The Role Of Neurotrophin Signaling In Urinary Bladder Dysfunction With Cyclophosphamide-Induced Cystitis

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THE ROLE OF NEUROTROPHIN SIGNALING IN URINARY BLADDER DYSFUNCTION WITH CYCLOPHOSPHAMIDE-INDUCED CYSTITIS

A Dissertation Presented

by

Harrison W. Hsiang

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of

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In Partial Fulfillment of the Requirements
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Specializing in Neuroscience

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Abstract

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic inflammatory pelvic pain syndrome characterized by urinary frequency and urgency, bladder discomfort, decreased bladder capacity, and pelvic pain. A positive feedback loop of bladder inflammation and afferent hypersensitization is currently thought to underlie IC/BPS. Inflammation increases bladder afferent excitability, which in turn releases inflammatory neuropeptides, growth factors, cytokines, and chemokines throughout the micturition pathway, leading to altered bladder function and sensation. There currently exists no effective therapy for IC/BPS.

While its etiology remains unknown, a large body of evidence suggests a role for changes in neurotrophin signaling, particularly that of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Both are increased in the urine and bladders of humans and animals with cystitis. Administration or overexpression of NGF in the bladder produces changes in bladder function consistent with cystitis; complementarily, pharmacological disruptions of both NGF and BDNF are associated with improved bladder function in models of bladder inflammation. However, severe side effects associated with anti-NGF treatment have hampered attempts to develop effective therapies, highlighting the need for additional therapeutic targets. Additionally, neurotrophin signaling is complex and has not been thoroughly characterized in the bladder.

Here, we instead target neurotrophin signaling at the receptor level. Using conscious, open-outlet cystometry, we demonstrate bladder function improvement in a mouse model of cyclophosphamide (CYP)-induced cystitis as a consequence of treatment with novel pharmacological inhibitors for the NGF receptor TrkA, the BDNF receptor TrkB, and the pan-neurotrophin receptor p75NTR. Additionally, using immunohistochemistry and enzyme-linked immunosorbent assays, we demonstrate changes in a variety of NGF signaling–related proteins (NGF, TrkA, p75NTR, p-JNK, p-ERK) as a consequence of CYP treatment, inducing cystitis, and in response to subsequent treatment with TrkA and p75NTR inhibitors. Our findings demonstrate that these receptors represent additional potent therapeutic targets in mice with cystitis and reveal additional novel therapeutic targets that may be useful in the treatment of IC/BPS and lower urinary tract symptoms in other inflammatory disorders of the bladder.
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Chapter 1: Comprehensive Literature Review

1.1. The Lower Urinary Tract (LUT)

Though often taken for granted, proper urinary function and control is indispensable. The micturition reflex is enabled by complex neural signaling pathways that coordinate lower urinary tract function, sensation, and activation (Fig 1a). There are many ways this system can become compromised, however, resulting in chronic pelvic pain syndromes such as interstitial cystitis/bladder pain syndrome (IC/BPS) and, subsequently, discomfort and decreased quality of life.

1.1.1. Anatomy

Within the renal system, the lower urinary tract (LUT) – comprised of the urinary bladder and urethra – allows passive storage of kidney byproducts until it is appropriate to void. Accordingly, the urinary bladder can flexibly expand and dynamically contract.

There are several major external features of the bladder (Fig 1b). Urine enters the bladder through the ureters. On the anterior surface of the bladder, the apex, or vertex, features ligament remnants attached to the umbilicus. The posterior surface is known as the fundus, and its most inferior aspect known as the base of the bladder. The luminal region between is referred to as the body of the bladder. The internal urethral orifice provides an exit for stored urine through the bladder neck at the most caudal end of the bladder [1].

The urinary bladder wall is organized into three layers: 1) the outermost adventitia/serosa, 2) the muscularis propria (including the detrusor muscle), and 3) the
innermost urothelium. The mucosa is further subdivided into 1) the outermost lamina propria, 2) the basement membrane, and 3) the innermost urothelium (Fig 1c) [2]. The urothelium has been the focus of much study as it is capable not only of sensing chemical, mechanical, and thermal stimuli in the bladder but also of responding through the release of chemical mediators. Given the proximity of urothelial cells and bladder afferent nerves, the possibility of dynamic, reciprocal communication between the two has been suggested [3]. The urothelium is composed of a basal cell layer, which attaches to the basement membrane; an intermediate layer; and an apical layer containing large (25-250 µm) hexagonal “umbrella” cells. Tight junctions formed by multiple proteins, such as occludins, claudins, and plaques of uroplakin proteins, allow the urothelium to serve as an impermeable barrier. In addition, the apical surface of umbrella cells are coated by a protective glycosaminoglycan layer [2,3].

The lamina propria is an extracellular matrix containing a variety of interstitial cells, nerve terminals, and vasculature. Based on its innervations and location between the urothelium and the muscularis propria, the lamina propria is thought to play a role in the integration of epithelial and smooth muscle function [4,5]. Within the muscularis propria, there are three smooth muscle layers referred to in aggregate as the detrusor; the fibers of the outermost layers are arranged longitudinally, while the fibers of the middle layer are circular, allowing the bladder to contract during the elimination of urine [5]. The muscularis propria is surrounded by a vascularized outer connective tissue layer termed the adventitia (inferiorly) and the serosa (superiorly) [6].

The urinary bladder empties into the urethra. Urethral organization mimics that of the bladder, with an outermost adventitia, a muscularis propria, and an innermost mucosa.
Near the bladder neck, the mucosa is a transitional epithelium, but more distally, nonkeratinized, stratified squamous cells dominate [6,7]. Like the bladder, fibers in the muscularis propria are arranged longitudinally (inner layer) or circularly (outer layer) [6]. In males, bladder and urethral circular smooth muscle fascicles join to form an internal urethral orifice [6], a feature absent from the female urethra [6,7]. External urethral sphincter, formed by skeletal muscle in the urethral wall, anatomy differs by sex as well; in females, the sphincter urethrae, compressor urethrae, and sphincter urethrovaginalis combine to maintain urinary continence through urethral and vaginal closure [8]. Proper bladder function requires the coordination of the urethral sphincter and detrusor, allowing one to relax while the other contracts and vice versa, to facilitate voiding and filling.

**Figure 1** Overview of the bladder sensory pathway and bladder anatomy. A Mechanical and chemical stimuli are transduced to convey the state of the bladder, ascending via the hypogastric (T10, L1, and L2) and pelvic (L6, S1) nerves to the periaqueductal gray (PAG) and other supraspinal sites such as the pontine micturition center (PMC). B Major anatomy of the urinary bladder. C The layers of the bladder, traveling up from the outermost adventitia/serosa to the lumen.
1.1.2. Neural Control

Normal bladder function requires complex coordination of afferent sensory information from the LUT and efferent excitatory and inhibitory information from the central nervous system, ultimately placing the micturition reflex under conscious control. The bladder exhibits “switch-like” behavior between storage and elimination (for a detailed review, see Fowler et al. [9]). In brief: the mature micturition reflex is a spinobulbospinal reflex pathway triggered by sensations of bladder fullness (Fig 2) [9]. During storage, sympathetic input to the LUT causes detrusor relaxation and bladder neck/urethral contraction, allowing the bladder to expand with increased volume; voiding occurs when sympathetic input is released, relaxing the urethral sphincter, and parasympathetic activation causes the detrusor to contract [10,11].

Neural organization of the storage phase begins with sympathetic input to the LUT. Preganglionic sympathetic fibers in the intermediolateral cell column of the thoracolumbar (T10-L2) spinal cord are activated by spinal interneurons. These fibers form the thoracic and lumbar splanchnic nerves [9,12]. After synapsing onto the prevertebral inferior mesenteric ganglia or paravertebral ganglia, the sympathetic outflow continues along the hypogastric and pelvic nerves respectively [13]. Upon reaching the urinary bladder, this sympathetic input promotes bladder wall relaxation and accommodation through activation of bladder smooth muscle β-adrenergic receptors and urethral contraction through activation of internal urethral sphincter α-adrenergic receptors. In humans, urethral contraction is further elicited via α-motoneuron activity from Onuf’s nucleus in the ventrolateral horn of the sacral (S2-S4) spinal cord, which
travels along the pudendal nerve to skeletal muscle nicotinic acetylcholine receptors, providing conscious control of continence [12].

In contrast, the elimination phase depends on supraspinal circuitry [9,14]. Once the tension threshold is reached, activity from bladder afferent Aδ-fibers projects via the pelvic and hypogastric/splanchnic nerves to the dorsal horn of the thoracolumbar (T10-L2) and lumbosacral (L5-S1) spinal cord before traveling to the mesencephalic periaqueductal gray (PAG). Following cortical processing, the PAG sends excitatory input to the pontine micturition center (PMC) [13]. Descending cortical projections from the PMC synapse onto preganglionic parasympathetic neurons and inhibitory interneurons arising from the intermediolateral cell column of the human sacral (S2-S4) spinal cord [9,15]. Parasympathetic outflow exits through the hypogastric and pelvic plexus via the pelvic and pudendal nerves, eventually synapsing onto terminal ganglia and innervating the detrusor smooth muscle and urethra [10,12]. Cholinergic and non-cholinergic activation of the bladder smooth muscle muscarinic acetylcholine receptors and purinergic receptors promote bladder wall contraction, while urethral outlet relaxation is accomplished by nitrous oxide release to the internal urethral sphincter [9]. Elimination is further facilitated by the removal of sympathetic input to the LUT. PMC projections onto inhibitory interneurons reduce sympathetic and α-motoneuron activity in the LUT, relaxing the urethral sphincter to allow urine flow [15]. As the bladder empties, ascending distension-induced signaling to the PAG decreases, and the storage phase resumes [13].

Bladder afferents from the detrusor and urothelium allow not only the sensation of mechanical stimuli but also infection, inflammation, and barrier breakdown. They also
express a range of anti- and pro-nociceptive receptors and ion channels. Together, this allows the transduction of sensations ranging from fullness to pain from the complex signaling environment of the bladder. A subset of bladder afferent nerves does not fire under normal conditions but may become sensitized following central and peripheral changes in response to insult or injury, small-diameter unmyelinated fibers sensitive, sensitive to chemical and thermal stimuli, known as C-fibers [13,16].
Figure 2 Neural control of micturition during storage and filling. A Relevant regions in the brainstem and spinal cord. Supraspinal control is provided by the periaqueductal gray (PAG) in the midbrain and the pontine micturition center (PMC) in the pons. A region of the pons, the pontine storage center (PSC), may also influence urethral sphincter contraction. Sympathetic outflow to the bladder begins with preganglionic cells in the intermediolateral (IML) cell column in the thoracolumbar (T10-L2) spinal cord. α-motoneurons originating from Onuf’s nucleus in the ventral dorsal horn of the sacral spinal cord (S2-S4) innervate the external urethral sphincter, providing conscious control of continence. In mice and rats, the dorsolateral nucleus, also located in the ventral horn, fulfills a similar function. B During storage, sympathetic outflow (thick white lines) to the bladder via the hypogastric and pudendal nerves promotes bladder wall relaxation and bladder outlet contraction. Sympathetic outflow is prompted by low-level visceral afferent activity via the pelvic nerve. Descending input from the PSC (thin white line) may promote additional urethral contraction via the pudendal nerve, ensuring continence. C Elimination is initiated when the tension threshold is exceeded, initiating the spinobulbospinal pathway (dashed lines) through the PMC. This information may pass through relay neurons in the PAG first. Descending input from the PMC activates parasympathetic outflow (black lines) to the bladder (promoting bladder wall contraction and bladder outlet relaxation) and attenuates sympathetic outflow (demoting bladder wall relaxation and outlet and urethral sphincter contraction), initiating voiding. Additional supraspinal and cortical circuitry is likely involved in the generation of conscious bladder sensations and the switch between storage and elimination, not depicted here. Adapted from Fowler et al. [13].
1.2. Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS)

1.2.1 Background

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic inflammatory pelvic pain condition of unknown etiology characterized by increased urinary urgency and frequency, bladder discomfort, decreased bladder capacity, and pelvic pain [17,18]. Symptomatologies consistent with IC/BPS as it is understood today were first recognized by Drs. Phillip Syng Physick and Joseph Parish in the early 1800s, although there may be even earlier observations of similar afflictions [19]. The term “interstitial cystitis” was coined in 1887 by Dr. Alexander Skene to describe bladder overactivity and pelvic pain accompanied by urothelial ulceration and bladder wall inflammation [20]. In the ensuing decades, focal, ulcerative bleeding in the bladder wall described by Dr. Guy LeRoy Hunner would become a hallmark symptom of IC, while urinary urgency and pelvic pain in the absence of cystoscopic and histological features was termed “bladder pain syndrome” [21].

Today, the American Urological Association Interstitial Cystitis Guidelines Panel defines IC/BPS as six weeks or more of LUT symptoms accompanied by unpleasant sensations (pain, pressure, or discomfort) perceived in relation to the urinary bladder, absent any other clinically identifiable sources [21]. The term unites the previously distinguished IC — distinguished by the presence of cystoscopic features including urothelial ulceration and bladder wall inflammation — and BPS [21,22], now known as ulcerative and non-ulcerative IC/BPS. Overreliance on cystoscopic indicators in the
diagnosis of IC/BPS has historically led to under- or misdiagnosis of IC/BPS [21,23]; it is now known that non-ulcerative IC/BPS accounts for ~90% of cases [24].

1.2.2 Epidemiology
The prevalence of IC/BPS in the general population has typically been estimated between one and five percent, although estimates as high as 17% have been reported [21,25,26]. It is estimated to be five to ten times more common in women than men [23,27], and tends to present after the age of forty [26]. In the United States alone, it is estimated that as many as 3.3 to 7.9 million women fulfill the criteria for diagnosis of IC/BPS [28]. However, chronic prostatitis/pelvic pain syndrome in men has considerable clinical overlap with IC/BPS with recent suggestion that male IC/BPS may be under- and misdiagnosed [29,30]. IC/BPS has a direct annual cost of $750 million in healthcare expenditure and places a significant economic burden on the individual in addition to its significant impact on quality of life [21]. There is currently no generally accepted treatment [31].

1.2.3. Pathophysiology
While the etiology of IC/BPS remains mysterious [32], the known mechanisms involved can be broadly organized into urothelial alterations, neuronal sensitization, and central pain amplification, with inflammation playing a critical role in each.

Extensive evidence has implicated alteration of the urothelium in IC/BPS and other functional pain syndromes. As previously discussed, the urothelium is a highly specialized epithelial layer that protects the bladder (and other elements of the urinary
tract) from ions, solute, pathogens, and water in the lumen. A variety of urothelial alterations are observed in IC/BPS, including denudation of the GAG layer, tears, ulceration, glomerulation, and thinning to 1-2 cell layers [21,22,33–35]. This can compromise barrier function, resulting in a “leaky” urothelium marked by increased movement of urea, potassium, fluorescein, and rhamnose [36,37]. Infiltration of these molecules has been hypothesized to depolarize muscle and nerve cells and promote inflammation and inflammatory mediator release [37]. Elevated amounts of inflammatory mediators — such as cytokines, chemokines, neuropeptides, and growth factors — in the urine and urinary bladder of IC/BPS patients are well-documented [38–41]. This, in turn, promotes mast cell proliferation and activation [42–44]. Significantly elevated mast cell expression is observed in the detrusor, mucosa, and submucosa, and bladder as a whole in both ulcerative and non-ulcerative IC/BPS patients [43].

Urinary cations and mast cell–derived molecules may subsequently contribute to afferent nerve sensitization and neurogenic inflammation [17,18,45,46]. Increasing evidence indicates urothelial excitability is mediated by a variety of neuropeptides and growth factors expressed in the urinary bladder, such as nerve growth factor (NGF), that further sensitize bladder afferents [47–49]. This may explain stress- and, in female patients, menstruation-related flares due to corticotropin-releasing factor– and histamine–mediated mast cell activation [50–52]. Vasoactive and inflammatory molecules, such as substance P and NGF, increase afferent nerve proliferation [53], a distinguishing characteristic of IC/BPS from overactive bladder disorder [54]. Inflammatory mediators have also been implicated in the sensitization of mechanosensitive Aδ- and (silent) C-fibers in the pelvic and hypogastric/splanchnic nerve innervations in the bladder.
Subsequently, these fibers signal at lower bladder volume thresholds during filling, causing urgency [55]. Hypersensitization of bladder afferents in turn leads to the release of inflammatory neurochemicals throughout the micturition pathway [45, 56], thus initiating the positive feedback loop of urinary inflammation and sensitization.

