG were analyzed by real-time quantitative PCR using primers specific for rat p75NTR. Transcript levels are normalized to the housekeeping gene, L32 and expressed as relative change compared to control (100%). n = 5 for all; *, p ≤ 0.01 versus control; **, p ≤ 0.01 versus 4 hour treatment.
Table 1: Oligonucleotide primer sequences for p75NTR and the housekeeping gene, L32.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primers sequences</th>
<th>Location</th>
<th>Annealing temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>p75NTR upper</td>
<td>TGCTGCTGCTGCTGATTCTA</td>
<td>47U20&lt;sub&gt;a&lt;/sub&gt;</td>
<td>65 °C</td>
</tr>
<tr>
<td>p75NTR lower</td>
<td>ACAAGGTCTTGGCTCTGGAGGA</td>
<td>65L21&lt;sub&gt;a&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>L32 upper</td>
<td>CCTGGCCTTGGGATTGGTG</td>
<td>83U20&lt;sub&gt;b&lt;/sub&gt;</td>
<td>60 °C</td>
</tr>
<tr>
<td>L32 lower</td>
<td>GAAAAGCCATCGTAGAAGA</td>
<td>129L20&lt;sub&gt;b&lt;/sub&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>: Primer positions are given as the nucleotide sequences for the *Rattus norvegicus* p75 precursor protein gene sequence with GenBank accession number NM_012610.

<sup>b</sup>: Primer positions are given as the nucleotide sequences for the *Rattus norvegicus* L32 precursor protein gene sequence with GenBank accession number NM_013226.
Literature Cited


Chapter 4: The Role of p75NTR in Female Rat Urinary Bladder with Cyclophosphamide (CYP)-Induced Cystitis
Abstract

Previous studies demonstrated changes in urinary bladder neurotrophin content and upregulation of neurotrophin receptors, TrkA and the p75 neurotrophin receptor (p75NTR), in micturition reflex pathways after cyclophosphamide (CYP)-induced cystitis. p75NTR can bind NGF and modulate NGF-TrkA binding and signaling. We examined p75NTR expression and the role of p75NTR in the micturition reflex in control and CYP-treated rats. p75NTR–immunoreactivity (IR) was present throughout the urinary bladder. CYP-induced cystitis (4 hour (h), 48 h, chronic) increased (p ≤ 0.05) p75NTR expression in whole urinary bladder as shown by western blotting. The role of p75NTR in bladder function in control and CYP-treated rats was determined using conscious cystometry and immunoneutralization or PD90780, a compound known to specifically block NGF binding to p75NTR. An anti-p75NTR monoclonal antibody or PD90780 was infused intravesically and cystometric parameters were evaluated. Both methods of p75NTR blockade significantly (p ≤ 0.05) decreased the intercontracton interval and void volume in control and CYP-treated rats. Intravesical infusion of PD90780 also significantly (p ≤ 0.001) increased intravesical pressure and increased the number of non-voiding contractions during the filling phase. Control intravesical infusions of isotype matched IgG and vehicle were without effect. These studies demonstrate: (1) ubiquitous p75NTR expression in urinary bladder and increased expression with CYP-induced cystitis; (2) p75NTR blockade at the level of the urinary bladder produces bladder hyperreflexia in control and CYP-treated rats. The overall activity of the urinary bladder reflects the balance of NGF-p75NTR and NGF-TrkA signaling.
**Introduction**

Nerve growth factor (NGF) is important in sensory and sympathetic neuronal development and maintenance (Huang et al., 1999); however, many recent studies suggest additional role(s) for NGF in painful somatic and visceral inflammatory conditions (Chien et al., 2007; Friedman, 1999 #151) (Allen and Dawbarn, 2006; Chuang et al., 2001b; Frossard et al., 2004; Pezet and McMahon, 2006). NGF upregulation occurs at sites of tissue injury and inflammation (Lindholm et al., 1987; Woolf et al., 1997) and changes in NGF levels in urine as well as urinary bladder have been documented in humans with painful bladder syndrome (PBS)/interstitial cystitis (IC) and rodents with bladder inflammation (Lowe et al., 1997; Oddiah et al., 1998; Okragly et al., 1999; Vizzard, 2000b). A number of addition and subtraction studies have begun to demonstrate the role(s) of NGF in inflammatory conditions of the urinary bladder. For example, intravesical infusion or intramuscular detrusor administration of exogenous NGF results in increased bladder activity, sensitization of bladder afferents (Chuang et al., 2001b; Dmitrieva and McMahon, 1996; Dmitrieva et al., 1997), increased expression of Fos protein in lumbosacral spinal cord in response to bladder distention and increased expression of neuropeptides in lumbosacral spinal cord (Zvara and Vizzard, 2007). Strategies to reduce NGF in micturition reflex pathways reduce or eliminate these effects (Dmitrieva et al., 1997; Hu et al., 2005).

NGF signals through its specific receptor TrkA, as well as p75NTR. TrkA is a tropomyosin-related receptor tyrosine kinase receptor and p75NTR belongs to the tumor
necrosis factor-α family of receptors (Allen and Dawbarn, 2006; Barker, 2004; Bronfman and Fainzilber, 2004). p75NTR binds all neurotrophins, including brain-derived 
neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5. NGF-TrkA signaling is 
involved in some inflammatory effects of NGF (Pezet and McMahon, 2006), including 
sensitization of nociceptive afferents and the release of neuropeptides. TrkA expression 
is increased in bladder afferent cells in lumbosacral dorsal root ganglia (DRG) with acute 
and chronic cyclophosphamid (CYP)-induced bladder inflammation (Qiao and Vizzard, 
2002). In addition, p75NTR expression is also significantly upregulated in bladder afferent 
cells (2.4-2.8-fold) in lumbosacral DRG after CYP-induced cystitis (Klinger et al., 2008).

The pan-neurotrophin receptor p75NTR exhibits an ubiquitous distribution, has many 
functions and multiple binding partners and ligands (Barker, 2004; Bronfman and 
Fainzilber, 2004). In the absence of Trk expression, p75NTR is suggested to be involved 
in apoptosis and regulation of neuronal growth (Barker, 2004; Friedman and Greene, 
1999); however, in the presence of TrkA, p75NTR is suggested to function by enhancing 
Trk binding to neurotrophins (Barker, 2004; 2007; Wehrman et al., 2007). p75NTR 
expression has previously been identified in the urinary bladder (Vaidyanathan et al., 
1998; Wakabayashi et al., 1996; Wakabayashi et al., 1995) but few studies have 
examined the role of p75NTR in bladder function or in the context of urinary bladder 
inflammation (Wakabayashi et al., 1996). Although we do know that p75NTR expression 
is present in micturition reflex pathways in control rats and is regulated with CYP-
induced cystitis (Klinger et al., 2008), the potential role(s) of p75NTR in urinary bladder 
reflexes have not been explored. In this study we determined: (1) expression of p75NTR
in the urinary bladder with immunohistochemistry and western blot and regulation of \( p75^{NTR} \) expression after CYP-induced cystitis; (2) the effect of immunoneutralization with an anti- \( p75^{NTR} \) monoclonal antibody on bladder function in female rats without (non-inflamed) and with bladder inflammation (48 hr CYP treatment) using conscious cystometry; (3) the effect of intravesical infusion of PD90780, a compound known to specifically block NGF binding to \( p75^{NTR} \) (Colquhoun et al., 2004; Hefti et al., 2006; Spiegel et al., 1995), in female rats without (non-inflamed) and with bladder inflammation (48 hr CYP treatment) using conscious cystometry.
Materials and Methods

Cyclophosphamide-induced cystitis: acute, intermediate or chronic

Chemical cystitis was induced in adult (200-225 g), female Wistar rats by CYP treatment (Klinger et al., 2008; Murray et al., 2004; Vizzard, 2001; Vizzard and Boyle, 1999). CYP was administered in one of the following ways: 1) 4 hours (h) (150 mg/kg; i.p.) prior to euthanasia of the animals to elicit acute bladder inflammation; 2) 48 h (150 mg/kg; i.p.) prior to euthanasia to examine an intermediate inflammation; or 3) administered every 3rd day for 9 days to elicit chronic bladder inflammation (75 mg/kg; i.p.). All injections of CYP were performed under isoflurane (2%) anesthesia. Control animals were gender matched to the experimental groups and received a corresponding volume of saline (0.9%) or distilled water injected intraperitoneally under isoflurane (2%) anesthesia. Animals were euthanized by isoflurane anesthesia (5%) plus thoracotomy at the indicated time points, and the urinary bladder was harvested and weighed. The University of Vermont Institutional Animal Care and Use Committee approved all experimental procedures (protocol number 06-014) involving animal use. Animal care was under the supervision of University of Vermont College of Medicine's Office of Animal Care in accordance with American Association for Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. All efforts were made to minimize animal stress/distress and suffering and to use the minimum number of animals.

