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Characterization of the Expression of BDNF and CGRP and their Regulatory Pathways in Dorsal Root Ganglion during Cystitis.

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INTRODUCTION

1.1 Interstitial Cystitis

Interstitial cystitis is a chronic debilitating disease causing inflammation of the urinary bladder. It is characterized by excessive urgency and frequency of urination, suprapubic pain, dyspareunia and chronic pelvic pain in the absence of any identifiable cause (1, 2, 3). Onset of the disease usually occurs between the ages of 30 and 70 years (19). It is a chronic disease that persists throughout a person’s life and after years of remission and relapse, symptoms become more severe and constant (1, 2). The pain can become so intense that it may impact personal and professional aspects of a patient’s life (2). A population study in 1999, estimated the incidence of interstitial cystitis to be approximately 52-67 per 100,000 cases in the United States. They concluded that this estimate was 50% greater than previously reporter and 3-fold greater than that reported in Europe (5). This clearly shows that interstitial cystitis is not a rare disease and actually affects many people. Thus it is important to characterize this disorder to provide better treatment and quality of life to patients.

It is not yet clear what causes cystitis and there are several theories that have attempted to explain the etiology of the disease. One theory postulates that there is a decrease in the glycosaminoglycan component of the mucin layer that covers and protects the bladder urothelium. When this layer is compromised, potential irritating metabolites in the urine are exposed to the urothelium thus causing inflammation (1, 2, 3, 4). Another theory comes from the observation that patients with cystitis had abnormally high amounts of mast cells in the bladder
submucosa. It is postulated that these mast cells produce pharmacologically active mediators that have a significant effect on smooth muscle, vascular epithelium and inflammation (1, 2, 3, 4). A third theory results from histological examination of the bladder showing neural proliferation. Thus giving support for a possible neurogenic etiology of cystitis (2, 3, 4). Since the cause is currently unknown, this makes the clinical diagnosis of the disease particularly challenging. The National Institute of Diabetes and Digestive and Kidney Diseases developed inclusion and exclusion criteria for patients who are being considered for interstitial research (20). This table has helped to streamline the diagnosis of cystitis and thus useful in creating a homogenous population of interstitial cystitis patients in clinical research settings. However, there are several patients with a clinical diagnosis that do not meet the strict criteria (2).

1.2 Autonomic Control of Bladder Micturition

The peripheral nervous system (PNS) consists of sensory (afferent) and motor (efferent) communications with the central nervous system and is divided into the autonomic and somatic nervous systems. Micturition is under the control of both divisions of the PNS. There are two sphincters—internal and external that must relax in order to urinate. The external sphincter is under conscious control and thus under the control of the somatic nervous system. The internal sphincter is under autonomic control and works in conjunction with the smooth muscle found in the bladder wall. Normally smooth muscle contracts when stretched. However, the urinary bladder can stretch to fill with urine and does not spontaneously void. The smooth muscle is constantly contracting, but not simultaneously to allow the release of urine (21). The desynchronized contraction of the smooth muscles is due to the large number of gap junctions between the smooth muscle cells (22). During voiding, the smooth muscle of the bladder wall
must contract simultaneously and at the same time, the sphincters must relax. The nerves of the autonomic nervous system receive sensory information from the bladder wall and send their axons to the spinal cord to communicate with the central nervous system (CNS). The cell bodies of these afferent neurons are found at the dorsal root ganglion located next to the spinal cord. The autonomic nervous system can be further divided into the parasympathetic or sympathetic nervous systems.

The parasympathetic nerves (pelvic) send excitatory input to the bladder wall and inhibitory input to the urethra (23). In contrast, the sympathetic nerves (hypogastric) have excitatory inputs to the bladder neck and the urethra, blocking the passage of urine to be voided (24). There are also inhibitory effects of sympathetic fibers on the parasympathetic input (23). Once a certain threshold pressure is reached, the sympathetic input to the bladder will diminish and the parasympathetic input will become greater allowing for urination via relaxation of the sphincter and contraction of the bladder wall. Micturition is controlled by autonomic innervations as well as somatic. Thus the CNS must receive input from the bladder regarding pressure, tension and other information to determine when to void. Afferent nerve fibers travel within the pelvic, hypogastric and pudental nerves (25,27). There are two main afferents relaying information from the bladder to the CNS. They are small myelinated A-δ fibers and small unmyelinated C fibers (23, 25, 26). The A-δ fibers are mechanosensitive and are active during normal micturition (27, 26). The unmyelinated C fibers do not respond to passive distention, but more to nociception and are not mechanosensitive (28, 26). These fibers have their cell bodies in the DRG and terminate in the spinal cord.
1.3 Sensory Hypersensitivity and Transient Receptor Potential Vanilloid 1

Previous studies have shown that interstitial cystitis (IC) is a hypersensitive state where bladder fullness occurs at lower volumes than normal (29, 30). Normal females have a mean bladder capacity of 586mL and the first voiding desire occurs at a mean volume of 315mL (31). On the other hand, patients with IC have a mean bladder capacity of just 265mL and the first voiding desire at a mean volume of 74mL (30). This difference in mean volumes is due to the hypersensitization of afferent fibers innervating the bladder. This hypersensitization usually occurs after organ injury and contributes to the increase in pain sensation (32, 33). This sensory hypersensitivity explains both the sensation of pain as well as the increase in micturition frequency that is characteristic symptoms of IC.