Increased input from sensitized bladder afferents contributes to the release of neurochemicals in the spinal cord dorsal horn and, subsequently, central sensitization. A number of mediators, including substance P, calcitonin gene–related peptide, NGF, and brain-derived neurotrophic factor, have been implicated in spinal sensitization, potentially through N-methyl-D-aspartate (NMDA) receptor and nitrous oxide receptor potentiation [57–65]. The resulting changes in neurotransmitter synthesis and gene expression contribute to “wind-up” of the nervous system that manifests as symptoms of hyperalgesia (increased sensitivity to painful stimuli) and allodynia (painful response to normally innocuous stimuli).

The pathophysiology of IC/BPS is characterized by a clear positive feedback loop of inflammation and afferent hypersensitization. Previous investigations have implicated myriad inflammatory mediators and neuroactive chemicals. Of particular note is NGF, which contributes directly to both inflammation and afferent sensitization. Briefly, NGF is capable of initiating pro-inflammatory signaling cascades itself as well as through other mediators such as BDNF, substance P, and CGRP. It has well-documented effects on nociception and neuronal differentiation, proliferation, and survival. NGF also positively reinforces its own release, as it both activates and is released from mast cells. These actions, as well as the substantial evidence of its centrality to the pathophysiology of
IC/BPS and other inflammatory disorders of the bladder, will be further discussed in the following chapter.

1.2.4 Animal Models

We can further elucidate the mechanisms underlying IC/BPS and identify potential therapeutic targets through the study of animal models recapitulating its major pathophysiological features. The spectrum of symptomatologies encompassed by IC/BPS is broad, and incidentally there are myriad subchronic and chronic animal models which can be used to identify distinct mechanisms underlying the pathophysiology of IC/BPS. These models have been extensively reviewed previously (see Birder & Andersson [66] and Bjorling et al. [67]) and can be broadly organized into four categories: 1) bladder-centric models, 2) complex mechanism models, and 3) naturally occurring disease models, and 4) psychological and physical stressor models.

Bladder-centric models address circumstances in which toxic metabolites from the urine affect deeper layers of the bladder wall. These are typically induced by the instillation of an irritant — non-exhaustively: acetic acid; hydrochloric acid; acetone; cyclophosphamide or its active metabolite, acrolein; xylene; mustard oil; turpentine; protamine sulfate; lipopolysaccharide — into the bladder [66]. Bladder-centric models enable control of the timing, duration, and severity of inflammation [67]. This allows for various avenues of study: pathological changes in LUT signaling, in bladder voiding and pain behaviors, and the assessment of treatments intended to diminish the severity of pain, inflammation, and urinary dysfunction. An alternative to irritant-based models is
altered expression of urothelial targets, such as claudins, antiproliferative factors, and uroplakin II [17,66].

The most widely employed bladder-centric model is cystitis induced by cyclophosphamide (CYP). CYP is an anti-neoplastic alkylating agent used in chemotherapy treatment for several human cancers and whose dose-limiting effect is cystitis [68–70]. CYP is itself inert, but is metabolically activated in the liver to phosphamide mustard and acrolein, which are then excreted in urine [71]. Cystitis results from the accumulation of these toxic metabolites in the bladder prior to expulsion [71], although this is notably confined to the bladder — other organs of the urinary tract and viscera are spared [67,68]. Hemorrhagic cystitis is the only observable effect of CYP administration in mice at short time points, although longer durations produce immunosuppression and lung fibrosis [71].

CYP-induced cystitis is a reliable, well-validated, and extensively characterized model that reproduces the neurochemical and functional changes and localized bladder inflammation symptoms of IC/BPS [66–76]. Rodents with acute CYP-induced cystitis exhibit increased pain-related behaviors, bladder overactivity, and inflammation; edema and hemorrhage of the bladder, massive inflammatory cell infiltration, and mucosal destruction [66,68,71]. The pronounced inflammation and damage to bladder tissue observed in rats after even low doses of CYP are atypical in nonulcerative IC/BPS patients. Chronic models of milder bladder pain that produce inflammation without dramatic behavioral alterations have been described in both mice and rats, induced via multiple systemic administrations of CYP at lower doses [66,67,72,74,75]. Mice appear to be more resilient against the effects of systemic CYP administration than rats. The
mouse model of chronic CYP-induced cystitis described by Boudes et al. [74] recapitulates symptoms of detrusor overactivity, increased urinary frequency, and lower abdominal hyperalgesia without severe changes in physiological state, body temperature, weight, or overt damage to the bladder tissue. There is mild inflammatory response in the bladder characterized by edema of the lamina propria, moderate increases in gene expression of proinflammatory cytokines, mastocytosis, activation of proliferative signaling cascades, and decreased expression of urothelium-specific markers [75]. The bladder exhibits urothelial hyperplasia instead of denudation, erosion, or thinning, atypical of IC/BPS [75]. For this reason, the mouse model of chronic CYP-induced cystitis has been identified of particular relevance to nonulcerative and preclinical IC/BPS research. Both the acute and chronic models of CYP-induced cystitis have been used extensively to characterize inflammatory and nociceptive pathways and identify new mediators [46,76–81].

Bladder-centric models limit their focus to what changes are occurring in the bladder. There are several clear additional limitations. The hemorrhagic cystitis that results from acute CYP administration is atypical of IC/BPS, as is the urothelial hyperplasia observed in the mouse model of chronic CYP-induced cystitis. The chronic model may additionally be more aptly characterized as a repeat acute induction model, at least in rats [66]. These may present obstacles in the translational significance of findings. Nonetheless, bladder-centric models such as CYP-induced cystitis are of clear importance in studying the changes and mechanisms in the bladder underlying the pathophysiology of chronic inflammation.
Models with complex mechanisms instead propose that changes in the bladder are ultimately initiated elsewhere, such as in the central nervous system. These models are typically initiated by the injection of pseudorabies virus into the abductor caudalis dorsalis muscle at the base of the tail, chemically altering central circuits to produce neurogenic cystitis characterized by increased pelvic pain and mast cell infiltration in the lamina propria. While potentially illuminating more central mechanisms driving IC/BPS symptoms, the translational value of these models is not obvious [66].

Naturally occurring feline interstitial cystitis (FIC) is notable for the fidelity with which it recapitulates the underlying central and peripheral alterations, symptoms of increased pain and bladder overactivity, and comorbidities (such as irritable bowel syndrome) of IC/BPS as experienced by patients [82]. Despite this, scarce availability of animals and its inherently spontaneous nature have limited its use [66].

Psychological and physical stressor models such as water avoidance stress (WAS) and repeat variant stress (RVS) are alternative options. These models can induce hyperalgesia or analgesia, bladder overactivity, and a number of comorbidities, such as fibromyalgia, skin diseases, asthma, and irritable bowel syndrome, in rodents [66]. Increased inflammatory cell infiltration of the bladder, urothelial breakdown, peripheral and central sensitization, and engagement of voiding-associated areas of the micturition circuit associated with voiding [83], as well as moderate increases in plasmatic and urinary NGF concentrations [84] have been demonstrated in the WAS model, providing preliminary indications of its suitability as an animal model of IC/BPS.
1.3. Nerve Growth Factor (NGF)

1.3.1 Background

Nerve growth factor (NGF) is a member of the small family of dimeric secretory proteins known as neurotrophins, whose other members include, in mammals, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5), all of which affect many aspects of neurons, including their survival, shape, and function [85]. NGF was initially discovered by Nobel Laureates Rita Levi-Montalcini and Stanley Cohen in 1956. During development, NGF plays a critical role in the growth, differentiation, and survival of sympathetic and sensory afferent neurons [86,87]; however, it is its role in adulthood as a potent modulator of nociception that is of particular relevance here.

NGF is initially translated as the 7S, 130-kDa precursor protein complex proNGF before being cleaved by endoproteases. It can be cleaved intracellularly by furin or extracellularly by plasmin or matrix metalloproteinases. Alternatively, it can remain intact and signal as proNGF [88–90], but blocking proNGF processing prevents secretion of the mature product ([91]. “Nerve growth factor” used here refers to the mature, 2.5S, 26-kDa β-NGF subunit.

Under normal conditions in adulthood, NGF expression is low. While initially reported to be synthesized in mast cells, macrophages, and keratinocytes, subsequent RNA sequencing studies have found NGF transcripts virtually absent from a variety of potential sources including mast cells [92–94], keratinocytes [95,96], microglia [97], peripheral sensory neurons [98,99], and trigeminal and dorsal root ganglia [100], leaving the site of NGF synthesis in the healthy adult organism unknown [87]. Mast cells,
macrophages, and keratinocytes do manufacture NGF under pathological – specifically inflammatory – conditions, however [86,87].

1.3.2. NGF Signaling

Like all neurotrophins, NGF signals through two distinct receptors, a high-affinity tyrosine receptor kinase (Trk) and the tumor necrosis family receptor p75NTR. Each of the Trk receptors prefers a specific neurotrophin: TrkA is highly selective for NGF (and, in the absence of p75NTR, NT3), TrkB binds BDNF and NT4/5, and TrkC binds NT-3.

p75NTR binds all mature neurotrophins with approximately equal affinity while also displaying much higher affinity for their uncleaved pro-forms [85,86,101].

TrkA promotes cell survival, differentiation, neurite outgrowth, and synaptic plasticity. When NGF binds to the extracellular region of TrkA, the receptor dimerizes, auto-phosphorylates, and initiates signaling events by docking and phosphorylating downstream proteins [85,101–103]. TrkA regulates three major downstream signaling pathways: 1) Ras, which results in activation of the MAPK/ERK cascade promoting neuronal differentiation and neurite outgrowth; 2) PI3K, also activated through Ras or Gab1, which, in addition to promoting neuronal survival and growth, has also been implicated in the phosphorylation, membrane localization, and activation of the nociceptive cation channel transient receptor potential vanilloid 1 (TRPV1) [104], and 3) PLC-γ1, which promotes synaptic plasticity through calcium- and protein kinase C (PKC)-regulated pathways (Fig 3A) [102]. The NGF-TrkA complex is then endocytosed and transported retrogradely, modulating gene expression, or recycled or degraded [85,101–103]. Intracellularly localized TrkA can also be activated by G protein-coupled
receptor ligands, such as adenosine or pituitary adenylate cyclase-activating protein (PACAP) [105].

By contrast, p75NTR promotes cell death. p75NTR lacks a catalytic domain, but its signaling is complex for a number of reasons: Firstly, p75NTR acts synergistically with TrkA when co-expressed at high TrkA:p75NTR ratios [106–108], increasing the specificity and affinity of TrkA for NGF by suggested mechanisms including distinct but converging downstream signaling pathways, ligand sharing, and p75NTR-induced conformational changes in the TrkA receptor (Fig. 3B) [86,109,110]. Secondly, there are a variety of factors affecting pro-apoptotic p75NTR actions. p75NTR regulates three major downstream pathways, most notably 1) the pro-apoptotic Jun kinase (JNK) pathway, but also 2) Rho, which controls growth cone motility, and 3) NF-κB, which controls the transcription of multiple cell survival–promoting genes [102] and seems to respond only to NGF binding (Fig. 3A) [111]. In fact, p75NTR seems to have an intrinsic ability to distinguish between its various ligands based on differing effects, degrees of cooperativity and binding kinetics [112]. Only NGF (of the mature neurotrophins), in a study conducted in cultured oligodendrocytes, seems capable of producing sustained release of ceramide and accompanying activation of the JNK and, importantly, producing cell death [113]. However, mature neurotrophins are not effective activators of pro-apoptotic signaling at physiological doses [101]. Instead, pro-apoptotic actions through p75NTR seem to require activation via pro-neurotrophins, such as proNGF and proBDNF, and the coexpression of sortilin [101,114], one of a number of auxiliary receptors with which p75NTR can form heterodimers (Fig. 3B). p75NTR may additionally promote apoptosis via an alternative signaling pathway following (potentially Trk-mediated) ADAM10- or ADAM17-
dependent cleavage [101]. Activation of TrkA generates an anti-apoptotic signal that dominates over any pro-apoptotic signals [112], making pro-apoptotic signaling unlikely when TrkA is coexpressed. NGF binding of p75NTR-sortilin heterodimers reduces growth [115,116].

Thus, neurotrophin signaling depends heavily on the presence and expression of various coreceptors, ligand availability, and cellular context.

**Figure 3** Overview of p75NTR- and Trk-mediated signaling. A Major downstream signaling cascades of p75NTR and Trk (adapted from Reichardt [102]). Signaling through Trk promotes cell survival and differentiation through downstream pathways including the MAP kinase pathway. Pro-apoptotic signaling through p75NTR is thought to be primarily mediated by the Jun kinase pathway. Dimerization is not depicted. B The specificity and affinity of TrkA for NGF is enhanced by coexpression with p75NTR through a yet unknown mechanism. Pro-apoptotic signaling through p75NTR is currently thought to depend on its coexpression with sortilin and activation via immature proneurotrophins. p75NTR can form heterodimers with a number of other coreceptors. Both TrkA and p75NTR can alternatively be activated intracellularly as well (adapted from Bothwell [101]).
1.3.3. NGF in Nociception

Though originally discovered for its role in development, considerable evidence since indicates that NGF acts as a pain mediator in adulthood (reviewed in Barker et al. [86]), primarily through TrkA [117–120]. Briefly: NGF levels are increased in a number of chronic pain conditions and animal models of pain and inflammatory conditions, such as interstitial cystitis; blockage of NGF signaling attenuates pain-related symptoms and behaviors in the same, although notably anti-NGF treatment trials have been halted in the past due to severe side effects, a consequence of the ubiquity of NGF signaling throughout the body [117]; and administration or overexpression of NGF results in hyperalgesia (increased sensitivity to pain), allodynia (pain in response to normally innocuous stimuli), and decreased nociceptor activation thresholds [86]. NGF is clearly implicated in changes in nociception, and subsequent studies have further elucidated the mechanisms by which these changes occur.

Following NGF binding, TrkA is internalized, initiating various intracellular signaling cascades before being transported retrogradely to the nucleus and modulating the expression of genes that influence nociceptor function [117]. Broadly, NGF affects nociceptors in three ways: 1) sensitization of peripheral nociceptive terminals; 2) alteration of nociception-related transcription; and 3) sprouting of nociceptors [86].

NGF rapidly sensitizes peripheral nociceptive terminals through a variety of downstream signaling actions on ion channels and receptors. Its administration to cultured primary sensory nociceptive neurons increases sensitivity to thermal [104], mechanical [121], and chemical (such as capsaicin) [122] stimuli through PI3K- and MAPK/ERK-mediated increases in the trafficking and phosphorylation of the pro-
nociceptive cation channel TRPV1 [104,123]. Complementarily, naked mole-rats, in which NGF administration fails to produce hyperalgesia, owe this to hypofunction of the TrkA receptor and, correspondingly, decreased sensitization of TRPV1 [124]. NGF modulates the ATP-gated P2X3 receptor, potentially through TrkA-mediated activation of PKC [125,126]. NGF also increases cell excitability by enhancing tetrodotoxin-resistant Na$^+$ currents and suppressing delayed rectifying K$^+$ currents [127]. While the mechanism by which this occurs remains unknown, ERK1/2 and p38 MAPK notably increase activation of the sodium channels Na$\text{v}_{1.7}$ and Na$\text{v}_{1.8}$ respectively [128,129].

Following retrograde transport to the nucleus, NGF also regulates the transcription of several important nociceptor genes that influence the expression of, non-exhaustively, several receptors (TRPV1 [130–132], P2X3 [132–134], ASIC3 [135–137], NMDAR2B [138]), transmitters (substance P [139,140], CGRP [139], BDNF [141]), and ion channels (Na$\text{v}$s, Ca$\text{v}$s [142]), with a duration of several weeks [143,144].

NGF clearly also increases axonal growth and subsequently nociceptor hyperinnervation [145]. Increased sprouting of sensory nerves may increase nociceptor terminal density in peripheral tissue, contributing to observed increases in sensitivity and perception of painful stimuli [86]. Although the functional contribution remains unclear, this anatomical remodeling at sites of injury or inflammation is evident in several pain conditions, such as bone cancer and arthritis, and their animal models [146,147]. Notably, sensory hyperinnervation and increased urothelial p75$^{NTR}$ expression are distinguishing factors in IC/BPS compared to overactive bladder disorder [54].

These actions are not just limited to peripheral sensitization. Increased sprouting in the DRG and dorsal horn may also contribute to more persistent central sensitization and
wind-up. Rats overexpressing NGF display increased axonal expression of peptidergic nociceptive neurons in the dorsal horn as well as thermal and mechanical hyperalgesia [148,149]. NGF stimulates increased release of CGRP and substance P — which are known to potentiate NMDA receptor activity [61–63] — at central afferent terminals of sensory neurons [64], potentially leading to increased spinal and dorsal horn excitability.