Whole-mount bladder preparation and immunohistochemistry
The urinary bladder from control \((n = 5)\) and CYP treatment \((n = 5)\) was dissected and placed in Krebs solution. The bladder was cut open along the midline and pinned to a Sylgard-coated dish. The bladder was incubated for 3 h at room temperature in cold fixative (2% paraformaldehyde + 0.2% picric acid), and the urothelium was removed as previously described (Zvarova et al., 2004). Urothelium and bladder musculature were processed separately for \(p75^{\text{NTR}}\)-IR. Control and CYP-treated tissues were incubated overnight at room temperature in rabbit anti-\(p75^{\text{NTR}}\) antiserum (1:3000; Advanced Targeting Systems (ATS), San Diego, CA) in 1% goat serum and 0.1 M KPBS (0.1 M phosphate buffered saline (PBS) with potassium) and then washed (3 × 15 minutes) with 0.1 M KPBS, pH 7.4. After washing, the tissue was incubated in a species-specific secondary antibody (1:500; Cy3-conjugated goat anti-rabbit; Jackson Immunoresearch, West Grove, PA) for 2 h at room temperature, followed by washing and coverslipping with Citifluor (London, UK). Control tissues incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining was observed. The specificity of the \(p75^{\text{NTR}}\) antiserum was previously established (Klinger et al., 2008). Some whole-mount preparations were stained with the pan neuronal marker, protein gene product 9.5 (Abcam, Cambridge, MA; 1:15) to visualize nerve fibers in the suburothelial plexus and to demonstrate that suburothelial nerve fibers expressed \(p75^{\text{NTR}}\)-IR.

\(p75^{\text{NTR}}\) localization in urinary bladder sections after intravesical \(p75^{\text{NTR}}\) infusion
Immediately after cystometric analyses, urinary bladders were harvested from rats that had received intravesical infusion of monoclonal antibody to p75<sup>NTR</sup> and those that had received intravesical infusion of protamine sulfate. Animals were deeply anesthetized with isoflurane (5%) and euthanized via thoracotomy. Bladders were quickly removed and postfixed in 4% paraformaldehyde overnight. Tissues were cryoprotected by immersion in 30% sucrose (in 0.1 M phosphate-buffered saline) overnight. Bladders were sectioned (20 µm) on a cryostat and directly mounted on gelled (0.5%) microscope slides. Tissue was incubated in secondary antibody (Cy2-conjugated goat anti-mouse; Jackson ImmunoResearch, West Grove, PA) for two h and washed (3 ×15 minutes) at room temperature with 0.1 KPBS (pH 7.4). Slides were coverslipped with Citifluor (London, UK).

**Assessment of positive staining in urinary bladder**

Staining observed in experimental tissue was compared with that observed from experiment-matched negative controls. Tissues exhibiting immunoreactivity that was greater than the background level observed in experiment-matched negative controls were considered positively stained.

**Imaging and Visualization of Bladder Sections**

Tissues were examined under an Olympus fluorescence photomicroscope (Optical Analysis, Nashua, NH) for visualization of Cy2. Cy2 was visualized with a filter with an excitation range of 470-490 and an emission range from 510-530. Images of bladder
sections were captured through a video camera attachment to the microscope with the exposure time, brightness, and contrast being held constant.

**Imaging and visualization of bladder whole-mounts**

Tissue was examined and optical sections were acquired using a Zeiss LSM 510 confocal scanning system attached to a Zeiss LSM 510 microscope using a plan Fluor 20x or 10x objective. An excitation wavelength of 543 nm was used for visualization of p75NTR. Bladder whole-mount images were captured through a video camera attachment to the microscope with the exposure time, brightness, and contrast held constant.

**Western blotting for p75\textsuperscript{NTR} expression in whole urinary bladder**

Whole urinary bladders were homogenized separately in tissue protein extraction agent with protease inhibitors (T-PER; Roche, Indianapolis, IN), and aliquots were removed for protein assay. Samples (23 µg) were suspended in sample buffer for fractionation on gels and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and efficiency of transfer was evaluated. Membranes were blocked overnight in a solution of 5% milk, 3% bovine serum albumin in Tris-buffered saline with 0.1% Tween. Membranes were incubated in rabbit anti-p75\textsuperscript{NTR} (1:2000; ATS) overnight at 4°C. Washed membranes were incubated in a species-specific secondary antibody (1:7000; goat anti-rabbit horseradish peroxidase) for 2 h at room temperature for
enhanced chemiluminescence detection (Pierce, Rockford, IL). Blots were exposed to Biomax film (Kodak, Rochester, NY) and developed. The intensity of each band was analyzed, and background intensities were subtracted using Un-Scan It software (Silk Scientific, Orem, UT). Western blot analysis of erk1 and erk2 (1:2000; Cell Signaling Technology, Danvers, MA) in samples was used as a loading control. The specificity of the p75<sup>NTR</sup> antiserum was previously established (Klinger et al., 2008). In pilot studies, the concentration of p75<sup>NTR</sup> antiserum used for western blotting studies of urinary bladder was titrated. The p75<sup>NTR</sup> antiserum concentration selected for experimentation did not result in saturation in control tissues and permitted changes in p75<sup>NTR</sup> expression with CYP treatment to be evaluated semi-quantitatively.

**Intravesical catheter placement**

A lower midline abdominal incision was performed under general anesthesia with 2-3% isoflurane using aseptic techniques. Polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ) with the end flared by heat was inserted into the dome of the bladder and secured in place with a 6-0 nylon purse-string suture (Hu et al., 2003; Studeny et al., 2008). The distal end of the tubing was sealed, tunneled subcutaneously, and externalized at the back of the neck, out of the animal’s reach. Animals were maintained for 72 h after surgery to ensure complete recovery.

**Cystometry**
The effects of p75$_{NTR}$ blockade on bladder function in control (no inflammation) and CYP-treated rats (48 h) were evaluated by immunoneutralization with intravesical infusion of anti-p75$_{NTR}$ monoclonal antibody (ATS; 100 µg/ml) or PD90780 (Pfizer; 10-100 µM), known to specifically block NGF- p75$_{NTR}$ (Colquhoun et al., 2004; Hefti et al., 2006; Spiegel et al., 1995) using conscious cystometry and continuous infusion of intravesical saline. Intravesical instillation of an isotype-matched IgG and saline (room temperature, 0.9%) were used as controls. Animals were placed conscious and unrestrained in recording cages with a balance and pan for urine collection and measurement placed below (Braas et al., 2006; Hu et al., 2005; Zvara and Vizzard, 2007). Intravesical pressure changes were recorded using a Small Animal Cystometry System (Med Associates, Inc.). Saline at room temperature was infused at a rate of 10 ml/h to elicit repetitive bladder contractions. At least four reproducible micturition cycles were recorded after an initial stabilization period of 25 - 30 minutes. Voided saline was collected to determine voided volume. Intercontraction interval, maximal voiding pressure, pressure threshold for voiding and baseline resting pressure were measured (Maggi et al., 1986). The number of non-voiding bladder contractions (NVCs) per voiding cycle during the filling phase was determined. For these studies, NVCs were defined as rhythmic intravesical pressure rises greater than 7 cm H$_2$O from baseline pressure without a release of fluid from the urethra. Exclusion criteria Rats were removed from study when adverse events occurred that included: ≥ 20% reduction in body weight post surgery, a significant post-operative event, lethargy, pain or distress not relieved by our IACUC-approved regimen of post-operative analgesics or hematuria in control.
rodents. In the present study, no rats were excluded from the study or from analysis due to any of these exclusion criteria. In addition, behavioral movements such as grooming, standing, walking, and defecation rendered bladder pressure recordings during these events unusable (Streng et al., 2006). Experiments were conducted at similar times of the day to avoid the possibility that circadian variations were responsible for changes in bladder capacity measurements (Dorr, 1992). Rats were euthanized at the conclusion of study and urinary bladder harvested as described above.

**Drug treatments**

The effects of p75NTR blockade on bladder function in control (no inflammation) and CYP-treated rats (48 h) were evaluated by immunoneutralization with intravesical infusion of anti-p75NTR monoclonal antibody (ATS; 100 µg/ml) or PD90780 (Pfizer; 10-100 µM), known to specifically block NGF-p75NTR (Colquhoun et al., 2004; Hefti et al., 2006; Spiegel et al., 1995) using conscious cystometry. Immediately before cystometric analysis, rats were anesthetized (1-2% isoflurane) and bladders were manually emptied with the Credé maneuver. A solution of protamine sulfate (Sigma-Aldrich, St. Louis, MO; 10 mg/ml in sterile saline) was then infused into the bladder (≤ 1 ml) and maintained (45 minutes (min)) in the bladder while the rat was anesthetized with isoflurane (1-2%) to prevent voiding and expulsion of bladder contents (Corrow and Vizzard, 2007b). Protamine sulfate is used to disrupt urothelial cell-to-cell contact and increase urothelial permeability (Nishiguchi et al., 2005). This concentration of protamine sulfate does not affect voiding frequency (Chuang et al., 2003; Fraser et al.,
2003). The bladder was then flushed with 0.9% sterile saline and emptied. Rats were then infused (≤ 1 ml) with either anti-p75NTR monoclonal antibody (n = 9) or with PD90780 (n = 24) and maintained (30 min) in the bladder while the rat was anesthetized (isoflurane, 1-2%) to prevent voiding and expulsion of bladder contents (Braas et al., 2006; Corrow and Vizzard, 2007b). The concentrations and duration of PD90780 infusion chosen for this study were based on previous experiments with PD90780 (Allington et al., 2001; Colquhoun et al., 2004; Du et al., 2006; Spiegel et al., 1995; Wang et al., 2001). The concentration anti-p75NTR monoclonal antibody used for intravesical infusion was based on previous studies using anti-p75NTR or anti-NGF in intrathecal infusion or cell culture applications (Du et al., 2006; Ito et al., 2003; Obata et al., 2006; Seki et al., 2002; Seki et al., 2004).

Figure preparation

Digital images were obtained using a charge-coupled device camera (MagnaFire SP, Optronics; Optical Analysis, Nashua, NH) and LG-3 frame grabber attached to an Olympus microscope (Optical Analysis). Exposure times were held constant when acquiring images from control and experimental animals processed and analyzed on the same day. Images were imported into Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA) where groups of images were assembled and labeled.

Materials
All standard chemicals were obtained from Sigma-Aldrich or Fisher and were either analytical or laboratory grade. PD90780 was obtained through a compound transfer agreement with Pfizer, Inc. (Groton, CT).

Statistical Analyses

All values represent mean ± S.E.M. Data were compared one-way analysis of variance (ANOVA) or two-way ANOVA, where appropriate. When F ratios exceed the critical value (p ≤ 0.05), the Dunnett’s post-hoc test was used to compare group means.
Results

*p75$^{NTR}$ immunoreactivity (IR) in urinary bladder*

To determine the localization of p75$^{NTR}$ expression in the urinary bladder, whole-mount preparations were prepared in control (Fig. 1A-J), and 4 hour (h) (not shown) and chronic CYP-treated rats (not shown). p75$^{NTR}$ expression was present in the urothelium and neuronal fibers in the urinary bladder of control rats and CYP-treated rats. p75$^{NTR}$ – IR was seen in urothelial cells (Fig. 1A, B, D), nerve fibers (Fig. 1B-J) in the suburothelial plexus, and in nerve fibers in the detrusor in both control and CYP-treated rats. Nerve fibers adjacent to and encircling suburothelial vasculature also exhibited p75$^{NTR}$ –IR (Fig. 1C, D, H-J). p75$^{NTR}$-immunoreactive suburothelial nerve fibers and nerve fibers associated with the suburothelial vasculature also exhibited immunoreactivity for the pan neuronal marker, protein gene product (PGP9.5) (Fig. 1E-J). Given the widespread and overlapping nature of the p75$^{NTR}$ –IR in the defined structures, it was not possible to quantify changes in p75$^{NTR}$ –IR in individual structures with CYP-induced cystitis. Rather, we examined regulation of p75$^{NTR}$ protein expression in whole urinary bladder using western blotting techniques.

*p75$^{NTR}$ protein expression in urinary bladder with CYP-induced cystitis*

p75$^{NTR}$ protein expression in whole urinary bladder as determined by Western blot analysis significantly ($p \leq 0.05$) increased after 4 h (7.7-fold), 48 h (11.1-fold) or chronic CYP-treatment (3.3-fold) (Fig. 2A, B).
Effects of immunoneutralization of p75<sup>NTR</sup> on cystometry in rats with and without CYP-induced cystitis with intravesical instillation of anti-p75 monoclonal antibody

**Control (no inflammation)** Intravesical infusion of an anti-p75<sup>NTR</sup> monoclonal antibody (100 µg/ml; Fig. 3B, D) increased voiding frequency, with a decreased intercontraction interval (ICI) (p ≤ 0.001) and decreased void volume (p ≤ 0.001) compared to control rats (Fig. 3A) without intravesical anti-p75<sup>NTR</sup> instillation. Intravesical infusion of a monoclonal antibody to p75<sup>NTR</sup> did not affect baseline, micturition or threshold pressure or non-voiding contractions during the filling phase (Table 1).

**CYP treatment** As previously demonstrated, CYP-treatment (48 h) decreased ICI (p ≤ 0.001) and void volume (p ≤ 0.001; Fig. 4) and increased micturition (p ≤ 0.001) and threshold pressure (p ≤ 0.001; Table 1). Intravesical infusion of anti-p75<sup>NTR</sup> monoclonal antibody (100 µg/ml) in 48 h CYP-treated rats (Fig. 3D) resulted in an additional increase in voiding frequency with associated decreased ICI (p ≤ 0.001) and decreased void volume (p ≤ 0.005; Fig. 4A, B, Table 1). There were no significant changes in micturition pressure, threshold pressure or non-voiding contractions as compared to rats with only 48 h CYP-treatment (Fig. 3C) (Table 1). Control experiments with intravesical infusion of an isotype-matched monoclonal IgG (100 µg/ml) or protamine sulfate (10 mg/ml) showed no effects on bladder function in control or CYP-treated rats (Figs. 3, 4; Table 1) compared to rats without protamine sulfate or antibody infusion in control or CYP-treated rats. In pilot studies performed without the initial infusion of protamine sulfate, no changes in bladder function with subsequent intravesical infusion of the anti-
p75\textsuperscript{NTR} monoclonal antibody (100 µg/ml) were observed. Therefore, all subsequent experiments included protamine sulfate intravesical infusion. The duration of anti-p75NTR monoclonal antibody effects on all cystometric parameters evaluated in control and CYP-treated rats was 30-40 min.

\textit{p75\textsuperscript{NTR} immunoreactivity (IR) in urinary bladder after intravesical instillation of anti-p75\textsuperscript{NTR} monoclonal antibody}

\textit{p75\textsuperscript{NTR}-IR was examined after intravesical instillation of anti-p75\textsuperscript{NTR} monoclonal antibody by application of a species-specific secondary antibody to cryostat sections (20 µm). p75\textsuperscript{NTR}–IR was present in urothelium and lamina propria (Fig. 5A). p75\textsuperscript{NTR}–IR decreased in intensity with increasing distance from the bladder lumen. Urinary bladder sections from rats not receiving intravesical instillation of anti-p75\textsuperscript{NTR} monoclonal antibody exhibited no immunoreactivity above background levels (Fig. 5B).}

\textit{Effects of NGF-p75\textsuperscript{NTR} blockade on cystometry in rats with and without CYP-induced cystitis with intravesical instillation of PD90780}

\textbf{Control (no inflammation) rats} PD90780, a compound that specifically blocks NGF binding to p75\textsuperscript{NTR} (Colquhoun et al., 2004; Hefti et al., 2006; Spiegel et al., 1995), was infused intravesically (10 µM, 25 µM, 50 µM, and 100 µM) in rats without CYP-induced cystitis and the effects on cystometric parameters were determined (Fig. 6A-D). Intravesical instillation of PD90780 decreased void volume (p ≤ 0.002) at all concentrations evaluated, and decreased ICI at 10 µM (p ≤ 0.05; Fig. 7) and 50 µM (p ≤
0.05) (Fig. 6A, B; Fig. 7). PD90780 instillation increased threshold pressure at 25 µM and 50 µM (p ≤ 0.02) and increased micturition and baseline pressure at all concentrations (p ≤ 0.002; Table 2) evaluated. Effects of intravesical infusion of PD90780 in control rats (no inflammation) on void volume were more variable than that observed in CYP-treated rats (below) (data not shown); there was a disparity between void volume and infused volume suggestive of potential PD90780 effects on urethral outlet function.

**CYP treatment** Intravesical infusion of PD90780 in CYP-treated (48 h) rats significantly increased voiding frequency (Fig. 6C, D) with a decreased void volume (p ≤ 0.002; Fig. 8B) and ICI (p ≤ 0.002; Fig. 8A) compared to rats with CYP-treatment (48 h) alone. PD90780 (10 µM, 50 µM, 100 µM) significantly (p ≤ 0.001) increased threshold, baseline and micturition pressure (Table 2). The numbers of non-voiding contractions per micturition cycle during the filling phase were significantly (p ≤ 0.002) increased at 10 µM and 50 µM PD90780 concentrations (Table 2). The duration of PD90780 effects for all concentrations examined for all cystometric parameters evaluated in control and CYP-treated rats was 30-40 min.
Discussion

We have demonstrated several novel findings with respect to the expression and regulation of \( p75^{\text{NTR}} \) in urinary bladder with CYP-induced cystitis and the role of \( p75^{\text{NTR}} \) in urinary bladder function in control (no inflammation) and CYP-treated rats. \( p75^{\text{NTR}} \) is expressed throughout the urinary bladder and is present in nerve fibers of the detrusor smooth muscle, the suburothelial nerve plexus, urothelial cells, and nerve fibers associated with the suburothelial bladder vasculature. Total urinary bladder \( p75^{\text{NTR}} \) expression increased after acute (4 h), intermediate (48 h) and chronic CYP-induced cystitis. \( p75^{\text{NTR}} \) blockade via immunoneutralization and PD90780, known to block NGF-\( p75^{\text{NTR}} \) interactions (Colquhoun et al., 2004; Hefti et al., 2006; Spiegel et al., 1995), resulted in bladder hyperreflexia in control rats and further enhanced bladder hyperreflexia in CYP-treated (48 h) rats associated with decreased void volumes and intercontraction intervals (ICI) and increased number of non-voiding contractions for PD90780. These studies suggest that \( p75^{\text{NTR}} \) interactions can affect overall bladder function.