NGF treatment also increases peripheral levels of BDNF, which is then transported anterogradely to peripheral and central sensory nerve terminals [141], and enhances BDNF release in the dorsal horn [65]. BDNF-p75NTR signaling through sphingosine kinase increases sensory neuron excitability [150], and, notably, BDNF-mediated central sensitization is thought to underlie many pain conditions [151].

Finally, NGF seems to have a reciprocal relationship with inflammatory mediators. NGF triggers mast cells to release granules of inflammatory mediators including prostaglandins, bradykinin, histamine, and NGF itself, which can act on receptors and ion channels in peripheral nociceptor terminals to produce increased cell excitability and, ultimately, thermal and mechanical hyperalgesia [152]. Whether this occurs in rodents is disputed; one study found that NGF administration did not activate mast cells [153], and another found that rodent mast cells do not express NGF receptors, though humans do express TrkA [154]. Even NGF alone is not sufficient to produce mast cell degranulation, it will potentiate the actions of mediators released during inflammation and injury by ruptured cells and invading inflammatory cells. For example, NGF potentiates DRG sensitivity to bradykinin in rats [155]. What’s more, inflammatory mediators influence NGF levels and effects in turn. A study in cultured sciatic nerve explants suggests that IL-1β plays a role in increased NGF levels [156,157]; additionally, bradykinin modulates
NGF levels in the bladder urothelium and bradykinin-1 receptor inhibition prevents thermal hyperalgesia following NGF administration [158,159]. Taken together, these observations raise the possibility of a positive feedback loop in which NGF stimulates inflammatory mediator release and actions, which in turn stimulate the synthesis or release and effects of NGF [86], reminiscent of that thought to underlie the symptoms of IC/BPS [32].

1.3.4. NGF in Cystitis

NGF has a well-documented role in bladder sensory function and the development of referred hyperalgesia in response to urinary inflammation [39,160]. Several studies have noted increased NGF levels in the blood serum [161], urine [40,162], and bladder urothelium of patients with IC/BPS [163], as well as throughout the central and micturition pathways – including the bladder, spinal cord, and peripheral DRG – in animal models of cystitis [163–165]. Cystitis is also associated with changes in NGF and BDNF receptors: in rats with CYP-induced cystitis, TrkA and TrkB immunoreactivity and Trk phosphorylation increased in the urinary bladder and L1 and L6 DRG [76,166]. Increased urothelial p75<sub>NTR</sub> expression is a distinguishing factor between IC/BPS and overactive bladder disorder [54], a finding that is recapitulated in animal models: urinary bladder p75<sub>NTR</sub> expression is increased in acute, intermediate, and chronic CYP-induced models of cystitis in rats [167].

Increasing LUT NGF recapitulates cystitis symptoms. Administration of NGF to the LUT increases bladder activity, sensitizes bladder afferents, and increases neuropeptide expression in the lumbosacral spinal cord [168,169], Correspondingly, urothelial NGF-
overexpressing (OE) mice display cystitis-associated symptoms including increased voiding frequency and changes in the expression of neurotrophins, their receptors, and nociceptive ion channels; relative to controls, NGF-OE mice display decreased TrkA, TrkB, and BDNF expression and increased TRPV4 and p75NTR expression, which the authors suggest may represent compensatory changes to restore function [165,170],

Studies that disrupt NGF-TrkA actions give complementary results. The NGF-scavenging agent REN1820 reduces voiding frequency and pain behaviors in rats with CYP-induced bladder inflammation [171]. Similarly, NGF sequestration via TrkA-IgG fusion molecules prevents the development of hyperalgesia in adult rats [172]. Trk inhibition via the nonselective Trk-inhibitor K525A reduces CYP-induced cystitis–associated changes in voiding frequency [170]. The p75NTR-inhibitor PD90780 produces bladder hyperreflexia in both control and CYP-treated mice only when infused with protamine sulfate, which disrupts urothelial barrier function [167].

Results from anti-NGF treatments in humans are less clear: while one study found that the anti-NGF monoclonal antibody tanezumab (at 200 µg/kg) reduced pain and urgency episode frequency in IC/BPS patients, additional studies at a variety of doses have failed to find an effect relative to the placebo [117]. However, evidence is limited as previous anti-NGF trials have been halted due to severe side effects, most notably rapid destruction of the joints [117]. The ubiquity of NGF signaling raises several additional concerns with anti-NGF treatments: impairment of possible NGF actions in nociceptive afferent and sympathetic neurons, altered sensation, and altered sympathetic ganglia neuron morphology, based on observations in preclinical trials [87], making the need for additional therapeutic targets clear.
1.4. Brain-Derived Neurotrophic Factor (BDNF)

1.4.1. Background and Signaling

Brain-derived neurotrophic factor (BDNF) is a 12.4 kDa-basic protein member of the neurotrophin family [85]. Although first noted for its role promoting the survival of some sensory neurons during development, BDNF is also important to learning and memory [173], and is an important modulator of pain in the mature nervous system [174].

BDNF is synthesized and secreted in an activity-dependent manner. The Bdnf gene is composed of nine exons under the control of nine promoters, the fourth of which, promoter IV, is driven by neuronal activity [175,176]. BDNF is synthesized and secreted in the central nervous system as well as by a number of peripheral cells, including neurons, microglia, astrocytes [177–181], monocytes, lymphocytes, and vascular endothelial and skeletal muscle cells [182,183]. Uniquely among the neurotrophins, BDNF is also synthesized in the cell bodies of peripheral DRG and transported anterogradely to terminals in the spinal cord [174,182]. It is initially synthesized as pre-proBDNF in the endoplasmic reticulum before being rapidly cleaved into the 35-kDa precursor protein proBDNF. The immature pro-form is subsequently cleaved into mature BDNF and collects in vesicles for secretion [175,176,182].

Like NGF, the pro- and mature forms of BDNF activate different receptors and pathways. BDNF homodimers bind the tropomyosin receptor kinase B (TrkB) with high affinity [85]. The receptor then dimerizes, autophosphorylates, and activates a number of intracellular pathways, including 1) the neuronal differentiation and neurite outgrowth–promoting ERK, via Ras, and subsequent downstream pathways such as 2) the pro-
survival and anti-apoptotic PI3K/Akt and 3) MAP kinase–interacting kinases, or MNK, 4) the mechanistic target of rapamycin (mTOR)-signaling, and 5) the synaptic plasticity–regulating PLCγ (Fig. 3a) [102]. While both BDNF and proBDNF are capable of binding the pan-neurotrophin receptor p75NTR, proBDNF does so with much higher affinity. p75NTR activates a number of downstream signaling pathways, including 1) the pro-apoptotic Jun kinase (JNK) pathway, but also 2) Rho, which controls growth cone motility, and 3) NF-κB, which controls the transcription of multiple cell survival–promoting genes (Fig. 3a) [102], although pro-apoptotic p75NTR actions seem to require binding of NGF or proNGF [112].

1.4.2. BDNF in Nociception and Cystitis

In addition to its roles in learning and memory and, during development, in influencing neuronal survival, substantial anatomical, functional, and behavioral evidence implicates BDNF in the modulation of nociception in the mature nervous system.

There are a number of central and peripheral neurons that synthesize and anterogradely transport BDNF. Central examples include the cerebral cortex and hippocampus [173,184]. In the periphery, small- to medium-sized peptidergic dark neurons — NGF-sensitive primary afferent sensory neurons that synthesize a number of pain-related neuropeptides such as substance P and CGRP — are of particular interest. These neurons synthesize and transport BDNF to central terminals in the dorsal horn of the spinal cord [185]. There, BDNF is stored in dense core vesicles alongside substance P and CGRP [186]. It can then be released in an activity-dependent fashion with putative neurotransmitter-like function [185]. BDNF release is observed from C-fiber terminals in
the spinal cord [65], and increased DRG BDNF expression and subsequent release in laminae I-II of the spinal cord dorsal horn is also observed following peripheral inflammation [65,187]. This increase in BDNF mRNA and protein occurs via an NGF-mediated mechanism in TrkA-expressing neurons [174], which constitute roughly two-thirds of small- to medium-sized peptidergic neurons under healthy conditions [185]. Both BDNF and TrkB are expressed throughout nociceptive pathways, including the sensory and dorsal root ganglia, as well as supraspinal centers (including relay centers, such as the thalamus, reticular formation, and PAG, as well as higher cortical areas) [60,174,175,185].

Functional evidence further implicates BDNF in nociception. There is some indication that BDNF modulates pain at the DRG-level. Its injection to the hindpaw produces acute thermal hyperalgesia [188] and its administration to the DRG produces mechanical allodynia in healthy rats [189]. Complementarily, in rats with sciatic nerve transection, delivery of an anti-BDNF antibody attenuates mechanical allodynia [189]. There is much greater evidence indicating a role for BDNF as a pain modulator in the spinal cord. BDNF is released in the spinal cord dorsal horn by peptidergic afferent fibers, especially when enhanced by NGF signaling [65,185]. Release of BDNF in the spinal cord by painful (noxious, chemical, mechanical, or thermal) but not innocuous stimuli phosphorylates TrkB in rodents [190,191]. ERK activation via BDNF-TrkB signaling is critical to the development of acute [192], inflammatory [193], and neuropathic pain [194], potentially through central sensitization [174]. BDNF administration increases evoked potentials in isolated spinal cord preparations [195]. That BDNF-deficient mice exhibit reduced wind-up [196] suggests this is true of
endogenously-released BDNF as well. BDNF sequestration via TrkB-IgG also reduces
evoked ventral root potentials in rats where BDNF is upregulated through NGF pre-
treatment [195]. BDNF also regulates NMDA receptor phosphorylation, channel
properties, and trafficking, likely through MEK/ERK, PLC/PKC, and other downstream
kinases [185]. Taken together, these findings suggest that BDNF potentiates excitatory
signaling, contributing to central sensitization.

Finally, BDNF is known to modulate pain behaviors in a number of normal and
pathological conditions [174,185]. Of particular interest are inflammatory pain
conditions, in which BDNF plays a clear pro-nociceptive role. BDNF is known to be
upregulated in the DRG and spinal cord as a consequence of peripheral inflammation
[197,198], and its sequestration reduces pain-related behaviors in animal models of
inflammatory pain [195,199].

BDNF is also well-implicated in acute inflammation of the bladder. BDNF and TrkB
are upregulated throughout the micturition pathway — particularly the urothelium — as a
consequence of bladder inflammation, spinal cord injury, and bladder outlet obstruction
[76,166,169,200–202]. At least in rats with turpentine-induced cystitis, this appears to be
short-lived; BDNF mRNA transcripts were significantly elevated two — but not six —
hours following the induction of cystitis [164]. Functionally, BDNF overexpression in the
bladder wall is associated with bladder overactivity and increased expression of
nociceptive TRPV1 and TRPA1 channels and cholinergic and purinergic signaling
proteins [203]; likewise, administration of exogenous BDNF intrathecally produces
bladder hyperactivity and pain, symptoms reduced by its blockade or sequestration [204].
Furthermore, its sequestration via TrkB-IgG improves bladder function and
downregulates markers of noxious input [200], such as cFOS and p-ERK [80,205,206], and TrkB inhibition via ANA-12 reduces mechanical allodynia in rats with CYP-induced cystitis [81]. The same study [81] demonstrated increased expression and activation of BDNF-TrkB signaling–related proteins as well as IL-1β and TNF-α in the dorsal spinal horn, suggesting that BDNF aggravates inflammation through activation of astrocytes and microglia. In patients with IC/BPS, treatment with botulinum toxin A is associated with subjective improvement and decreased urinary concentration of NGF and BDNF [201]; by contrast, only urinary NGF — not BDNF — concentration decreased in IC/BPS patients treated with hyaluronic acid [207].

1.5. Project Goals and Hypotheses

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic inflammatory pelvic pain syndrome characterized by increased urinary frequency, urgency, decreased bladder capacity, bladder discomfort, and pelvic pain. Despite the tremendous toll it exacts on patient quality of life, finances, and the economy as a whole, there currently exists no effective treatment. While its etiology remains unknown, an increasingly large body of evidence indicates a role for changes in neurotrophin signaling, particularly nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). NGF is a well-established modulator of nociceptive signaling, sensitizing the peripheral and central nervous system by a number of mechanisms and promoting further inflammation. NGF signaling also influences bladder function and is clearly implicated in cystitis by an increasingly large body of behavioral and functional evidence. NGF signaling also enhances BDNF synthesis and release, which in turn promotes peripheral and central sensitization. BDNF
plays a clear pro-nociceptive role in inflammatory pain conditions, with accumulating evidence directly implicating it in cystitis. However, neurotrophin signaling is ubiquitous and complex, depending heavily on the presence of various coreceptors, ligand availability, and cellular context, and its lack of characterization in the bladder has hampered attempts to provide effective therapeutic options for cystitis.

The objective of this dissertation research is to characterize the neurochemical alterations in neurotrophin signaling in the bladder associated with an experimental model of IC/BPS and identify novel therapeutic targets. Our central hypothesis is that the neurotrophins NGF and BDNF and their receptors, TrkA, TrkB, and p75NTR, contribute to changes in bladder function associated with cyclophosphamide (CYP)-induced cystitis.

Chapter 2 of this dissertation explores the neurotrophin receptors as targets for therapeutic intervention in urinary dysfunction with bladder inflammation. We hypothesized that pharmacological inhibition of TrkA, TrkB, and p75NTR would improve bladder functional changes associated with acute and chronic cystitis in mice. Chapter 3 explores changes in NGF signaling in the bladder that may underlie pathological changes in urinary function and sensation with bladder inflammation. We hypothesized that changes in NGF signaling in the bladder accompanying cystitis would be consistent with actions via TrkA. Taken together, these studies characterize NGF signaling in the bladder and provide functional evidence for the roles of NGF and BDNF signaling in bladder dysfunction. These studies not only improve our understanding of bladder dysfunction with inflammation, but may also aid in the development of effective therapies for individuals suffering from inflammatory bladder syndromes.
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Chapter 2: Effects of pharmacological neurotrophin receptor inhibition on bladder function in female mice with cyclophosphamide-induced cystitis

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Abstract

Interstitial cystitis/bladder pain syndrome is a chronic inflammatory pelvic pain syndrome of unknown etiology characterized by a number of lower urinary tract symptoms, including increased urinary urgency and frequency, bladder discomfort, decreased bladder capacity, and pelvic pain. While its etiology remains unknown, a large body of evidence suggests a role for changes in neurotrophin signaling, particularly that of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Here, we evaluated the effects of pharmacological inhibition of the NGF receptor TrkA, BDNF receptor TrkB, and pan-neurotrophin receptor p75NTR on bladder function in acute (4-hour) and chronic (8-day) mouse models of cyclophosphamide (CYP)-induced cystitis. TrkA inhibition via ARRY-954 significantly increased intermicturition interval and bladder capacity in control and acute and chronic CYP-treatment conditions. TrkB inhibition via ANA-12 significantly increased intermicturition interval and bladder capacity in acute, but not chronic, CYP-treatment conditions. Interestingly, intermicturition interval and bladder capacity significantly increased following p75NTR inhibition via LM11A-31 in the acute CYP-treatment condition, but decreased in the chronic condition, potentially due to compensatory changes in neurotrophin signaling or increased urothelial barrier dysfunction in the chronic condition. Our findings demonstrate that these receptors represent additional potent therapeutic targets in mice with cystitis and may be useful in the treatment of interstitial cystitis and other inflammatory disorders of the bladder.

Keywords: Interstitial Cystitis, Nerve Growth Factor, Brain-Derived Neurotrophic Factor, Urinary Bladder, Cyclophosphamide, p75, TrkA, TrkB
**Introduction**

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic inflammatory pelvic pain syndrome of unknown etiology characterized by a number of lower urinary tract (LUT) symptoms, including increased urinary urgency and frequency, bladder discomfort, decreased bladder capacity, and pelvic pain. It is currently thought that a positive feedback loop of urinary bladder inflammation and afferent hypersensitization underlies IC/BPS. Inflammation increases excitability of bladder afferents, which in turn release inflammatory neuropeptides, growth factors, cytokines, and chemokines throughout the micturition pathway, leading to altered bladder function (1, 2). There currently exists no effective therapy for IC/BPS and the disease exacts a tremendous financial burden on both individuals and the economy as a whole (3).

While the etiology of IC/BPS remains unknown (4), a large body of evidence suggests a role for changes in neurotrophin signaling, particularly that of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (5–8). Both NGF and BDNF are well-implicated in the LUT symptoms in overactive bladder and IC/BPS. Previous studies have shown upregulation of NGF at sites of tissue inflammation (9, 10), changes in its expression in the urine and bladders of both rodents and humans with cystitis (11–14), and changes in bladder function consistent with cystitis as a consequence of its urothelial overexpression (15, 16) or administration to the bladder (17, 18). BDNF is upregulated throughout the micturition pathway in both humans and rodents with cystitis (19–21) as a consequence of increased NGF synthesis (22, 23) and its reduction is associated with subjective improvement in IC/BPS patients undergoing treatment (20). Pharmacological disruptions of both NGF and BDNF in models of bladder inflammation
have given complementary results, improving bladder function (15, 24, 25). However, anti-NGF treatments for a variety of pain conditions have been halted due to severe side effects (26, 27). Thus, there is a clear need for additional therapeutic targets.

Neurotrophin signaling can alternatively be targeted at the receptor level. NGF activates two distinct receptors, the pro-survival high-affinity tyrosine receptor kinase A (TrkA) receptor and the pro-apoptotic low-affinity pan-neurotrophin receptor p75\textsuperscript{NTR} (28–31). Under certain ratios of coexpression, p75\textsuperscript{NTR} increases TrkA affinity for NGF, thus modulating NGF signaling (32). Most BDNF interactions are mediated through the high-affinity TrkB receptor, although it can also activate p75\textsuperscript{NTR} (28–30). Here, we show the effects of pharmacological inhibition of p75\textsuperscript{NTR}, TrkA, and TrkB on bladder function in a mouse model of cyclophosphamide (CYP)-induced cystitis. Our findings demonstrate that these receptors represent additional potent therapeutic targets in mice with cystitis and may be useful in the treatment of IC/BPS and LUT symptoms in other inflammatory disorders of the bladder.

Methods

Animals

Female C57BL/6 wildtype (WT) mice used in this study were bred locally at the Larner College of Medicine at the University of Vermont. The litters were of normal size, weight, and activity (feeding, drinking, behaviors). The average litter size was 6-8 mice. Mouse litters were undisturbed and not manipulated. Mice from different litters were assigned with simple randomization by distributing experimental groups across multiple
cages and litters. The UVM Institutional Animal Care and Use Committee approved all experimental protocols involving animal usage (IACUC #X9-020). Animal Care was under the supervision of the UVM Office of Animal Care Management in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health (NIH) guidelines. Estrous cycle status was not determined in female mice before use. All efforts were made to minimize the potential for animal pain, stress, or distress. Separate groups of littermate WT were used in the following experiments.

**Bladder catheter implantation**

Female adult WT mice (3-4 months) were anesthetized with 2-3% isoflurane in oxygen. A mid-abdominal incision was made, allowing access to the urinary bladder, into the dome of which a small hole was made and flared-tip PE-50 tubing inserted. The tubing was run subcutaneously to the nape of the neck and coiled. A small incision was made and a wax-sealed port anchored via stitching to the nape of the neck, allowing access to the tubing. All incisions were stitched up and the mice given a three-day recovery period. During this time, the mice also received a postoperative analgesic, carprofen, administered subcutaneously (s.c.; 0.1 mL/10g) for 48 hours following surgery. Induction of cystitis via CYP began following this period. Cystometry was conducted following appropriate incubation periods for both acute and chronic CYP treatments.
**CYP-induced cystitis**

Mice (N = 5-8, per treatment group) received cyclophosphamide (CYP) intraperitoneally (i.p.) to create acute (4-hour incubation, 200 mg/kg) or chronic (75 mg/kg every third day for a total of three injections) treatment groups (14, 33, 34). CYP is metabolized to acrolein, an irritant then expelled in the urine. (35) Injections were performed under 3% isoflurane anesthesia. The control group received no CYP treatment.

**Conscious, open-outlet cystometry**

Following the recovery period, tubing was exteriorized and the mice were placed, unrestrained, in a wire-bottomed recording cage and the catheter connected, via a T-tube, to a pressure transducer (Crass Model PT300, West Warwick RI, USA) and microinjection pump (Harvard Apparatus 22, South Natick MA, USA). Room temperature saline (0.9%) was infused into the bladder (25 μL/minute). The following urodynamic parameters were recorded using a Small Animal Cystometry Lab Station (MED Associates, St Albans VT, USA): bladder pressure (threshold, maximum, minimum, and average), infused volume (IV; the volume of saline infused into the bladder since the last void), and intermicturition interval (IMI). Bladder capacity is defined as the IV necessary to elicit a micturition event. At least six reproducible micturition cycles were recorded per mouse following a 15-minute acclimation period (33, 35). Mice in these studies had residual volume of less than 5 μL, meaning infused volume and void volume were similar. Testing was conducted at similar times of day to mitigate any impact of circadian rhythm. Bladder pressure measurements between mice
displayed high variability and no statistical differences were observed between groups in the present study.

*Intravesical administration of pharmacological inhibitors*

Following initial cystometry, each treatment group received intravesical delivery of: 30 mg/kg ARRY-952 selective TrkA inhibitor in 20% Captisol vehicle (AR; Pfizer, New York NY, USA), 100 mg/kg selective p75NTR inhibitor LM11A-31 (LM; Ricerca Biosciences, Painesville OH, USA) in sterile, injectable water, 100mg/kg selective TrkB inhibitor ANA-12 (ANA; MedChem Express, Monmouth Junction NJ, USA) in 10% dimethyl sulfoxide (DMSO) over thirty minutes. 0.5 mL of inhibitor was delivered to the bladder over a 30-minute period for each treatment group. Vehicle controls found no effects of the Captisol vehicle alone (Figure 3). Subsequently, the mice then underwent another session of cystometry, allowing each mouse to serve as its own baseline pre- and post-administration of inhibitors. Following the final cystometry session, mice were deeply anesthetized with 5% isoflurane in oxygen and euthanized via thoracotomy.

*Exclusion criteria*

Mice were withdrawn from the study due to postoperative complications or pain, lethargy, or distress that could not be addressed with postoperative analgesics. Some cystometry recordings could not be used due to artifacts from excessive behavioral movements (such as grooming and chewed tubing); in CYP-treatment conditions, mice exhibiting neither bladder functional impairment nor signs of bladder inflammation (blistering of the urothelium, edema, and sloughing of urothelial cells) on visual
inspection after euthanasia were excluded as well. Approximately 10% of mice were removed from the study.

*Cystometry analysis*

Cystometry traces were analyzed offline using MED-CMG software (Med Associates, St Albans VT, USA) and R. Values of functional bladder parameters were averaged before and after treatment for each mouse and averaged for each treatment group. Results were statistically analyzed using Welch’s paired t-tests and one-way analysis of variance (ANOVA) with Tukey’s honestly significant difference (HSD) post-hoc analysis where appropriate. P-values less than or equal to 0.05 were considered statistically significant. Asterisks (*, **, ***) indicate statistical differences at the p ≥ 0.05, 0.01, and 0.001 levels. Data is presented as boxplots with range and individual slope graphs. Boxplots display the median, interquartile range, maximum, minimum, and outliers (indicated with dots). The range is indicated by the corresponding bolded portions of the y-axis. Slope graphs indicate the change of individual mice before and after treatment. Red dotted lines indicate the change in mean values for each condition before and after treatment.

**Results**

*Both acute (4-hour) and chronic (8-day) CYP-treatment reduced intermicturition interval and bladder capacity*

Bladder function was assessed through conscious, open-outlet cystometry (Figure 1). As previously demonstrated (14, 33, 34), intermicturition interval (IMI) and infused volume
(IV) were statistically significantly reduced in both the acute (4-hour, 200 mg/kg i.p.) and chronic (8-day, 75 mg/kg i.p.) cyclophosphamide (CYP)-treatment conditions when compared to the control conditions (Figure 2 and Table 1). One-way ANOVA revealed a statistical difference in IMI by condition (F(2,15) = 40.95, p = 8.38x10^-7) and Tukey’s HSD Test for multiple comparisons revealed that the mean IMI value was significantly different between control and acute CYP (p = 0.0000014) and control and chronic CYP conditions (p = 0.0000098). One-way ANOVA also revealed a statistical difference in IV by condition (F(2,15) = 40.83, p = 8.54x10^-7) and Tukey’s HSD Test for multiple comparisons revealed that the mean IV value was significantly different between control and acute CYP (p = 0.0000014) and control and chronic CYP conditions (p = 0.00001; Table 1).

Figure 1 Bladder function was assessed via conscious, open-outlet cystometry. (A) Tubing is surgically implanted in the bladder and run subcutaneously to a port at the nape of the neck, allowing direct infusion into the bladder. (B) During cystometry, the mouse is placed in a wire-bottomed recording chamber. Saline is infused into the bladder at a constant rate of 25 μL/minute, allowing the measurement of various urodynamic parameters: bladder pressure (threshold, maximum, minimum, and average), infused volume (IV; the volume of saline infused into the bladder since the last void), and intermicturition interval (IMI; time between voids). Original illustrations by Harrison Hsiang.
**Figure 2** Increased voiding frequency following acute (4-hour) and chronic (8-day) CYP-treatment. (A) Representative traces of bladder pressure (mm Hg) over time (seconds) for control, acute and chronic CYP-treatment during constant intravesical infusion of saline. Bladder pressure increases with filling, spiking when the bladder contracts during micturition. Intermicturition interval is visibly decreased (increased frequency) in acute and chronic CYP-treatment conditions. (B) Both acute and chronic CYP-treatment increased voiding frequency. Intermicturition interval and infused volume were significantly reduced in acute (p = 0.0000014) and chronic (p = 0.0000098) CYP-treatment conditions. Circles mark the individual data points for each condition. N = 6 for all.

**Table 1** Mean ± SEM values for intermicturition interval and infused volume for control and acute (4-hour) and chronic (8-day) CYP-treatment conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Intermicturition interval (ms)</th>
<th>Infused volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>262.48±12.97</td>
<td>109.51±5.42</td>
</tr>
<tr>
<td>4-hour CYP</td>
<td>112.38±11.08***</td>
<td>46.99±4.61***</td>
</tr>
<tr>
<td>8-day CYP</td>
<td>135.00±55.12***</td>
<td>56.46±23.05***</td>
</tr>
</tbody>
</table>

Intermictrurition interval and infused volume were significantly reduced in acute (p = 0.0000014) and chronic (p = 0.0000098) CYP-treatment conditions. N = 6 for all.
TrkA inhibition via AR reduced void frequency and increased bladder capacity in both the acute and chronic CYP-induced cystitis models

Intravesical administration of ARRY-954 (AR) statistically increased IMI and IV in control (p = 0.01622; p = 0.01633) and acute (p = 0.001935; p = 0.01954) and chronic (p = 0.02549; p = 0.02548) CYP-induced cystitis conditions. IMI interval increased 1.18-fold in control mice, 1.56-fold in acute CYP-treated mice, and 1.68-fold in chronic CYP-treated mice (Figure 3 and Table 2). AR was delivered intravesically in 20% Captisol. Vehicle controls found no effect of vehicle alone (p > 0.05; Figure 4).

**Figure 3** Decreased voiding frequency following intravesical treatment with AR. Intermicturition interval was statistically increased following intravesical administration of AR in control (1.18-fold, p = 0.01622, N = 6) and acute (4-hour; 1.56-fold, p = 0.001935, N = 5) and chronic (8-day; 1.68-fold, p = 0.02549, N = 6) CYP-treated mice. Slope graphs indicate the change of individual mice before and after treatment. Red dotted lines indicate the change in mean values for each condition before and after treatment.
Table 2 Mean ± SEM values for intermicturition interval and infused volume before and after AR treatment.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Intermicturition Interval (ms)</th>
<th>Infused Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Control</td>
<td>192.56±29.13</td>
<td>227.45±30.17*</td>
</tr>
<tr>
<td>4-hour CYP</td>
<td>78.76±14.69</td>
<td>124.03±19.65**</td>
</tr>
<tr>
<td>8-day CYP</td>
<td>77.94±7.76</td>
<td>131.17±22.61*</td>
</tr>
</tbody>
</table>

Intermicturition interval and infused volume were statistically increased following intravesical administration of AR in control (p = 0.01622, p = 0.01633, N = 6) and acute (4-hour; p = 0.01954, p = 0.001935, N = 5) and chronic (8-day; p = 0.02549, p = 0.02548, N = 6) CYP-treated mice.

Figure 4 No effect on bladder function following intravesical treatment with 20% Captisol (p > 0.05, N = 6). Slope graphs indicate the change of individual mice before and after treatment. Red dotted lines indicate the change in mean values for each condition before and after treatment.

p75NTR inhibition via LM reduced void frequency and increased bladder capacity in acute CYP-treated mice, but reduced function in chronic CYP-treated mice

Intravesical administration of LM11A-31 (LM) statistically increased IMI (p = 0.001702) and IV (p = 0.001676) in acute (4-hour) CYP-treated mice, a 1.83-fold increase.

However, LM administration statistically decreased IMI (p = 0.007845) and IV (p =
0.007725) in chronic (8-day) CYP-treated mice 0.63-fold. No change was observed following treatment in the control condition (p > 0.05; Figure 5 and Table 3).

**Figure 5** Decreased voiding frequency following intravesical treatment with LM in acute CYP-treatment conditions. Intermicturition interval was statistically increased following intravesical administration of LM in acute (4-hour) CYP-treated mice (1.83-fold, p = 0.001702, N = 7). Intermicturition interval was statistically decreased following intravesical administration of LM in chronic (8-day) CYP-treated mice (0.63-fold, p = 0.007845, N = 5). There was no statistical difference before and after LM treatment in the control condition (p > 0.05; N = 6). Slope graphs indicate the change of individual mice before and after treatment. Red dotted lines indicate the change in mean values for each condition before and after treatment.

**Table 3** Mean ± SEM values for intermicturition interval and infused volume before and after LM treatment.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Intermicturition Interval (ms)</th>
<th>Infused Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Control</td>
<td>248.18±14.58</td>
<td>240.73±15.61</td>
</tr>
<tr>
<td>4-hour CYP</td>
<td>104.45±12.27</td>
<td>191.62±19.19**</td>
</tr>
<tr>
<td>8-day CYP</td>
<td>144.74±11.88</td>
<td>91.22±13.33***</td>
</tr>
</tbody>
</table>

Intermicturition interval and infused volume were statistically increased following intravesical administration of LM in acute (4-hour) CYP-treated mice (p = 0.001702, p = 0.001676, N = 7). Intermicturition interval was statistically decreased following intravesical administration of LM in chronic (8-day) CYP-treated mice (p = 0.007845, p = 0.007725, N = 5). There was no statistical difference before and after LM treatment in the control condition (p > 0.05; N = 6).
TrkB inhibition via ANA reduced void frequency and increased bladder capacity in acute but not chronic CYP-treated mice

Intravesical administration of ANA statistically increased IMI (p = 0.001114) and IV (p = 0.001113) in the acute CYP-treatment condition. No change was found in the control and chronic CYP-treatment conditions (p > 0.05; Figure 6 and Table 4).

Figure 6 Decreased voiding frequency following intravesical treatment with ANA. Intermicturition interval was statistically increased following intravesical administration of ANA in acute (4-hour) CYP-treated mice (1.6-fold, p = 0.001114, N = 8). There was no statistical difference following treatment in the control and chronic (8-day) CYP-treatment conditions (p > 0.05, N = 8, 7). Slope graphs indicate the change of individual mice before and after treatment. Red dotted lines indicate the change in mean values for each condition before and after treatment.

Table 4 Mean ± SEM values for intermicturition interval and infused volume before and after ANA treatment.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Intermicturition Interval (ms)</th>
<th>Infused Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Control</td>
<td>195.45±23.16</td>
<td>216.12±23.00</td>
</tr>
<tr>
<td>4-hour CYP</td>
<td>66.71±12.48</td>
<td>107.03±15.60***</td>
</tr>
<tr>
<td>8-day CYP</td>
<td>147.71±25.06</td>
<td>164.69±32.78</td>
</tr>
</tbody>
</table>

Intermicturition interval and infused volume were significantly increased in the acute (4-hour, p = 0.001114, p = 0.001113, N = 8) but not control or chronic CYP-treatment conditions (p > 0.05, N = 8,7).
Discussion

This study demonstrates the different effects of p75NTR, TrkA, and TrkB inhibition on bladder function in control and acute or chronic CYP-treated mice.