NGF binds to two receptors, the \( p75^{\text{NTR}} \) receptor, a member of the tumor necrosis factor family of receptors and TrkA, a receptor tyrosine kinase (Allen and Dawbarn, 2006; Barker, 2007; Frossard et al., 2004). However, little information exists concerning the underlying interactions between neurotrophins, TrkA and \( p75^{\text{NTR}} \) receptors in mediating inflammatory-induced changes in micturition pathways. The majority of NGF-responsive cells express both \( p75^{\text{NTR}} \) and TrkA (Chao and Hempstead, 1995) and
TrkA is exclusively expressed with p75\textsuperscript{NTR} in rat DRG (Wright and Snider, 1995). NGF selectively binds TrkA whereas p75\textsuperscript{NTR} can bind all neurotrophins with the same affinity (Huang et al., 1999). p75\textsuperscript{NTR} also binds neurotrophin precursor molecules with high affinity, including proNGF (Huang et al., 1999). The ability of NGF to bind two very distinct receptors and the resulting signaling via this receptor complex has puzzled researchers (Barker, 2007). Much of the recent attention on p75\textsuperscript{NTR} has been focused on its role in apoptosis and neuronal growth inhibition. However, the original function attributed to p75\textsuperscript{NTR} was as an accessory protein that modulates Trk receptors to regulate their affinity for their appropriate ligand (Barker, 2007; Chao et al., 2006). The co-expression of TrkA and p75\textsuperscript{NTR} in heterologous cells results in high-affinity NGF binding sites (Barker, 2004; Verdi et al., 1994). Thus, it has become a prevailing concept that p75\textsuperscript{NTR} and TrkA combine to form a receptor complex that results in high-affinity NGF binding (Chao et al., 2006). However, a recent study (Wehrman et al., 2007) provides structural and mechanistic evidence for a ligand-passing model in which NGF rapidly associates with p75\textsuperscript{NTR} and then is presented to TrkA (Wehrman et al., 2007). In the model presented, blocking NGF binding to p75\textsuperscript{NTR} attenuates NGF binding to TrkA and subsequent TrkA activation (Wehrman et al., 2007). It is also suggested that convergent signaling pathways activated by p75\textsuperscript{NTR} and TrkA could underlie the complex crosstalk between p75\textsuperscript{NTR} and TrkA (Barker, 2007; Wehrman et al., 2007).

We have recently demonstrated upregulation of p75\textsuperscript{NTR} in dye-labeled lumbosacral bladder afferents and in the superficial laminae of the dorsal horn in central
micturition reflex pathways after acute and chronic CYP-induced cystitis (Klinger et al., 2008). Few studies have examined the regulation of p75NTR in rat urinary bladder after experimentally induced nerve regeneration or urinary bladder inflammation (Elsasser-Beile et al., 2000; Jahed and Kawaja, 2005; Wakabayashi et al., 1998; Wakabayashi et al., 1996). To our knowledge, no study has addressed the functional role of p75NTR in micturition reflex pathways.

It is well documented that NGF is important in a number of inflammatory conditions including urinary bladder, colon and lung inflammation (Delafoy et al., 2006; Freund-Michel and Frossard, 2008; Stanzel et al., 2008; Vizzard, 2000b). The exact contribution of NGF to bladder function is not known but a role for NGF in bladder hyperreflexia and bladder overactivity has been suggested (Chuang et al., 2001b; Clemow et al., 1998; Dmitrieva et al., 1997; Guerios et al., 2006; Hu et al., 2005; Oddiah et al., 1998; Zvara and Vizzard, 2007). Experimentally induced cystitis induces bladder hyperreflexia and increases bladder NGF protein and transcript (Murray et al., 2004; Vizzard, 2000b). In the spontaneously hypertensive rat model, increased expression of bladder NGF is associated with hyperactive voiding (Clemow et al., 1998). Bladder outlet obstruction is associated with increased expression of bladder NGF (Steers et al., 1991; Steers and Tuttle, 2006; Zvara et al., 2002). Intrathecal (Yoshimura et al., 2006), intravesical delivery of NGF (Dmitrieva et al., 1997) or adenovirus-mediated NGF overexpression (Lamb et al., 2004) in the bladder induces bladder hyperreflexia and bladder afferent cell hyperexcitability (Yoshimura et al., 2006; Yoshimura et al., 2002) in
control rats. NGF scavenging methods (Dmitrieva et al., 1997; Hu et al., 2005) reduce bladder hyperreflexia in rats with experimentally induced inflammation. Elevated levels of neurotrophins are detected in the urine of women with painful bladder syndrome (Okragly et al., 1999) (PBS)/interstitial cystitis (IC) or in the urothelium of individuals with neuropathic bladder (Lowe et al., 1997). However, a recent study failed to demonstrate an association between increased urothelium/suburothelium NGF with detrusor overactivity or bladder sensation (Birder et al., 2007). More recently, it has been demonstrated that urinary NGF levels are increased in overactive bladder (OAB), urinary incontinence and decreased in patients responding to botulinum toxin-A treatment (Liu and Kuo, 2008a; b). Thus, NGF may be a potential biomarker for OAB.

Neurotrophins, through interactions with Trk and/or p75NTR receptors, may contribute to neurochemical (Vizzard, 2000b; 2001), electrophysiological (Yoshimura et al., 2002) and organizational (Steers and Tuttle, 2006; Vizzard, 2000b) plasticity of micturition pathways after cystitis. Neurotrophin/Trk and/or p75NTR interactions could induce long-term changes in cells (Steers and Tuttle, 2006), including: (1) mediating neurotransmitter phenotype; (2) influencing dendrite size and synaptic reorganization; (3) increasing synaptic efficacy; and (4) controlling innervation density and target organ function. Consistent with the effects of exogenous NGF on urinary bladder function, it has also been shown that sequestration of NGF or TrkA decreases bladder hyperreflexia and improves animal well being (Dmitrieva et al., 1997; Hu et al., 2005). Systemic administration of REN1820, an NGF sequestering protein, decreased bladder
hyperreflexia associated with CYP-induced cystitis (Hu et al., 2005). Sequestration of NGF with a TrkA fusion protein also reduced bladder hyperreflexia following turpentine oil intravesical instillation (Dmitrieva et al., 1997). Intravenous administration of k252a, an inhibitor of Trk receptors, reduced referred somatic hypersensitivity of rat hindpaws induced by CYP-induced cystitis (Guerios et al., 2006). Intravesical instillation of k252a in CYP-treated rats also reduces bladder hyperreflexia (Vizzard et al., in preparation). Blockade of the NGF-TrkA pathway in respiratory inflammation models with k252a (Mohtasham et al., 2007) or NGF blocking antibodies decreased recruitment of inflammatory cells and bronchial hyperresponsiveness, respectively (Frossard et al., 2004). Immunoneutralization of p75NTR in adult rat sensory neurons inhibits upregulation of substance P induced by NGF application (Skoff and Adler, 2006). Studies using p75NTR knock-out (KO) mice or immunoneutralization of p75NTR reduced bronchial hyperresponsiveness and substance P release in the airway (Farraj et al., 2006; Kerzel et al., 2003; Tokuoka et al., 2001).

In contrast to reduced hyperresponsiveness with p75NTR blockade or absence (p75NTR KO) in the pulmonary system (Farraj et al., 2006; Kerzel et al., 2003; Tokuoka et al., 2001), the present studies demonstrate bladder hyperreflexia or enhanced hyperreflexia in control or CYP-treated rats following intravesical blockade of p75NTR by two different approaches. Immunoneutralization of p75NTR via intravesical instillation of an anti-p75NTR monoclonal antibody increased urinary bladder frequency (decreased intercontraction interval (ICI), decreased void volume) in both control (no inflammation)
and CYP-treated rats whereas intravesical pressures (baseline, micturition, threshold) were not affected. To confirm that the observed changes in bladder function were not associated with non-specific actions of immunoglobulin, intravesical instillation of isotype-matched IgG was infused and was without effect on any cystometric parameter evaluated. Intravesical infusion of PD90780, known to block NGF-p75NTR interactions (Colquhoun et al., 2004; Hefti et al., 2006; Spiegel et al., 1995), produced similar but also additional changes in the cystometric parameters evaluated. PD90780 increased voiding frequency (decreased ICI, decreased void volume) in control and CYP-treated rats, but also altered threshold, baseline and micturition pressures; vehicle infusions were without effect. This study and previous studies have demonstrated that CYP-treatment produces bladder hyperreflexia in addition to a number of neurochemical, electrophysiological and organizational changes of the micturition reflex (Hu et al., 2003; Hu et al., 2005; Vizzard, 2000a; 2001; Vizzard and Boyle, 1999; Yoshimura and de Groat, 1999). The demonstration that p75NTR blockade further increases bladder hyperreflexia shows that the frequency of the micturition reflex is not saturated with CYP treatment but can be further modulated.

Intravesical infusion of PD90780 produced some differential effects between control (no inflammation) and CYP-treated rats in contrast to the effects produced by immunoneutralization of p75NTR with an anti-p75NTR monoclonal antibody that were similar in control and CYP-treated rats. In control rats treated with intravesical instillation of PD90780, there was evidence of incomplete voiding as rats exhibited small
void volumes in comparison to infused volume. This mismatch between void and infused volumes was not observed in CYP-treated rats infused with PD90780 or in control or CYP-treated rats infused with anti-p75NTR monoclonal antibody. This mismatch between void volume and infused volume may suggest PD90780 effects on the urethral outlet. Given the ubiquitous expression of p75NTR throughout the urinary bladder demonstrated in the present studies and the peripheral and central nervous system in general (Friedman and Greene, 1999; Frossard et al., 2004; Wakabayashi et al., 1998; Wakabayashi et al., 1996), it would not be surprising that the urethra expressed p75NTR. Why the potential effects of PD90780 on the urethra are exhibited in control but not CYP-treated rats may be related to p75NTR and NGF expression in the urinary bladder with CYP-induced cystitis. Previous and present studies demonstrated significant increases in total p75NTR and NGF bladder expression with CYP-induced cystitis (Hu et al., 2005; Klinger et al., 2008; Murray et al., 2004; Vizzard, 2000b). In control rats with less p75NTR and NGF expression in the urinary bladder, infusion of PD90780 may exert bladder and urethral effects because the drug may be more available and have broader effects and affect NGF-p75NTR interactions at the urethra. In contrast, with CYP-induced cystitis and increased p75NTR and NGF expression in the urinary bladder, effects of PD90780 infusion may be less available and restricted to the urinary bladder. PD90780 effects on ICI and void volume in control rats were also demonstrated at the lowest concentration (10 µM) evaluated whereas PD90780 effects in CYP-treated rats (48 h) were observed at 25 µM PD90780. Thus, differences in p75NTR and NGF expression in control versus inflamed urinary bladder may also underlie differences in effective doses of PD90780. Another
possibility underlying differential effects of PD90780 in control and CYP-treated rats may be due to variable penetration of PD90780 in the inflamed urinary bladder with CYP-treatment.

Cystometric effects of p75\textsuperscript{NTR} blockade with intravesical instillation of an anti-p75\textsuperscript{NTR} monoclonal antibody may be due to blockade of other neurotrophins, in addition to NGF, binding to p75\textsuperscript{NTR} since brain derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4 may also bind p75\textsuperscript{NTR}, are also present in urinary bladder, and expression of BDNF, NT-3 and NT-4 is altered by CYP-induced cystitis (Vizzard, 2000b). However, the PD90780 compound, which specifically blocks NGF binding to p75\textsuperscript{NTR} (Colquhoun et al., 2004; Hefti et al., 2006; Spiegel et al., 1995), produced similar and broader cystometric changes. Thus, we suggest that the observed cystometric effects are attributable to blockade of p75\textsuperscript{NTR}-NGF interactions.

These studies demonstrate widespread p75\textsuperscript{NTR} expression in many cell types in the urinary bladder and increased total p75\textsuperscript{NTR} expression in the urinary bladder with CYP-induced cystitis. This makes it difficult to determine the exact site(s) of action in the urinary bladder of the p75\textsuperscript{NTR} antibody. We do know that intravesical infusion of anti-p75\textsuperscript{NTR} monoclonal antibody without infusion of protamine sulfate showed no cystometric effects. This likely indicates a site of p75\textsuperscript{NTR} action deep to the urothelium, since protamine sulfate is used to disrupt cell-to-cell contact of urothelial cells (Chuang et al., 2003; Stein et al., 1996). Our demonstration of p75\textsuperscript{NTR}–IR after intravesical infusion
of anti- \( p75^{NTR} \) supports this suggestion as \( p75^{NTR} \)-IR is present in the lamina propria. Potential targets include the suburothelial nerve plexus, interstitial cells of Cajal and myofibroblasts all distributed beneath the urothelium (Andersson and Arner, 2004) as well as inflammatory cell infiltrates in bladders from CYP-treated rats and nerve fibers innervating the detrusor smooth muscle.

\( p75^{NTR} \) receptor blockade at the level of the urinary bladder produced changes in ICI, void volume, changes in threshold and micturition pressure and increases in the number of non-voiding contractions during the filling phase. Changes in ICI resulting in changes in void volume, and changes in threshold pressure are suggestive of changes on the afferent limb of the micturition reflex. Changes in micturition pressure are thought to be dependent on the efficiency of neurotransmission of bladder efferents (Maggi et al., 1986). Changes in non-voiding contractions observed with PD90780 infusions in CYP-treated rats may have both myogenic and neurogenic components (Gillespie, 2005; Herrera et al., 2003; Streng et al., 2006). For example, myogenic contractions could trigger afferent firing which then evokes a reflex efferent discharge that amplifies the myogenic contraction. Effects of intravesical anti-\( p75^{NTR} \) infusion were limited to ICI and void volume without effects on intravesical pressures suggesting that the afferent limb of the micturition reflex is targeted. Greater concentrations of anti-\( p75^{NTR} \) were not evaluated in the present study so it is not known if additional cystometric changes would have emerged suggestive of effects on the efferent limb of the micturition reflex. In
contrast, effects of PD90780 on ICI, void volume and intravesical pressures suggest that both the afferent and efferent limbs of the micturition reflex are affected.

The present experiments show that p75NTR blockade at the level of the urinary bladder increases voiding frequency in control rats and further increases voiding frequency in CYP-treated rats. Thus, one function of p75NTR and NGF-p75NTR interactions in vivo may be to reduce bladder activity or to offset bladder hyperreflexia induced by CYP-induced cystitis. Whereas it is known that p75NTR expression can enhance TrkA-NGF binding in some systems (Allen and Dawbarn, 2006; Chao and Hempstead, 1995), it is also possible that NGF binding to p75NTR could reduce NGF-TrkA signaling by sequestering NGF away from TrkA (Dhanoa et al., 2006; Hannila and Kawaja, 2005; Nykjaer et al., 2005). The importance of p75NTR in bladder function may depend on the balance of NGF/TrkA and NGF/p75NTR signaling. Although perhaps less clinically relevant than OAB, detrusor overactivity or bladder hyperreflexia, detrusor underactivity is a clinical problem that is seen most often in men although women are also affected (Cucchi et al., 2008). It consists of reduced muscle contraction strength or velocity and often results in incomplete voiding, post-void residual, prolonged emptying and reduced free uroflow (Cucchi et al., 2007; Cucchi et al., 2008). Detrusor underactivity can be neurogenic, idiopathic or a result of bladder outflow obstruction (Cucchi et al., 2007; Murakami et al., 2008). Whether blockade of p75NTR in this context would be beneficial or whether p75NTR receptors are regulated by detrusor underactivity awaits further study.
Acknowledgements

The authors gratefully acknowledge the technical expertise and support provided by the VT Cancer Center DNA Analysis Facility. The authors thank Abbey Dattilio, Kristen Schutz and Susan Malley for technical assistance.

Grants

This work was funded by NIH grants DK051369, DK060481, and DK065989. NIH Grant Number P20 RR16435 from the COBRE Program of the National Center also supported the project for Research Resources.
Table 1: Effects of intravesical infusion of an anti-p75NTR monoclonal antibody on cystometric parameters.

Effects of intravesical infusion of an anti-p75NTR monoclonal antibody on baseline pressure, threshold pressure, micturition pressure and numbers of non-voiding contractions (NVCs) per micturition cycle are indicated. Threshold pressure and micturition pressure were significantly increased after 48 h CYP treatment. No effects of anti-p75NTR monoclonal antibody on threshold pressure, baseline pressure or peak micturition pressure were observed. The no antibody group received intravesical infusion of protamine sulfate (10 mg/ml), which did not differ from rats receiving intravesical infusion of protamine sulfate and isotype-matched IgG. Values are means ± S.E.M. *, p ≤ 0.001 compared to isotype-matched IgG infusion in control rats. n = 4-11 rats in each group.

<table>
<thead>
<tr>
<th>Intravesical Infusion</th>
<th>Threshold Pressure (cm H2O)</th>
<th>Micturition Pressure (cm H2O)</th>
<th>Baseline Pressure (cm H2O)</th>
<th>Non-voiding Contractions per Micturition Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no CYP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no antibody</td>
<td>15.1 ± 0.9</td>
<td>38.5 ± 1.3</td>
<td>15.1 ± 0.9</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>anti-p75NTR monoclonal antibody</td>
<td>14.2 ± 1.0</td>
<td>39.5 ± 2.0</td>
<td>15.0 ± 0.6</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>48 h CYP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no antibody</td>
<td>19.3 ± 0.7*</td>
<td>46.7 ± 1.4*</td>
<td>20.0 ± 0.8*</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>anti-p75NTR monoclonal antibody</td>
<td>13.9 ± 1.1</td>
<td>44.1 ± 2.0</td>
<td>14.0 ± 1.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Intravesical Infusion</td>
<td>Threshold Pressure (cm H₂O)</td>
<td>Micturition Pressure (cm H₂O)</td>
<td>Baseline Pressure (cm H₂O)</td>
<td>Non-Voiding Contractions per Micturition Cycle</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------------------</td>
<td>-------------------------------</td>
<td>---------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>no CYP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS only</td>
<td>15.1 ± 0.9</td>
<td>38.5 ± 1.3</td>
<td>15.1 ± 0.9</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>10 µM PD90780</td>
<td>17.0 ± 1.3</td>
<td>47.1 ± 3.0 *</td>
<td>18.4 ± 1.2 *</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td>25 µM PD90780</td>
<td>20.2 ± 1.5 *</td>
<td>50.8 ± 1.8 *</td>
<td>21.5 ± 1.3 *</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>50 µM PD90780</td>
<td>19.4 ± 1.1 *</td>
<td>47.1 ± 2.6 *</td>
<td>21.5 ± 1.0 *</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>100 µM PD90780</td>
<td>19.1 ± 1.2</td>
<td>51.0 ± 2.1 *</td>
<td>21.3 ± 1.4 *</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>48 h CYP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS only</td>
<td>19.3 ± 0.7</td>
<td>46.7 ± 1.4</td>
<td>20.0 ± 0.8</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>10 µM PD90780</td>
<td>25.1 ± 0.8 *</td>
<td>57.8 ± 2.8 *</td>
<td>24.3 ± 0.6 *</td>
<td>1.1 ± 0.3 *</td>
</tr>
<tr>
<td>25 µM PD90780</td>
<td>22.1 ± 1.7</td>
<td>49.1 ± 3.1</td>
<td>21.8 ± 1.7</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>50 µM PD90780</td>
<td>25.7 ± 0.8 *</td>
<td>61.2 ± 2.5 *</td>
<td>27.7 ± 1.3*</td>
<td>0.6 ± 0.2 *</td>
</tr>
<tr>
<td>100 µM PD90780</td>
<td>24.0 ± 0.8 *</td>
<td>56.1 ± 2.6 *</td>
<td>24.3 ± 0.8 *</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