TrkA inhibition via intravesical administration of AR was associated with improved bladder function, with statistical increases in both the intermicturition interval (IMI) and infused volume (IV), in control and both acute (4-hour) and chronic (8-day) CYP-treatment conditions. Unsurprisingly, accumulating evidence suggests that NGF actions in cystitis are primarily TrkA-mediated. Its sequestration has prevented the development of hyperalgesia and reduced cystitis-associated changes in voiding frequency in CYP-treated animals (16, 24, 36). A previous study has also shown functional improvement following treatment with pan-Trk inhibitor K252A in rats with CYP-induced cystitis (15). TrkA and TrkB immunoreactivity and phosphorylation are increased in the urinary bladder and its afferents following bladder inflammation (21, 37). TrkA is also implicated in the expression of the nociceptive TRPV1 receptor (38), known to be upregulated in cystitis (39, 40), as well as in the development of mechanical and thermal hyperalgesia (41–46), all of which are associated with cystitis. TrkA inhibition increased IMI and IV in the control condition, suggesting that NGF-TrkA signaling may modulate bladder function even under noninflammatory conditions.

p75NTR inhibition via intravesical administration of LM also improved bladder function in the acute CYP-treatment condition. Given that NGF actions during cystitis may be primarily TrkA-mediated, this finding is consistent with the understanding that p75NTR modulates NGF-TrkA actions when the two receptors are coexpressed, potentially even when activated by immature proneurotrophins (30, 32). In rats with thermal
hyperalgesia, the magnitude of the acute response is TrkA-mediated while the duration of the response is $p75^{NTR}$-mediated. (47) However, $p75^{NTR}$ can also enact pro-apoptotic and growth-limiting actions independently of TrkA (28, 29, 47). $p75^{NTR}$ and its downstream effectors are essential for the development of NGF-mediated mechanical hyperalgesia in the rat hindpaw (43). NGF-$p75^{NTR}$ signaling–mediated changes in ceramide (via the sphingomyelin cycle) have been demonstrated to occur independently of TrkA in cell culture (48). NGF binding to $p75^{NTR}$-sortilin complexes reduces growth (47).

Additionally, $p75^{NTR}$ activates Jun kinase (JNK) (28), which influences a number of cystitis-relevant cellular functions including apoptosis, inflammation, cytokine production, and cellular differentiation and proliferation. In thermal hyperalgesia, it seems the magnitude of the acute response is TrkA-mediated while the duration of the response is $p75^{NTR}$-mediated (46). These studies, alongside our demonstration that $p75^{NTR}$ inhibition improved bladder function in CYP-treated mice, suggests that $p75^{NTR}$ may be a potent modulator of NGF-TrkA signaling–mediated bladder dysfunction and peripheral sensitization in cystitis.

Interestingly, while $p75^{NTR}$ inhibition improved bladder function in the acute CYP-treatment condition, it was associated with reduced bladder function in the chronic condition, with both IMI and IV decreasing after treatment. This may result from compensatory changes in protein expression under chronic inflammation conditions. Girard et al. (16) observed reduced expression of TrkA and TrkB and increased expression of $p75^{NTR}$ in urothelium-specific NGF-overexpressing mice, which the authors postulated may represent compensatory, concomitant changes to reduce urinary frequency. These compensatory changes in protein expression likely disrupt $p75^{NTR}$-
mediated facilitation of NGF-TrkA actions, which depend on high TrkA:p75\textsuperscript{NTR}
coexpression (30, 32), instead allowing p75\textsuperscript{NTR} actions to oppose NGF-TrkA actions.
p75\textsuperscript{NTR} inhibition under these conditions may undermine natural compensatory changes
and consequently reduce bladder function. An alternative explanation may be that the
effects of p75\textsuperscript{NTR} inhibition differ between the urothelium and regions of the bladder deep
to the urothelium; Klinger et al. (49) found that PD90780, known to disrupt NGF-p75\textsuperscript{NTR}
signaling, produces bladder overactivity in control and CYP-treated rats only when
infused with protamine sulfate, which disrupts urothelial barrier function. Here, reduced
bladder function following LM infusion may result from potentially increased urothelial barrier
dysfunction at the chronic timepoint, allowing LM to penetrate deeper into the bladder wall. Chronic CYP-treated mice display urothelial hyperplasia without
commensurate increases in mRNA expression for urothelial tight junction proteins,
suggesting urothelial barrier function is indeed compromised in the chronic condition
(50), which may influence the penetration rate of the inhibitors tested here.

TrkB inhibition via intravesical administration of ANA improved bladder function in
the acute but not chronic CYP-treatment condition. This is consistent with previous
studies. BDNF is well-implicated in acute inflammatory responses in the bladder.
Expression of BDNF and TrkB increase throughout the micturition pathway –
particularly the urothelium – in response to bladder inflammation, spinal cord injury, and
bladder outlet obstruction (19–21, 37, 51, 52); furthermore, overexpression of BDNF in
the bladder wall induces bladder overactivity and increases expression of nociceptive
TRPV1 and TRPA1 channels and cholinergic and purinergic signaling proteins (53) and
its sequestration improves bladder function and reduces expression of cFOS and
phosphorylated ERK, known markers of noxious input (54–56), in rats with acute CYP-induced cystitis (19). However, there is some indication that BDNF actions in the bladder are short-lived. Oddiah et al. (12) found that BDNF expression was increased when measured two hours after turpentine-induced inflammation but not at six hours after. This is consistent with our finding that TrkB inhibition improved bladder function in the acute (4-hour) but not chronic (8-day) CYP-treatment conditions. A possible explanation is increased retrograde transport of BDNF to the dorsal root ganglia and spinal dorsal horn at later time points in cystitis, contributing to central windup. BDNF is upregulated in the spinal dorsal horn following CYP treatment, promoting astrocyte and microglia activation and, in turn, increased inflammation and mechanical allodynia (57); delivery of exogenous BDNF intrathecally reproduces symptoms of bladder hyperactivity and pain, and its blockade or sequestration reduces them. NGF is known increase peripheral and spinal levels of BDNF, which is then transported retrogradely to central and peripheral nerve terminals (58). BDNF notably increases sensory neuron excitability through p75NTR (59), and its role in central sensitization is thought to underlie many pain conditions (60). Notably, BDNF actions in the spinal cord only persist 10-20 minutes after administration (61), likely due to receptor internalization and diffusion or degradation of BDNF (22, 62). Here, due to the intravesical delivery of inhibitors, it is likely treatment primarily affected receptors expressed in the bladder, however, a number of studies have demonstrated effects of inhibition of various neurotrophin signaling–related proteins at the DRG level as well (41, 63, 64). These studies implicate neurotrophin signaling at the DRG level in the modulation of pain sensation in addition to its functional effects at the level of the bladder, demonstrated here. Future studies addressing intrathecal or systemic
administration of agents will help to evaluate contributions from receptors expressed in the DRG and more widely throughout the body.

There are several limitations to the present study. First, the effects of these inhibitors were only evaluated in a mouse model of cystitis. CYP-induced cystitis is a reliable, well-validated, and extensively characterized model that reproduces the neurochemical and functional changes and localized bladder inflammation symptoms of IC/BPS (50, 65–67); of particular relevance, mice appear to be more robust to systemic CYP treatment than rats, exhibiting detrusor overactivity, increased urinary frequency, and lower abdominal hyperalgesia without dramatic alterations in physiological state, body temperature, or weight (68), more closely modeling the chronic nature of IC/BPS. However, we acknowledge that the 8-day model is more akin to repeat acute inductions rather than a true chronic condition; there is currently a paucity of chronic models for cystitis. Nonetheless, all animal models have limitations that may limit therapeutic translation of these results. Cross-validation in a number of available alternative animal models such as other irritant-induced cystitis models, stress models, and naturally occurring cystitis in cats (67, 69), will provide additional evidence as to the role of neurotrophin signaling in cystitis and other inflammatory disorders of the bladder. Additionally, these studies were conducted solely in female mice, which may leave sex differences unaccounted for. IC/BPS disproportionately affects women over men at a rate of 10:1 (70, 71); however, chronic prostatitis/pelvic pain syndrome in men has considerable clinical overlap with IC/BPS with recent suggestion that male IC/BPS may be under- and misdiagnosed (72). Future studies will benefit from incorporating male subjects. Finally, we did not investigate the effects of the various inhibitors in combination, which may reveal
synergistic effects and allow for lower doses of each individual inhibitor to achieve the same effect.

The present study demonstrates that p75\textsuperscript{NTR}, TrkA, and TrkB are potent therapeutic targets in the treatment of cystitis. Pharmacological inhibition of all three receptors was associated with strong improvement in LUT symptoms in the acute cystitis condition. TrkA inhibition improved bladder function in the chronic cystitis condition as well, while findings resulting from p75\textsuperscript{NTR} and TrkB inhibition provide further insight into the roles of NGF and BDNF signaling in sustained conditions of bladder inflammation.

**Funding**

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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Chapter 3: Changes in nerve growth factor signaling in female mice with cyclophosphamide-induced cystitis

Harrison W Hsiang, Beatrice M Girard, and Margaret A Vizzard

Frontiers in Urology, accepted December 28, 2022
Abstract

IC/BPS is a chronic inflammatory pelvic pain syndrome characterized by lower urinary tract symptoms including unpleasant sensation (pain, pressure, or discomfort) in the suprapubic or bladder area, as well as increased urinary frequency and urgency, and decreased bladder capacity. While its etiology remains unknown, increasing evidence suggests a role for changes in nerve growth factor (NGF) signaling. However, NGF signaling is complex and highly context dependent. NGF activates two receptors, TrkA and p75NTR, which activate distinct but overlapping signaling cascades. Dependent on their coexpression, p75NTR facilitates TrkA actions. Here, we show effects of CYP treatment and pharmacological inhibition of p75NTR (via LM11A-31) and TrkA (ARRY-954) on NGF signaling–related proteins: NGF, TrkA, phosphorylated (p)-TrkA, p75NTR, p-ERK1/2, and p-JNK. Cystitis conditions were associated with increased urothelial NGF expression and decreased TrkA and p75NTR expression as well as altering their co-expression ratio; phosphorylation of ERK1/2 and JNK were also altered. Both TrkA and p75NTR inhibition affected the activation of signaling pathways downstream of TrkA, supporting the hypothesis that NGF actions during cystitis are primarily TrkA-mediated. Our findings, in tandem with our recent companion paper demonstrating the effects of TrkA, TrkB, and p75NTR inhibition on bladder function in a mouse model of cystitis, highlight a variety of potent therapeutic targets and provide further insight into the involvement of NGF signaling in sustained conditions of bladder inflammation.

Keywords: Interstitial Cystitis/Bladder Pain Syndrome, Nerve Growth Factor, Lower Urinary Tract, Neurotrophin, p75, TrkA, ERK1/2, JNK
**Introduction**

There is currently no effective therapy for interstitial cystitis/bladder pain syndrome (IC/BPS), despite the tremendous toll it exacts on patients and the economy as a whole (1). IC/BPS is a chronic inflammatory pelvic pain syndrome characterized by lower urinary tract (LUT) symptoms including urinary frequency and urgency, decreased bladder capacity, and unpleasant sensation (pain, pressure, or discomfort) relating to the urinary bladder. While its etiology remains unknown (2), a positive feedback loop of bladder inflammation and afferent hypersensitization is thought to underlie IC/BPS (3). Increased activity in sensitized bladder afferents stimulates the release inflammatory neuropeptides, growth factors, cytokines, and chemokines throughout the micturition pathway, in turn promoting further inflammation, neuronal hypersensitization, and central pain amplification, leading to urinary dysfunction (4,5).

An increasingly large body of evidence indicates neurotrophin signaling, particularly nerve growth factor (NGF), in the pathophysiology of IC/BPS. NGF is upregulated in the urine and bladders humans with cystitis, and animal models demonstrate changes in its transcription and expression of NGF throughout the LUT (6–9). Its administration or overexpression in the bladder produces changes in bladder function consistent with cystitis (10–13), and, complementarily, its disruption in models of bladder inflammation is associated with improved bladder function (10,14–16).

However, previous attempts at therapies targeting NGF have been hampered by severe side effects (15,17) and incomplete characterization of neurotrophin signaling in the urinary bladder. NGF signaling is complex. NGF can activate two distinct receptors, the high-affinity tyrosine receptor kinase (Trk) A and the pan-neurotrophin receptor
p75\textsuperscript{NTR}. TrkA promotes cell survival, neurite outgrowth, and synaptic plasticity through three major downstream signaling pathways: 1) Ras, which results in activation of the MAPK/ERK cascade promoting neuronal differentiation and neurite outgrowth; 2) PI3K, also activated through Ras or Gab1, promoting neuronal survival and growth (18), as well as mediating the nociceptive action channel TRPV1 (19); and 3) PLC-\(\gamma\)1, promoting synaptic plasticity through calcium- and protein kinase C (PKC)-regulated pathways (18).

p75\textsuperscript{NTR} is sensitive to all neurotrophins with approximately equal affinity, including immature pro-neurotrophins, and it is capable of partnering with a number of coreceptors (20). When coexpressed with TrkA at high TrkA: p75\textsuperscript{NTR} ratios (21–23), p75\textsuperscript{NTR} facilitates TrkA actions (24,25). However, p75\textsuperscript{NTR} can also act independently of TrkA, promoting cell death when activated by pro-neurotrophins and co-expressed with sortilin (26,27). p75\textsuperscript{NTR} regulates three major downstream pathways: 1) the pro-apoptotic Jun kinase (JNK) pathway; 2) Rho, which mediates growth cone motility; and 3) the cell survival–promoting NF-\(\kappa\)B (18). Thus, NGF signaling depends heavily on the presence and expression of various coreceptors, ligand availability, and cellular context.

Thorough characterization of neurotrophin signaling in the bladder and its alteration during cystitis presents a clear path toward the identification of novel therapeutic targets and effective therapies for IC/BPS. In our recent companion paper (16), we demonstrated that pharmacological inhibition of the TrkA and p75\textsuperscript{NTR} improves bladder function in a mouse model of cyclophosphamide (CYP)-induced cystitis. Previous studies have examined changes in NGF signaling, which is notably complex and tissue- and context-specific, sporadically at the level of the whole bladder across various species and models of cystitis. Here, we comprehensively demonstrate the effects of CYP treatment at two
timelines, acute and chronic, and subsequent $p75^{NTR}$ and TrkA inhibition via novel, selective pharmacological inhibitors on the expression and activation of various NGF signaling–related proteins between the bladder urothelium and detrusor: NGF, TrkA, $p75^{NTR}$, ERK1/2 and JNK.

**Methods**

**Animals**

Female C57BL/6 wildtype (WT) mice used in this study were purchased at five months of age from Jackson Labs (Bar Harbor ME, USA). Mice were of normal size, weight, and activity (feeding, drinking, behaviors). A three-day acclimation period was observed following arrival. The UVM Institutional Animal Care and Use Committee approved all experimental protocols involving animal usage (IACUC #X9-020). Animal Care was under the supervision of the UVM Office of Animal Care Management in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health (NIH) guidelines. Estrous cycle status was not determined in female mice before use. All efforts were made to minimize the potential for animal pain, stress, or distress. Separate groups of littermate WT were used in the following experiments.

**CYP-Induced Cystitis**

Mice received cyclophosphamide (CYP) intraperitoneally (i.p.) to create acute (4-hour incubation, 200 mg/kg) and chronic (75 mg/kg every third day for a total of three
injections) treatment groups (9,28,29). CYP is metabolized to acrolein, an irritant then expelled in the urine (29). Injections were performed under 3% isoflurane anesthesia. The control group received no CYP treatment.

**Transurethral Catheterization**

A transurethral catheter (PE-10; Clay Adams, Parsippany NJ, USA) lubricated with veterinary eye lubricant was carefully inserted into the bladder through the urethra. Animals were anesthetized with 3% isoflurane and the catheter was then positioned in the bladder without contacting the bladder wall. Mice then received either 30 mg/kg ARRY-952 selective TrkA inhibitor in 20% Captisol vehicle (AR; Pfizer, New York NY, USA) or 100 mg/kg selective p75NTR inhibitor LM11A-31 (LM; Ricerca Biosciences, Painesville OH, USA) in sterile, injectable water based on our previous study, in press (15). Saline was administered as a treatment control, as well as an additional 20% Captisol vehicle control treatment. Anesthesia was maintained to prevent expulsion of the inhibitors or vehicle controls via voiding reflex for 30 minutes; the mice were then deeply anesthetized with 5% isoflurane, euthanized via cervical dislocation, and the urinary bladders harvested.

**Enzyme-Linked Immunosorbent Assays (ELISA)**

For protein assay, the urothelium and detrusor were dissected from harvested urinary bladders. Tissue was pinned onto a dissection dish with small dissection pins (Watkins, Doncaster UK) and kept wet with saline during dissection. We have previously verified
the specificity of the split bladder preparations was examined for the presence of a-smooth muscle actin (1:1000; Abcam, Cambridge, MA) and uroplakin II (1:25; American Research Products, Belmont, MA) by western blotting or Q-PCR (30,31). Dissected urothelium and detrusor tissue were then placed in collection tubes with Tissue Protein Extraction Reagent (250 µL for phospho-/total JNK ELISAs; 450 µL for all else) with complete protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany) and stored at -20°C. ELISA kits were used to detect NGF (BioSensis, Thebarton SA, Australia); TrkA, phospho-TrkA, and p75NTR (Bio-Techne, Minneapolis MN, USA); JNK, phospho-JNK, ERK, and phospho-ERK (ThermoFisher Scientific, Waltham MA, USA). All were mouse-specific or exhibited cross-reactivity. The assays were performed according to the manufacturers’ instructions. Bradford assays were performed as previously described (32–35).

**ELISA Analyses**

The standards provided generated linear standard curves for each protein measured. Background absorbance at 570 nm was subtracted from sample and standard absorbance values at 450 nm. Curve-fitting of sample protein content values to standard values was estimated with a least-squares fit analysis as previously described (32–35). Figures were prepared in R and Adobe Illustrator.

**Statistical Analyses**

For each sample, the protein of interest was evaluated relative to the total protein present. Phospho-/total JNK and ERK were detected using ThermoFisher InstantOne
MultiSpecifies ELISA kits, which do not provide standards, and are presented as the optical density (OD) of the phosphorylated protein relative to the OD of the protein regardless of phosphorylation (e.g. p-JNK OD/total JNK OD). Outliers were removed using Dixon’s Q-Test. Less than 1% of data points were outliers. Results were statistically analyzed using linear models with pairwise comparisons using estimated marginal means. For single comparisons in vehicle controls, Welch’s t-tests were used. P-values less than or equal to 0.05 were considered statistically significant. Asterisks (*, **, ***,***) indicate statistical differences at the p ≤ 0.05, 0.01, 0.001, and 0.0001 levels. All analyses were performed in R.

**Immunohistochemistry (IHC)**

IHC was performed as previously described (32,34,35). Bladders were fixed in 4% paraformaldehyde and conditioned in 10%, 20% and then 30% sucrose, then embedded in optimal cutting temperature (OCT) compound and sectioned at 20µm. Bladder sections were randomly selected from all regions of the urinary bladder. Primary and secondary antibodies (Table 1) were used to identify TrkA, p75NTR, p-JNK, and p-ERK expression; TrkA and p75NTR were co-stained to identify coexpression. Methodological and procedural controls included incubation without primary or secondary antibodies (blocking buffer only); with primary but without secondary; and without primary but with secondary. Antibody specificity was assured by the manufacturer. All processing was conducted simultaneously across conditions and treatments.
**IHC Figure Preparation**

Digital images were captured with an Olympus fluorescence photomicroscope. Imaging settings were consistent for acquisition and assembly across conditions for each protein of interest. Calibration bar represents 25 μm. Images were assembled and labeled in Adobe Photoshop.

**Table 1** Primary antibody manufacturers, hosts, catalog #s, and dilutions with secondary antibody pairings and dilutions.

<table>
<thead>
<tr>
<th>Primary</th>
<th>Manufacturer</th>
<th>Host</th>
<th>Catalog #</th>
<th>Dilution</th>
<th>Secondary</th>
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<td>Sheep</td>
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<td>cy3-DAS</td>
<td>1:150</td>
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<tr>
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<td>FITC-DAR</td>
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<tr>
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<td>Rabbit</td>
<td>9102</td>
<td>1:750</td>
<td>cy3-GAR</td>
<td>1:500</td>
</tr>
<tr>
<td>p-JNK</td>
<td>Cell Signaling</td>
<td>Rabbit</td>
<td>4668</td>
<td>1:750</td>
<td>cy3-GAR</td>
<td>1:500</td>
</tr>
</tbody>
</table>

DAS, donkey anti-sheep; DAR, donkey anti-rabbit; GAR, goat anti-rabbit; FITC, Fluorescein 5-isothiocyanate; cy3, Cyanine 3.
Results

_Urothelial NGF expression increased under acute CYP conditions_

NGF expression in the urothelium was significantly increased following induction of acute CYP-induced cystitis (fig 1). Analysis with a linear model found a significant main effect of condition ($F(2,33) = 41.16, p = 1.081 \times 10^{-9}$). Pairwise comparisons using estimated marginal means showed that NGF expression was significantly elevated in the acute CYP condition compared to control ($p = 1 \times 10^{-4}$) and chronic CYP ($p = 1 \times 10^{-4}$) conditions.

An effect of condition was also found in the detrusor. Analysis with a linear model found a significant main effect of condition ($F(2,28) = 5.05, p = 0.013$). However, pairwise comparisons with estimated marginal means revealed that while detrusor NGF expression differed significantly between acute and chronic CYP conditions ($p = 0.01$), neither CYP condition differed significantly from the control condition ($p > 0.05$ for both). Vehicle controls found no statistical differences in NGF expression between saline and Captisol vehicle treatment under control conditions in urothelium and detrusor tissue ($p > 0.05$ for both).
Figure 1  Acute CYP treatment increases urothelial NGF expression. Analysis with a linear model found a significant main effect of condition ($F(2,33) = 41.1632$, $p = 1.081 \times 10^{-9}$). Pairwise comparisons using estimated marginal means showed that NGF expression was significantly elevated in the acute CYP condition compared to control ($p = 1 \times 10^{-4}$) and chronic CYP ($p = 1 \times 10^{-4}$) conditions.

Urothelial $p75^{NTR}$ expression decreased under acute and chronic CYP conditions

Urothelial $p75^{NTR}$ expression decreased as a consequence of CYP treatment (fig 2). Analysis with a linear model found a significant main effect of condition ($F(2,34) = 151.05$, $p = 2 \times 10^{-16}$). Pairwise comparisons with estimated marginal means found that $p75^{NTR}$ expression differed significantly between acute CYP and control conditions ($p = 0.00064$), acute CYP and chronic CYP conditions ($p = 1 \times 10^{-4}$), and chronic CYP and control conditions ($p = 1 \times 10^{-4}$). In the detrusor, no significant effect of either condition ($F(2,34) = 0.38$, $p > 0.05$) or treatment ($F(3,34) = 0.71$, $p > 0.05$) was found. Vehicle controls found no statistical differences in $p75^{NTR}$ expression between saline and Captisol vehicle treatment under control conditions in urothelium and detrusor tissue ($p > 0.05$ for both).
Figure 2 Urothelial p75NTR expression decreased as a consequence of CYP treatment. A) p75NTR immunoreactivity (IR) in cryostat sections of urinary bladder from mice in control, acute (4-hour) CYP, and chronic (8-day) CYP conditions. Note the decreasing urothelial IR in CYP conditions. Lumen (L), lamina propria (LP), and urothelium (U) of the bladder as indicated. B) Analysis with a linear model found a significant main effect of condition (F(2,34) = 151.0538, p = 2x10^{-16}). Pairwise comparisons with estimated marginal means found that p75NTR expression differed significantly between acute CYP and control conditions (p = 0.000643), acute CYP and chronic CYP conditions (p = 1x10^{-4}), and chronic CYP and control conditions (p = 1x10^{-4}).

Urothelial TrkA expression decreased under chronic CYP conditions

Urothelial TrkA expression decreased as a consequence of chronic CYP treatment (fig 3). Analysis with a linear model found a significant main effect of condition (F(2,34) = 36.49, p = 3.54x10^{-9}). Pairwise comparisons with estimated marginal means revealed that urothelial TrkA expression in chronic CYP conditions was significantly reduced compared to control (p = 1x10^{-4}) and acute CYP conditions (p = 1x10^{-4}). Detrusor TrkA expression increased as a consequence of CYP treatment. Analysis with a linear model
found a significant main effect of condition \((F(2,34) = 5.37, p = 0.0094)\). Pairwise comparisons with estimated marginal means revealed that detrusor TrkA expression was significantly elevated under acute \((p = 0.045)\) and chronic \((p = 0.012)\) CYP conditions when compared to the control condition. Vehicle controls found no statistical differences in TrkA expression between saline and Captisol vehicle treatment under control conditions in urothelium and detrusor tissue \((p > 0.05\) for both).

**AR treatment significantly reduced urothelial TrkA phosphorylation in CYP conditions**

AR treatment significantly reduced TrkA phosphorylation in the urothelium under CYP conditions (fig 4). Analysis with a linear model found a significant main effect of treatment \((F(2,24) = 4.16, p = 0.029)\). Pairwise comparisons with estimated marginal means revealed that p-TrkA expression was significantly reduced following AR treatment when compared to saline under acute \((p = 0.022)\) and chronic \((p = 0.022)\) CYP conditions.
Figure 3 Urothelial TrkA expression decreased as a consequence of chronic CYP treatment. A) TrkA immunoreactivity (IR) in cryostat sections of urinary bladder from mice in control, acute (4-hour) CYP, and chronic (8-day) CYP conditions. Note the decreased urothelial TrkA IR in the chronic CYP condition. Urothelial hyperplasia is also evident in the CYP conditions. Lumen (L), lamina propria (LP), and urothelium (U) of the bladder as indicated. B) Urothelial TrkA expression decreased as a consequence of chronic CYP treatment. Analysis with a linear model found a significant main effect of condition (F(2,34) = 36.4875, p = 3.535x10^-9). Pairwise comparisons with estimated marginal means revealed that urothelial TrkA expression in chronic CYP conditions was significantly reduced compared to control (p = 1x10^-4) and acute CYP conditions (p = 1x10^-4). C) Detrusor TrkA expression increased as a consequence of CYP treatment. Analysis with a linear model found a significant main effect of condition (F(2,34) = 5.3729, p = 0.009383). Pairwise comparisons with estimated marginal means revealed that detrusor TrkA expression was significantly elevated under acute (p = 0.0447) and chronic (p = 0.0115) CYP conditions when compared to the control condition.
Figure 4 AR treatment significantly reduced TrkA phosphorylation in CYP conditions. Analysis with a linear model found a significant main effect of treatment (F(2,24) = 4.1631, p = 0.02885). Pairwise comparisons with estimated marginal means revealed that p-TrkA expression was significantly reduced following AR treatment when compared to saline under acute (p = 0.0216) and chronic (p = 0.0216) CYP conditions.

Urothelial TrkA:p75NTR expression ratio is significantly altered in the chronic CYP condition

Under chronic CYP conditions, the expression ratio of TrkA:p75NTR in the urothelium is significantly altered (fig 5). Analysis with a linear model found a significant main effect of condition (F(2,34) = 11.81, p = 0.00013). Pairwise comparisons with estimated marginal means revealed that TrkA:p75NTR expression ratio was significantly elevated in the chronic CYP condition when compared to control (p = 0.014) and acute CYP (p = 0.0005) conditions. In the detrusor, no significant effect of either condition (F(2,34) = 1.95, p > 0.05) or treatment (F(3,34) = 0.79, p > 0.05) was found.
Figure 5 Urothelial TrkA:p75NTR expression ratio was significantly altered in the chronic CYP condition. A) Merged TrkA and p75NTR immunoreactivity (IR) in cryostat sections of urinary bladder from mice in control, acute (4-hour) CYP, and chronic (8-day) CYP conditions. Note the dominance of TrkA IR in the urothelium in the chronic CYP condition. Urothelial hyperplasia is especially evident in the chronic CYP condition. Lumen (L), lamina propria (LP), and urothelium (U) of the bladder as indicated. B) Analysis with a linear model found a significant main effect of condition (F(2,34) = 11.8115, p = 0.0001264). Pairwise comparisons with estimated marginal means revealed that TrkA:p75NTR expression ratio was significantly elevated in the chronic CYP condition when compared to control (p = 0.0135) and acute CYP (p = 0.0005) conditions.
**ERK1/2 phosphorylation is significantly increased under acute CYP conditions, but not following LM or AR treatment**

Urothelial p-ERK1/2 expression is significantly elevated under acute CYP conditions when treated with saline but not with LM or AR (fig 6). Analysis with a linear model found significant main effects of condition (F(2,32) = 32.77, p = 1.79x10⁻⁸) and treatment (F(2,32) = 2.97, p = 0.046), and the interaction was significant (F(2,32) = 8.49, p = 0.0011). Pairwise comparisons with estimated marginal means revealed that p-ERK1/2 expression was significantly elevated under acute CYP conditions when compared to control (p < 0.0001) and chronic CYP (p < 0.0001) conditions when treated with saline; however, under acute CYP conditions, p-ERK1/2 expression was significantly reduced following both AR (p = 0.0001) and LM (p = 0.046) treatment when compared to saline. In the detrusor, no significant effect of either condition (F(2,33) = 2.91, p > 0.05) or treatment (F(3,33) = 1.94, p > 0.05) was found. Vehicle controls found no statistical differences in p-ERK1/2 expression between saline and Captisol vehicle treatment under control conditions in urothelium and detrusor tissue (p > 0.05 for both).
Figure 6 ERK1/2 phosphorylation is significantly increased under acute CYP conditions, but not following LM or AR treatment. A) p-ERK1/2 immunoreactivity (IR) in cryostat sections of urinary bladder from mice in control, acute (4-hour) CYP, and chronic (8-day) CYP conditions. Note the increased pERK1/2 IR in the urothelium in the chronic CYP condition. Lumen (L), lamina propria (LP), detrusor (D), and urothelium (U) of the bladder as indicated. B) Analysis with a linear model found significant main effects of condition ($F(2,32) = 32.7676, p = 1.785 \times 10^{-8}$) and treatment ($F(2,32) = 2.9739, p = 0.046250$), and the interaction was significant ($F(2,32) = 8.4870, p = 0.001104$). Pairwise comparisons with estimated marginal means revealed that p-ERK1/2 expression was significantly elevated under acute CYP conditions when compared to control ($p < 0.0001$) and chronic CYP ($p < 0.0001$) conditions when treated with saline; however, under acute CYP conditions, p-ERK1/2 expression was significantly reduced following both AR ($p = 0.0001$) and LM ($p = 0.0464$) treatment when compared to saline.
Urothelial and detrusor JNK phosphorylation changed as a consequence of condition and treatment

In the urothelium, p-JNK expression was significantly elevated in the acute CYP condition (fig 7). Analysis with a linear model found a significant main effect of condition (F(2,32) = 13.27, p = 6.39x10^{-5}). Pairwise comparisons with estimated marginal means revealed that urothelial p-JNK expression was significantly elevated when compared to control (p = 0.0058) and chronic CYP (p = 0.00028) conditions.

Effects in the detrusor were more complex, with altered p-JNK expression under chronic CYP conditions as well as a consequence of LM treatment under both CYP conditions (fig 7C). Analysis with a linear model found significant main effects of condition (F(2, 33) = 20.63, p = 1.55x10^{-6}) and treatment (F(3,33) = 6.01, p = 0.0022) on detrusor p-JNK expression. The interaction was not significant. Pairwise comparisons with estimated marginal means revealed that detrusor p-JNK expression was significantly elevated under chronic CYP conditions when compared to control (p = 0.012) and acute CYP (p = 1x10^{-4}) conditions, but p-JNK expression was significantly reduced following LM treatment when compared to saline (p = 0.016) and AR (p = 0.0018) treatments. Vehicle controls found no statistical differences in p-JNK expression between saline and Captisol vehicle treatment under control conditions in urothelium and detrusor tissue (p > 0.05 for both).
Figure 7 Urothelial and detrusor JNK phosphorylation changed as a consequence of condition and treatment. A) p-JNK immunoreactivity (IR) in cryostat sections of urinary bladder from mice in control, acute (4-hour) CYP, and chronic (8-day) CYP conditions. Note the increased p-JNK IR in the urothelium in the acute CYP condition. Urothelial hyperplasia is especially evident in the chronic CYP condition. Lumen (L), lamina propria (LP), and urothelium (U) of the bladder as indicated. B) Analysis with a linear model found a significant main effect of condition ($F(2, 32) = 13.2701$, $p = 6.385 \times 10^{-5}$) on urothelial p-JNK expression. Pairwise comparisons with estimated marginal means revealed that urothelial p-JNK expression was significantly elevated when compared to control ($p = 0.005848$) and chronic CYP ($p = 0.000283$) conditions. C) Analysis with a linear model found significant main effects of condition ($F(2, 33) = 20.6250$, $p = 1.545 \times 10^{-6}$) and treatment ($F(3, 33) = 6.0107$, $p = 0.002199$) on detrusor p-JNK expression. The interaction was not significant. Pairwise comparisons with estimated marginal means revealed that detrusor p-JNK expression was significantly elevated under chronic CYP conditions when compared to control ($p = 0.0116$) and acute CYP ($p = 1 \times 10^{-4}$) conditions, but p-JNK expression was significantly reduced following LM treatment when compared to saline ($p = 0.01641$) and AR ($p = 0.00183$) treatments.
Discussion

This study comprehensively demonstrates changes in NGF signaling–related protein expression and activation in the bladder in acute (4-hour) and chronic (8-day) CYP-treated mice with TrkA or p75NTR inhibition (Table 2).