Table 2: Effects of intravesical infusion of PD90780 on cystometric parameters. Effects of intravesical infusion of PD90780 on baseline pressure, threshold pressure, micturition pressure and non-voiding contractions (NVCs) per micturition cycle are indicated. In control rats (no CYP treatment), intravesical infusion of PD90780 significantly increased micturition pressure and threshold pressure. In CYP-treated rats (48 h), PD90780 infusion significantly increased threshold pressure, micturition pressure and NVCs. PS, protamine sulfate (10 mg/ml). Values are means ± S.E.M. *, p ≤ 0.001 compared to PS (10 mg/ml) and saline infusion in control rats. n = 4-11 rats in each group.
Figure 1: Urinary Bladder whole-mount preparations
p75NTR and pan-neuronal marker, protein gene product 9.5 (PGP9.5) immunoreactivity (IR) in urinary bladder whole-mount preparations with the urothelium/suburothelium dissected from the detrusor smooth muscle. A. Confocal image of p75NTR–IR in urothelial cells. B. Epifluorescence image of p75NTR–IR suburothelial nerve fibers (yellow arrows) in close proximity to p75NTR–IR urothelial cells (white arrows). C. p75NTR–IR in suburothelial nerve fibers (white arrows) and vasculature (yellow arrows). D. p75NTR–IR in vasculature (yellow arrows), nerve fibers (white arrows) and urothelial cells out of the focal plane (asterisks). p75NTR-immunoreactive suburothelial fibers (arrows E, H) also expressed PGP9.5-IR (arrows F, I). Panels G and J show merged images of E, H and F, I, respectively. Calibration bar represents 100 µm.
Figure 2: Western blot for p75\textsuperscript{NTR} expression in whole urinary bladder

A. Representative western blot of whole urinary bladder (23 μg) for p75\textsuperscript{NTR} expression in control rats and those treated with cyclophosphamide (CYP) for varying duration. Erk1 staining was used as a loading control.

B. Histogram of relative p75\textsuperscript{NTR} band density in all groups examined normalized to Erk1 in the same samples. p75\textsuperscript{NTR} expression in whole urinary bladder is significantly increased with acute (4 h), intermediate (48 h) and chronic CYP treatment. *, p ≤ 0.05. n = 4 for all groups.
Figure 3: Intravesical infusion of anti-p75NTR monoclonal antibody

Intravesical infusion of anti-p75NTR monoclonal antibody in control and in CYP-treated (48 h) rats increased voiding frequency (decreased intercontraction interval). Representative cystometrogram recordings in rats treated with (A) intravesical protamine sulfate in control rats, (B) protamine sulfate and anti-p75NTR monoclonal antibody (100 µg/ml) in control rats, (C) protamine sulfate in CYP-treated (48 h) rats, (D) protamine sulfate and anti-p75NTR monoclonal antibody (100 µg/ml) in CYP-treated (48 h) rats.
Figure 4: Voiding frequency and inter-contraction interval

Intravesical infusion of anti-p75NTR monoclonal antibody (100 µg/ml) significantly increased voiding frequency in control and in CYP-treated (48 h) rats. Both inter-contraction interval (A) and void volume (B) were significantly (p ≤ 0.001) reduced with intravesical infusion of anti-p75NTR. n = 4-11 for all groups.
Figure 5: p75NTR-IR in urinary bladder sections after intravesical infusion of anti-p75NTR

p75NTR-IR in urinary bladder sections after intravesical infusion of anti-p75NTR and application of species-specific secondary antibodies to demonstrate penetration of the antibody into the urinary bladder with intravesical infusion. Epifluorescence images of (A) rat urinary bladder after intravesical infusion of anti-p75NTR (100 µg/ml) and (B) rat urinary bladder after only intravesical infusion of protamine sulfate. A. p75NTR-IR is observed in urothelium and lamina propria, with staining reduced with increasing distance from the bladder lumen. B. No p75NTR-IR was observed in rat urinary bladder in the absence of intravesical antibody infusion. U, urothelium; LP, lamina propria; l, lumen. Calibration bar represents 120 µm.
Figure 6: Intravesical infusion of PD90780

Intravesical infusion of PD90780, a compound that specifically blocks NGF binding to \( p75^{NTR} \), results in decreased bladder capacity in control (no inflammation) and in 48 h CYP-treated rats. Representative cystometrogram recordings in rats treated with (A) intravesical protamine sulfate in control rats, (B) protamine sulfate and PD90780 (100 µM) in control rats, (C) protamine sulfate in CYP-treated (48 h) rats, (D) protamine sulfate and PD90780 (100 µM) in CYP-treated (48 h) rats.
Figure 7: Voiding frequency and inter-contraction interval in control rats
Summary histograms of the effects of intravesical infusion of PD90780 in control rats (no CYP treatment). 
A. Infusion of PD90780 (10, 50 µM) significantly (p ≤ 0.05) reduced inter-contraction interval. B. Void volume in control rats was also significantly (p ≤ 0.05) reduced at all PD90780 concentrations evaluated (10 µM, 25 µM, 50 µM, 100 µM). n = 4-6 for all groups.
Figure 8: Voiding frequency and inter-contraction interval in CYP-treated rats

Summary histograms of the effects of intravesical infusion of PD90780 in CYP-treated (48 h) rats. Rats were treated with CYP 48 h prior to intravesical infusion of PD90780 and analysis with conscious cystometry. A. Intravesical infusion of PD90780 (25 µM, 50µM, 100 µM) significantly (p ≤ 0.002) reduced inter-contraction interval in rats treated with CYP (48 h). B. Void volume in CYP- treated (48 h) rats was also significantly (p ≤ 0.002) reduced at these same concentrations. n = 5-11 for all groups.
References


Chapter 5: Summary and Conclusions

CYP-induced bladder inflammation is an important model that allows the study of the effects of acute and chronic bladder inflammation. The results of experiments done in CYP-treated animals may have relevance to human conditions that involve bladder inflammation, including IC/PBS (Westropp and Buffington, 2002). CYP cystitis results in many changes in urinary bladder reflex pathways and many are not understood. The overall goal for this dissertation was to further understand and characterize the changes associated with bladder inflammation and specifically, the roles that both cyclooxygenase-2 (COX-2) and the neurotrophin receptor p75^Neurotrophin receptor (NTR) play.

Using the CYP model of bladder inflammation, we first examined COX-2 expression in the bladder tissues of control rats and of rats with varying duration of CYP-induced cystitis. By separating the detrusor from the urothelium and suburothelium and using Western blot analysis, we showed that COX-2 expression is significantly increased in both compartments, and also that COX-2 expression increased with a greater fold change in the urothelium/suburothelium than in the detrusor smooth muscle. Upon further examination with immunostaining, COX-2 expression was indeed found to be upregulated in the urothelium, as well as in inflammatory infiltrates and nerve fibers in the suburothelial plexus with acute and chronic cystitis. Inflammatory infiltrates were also present in the detrusor smooth muscle. These results identify possible cellular targets for COX-2 inhibitors that have been used to attenuate bladder hyperreflexia with CYP in previous studies, and also identify the localization of COX-2 upregulation
reported previously (Hu et al., 2003; Wheeler et al., 2001). These results suggest that COX-2 activation contributes to bladder inflammation in acute and chronic cystitis and, more specifically, may have its effect on the production of prostaglandins in urothelium, and nerve fibers and macrophages in the suburothelial plexus. Prostaglandins are known to modulate the basal tone of the detrusor and influence detrusor contraction as well as target primary bladder afferents, thus influencing the micturition reflex (Angelico et al., 2006; Maggi, 1992). Application of prostaglandins to the detrusor stimulates the micturition reflex (Maggi et al., 1984). However, the story of CYP-induced bladder inflammation goes beyond COX enzymes and prostaglandins; COX-2 expression can be stimulated in part by growth factors and inflammatory cytokines.

Since NGF and its receptor TrkA have been implicated in the progression of CYP-induced urinary bladder inflammation, the next step of this project was to explore the expression of the pan-neurotrophin receptor p75NTR in rat urinary bladder sensory neurons and spinal cord with CYP-induced cystitis. p75NTR expression was examined using immunohistochemistry in lumbosacral spinal cord and DRG. p75NTR was present in control and significantly increased in spinal cord dorsal horn at lumbosacral levels known to be involved in micturition reflexes in rats (L1-L2; L6-S1), suggesting that p75NTR could be involved in central processing of afferent information. p75NTR immunoreactivity was also noted in control lumbosacral DRG cell bodies and staining surrounding neuronal cell bodies. This staining was increased with acute, intermediate and chronic CYP treatment. Upon examination with confocal microscopy, pericellular p75NTR staining did not co-label with GFAP, suggesting that this staining is more likely associated with the
neuronal cell membrane, and not of glial origin. p75NTR mRNA expression increased with acute, intermediate and chronic CYP treatment in L6 DRG. The upregulation of p75NTR mRNA and protein expression in whole DRG could suggest p75NTR upregulation in the afferents of other organs as well, including colon and urogenital structures, which also have afferents in lumbosacral DRG. It is interesting to note that cross-talk can occur between these visceral organs, with convergence of sensory information occurring in the dorsal root ganglia, spinal cord and brain (Malykhina, 2007). This cross-talk can result in cross-sensitization between visceral organs and may contribute to chronic pain experienced in both bladder, urogenital organs and colon (Malykhina, 2007). Cross-sensitization may be a factor in the co-morbidity of such conditions as Irritable Bowel Syndrome and Interstitial Cystitis/Painful Bladder Syndrome.