Acute and chronic CYP conditions were associated with changes in a number of NGF signaling–related proteins. As expected, NGF expression in the urothelium was significantly elevated under the acute CYP condition. A preponderance of evidence from both humans and animal models implicates NGF in cystitis. It is upregulated in the blood serum, urine, and bladders — specifically the urothelium (6) — of IC/BPS patients (7,36–38). Animal models of cystitis have further demonstrated its upregulation throughout the micturition pathway, including the bladder, spinal cord, and peripheral DRG (6,8,10), although some studies have observed discrepancies between increases in transcription and protein expression in the whole bladders of rodents with CYP-induced cystitis (9, 44). Administration of NGF to the LUT increases bladder activity, sensitizes afferents, and increases neuropeptide expression in the lumbosacral spinal cord (39,40), and, similarly, its chronic overexpression in the urothelium produces cystitis-associated symptoms, such as increased voiding activity and changes in neurotrophin signaling–related proteins that include neurotrophin receptors TrkA, TrkB, and p75<sup>NTR</sup> and nociceptive ion channels like TRPV4, in mice (10,11). Disrupting NGF signaling gives complementary results. Its sequestration via TrkA-IgG fusion molecules prevents the development of hyperalgesia (41), and treatment with the NGF-scavenging agent REN1820 reduces voiding frequency and pain behaviors in rats with CYP-induced cystitis (42). It is clear that the upregulation of urothelial NGF is well-implicated in
altered urinary function and pain sensation with cystitis, and our findings here further reinforce its importance to the pathophysiology of cystitis.

We did not find a statistical increase in NGF expression under chronic CYP conditions from control levels. It is possible that the 75 mg/kg dose of CYP used to induce cystitis was insufficient to produce statistically evident increases in NGF expression in the present study. Boudes et al. (43), which initially described the chronic CYP-induced cystitis model in mice, noted that doses of 80 mg/kg — but not 40 mg/kg — CYP increased NGF concentration in the urine. Nonetheless, even the 40 mg/kg group exhibited other hallmarks of cystitis, such as inflammation, urothelial hyperplasia, and referred hyperalgesia. Here, we see consistent visual evidence of urothelial hyperplasia as well as condition-dependent changes in NGF signaling–related proteins (TrkA, p75\textsuperscript{NTR}, and p-JNK). Additionally, our recent companion paper demonstrated that reductions in the bladder functional parameters intermicturition interval (time between voids) and infused volume (volume of saline infused into the since last void) under the chronic CYP condition are reversed by pharmacological inhibition of the NGF receptor TrkA (16), suggesting alterations in NGF signaling have relevance to bladder functional parameters in the chronic CYP condition despite the lack of a statistical increase observed here.

Interestingly, expression of both NGF receptors, p75\textsuperscript{NTR} and TrkA, decreased with cystitis. TrkA expression in the urothelium was significantly decreased under the chronic CYP condition. This is consistent with previous studies: urothelial TrkA-IR is reduced in both rats with CYP-induced cystitis (44) and urothelium-specific NGF-overexpressing (NGF-OE) mice (11), although TrkA expression may increase in bladder afferents, major pelvic ganglia, and DRG as a consequence of increased NGF availability (44,45).
Unsurprisingly, urothelial TrkA phosphorylation decreased following treatment with selective TrkA inhibitor AR.

More surprisingly, $p75^{NTR}$ expression in the urothelium was significantly decreased under the acute CYP condition and decreased further under the chronic CYP condition. This is unexpected, given that previous observations suggest $p75^{NTR}$ is upregulated with cystitis. Klinger and Vizzard (46) found clear increases in whole bladder $p75^{NTR}$ expression in rats with CYP-induced cystitis at the acute (4-hour), intermediate (48-hour), and chronic (8-day) timepoints. Whole bladder $p75^{NTR}$ expression is also increased in NGF-OE mice (11). In humans, increased $p75^{NTR}$ expression has been identified as a distinguishing feature between IC/BPS and overactive bladder syndrome (47). Nevertheless, our results demonstrate a clear reduction in urothelial $p75^{NTR}$ expression with cystitis, highlighting the complexity of NGF signaling in the bladder and the necessity of its thorough characterization in models of cystitis.

Increasing evidence indicates that NGF actions in cystitis are primarily TrkA-mediated. TrkA sequestration reduces bladder overactivity and hyperalgesia in CYP-treated animals (41,48), and pan-Trk inhibition via K252A produces functional improvement in CYP-treated rats (49,50). TrkA also regulates the expression of the nociceptive TRPV1 receptor (19,51), likely through PI3K (19) and the MAPK/ERK pathway (52). TRPV1 contributes to the development of mechanical and thermal hyperalgesia (49,53–57) and is known to be upregulated in cystitis (58,59). $p75^{NTR}$ is instead understood to modulate TrkA actions through an unknown mechanism when the two receptors are coexpressed (26,60). For example, NGF-mediated mechanism hyperalgesia in the rat hindpaw depends on $p75^{NTR}$ and its downstream effectors (54);
similarly, in acute thermal hyperalgesia, TrkA seems to mediate the magnitude of the response while p75NTR modulates its duration (57). p75NTR can signal independently of TrkA to promote apoptosis, but activation of TrkA generates an anti-apoptotic signal that dominates over any pro-apoptotic signals (61).

Consistent with the hypothesis that p75NTR facilitates TrkA actions in the bladder, our recent companion paper demonstrated that local pharmacological inhibition of either TrkA or p75NTR produces bladder function improvement in a mouse model of cystitis (16). Intravesical treatment with AR or LM significantly increased intermicturition interval and infused volume in mice with acute CYP-induced cystitis. However, under the chronic condition, p75NTR inhibition via LM instead reduced intermicturition interval and infused volume.

Our findings in the present study may lend insight into the effect of p75NTR inhibition on bladder functional parameters observed there. Here, we found that the urothelial TrkA:p75NTR expression ratio was significantly altered under the chronic CYP condition. p75NTR-mediated facilitation of TrkA actions notably depends on the ratio of TrkA and p75NTR coexpression (26,60). Girard et al. (11) demonstrated that, in mice chronically overexpressing NGF in the urothelium, whole bladder TrkA expression decreased while p75NTR expression increased, which the authors suggest may represent concomitant, compensatory changes to reduce NGF-mediated increases in urinary frequency. The change in urothelial TrkA:p75NTR expression ratio demonstrated in the present study may similarly arise from compensatory changes in TrkA and p75NTR expression induced by chronic cystitis conditions.
There were also changes in the activation of proteins downstream of TrkA and p75<sup>NTR</sup>. In our previous study (16), we demonstrated that TrkA and p75<sup>NTR</sup> inhibition via AR and LM respectively improved bladder functional parameters in the same mouse models of cystitis as used presently. For this reason, effects of the same inhibitors on NGF signaling–related protein expression and activation was evaluated here.

Phosphorylation of ERK1/2 in the urothelium increased significantly under the acute CYP condition. The MAPK/ERK cascade is a major signaling pathway downstream of TrkA that promotes neuronal differentiation and outgrowth (18). ERK1/2 activation is implicated in altered urothelial sensory mechanisms and the development of chronic bladder pain in response to a number of insults, including noxious stimuli, bladder distension, and inflammation (30,62–65). Rodents with CYP-induced cystitis display elevated p-ERK1/2 expression in the urinary bladder and lumbosacral spinal cord (30,63), and upstream inhibition of ERK phosphorylation via U0126 significantly increases bladder capacity in CYP-treated rats (30). ERK1/2 may also be involved in rapid sensitization of peripheral nociceptive terminals. The MAPK/ERK pathway is known to increase the trafficking and phosphorylation of TRPV1 (52), and ERK1/2 is capable of increasing sodium channel activation (66,67), consistent with observations that NGF increases cell excitability through enhancement of Na<sup>+</sup> currents (66).

Urothelial ERK1/2 phosphorylation, significantly increased under the acute CYP condition, was significantly reduced in groups treated with LM and AR. Given that AR treatment reduces TrkA phosphorylation, it is unsurprising that phosphorylation of ERK1/2, downstream of TrkA (18), is also affected. However, treatment with the p75<sup>NTR</sup> inhibitor LM also reduced urothelial ERK1/2 phosphorylation. This is likely due to
removal of p75NTR-mediated facilitation of TrkA with p75NTR inhibition. While the specific mechanism by which this facilitation occurs remains unknown, numerous studies have demonstrated that NGF must bind p75NTR in order to facilitate NGF-TrkA actions (25,26,60).

Phosphorylation of JNK was also significantly altered as a consequence of CYP treatment. In the urothelium, p-JNK expression increased significantly under the acute CYP condition. JNK is a member of the MAPK superfamily downstream of p75NTR implicated in the development of inflammation. The JNK pathway has been implicated in a number of chronic pain disorders including IC/BPS (68). Dugan et al. (69) also demonstrated increased JNK phosphorylation in whole bladders of rats with acute (4-hour) and intermediate (48-hour) CYP-induced cystitis; treatment with SP600125, which blocks JNK phosphorylation, subsequently improved bladder function and reduced neuropeptide (substance P, CGRP) expression.

p-JNK expression was also increased in the detrusor under the chronic CYP condition. Significant increases in the expression of p-JNK and p-c-jun have been shown in the bladder muscle layer of IC/BPS patients, suggested to result from structural damage to the bladder and urothelial barrier function compromise, allowing inflammatory mediators and mast cells to infiltrate (68). Here, p75NTR inhibition via LM treatment significantly reduced detrusor p-JNK expression under both the acute and chronic CYP conditions. Previous studies have also indicated the relevance of urothelial barrier dysfunction to targeting p75NTR signaling in cystitis. Klinger et al. (46) demonstrated that p75NTR inhibition via PD90780 produces bladder overactivity in control and CYP-treated rats only when infused with protamine sulfate, which disrupts
urothelial barrier function. In our recent companion paper, we demonstrated that while p75NTR inhibition via LM improves bladder function in acute CYP-treated mice, it reduces bladder function in chronic CYP-treated mice, possibly owing to reduced urothelial barrier function and deeper penetration of LM into the bladder wall (16). These findings raise the possibility that the contributions of p75NTR signaling to bladder function differ between the urothelium and deeper layers of the bladder.

There are several limitations to the present study. While CYP-induced cystitis is a reliable, extensively characterized, and well-validated model known to recapitulate the neurochemical and functional changes and localized bladder inflammation symptoms of IC/BPS (70–73), chronic models of cystitis are limited, and the paradigm used here may more accurately constitute repeat acute inflammation inductions. As previously noted, there is particular sensitivity to CYP dose in the chronic paradigm (43). In mice, the chronic CYP-treatment paradigm produces urothelial hyperplasia, activation of proliferative signaling cascades, and decreased expression of urothelium-specific markers, but not massive infiltration of inflammatory mediators, hemorrhage, mucosal alteration, and loss of the urothelium. For these reasons, Golubeva et al. (70) suggests that the mouse model of chronic CYP-induced cystitis may have more relevance to nonulcerative IC/BPS. Cross-validation of these findings in alternative models of cystitis via a number of induction methods and a range of species, such as other irritant-induced cystitis models, stress models, and naturally occurring cystitis in cats (70,73–75), is especially prudent given the evident complexity of neurotrophin signaling in the bladder and spectrum of symptomatologies encompassed by IC/BPS. This study is also limited for being conducted solely in female mice. Although the transurethral catheterization
method used here is far more easily performed in female mice, this potentially fails to account for sex differences. IC/BPS is estimated to be more prevalent in women than men 10:1 (76,77), but there is increasing indication that male IC/BPS may be under- and misdiagnosed given its considerable clinical overlap with chronic prostatitis/pelvic pain syndrome in men (78).

The present study demonstrates the effects of CYP treatment and subsequent TrkA and \( p75^{NTR} \) pharmacological inhibition on various NGF signaling–related proteins. Cystitis conditions were associated with increased expression of NGF and decreased the expression of its two receptors, TrkA and \( p75^{NTR} \), as well as altering their co-expression ratio; phosphorylation of downstream signaling molecules ERK1/2 and JNK were also altered. Both TrkA and \( p75^{NTR} \) inhibition affected the activation of signaling pathways downstream of TrkA, supporting the hypothesis that NGF actions during cystitis are primarily TrkA-mediated. These findings, especially in tandem with our recent companion paper (16), highlight a variety of potent therapeutic targets in the treatment of cystitis and provide further insight into the involvement of NGF signaling in sustained conditions of bladder inflammation.

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<tr>
<th>Tissue</th>
<th>Condition</th>
<th>NGF</th>
<th>TrkA</th>
<th>( p75^{NTR} )</th>
<th>p-ERK1/2</th>
<th>p-JNK</th>
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<tbody>
<tr>
<td>Urothelium</td>
<td>Acute CYP</td>
<td>↑</td>
<td>↓*</td>
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<td>Chronic CYP</td>
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<td>Detrusor</td>
<td>Acute CYP</td>
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<td>Chronic CYP</td>
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Table 2. Summary of changes in urothelial and detrusor relative expression of NGF, TrkA, \( p75^{NTR} \), p-ERK1/2, and p-JNK as a consequence of CYP treatment at the acute (4-hour) and chronic (8-day) CYP treatment timelines when compared to the control condition. Non-statistically significant changes are denoted with *. 
Funding

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Chapter 4: Conclusions and Future Directions

4.1 Overview

IC/BPS is a chronic inflammatory pelvic pain syndrome characterized by bladder overactivity and pain or discomfort perceived to be related to the bladder. Despite its tremendous burden on individual quality of life, finances, and the economy as a whole, there are currently no effective therapies for IC/BPS and its etiology remains unknown [21]. Substantial evidence from humans and animal models points towards a central role of changes in neurotrophin signaling, especially NGF, in the pathophysiology of IC/BPS [158,162–166,168–170,201,208–214]. NGF signaling is relatively ubiquitous, complex, and highly context-dependent [85,102,112,174]. These factors, alongside its lack of characterization in the bladder, are continuing obstacles in the development of therapeutic options for IC/BPS. We propose that changes in neurotrophin signaling in the urinary bladder contribute to inflammation and peripheral afferent hypersensitization underlying bladder dysfunction. These studies characterize changes in NGF signaling in mouse models of acute and chronic cystitis and demonstrate that several associated proteins may represent potential diagnostic markers or therapeutic targets in the bladder to improve function with urinary bladder inflammation.

4.2 Summary of results

These studies demonstrate a central role for changes in NGF signaling in bladder function and dysfunction in mice with CYP-induced cystitis.

Specifically, in acute (4-hour) CYP-induced cystitis, there were a number of alterations to NGF signaling–related proteins in the urothelium: NGF expression
increased, p75NTR expression decreased, and ERK1/2 and JNK phosphorylation increased; TrkA expression was significantly increased in the detrusor. These changes were accompanied by substantial reductions in bladder function as evident by decreased intermicturition interval (IMI) and infused volume (IV) of saline between voids. In chronic (8-day) CYP-induced cystitis, urothelial TrkA and p75NTR expression both decreased, altering their ratio of expression, and detrusor TrkA expression and JNK phosphorylation both increased. These changes were similarly accompanied by decreases in IMI and IV, although not as dramatically as those seen under acute cystitis. These findings demonstrate that bladder dysfunction in acute cystitis is associated with changes in urothelial NGF signaling and raise the possibility of compensatory changes in NGF signaling under sustained conditions of inflammation (see 4.5).

Complementarily, we demonstrated that pharmacological inhibition of neurotrophin receptors improved bladder function under acute cystitis conditions. TrkA inhibition via AR increased IMI and IV under control and acute and chronic cystitis conditions; in cystitis conditions, AR treatment was associated with reductions in phosphorylation of TrkA (as expected) as well as ERK1/2, downstream. p75NTR inhibition was also increased IMI and IV under acute cystitis with an associated decrease in ERK1/2 phosphorylation. These findings are consistent with the hypothesis that p75NTR facilitates NGF-TrkA actions in the urothelium (see 4.5). Interestingly, p75NTR inhibition decreased IMI and IV under chronic cystitis, with associated reductions in JNK phosphorylation (downstream of p75NTR) — which was notably increased in the detrusor under chronic cystitis — under both cystitis conditions. These findings raise the possibility that the contributions of p75NTR actions to bladder function differ between the urothelium and detrusor (see 4.6).
Finally, TrkB inhibition via ANA increased IMI and IV under acute cystitis. TrkB notably also influences the MAPK/ERK pathway [102] and its role in bladder function and especially sensation warrants further discussion (see 4.7).