A retrograde dye-tracing technique with Fastblue was used to determine p75NTR expression in bladder-projecting primary afferents. Bladder-projecting afferent cells constitutively expressed p75NTR and expression was also significantly increased with acute, intermediate and chronic CYP treatment, suggesting a role for this receptor in normal bladder afferent signaling, and possibly in mediating the effects of bladder inflammation. Overall, these results demonstrated constitutive expression of p75NTR in micturition reflexes and modification of this expression by bladder inflammation. The functional significance of p75NTR in bladder reflexes remained to be determined.

Upon examination of p75NTR expression in the urinary bladder, we have detected p75NTR immunoreactivity in urothelial cells, in nerve fibers in the suburothelial plexus, and in nerve fibers associated with the vasculature. No upregulation of this staining was
obvious with immunohistochemical techniques because of the large amount of overlapping structures expressing p75NTR expression in the urinary bladder, but Western blot analysis of whole bladder expression of p75NTR did show a significant increase in p75NTR protein expression after acute, intermediate, and chronic CYP cystitis.

Blockade of p75NTR in rat urinary bladder was achieved by intravesical infusion of anti-p75NTR monoclonal antibody as well as intravesical infusion of PD90780, a compound that specifically blocks NGF binding to p75NTR (Colquhoun et al., 2004; Hefti et al., 2006; Spiegel et al., 1995). p75NTR blockade was done in both control and CYP (48 hour)-treated rats. Bladder function was then studied using conscious cystometry with continuous intravesical infusion of saline. Infusion with each compound demonstrated a significant increase in voiding frequency (decreased inter-contraction interval, decreased void volume) in both control and CYP-treated rats. Changes in voiding frequency are suggestive of changes in the afferent limb of the micturition reflex. Changes in both threshold pressure and number of non-voiding contractions were seen with infusion of PD90780 in control and CYP-treated rats, suggestive of possible changes in the afferent pathway. Increased micturition pressure was also noted, indicating an effect on the efficiency of the efferent branch of the micturition reflex pathway with this treatment.

Based on previous results showing that blockade of Trk signaling with k252a attenuated the bladder hyperreflexia observed with CYP-induced cystitis, we had hypothesized that p75NTR blockade in the urinary bladder would also decrease bladder
Cyclophosphamide-induced bladder inflammation results in increased NGF expression in the urinary bladder. NGF signaling through TrkA results in a number of inflammatory effects, including the sensitization of bladder afferents. This in turn results in increased overall bladder activity. We have used anti-p75NTR monoclonal antibody and PD90780, a compound that specifically blocks NGF binding to p75NTR, to block NGF signaling through p75NTR. This resulted in increased bladder voiding activity in both control and CYP-treated rats. We hypothesize that blockade of p75NTR increases the amount of NGF available to bind TrkA, thus increasing NGF-TrkA signaling and increasing inflammatory effects and sensitization of bladder afferents.

hyperreflexia with cystitis. This hypothesis was based on data that demonstrated a role for p75NTR in enhancing TrkA binding NGF, thus increasing NGF-TrkA signaling, meaning that p75NTR and TrkA would have complementary roles (Allen and Dawbarn, 2006). In this model, increased NGF-TrkA signaling would result in a cascade of inflammatory effects, including sensitization of bladder afferents (Fig. 1) (Chuang et al., 2001b). The upregulation of p75NTR in bladder afferents with cystitis is more widespread
than the upregulation of TrkA expression after cystitis; TrkA was only upregulated in L1 and L6 DRG, and not with all durations of CYP treatment (Qiao and Vizzard, 2002). The upregulation of TrkA in bladder afferents could occur as a result of an increase in NGF levels in the urinary bladder with cystitis (Vizzard, 2000b), and could be evidence of increased NGF-TrkA signaling that contributes to the inflammatory effects and bladder hyperreflexia associated with cystitis. In Chapter 3, we presented evidence that p75NTR is upregulated in bladder afferents with cystitis, and in Chapter 4, we showed that blockade of NGF-p75NTR actually increases voiding frequency in control rats and further increases bladder hyperreflexia in rats with CYP-induced cystitis. This suggests that NGF-p75NTR signaling in the urinary bladder may actually serve to modify bladder activity in an opposite way to NGF-TrkA signaling, thereby reducing inflammation-related hyperreflexia or to maintain reflex function under control conditions as well. p75NTR has long been considered as a binding partner to TrkA, with cooperation between the two resulting in NGF binding TrkA with greater affinity. However, p75NTR might also bind NGF on its own, therefore sequestering NGF and leaving less to bind to TrkA, and therefore less NGF-TrkA signaling. Furthermore, the upregulation of p75NTR in urinary bladder afferents seen with acute, intermediate and chronic durations of cystitis may serve as a compensatory mechanism of signaling, in order to attenuate inflammation-related bladder hyperreflexia.

**Future Directions**

This dissertation research leads to many questions for further study. The study of bladder function in p75NTR–null mice is an obvious next step for further deciphering the
function of p75NTR in micturition reflexes. Appendix A contains some preliminary data obtained for this dissertation project in p75NTR—null and in mice hemizygous for the p75NTR null gene. Even with preliminary results, it seems as though these mice present a complex phenotype of bladder function, some with bladder hyperreflexia and others with urinary incontinence. p75NTR transgenic mice have much to offer in the way of future research. It would also be interesting to see the effects of over-expression of the p75NTR receptor, perhaps in a model of p75NTR—over-expressing mice. Based on our current data, it would be hypothesized that p75NTR over-expression would increase NGF binding p75NTR, sequestering NGF away from TrkA. This would then result in less NGF-TrkA signaling and decrease bladder activity or hyperreflexia.

We hypothesize that blockade of NGF-p75NTR signaling actually leads to more NGF available to bind TrkA, thus more NGF-TrkA signaling and bladder hyperreflexia (Fig. 1). We could test this by trying a combination experiment that includes blockade of p75NTR as well as antagonism of TrkA. If blockade of TrkA rescued the bladder hyperreflexia seen with p75NTR blockade, this would support our hypothesis and demonstrate that NGF signaling through TrkA is important for the increased bladder voiding shown with blockade of p75NTR shown in this dissertation.

It would also be interesting to try to determine the precise location of the action of the p75NTR blockade shown in this dissertation. It would be interesting to apply a p75NTR—neutralizing agent exclusively to the detrusor smooth muscle, perhaps by a mini osmotic pump as described in Zvara and Vizzard, 2007 in order to determine the importance of the smooth muscle to p75NTR signaling. Pre-treatment of experimental animals with
resiniferatoxin could also be used to desensitize many bladder afferent fibers. This could be done before CYP treatment or before intravesical infusion of antibody or PD90780, and might help determine the role that afferents may play in the NGF-p75NTR signaling.

The present study of intravesical p75NTR blockade examined bladder function only after 48 hours of CYP treatment. Previous studies involving COX-2 inhibition have examined bladder function after 4 and 48 hour CYP treatment (Hu et al., 2003). It would be interesting, and perhaps more clinically relevant, to examine bladder function using cystometry after the induction of chronic bladder inflammation as well.

This dissertation examined the importance of p75NTR signaling in the urinary bladder by blocking p75NTR in the urinary bladder itself. To examine the role of p75NTR in the central micturition reflex processing, intrathecal application of either anti-p75NTR monoclonal antibody or PD90780 at the level of lumbosacral spinal cord in experimental rats might be used. This could be done in control and CYP-treated rats as well.

Finally, it is unknown what the intracellular signaling pathway(s) of NGF-p75NTR in the urinary bladder might be. Further research into the fate of NGF after binding p75NTR in the bladder and subsequent signal transduction would be useful in understanding the function of p75NTR in the micturition reflex pathways. While some p75NTR signaling pathways are known, such as induction of apoptosis through activation of the mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinases (JNK), and induction of cell survival effects via the transcription factor NF-κB, this information may or may not be applicable to p75NTR signaling in the urinary bladder. It would be interesting to know whether or not internalization of p75NTR after NGF signaling would
result in lysosomal degradation of \( p75^{NTR} \), or in possible recycling of the receptor at the cell membrane, or in downstream signaling effects.
Comprehensive Bibliography


vanilloid receptor TRPV1 and pan-neuronal marker PGP9.5 immunoreactivity in patients with neurogenic detrusor overactivity after intravesical resiniferatoxin treatment. BJU Int 93(6):770-776.


Dickson A, Avelino A, Cruz F, Ribeiro-da-Silva A. 2006. Peptidergic sensory and parasympathetic fiber sprouting in the mucosa of the rat urinary bladder in a


Liu HT, Kuo HC. 2008b. Urinary Nerve Growth Factor Levels Are Increased in Patients with Bladder Outlet Obstruction with Overactive Bladder Symptoms and Reduced After Successful Medical Treatment. Urology.