These findings highlight various potent therapeutic targets in the treatment of cystitis within the NGF signaling system in the bladder and provide further insight into its critical role in altered bladder function and sensation under sustained conditions of inflammation (see 4.8).

### 4.3 Major limitations

These studies have provided preliminary characterization of the role NGF signaling plays in bladder function and dysfunction in mouse models of chronic cystitis. As previously discussed, there are a number of limitations to these studies owing largely to the complexity and context-dependence of NGF signaling, the heterogeneity of symptomatologies of IC/BPS and other inflammatory disorders of the bladder, and limitations inherent to the particular animal model of cystitis here used.

The complexity of NGF signaling — its dependence on ligand availability, coreceptor expression, and cellular context [86,215] — necessitates further characterization of its role in bladder function and dysfunction. There are a limited number of studies investigating cystitis-mediated changes in NGF signaling in the bladder (see Comprehensive Literature Review) [164–167,170,213,214], and broader observations must be drawn from a number of different animal models. At least one of the findings reported here notably differs from forecasted results based on previous studies: while urothelial NGF-OE mice [170] and rats with acute CYP-induced cystitis demonstrate
increased whole bladder $p75^{NTR}$ expression [167], here we demonstrated progressive decreases in $p75^{NTR}$ expression with acute and chronic CYP-induced cystitis in mice. These may result from inherent differences in the specific relevance of NGF signaling to bladder function and sensation in differing models of cystitis by species and method of induction, to be clarified and expanded by additional studies.

Some findings may result from limitations in the animal model used here. Foundational studies on the chronic CYP-induced cystitis model in mice noted dose-dependence in the upregulation of bladder NGF expression [74,75]. The 75 mg/kg dose of CYP used here was evidently insufficient to produce statistically legible changes in either urothelial or detrusor NGF expression, although other relevant hallmark symptoms of cystitis, including urothelial hyperplasia, changes in NGF signaling–related proteins, and reductions in urodynamic measures of bladder function, were still reproduced. Nonetheless, it may be more accurately characterized as a repeat acute cystitis induction model as opposed to a true “chronic” model of cystitis [66]. CYP-induced cystitis is a reliable, extensively characterized, and well-validated model of IC/BPS as previously discussed [68–70,72,74,75], but its therapeutic translatability is unclear without cross-validation in alternative animal models such as other irritant-induced cystitis models, stress models, and naturally occurring cystitis in cats [66,82,216]. This is especially relevant given indications in the present studies that the therapeutic effect of certain interventions, at least as demonstrated here in a mouse model of cystitis, may vary given the extent and duration of the dysfunction. For example, $p75^{NTR}$ inhibition improved bladder function under acute cystitis yet reduced function under chronic cystitis; this may be accounted for by changes in TrkA:$p75^{NTR}$ expression ratio, suggestive of
compensatory changes under sustained conditions of inflammation (see 4.5), or by differing contributions of p75NTR actions between the urothelium and detrusor made apparent by increased urothelial permeability in the chronic condition (see 4.6).

The present study is additionally limited by its use of exclusively female mice. While IC/BPS is typically estimated to occur at a 10:1 ratio in women to men [27,217] and the feasibility of the current study substantially influenced by the suitability of female mouse urethra anatomy for transurethral catheterization, allowing direct instillation of various compounds to the bladder, there is some recent suggestion that IC/BPS is under- and misdiagnosed in male populations [30].

Although no single model can account fully for the tessela of symptoms encompassed by IC/BPS, further study in each will aid in identifying the distinct mechanisms underlying its pathophysiology.

4.4 NGF signaling in bladder function and dysfunction
The present studies have provided characterization of the changes in NGF signaling in the bladder as a consequence of cystitis and their associated alterations in bladder function. NGF has a well-established role as a pain modulator, rapidly sensitizing peripheral nociceptive terminals through downstream actions including on PI3K- and MAPK/ERK-mediated increases in the trafficking and activation of the pro-nociceptive cation channel TRPV1 through TrkA [104,123,131], cell excitability [128,129], nociceptor hyperinnervation [145–147] on the regulation of various pro-nociceptive genes relevant to the previous [86,112,174], and influencing central sensitization through stimulating CGRP, substance P, and BDNF [86,112,174,182,185,218]. While NGF can
clearly contribute to altered sensation (and, subsequently, altered bladder function) through a number of mechanisms, further characterization of its specific actions in the mouse bladder is warranted given its previously discussed tissue specificity.

Specific examination of the roles NGF signaling plays in regulation of cystitis-implicated protein expression and activation, such as ERK1/2, JNK, p38, MEK, and TRPV4 (non-exhaustively) [79,80,165,219], will help to determine a specific mechanism and identify additional potential targets for clinical intervention. Interventions agonizing or antagonizing targets in the NGF signaling cascade through a variety of means, from genetic to pharmacological, will further determine the necessity and sufficiency of their contributions when paired with protein and behavioral assays. Additionally, the present studies are limited in their scope to the urinary bladder. Both NGF and BDNF, however, are known to contribute to central sensitization as well [60,86,112,174,182,185,218]. Further examination of changes in neurotrophin signaling throughout the LUT, such as in DRG and the spinal cord, will contribute to a more holistic understanding of central contributions to alterations in bladder function and sensation observed with inflammation.

4.5 p75NTR-mediated facilitation of NGF-TrkA actions in the urothelium

Here we demonstrated that acute cystitis was associated with increased urothelial expression of NGF and phosphorylation of ERK1/2 (downstream of TrkA) and JNK (potentially mediated by TrkA, p75NTR, or both [102,220]) and reduced bladder function; furthermore, both TrkA and p75NTR inhibition subsequently reduced cystitis-mediated increases in ERK1/2 and (in the case of p75NTR inhibition) JNK phosphorylation with
associated improvements in bladder function, consistent with the hypothesis that p75\textsuperscript{NTR} facilitates NGF-TrkA actions in the urothelium.

When coexpressed at certain ratios, p75\textsuperscript{NTR} seems to modulate NGF signaling by increasing the affinity and specificity of TrkA for NGF through an unknown mechanism. Previous suggestions have included distinct but converging downstream signaling pathways, ligand passing, and p75\textsuperscript{NTR}-induced conformation changes in the TrkA receptor [109,110,215,221]. Although once believed to occur via the formation of a trimolecular p75\textsuperscript{NTR}-NGF-TrkA high-affinity site, Wehrman et al. [110,215] indicates that, although feasible, this ternary complex does not seem to form under physiological conditions. Numerous studies have demonstrated that NGF must, however, bind p75\textsuperscript{NTR} in order to facilitate NGF-TrkA actions [101,114,215]. This facilitation notably depends heavily on the ratio of TrkA and p75\textsuperscript{NTR} coexpression [101,215]. p75\textsuperscript{NTR} can signal independently of TrkA to promote apoptosis, but activation of TrkA generates an anti-apoptotic signal that dominates over any pro-apoptotic signals [112]. Our finding that p75\textsuperscript{NTR} inhibition also influences ERK1/2 phosphorylation (and potentially other signaling downstream of TrkA) may lend mild support to hypothesized mechanisms of activation of converging downstream signaling pathways and ligand passing over p75\textsuperscript{NTR} induced conformational changes in TrkA regarding p75\textsuperscript{NTR}-mediated facilitation of NGF-TrkA actions.

The notion that NGF actions during cystitis are primarily TrkA-mediated is well-precedented, as previously discussed. Briefly: NGF sequestration prevents the development of hyperalgesia and bladder overactivity in CYP-treated animals [170,172,222], as does pan-Trk inhibition via K252A [165]. TrkA and TrkB
immunoreactivity and phosphorylation are known to increase in the bladder and its afferents following inflammation [76,166], and TrkA is known to regulate nociceptive cation channel TRPV1 expression, trafficking, and activation [104].

Bladder activity has been previously proposed to reflect the balance of NGF-TrkA and NGF-p75NTR signaling [167]; however, our findings here, in accordance with the current understanding of NGF signaling from the literature, may refine this understanding to suggest that it is instead the ratio of TrkA:p75NTR expression in the urothelium that is of particular relevance. Anti-apoptotic signals from TrkA are sufficient to dominate over pro-apoptotic signals from p75NTR [107,112] but only at high TrkA:p75NTR expression ratios [108]; it is unlikely that independent p75NTR actions in the urothelium, where both receptors are coexpressed, influence bladder function. Here we demonstrated significant alterations to the ratio of urothelial TrkA:p75NTR expression under chronic cystitis conditions. In urothelium-specific NGF-OE mice, whole bladder TrkA expression decreased while p75NTR increased [170] — ostensibly altering TrkA:p75NTR expression ratio — which the authors suggest may constitute compensatory, concomitant changes to reduce NGF-mediated increases in urinary frequency [170].

Here, we found that the TrkA:p75NTR expression ratio in the urothelium significantly increased under chronic cystitis conditions. It is unclear if this change contributes to increased NGF-TrkA actions or instead represents natural compensatory changes in response to sustained inflammation and increased NGF signaling. TrkA inhibition improved bladder function under chronic cystitis while p75NTR inhibition reduced it, potentially suggesting the presence of independent p75NTR-actions opposing NGF-TrkA actions. The differing effects of TrkA and p75NTR inhibition on bladder function under
chronic cystitis conditions may alternatively be explained by differing contributions of $p75_{\text{NTR}}$ actions between the urothelium and detrusor made apparent by increased urothelial permeability in the chronic condition. Further study manipulating the ratio of TrkA:$p75_{\text{NTR}}$ expression in the urothelium will clarify changes in the contributions of TrkA and $p75_{\text{NTR}}$ actions to bladder function under different conditions.

4.6 Role of $p75_{\text{NTR}}$ deep to the urothelium

One of the more interesting findings in the present studies was that $p75_{\text{NTR}}$ inhibition via LM improved bladder function under acute cystitis but worsened function under chronic cystitis. This may arise from disruption of compensatory changes in neurotrophin receptor expression under chronic inflammatory conditions or, alternatively/additionally, from deeper penetration of LM into the bladder wall, potentially revealing differing contributions of $p75_{\text{NTR}}$ actions between the urothelium and detrusor to bladder function.

That JNK phosphorylation in the detrusor was significantly increased under the chronic — but not acute — condition provides mild evidence toward the latter. Increased detrusor JNK activation could result from deeper penetration of inflammatory mediators (such as NGF) and mast cells into the bladder wall as a consequence of structural damage to the bladder and urothelial barrier function compromise [223]. p-JNK and p-c-jun expression are notably increased in the bladder muscle layer of IC/BPS patients [223]. Here, observed cystitis-mediated increases in p-JNK expression were notably reduced following LM treatment in association with reduced bladder function under the chronic condition. This is in accordance with a previous study from Klinger and Vizzard [167], which demonstrated that $p75_{\text{NTR}}$ inhibition in acute CYP-treated rats reduced bladder
function only when infused with protamine sulfate, a known disruptor of urothelial barrier function.

Disruption of urothelial barrier function, either by pharmacological methods (such as protamine sulfate in Klinger and Vizzard [167]) or models altering the expression of urothelial targets (such as claudins, antiproliferative factors, and uroplakin II [17,66]) will help to disambiguate between possible contributions to the differential effects of $p75^{NTR}$ inhibition in acute and chronic cystitis. Alternatively, treatment with GAG family components promoting urothelial layer recovery (such as heparin sulfate or hyaluronic acid as demonstrated in mice by Kyker et al. [224]) may lend insight if capable of alleviating $p75^{NTR}$ inhibition-mediated reductions in bladder function under chronic cystitis.

4.7 BDNF actions in CYP-induced cystitis

Hyperalgesia (increased sensitivity to painful stimuli) and allodynia (painful response to normally innocuous stimuli) are hallmark symptoms of IC/BPS, likely as a consequence of central sensitization.

Here, we demonstrated that inhibition of the BDNF conjugate receptor TrkB via ANA improved bladder function under acute cystitis conditions. This is in accordance with previously discussed studies: BDNF expression is increased following turpentine-induced bladder inflammation [164], and its intrathecal administration [204] or overexpression in the bladder wall [203] causes bladder overactivity and increased
expression of TRPV1, TRPA1, and purinergic and cholinergic signaling–related proteins, symptoms reduced by its blockade or sequestration [204].

However, BDNF signaling is even more extensively indicated in peripheral and central sensitization. Peripherally, BDNF is sufficient to produce thermal and mechanical hyperalgesia in rats [188,189] with the latter symptom of rats with sciatic nerve transection being alleviated by treatment with an anti-BDNF antibody [189]. Centrally, BDNF has been demonstrated to stimulate acute, inflammatory, and neuropathic pain [193,194,225], potentially through MEK/ERK- and PLC/PKC-mediated potentiation of excitatory signaling through NDMA phosphorylation, dynamics, and trafficking [185,196]. BDNF is a potent pain modulator [60,185,218] that likely contributes to altered pain sensation with cystitis.

The bladder-centric approach taken here is limited in its ability to account for central changes in BDNF (and NGF) signaling, such as in the DRG and spinal cord. Future studies should also investigate its contributions to altered pain sensation with cystitis. Attempts to measure visceral pain via von Frey filament testing in the present study were hampered by high animal-to-animal variability (unpublished observations) which may prohibitively limit the viability of this technique in mice with CYP-induced cystitis. von Frey filament testing may be more feasible in rats, which are larger and typically demonstrate less variability. Alternatively, visceral pain could be assessed using visceromotor response (VMR) testing, in which electrical activity in response to stimuli is recorded from surgically implanted wire electrodes in the external abdominal oblique muscles [226]. Such studies would substantially expand our understanding of the
contributions of neurotrophin signaling from throughout the LUT not only to bladder dysfunction but also visceral pain in response to inflammatory conditions.

4.8 Clinical implications

The micturition reflex relies on complex neural signaling pathways that coordinate LUT function, sensation, and activation, and, owing to this complexity, can become compromised in many ways. These studies used a preclinical model of CYP-induced cystitis in mice to characterize changes in NGF signaling and function. We demonstrated alterations in bladder function and in the expression and activation of NGF signaling–related proteins following cystitis that were reduced by pharmacological inhibition of the neurotrophin receptors TrkA, TrkB, and p75NTR. Previous anti-NGF trials have been halted due to severe side effects owing to the relative ubiquity of its expression, highlighting the need for additional therapeutic targets [117]. Notably, however, there is some indication that tanezumab, the anti-NGF monoclonal antibody, reduces pain and urinary urgency and frequency in IC/BPS patients [117]. Neurotrophin signaling can alternatively be targeted at the receptor-level. These findings demonstrate that the NGF-receptor TrkA, BDNF-receptor TrkB, and pan-neurotrophin–receptor p75NTR represent potent therapeutic targets in the treatment of inflammatory bladder dysfunction and may be useful in the treatment of IC/BPS and even LUT symptoms in other inflammatory disorders of the bladder.

Changes in NGF signaling in the urine, bladder urothelium, and LUT may additionally provide useful biomarkers for monitoring inflammation or treatment response. Earlier treatment intervention based on elevation of urine or serum proteins
may enable better disease outcomes and progression [227,228]. Increased urine, serum, and tissue expression of NGF has already been noted in IC/BPS patients [38,161–163]. Urinary concentrations of both NGF and BDNF have been demonstrated to decrease in association with subjective improvement in response to treatments in IC/BPS patients [201,207]. Both neurotrophins may serve as biomarkers for IC/BPS, although it is unclear if their upregulation is so specific to IC/BPS that it will allow differential diagnosis from other inflammatory bladder syndromes (such as overactive bladder disorder). Notably, altered p75NTR expression in the urothelium, however, does distinguish IC/BPS from overactive bladder disorder [54]. Here, we observed progressive alterations in TrkA and p75NTR expression in the bladder urothelium with acute and chronic CYP-induced cystitis, raising the possibility that these receptors could offer alternative methods to track IC/BPS progression. This may prove especially prescient given potential compensatory alterations in NGF signaling under sustained conditions of inflammation, such as changes in the urothelial TrkA:p75NTR expression ratio or potential urothelial barrier dysfunction seen here under chronic CYP conditions, that may affect treatment options. Further characterization of alterations in NGF signaling and other neurochemicals in inflammatory bladder syndromes will aid in the development of more specific treatment plans according to specific biomarker profiles.
References for Conclusions


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