240


Appendix A: Functional and sensory changes in p75<sup>NTR</sup><sup>-/-</sup> mice
**Introduction**

The pan-neurotrophin receptor p75\textsuperscript{NTR} has been implicated in many cellular processes, including neuronal growth regulation and apoptosis (Allen and Dawbarn, 2006). In cells expressing TrkA, p75\textsuperscript{NTR} also enhances the binding affinity of TrkA for NGF (Allen and Dawbarn, 2006). p75\textsuperscript{NTR} may also function to reduce NGF-TrkA signaling by sequestering NGF, leaving less available to bind TrkA (Coome et al., 1998; Dhanoa et al., 2006; Hannila et al., 2004; Nykjaer et al., 2005). Studies in p75\textsuperscript{NTR} exon III (p75\textsuperscript{NTRexonIII}) deficient mice have shown the importance of p75\textsuperscript{NTR} in regulating injury-induced sprouting in rat dorsal horn and trigeminal ganglia and cerebellum (Dhanoa et al., 2006; Hannila and Kawaja, 2005; Scott et al., 2005). The original phenotypical findings in these mice include decreased sensory innervation of the footpad skin, loss of heat sensitivity and the development of skin ulcers in distal extremities (Lee et al., 1992). Changes were not originally seen in sympathetic innervation in sympathetic cervical ganglia or iris (Lee et al., 1992). In examining p75\textsuperscript{NTRexonIII} null mice, we sought to (1) examine bladder function using conscious cystometry, and (2) to begin to characterize the expression of sympathetic, parasympathetic, and sensory innervation of the urinary bladder using immunohistochemistry. This work is preliminary and unpublished.
Materials and Methods

Genotyping

p75NTRexonIII null mice were used in this study (Lee et al., 1992). Adult male and female p75NTR-/-, p75NTR +/-, and p75NTR +/+ littermates were generated using a hemizygous (p75NTR +/- mice) breeding strategy (Fig. 1). To determine the genotypes of progeny, tail DNA samples were obtained from postnatal day 21 mice, digested with NucPrep, and amplified with primers for the p75NTR transgene (p75-1: 5’- CGATGCTCCTATGGCTACTA; p75-2: 5’-CCTCGCATTCGGCGTCAGCC; and PGK: 5’-GGGAACTTCCTGACTAGGGG)

Tube Implant Surgery and Cystometry

Please refer to the Materials and Methods section of Chapter 4.

Immunohistochemistry

Bladder whole-mount preparations were prepared as described in Chapter 3. Bladder cross sections were obtained and prepared as described in Chapter 2. Urinary bladder sections and bladder whole-mount preparations were incubated overnight at room temperature with rabbit anti–vasoactive intestinal polypeptide (VIP) antibody (1:2000, Incstar, Stillwater, MN), rabbit anti-tyrosine hydroxylase (TH) antibody (1:1000, Novus Biologicals, Littleton, CO), or rabbit anti-calcitonin gene-related peptide (CGRP) antibody (1:1000, Phoenix Pharmaceuticals, Inc., St. Joseph, MO) in 1% goat serum and 0.1 M KPBS (Phosphate Buffer Solution with potassium), and then washed (3×15 min) with 0.1 M KPBS, pH 7.4. Tissue was then incubated with Cy3-conjugated goat anti–rabbit IgG (1:500; Jackson ImmunoResearch) for 2 hr at room temperature. After several
rinses with 0.1 M KPBS, tissues were mounted with Citifluor (Citifluor, London, UK) on slides and coverslipped. Control tissues incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining was observed.
Results

Cystometry in mice with the $p75^{\text{NTRexonIII}}$ null mutation

$p75^{\text{NTRexonIII}}$ null mice displayed a continuum of bladder function symptoms, ranging from bladder hyperreflexia to complete incontinence. Incontinence involved subtle changes in pressure during filling and continual leaking, as evidence by the cystometrogram pressure tracing shown in Fig. 2B. Bladder function was also studied in $p75^{\text{NTR}+/-}$ mice, and these mice also displayed abnormal bladder function that ranged from hyperreflexia to incontinence (data not shown). Wild-type littermates had normal bladder function, with regular voiding events associated with a peak in bladder pressure followed by a decrease in bladder pressure for filling (Fig. 2A).

$TH$ expression in mice with the $p75^{\text{NTRexonIII}}$ null mutation

$TH$ was used as a marker for sympathetic innervation in bladder cryostat sections in $p75^{\text{NTRexonIII}}$ null mice and in wild-type (WT) littermates (Fig. 3A, B). $TH$ staining appeared brighter and was more frequently observed in nerve fibers associated with the detrusor smooth muscle in null mice (A) as opposed to WT mice (B).

$VIP$ expression in mice with the $p75^{\text{NTRexonIII}}$ null mutation

$VIP$-immunoreactivity in bladder whole-mount preparations showed that $VIP$, a marker for parasympathetic neurons, is present in nerve fibers associated with the detrusor smooth muscle. This staining appears to be decreased in null mice (Fig. 4B) compared to WT mice (Fig. 4A). The differences in staining intensity were most obvious in the bladder base and urethral region.

$CGRP$ expression in mice with the $p75^{\text{NTRexonIII}}$ null mutation
Immunostaining for CGRP, a marker for most sensory neurons, appeared to be decreased in null mice (Fig. 5B) compared to WT mice (Fig. 5A) in the lamina propria layer of the urinary bladder. CGRP-immunoreactivity can also be seen in nerve fibers associated with the urothelium and in nerve fibers associated with the detrusor smooth muscle.
Figure 1: Genotyping of $p75^{NTRexonIII}$ null mice, wildtype and heterozygous $p75^{NTRexonIII}$ mice

$p75^{NTR}$ –null mice display a band at 317 bp (homo). Wild-type mice display a band at 247 bp (WT). Mice heterozygous for the null mutation show a band at both 317 and 247 bp (het).
Figure 2: Cystometry in p75<sup>NTR</sup>exonIII null mice

(A) Cystometrogram data from a wild-type (WT) littermate. (B) p75<sup>NTR</sup>exonIII null mice demonstrate a continuum of bladder overactivity, ranging from complete incontinence to pronounced hyperreflexia as compared to wild-type littermates. Panel B illustrates an example of subtle pressure changes (center) not associated with void events.
Figure 3: Tyrosine hydroxylase (TH) expression in p75
 NTRexonIII null mice

TH, a marker for sympathetic neurons, appears to stain more intensely and with greater
frequency in nerve fibers in the detrusor smooth muscle of the bladder in null mice (A) as
opposed to WT mice (B).
**Figure 4: Vasoactive Intestinal Polypeptide (VIP) expression in p75\textsuperscript{NTR\textsubscript{exonIII}} null mice**

Whole-mount preparation showing VIP staining in detrusor smooth muscle. Immunostaining for VIP, a marker for parasympathetic neurons, appears to be decreased in null mice (B) as compared to WT mice (A) in nerve fibers associated with the detrusor smooth muscle. M: detrusor smooth muscle; U: urothelium.
Figure 5: Calcitonin gene-related peptide (CGRP) expression in p75\textsuperscript{NTR\textsuperscript{exonIII}} null mice

Immunostaining for CGRP, a marker for most sensory neurons, appears to be decreased in null mice (B) as compared to WT mice (A) in the lamina propria layer of the urinary bladder wall. Staining can also be seen in nerve fibers associated with the urothelium and in nerve fibers associated with the detrusor smooth muscle. M: detrusor smooth muscle; U: urothelium; LP: lamina propria.
Discussion

Mice with the \( p75^{\text{NTRexonIII}} \) null mutation are a valuable resource for future studies of the functional role that \( p75^{\text{NTR}} \) may play in micturition reflexes. So far, these mice present a complex story of bladder activity, with some null mice displaying urinary incontinence and some with milder bladder hyperreflexia (Fig. 2B). To determine whether these mice exhibit variations in urinary bladder innervation, we have used immunohistochemistry to begin to examine the expression of sympathetic, parasympathetic and sensory neurons in the urinary bladder. Although not quantified, there is an indication that tyrosine hydroxylase (TH) stained with greater intensity and frequency in \( p75^{\text{NTR}^{-/-}} \) mice than in their wildtype littermates. An increase in sympathetic innervation in \( p75^{\text{NTR}^{-/-}} \) mice has been previously reported (Hannila and Kawaja, 2005; Scott et al., 2005). Vasoactive intestinal peptide (VIP) was used as a marker for parasympathetic innervation and this staining appeared in nerve fibers associated with the detrusor smooth muscle. VIP-immunoreactivity appeared to be decreased in \( p75^{\text{NTR}^{-/-}} \) mice as compared to wildtype littermates (Fig. 4A, B). Finally, calcitonin gene-related peptide (CGRP) was used as a marker for sensory neurons and this staining was present in small nerve fibers in detrusor smooth muscle and associated with the urothelium (Fig. 5A, B). Changes in sensory innervation of the footpad skin in \( p75^{\text{NTR}^{-/-}} \) mice have been previously documented (Lee et al., 1992).

Our cystometry studies in \( p75^{\text{NTRexonIII}^{-/-}} \) mice indicate possible complex changes in bladder function with the \( p75^{\text{NTRexonIII}^{-/-}} \) phenotype. Changes in the urethral outlet or in the urinary bladder could be affecting bladder function and these changes could be
neurogenic or myogenic. If the change in function is due to an increase in TH expression (sympathetic innervation) in the urinary bladder, the experimental use of β-adrenergic blocking agents might remove the inhibition and normalize function. If the changes are due to an increase in TH expression in the urethral outlet, this could result in α-mediated constriction and outlet obstruction. The experimental use of an α-adrenergic antagonist could relieve and normalize function. Other future avenues for this research could include possible future myograph studies on muscle contractility in bladder and urethra.

These preliminary results also show interesting changes in autonomic and sensory innervation in p75<sup>NTR</sup><sup>−/−</sup> mouse bladders. Further study into this could consist of quantification of the immunostaining of TH, VIP and CGRP, as well as the use of western blotting or enzyme-linked immunoassays (ELISAs) to quantify the protein expression of parasympathetic, sympathetic or sensory neuron markers.
References