Another typical feature of IC is the absence of any identifiable clinical cause of the symptoms (1, 34). This is congruent with the theory that IC is a result of sensory hypersensitivity as there are no clinical markers that could test for this. However, there are ways in the lab to determine the sensitivity of afferent fibers innervating the bladder. Two ways to sensitize afferent fibers include alteration of channel properties or increase the expression of the pain receptors (35, 36). Transient receptor potential vanilloid 1 (TRPV1) is a ligand-gated ion channel that can bind capsaicin, the active component of chili peppers, as well as other noxious stimuli and transduces the sensation of pain. It is a key molecule for integrating painful stimuli (37). Studies have shown that TRPV1 plays an important role in several diseases associated with pain such as: interstitial cystitis, airway diseases, inflammatory bowel disease and pancreatitis (38-41). TRPV1 knock-out mice have also been studied and determined that this receptor is important in the modulation of pain during inflammatory conditions and tissue damage (42, 43). There are neurons located in the spinal afferents that respond only to noxious stimulus and
remain silent under normal conditions. These nociceptors can be activated when there is tissue injury or inflammation (47). TRPV1 positive cells are primarily located in small and medium sized DRG cells, which are characteristic of nociceptive fibers (44). Also TRPV1 has been shown to increase in expression in these nociceptive fibers when there is peripheral inflammation (45, 46). The expression of TRPV1 channels increases with diseases exacerbated by sensory hypersensitivity. Activation of TRPV1 also contributes to the development of bladder hyperreflexia and nociceptive transmission to spinal dorsal horn neurons in cystitis (7).

1.4 Brain Derived Neurotrophin Factor and Sensory Hypersensitivity

It has previously been reported that functional digestive disorders are associated with a decrease in visceral pain threshold thus indicating visceral hypersensitivity (8, 9) and interstitial cystitis is no different. Brain derived neurotrophin factor (BDNF) was chosen to be characterized in this model of CYP-induced cystitis. BDNF was originally isolated from pig brain and is a 12.4 kDa basic protein, part of the neurotrophin family (6). The neurotrophin family consists of nerve growth factor (NGF), BDNF and neurotrophin (NT)-3 and NT-4/5. Each neurotrophin has affinity for a particular receptor: NGF to tropomyosin-related kinase (Trk) A (11, 12), BDNF and NT 4/5 to TrkB (13) and NT-3 to TrkC (48). All three Trk receptors are expressed within adult DRG (49,50) and all neurotrophins show retrograde transport to the DRG from peripheral nerves (51). TrkB, the receptor specific for BDNF and NT 4/5, is abundant during development, but also present in the CNS of adult animals thus indicating a continuing role for BDNF in the adult nervous system. In the early stages of development, it has been shown through the use transgenic mice that BDNF plays a key role in promoting the survival of some sensory neurons during development (52).
Another role of BDNF is its role in pain sensitivity. Numerous studies show that BDNF is a modulator of pain in the inflammatory and neuropathic pain. The role BDNF plays in the transmission of pain was discovered when there were significant increases in the level of BDNF expression in the spinal cord after tissue or nerve injury (53, 54). BDNF was also found to be a key player in the central sensitization that underlies many forms of hyperalgesia (55). BDNF’s role in the transmission of pain was confirmed when experimental manipulation of either BDNF or the receptor, TrkB, significantly altered nociceptive processing (56). Specifically in inflammatory pain, BDNF has shown to be an important modulator. Lung hypersensitivity in mice appears to be partially mediated by BDNF (57) and during pancreatitis, BDNF is expressed in significantly higher levels in ductular complexes and in the perineurium of enlarged nerves (58). The pancreatic content level of BDNF was also strongly correlated with pain intensity in patients suffering from the condition (58).

Up-regulation of BDNF in the DRG seems to be modulated in a NGF dependent mechanism (59). This is also true for peripheral inflammation models. BDNF mRNA and protein were both upregulated by an NGF dependent mechanism in TrkA-expressing sensory neurons (59). Recent studies investigating the role of intracellular signaling pathways in the development of inflammatory and neuropathic pain have shown that the MAP kinases ERK and p38 are important mediators of BDNF up-regulation and in the changes in pain-related behavior (60). Indeed phosphorylation of the MAPK/ERK in the spinal dorsal horn neurons has been proven to be an important step in the transmission of inflammatory pain (61). Thus any changes to the signaling pathway could play a role in regulating the expression of BDNF in nociceptive neurons in the DRG.
1.5 Sensory Expression of Calcitonin Gene-Related Peptide

There are two forms of calcitonin gene-related peptide (CGRP), namely αCGRP and βCGRP that were isolated from the thyroid and other tissues (62, 63). The effects of the two types of CGRP are through similar signaling cascades—they activate adenyl cyclase and increase the intracellular cAMP concentration (64). Both of the two forms are present in peripheral tissues and in the nervous system (65). CGRP is especially rich in the DRG, trigeminal ganglion neurons and fibers originating from the ganglion projecting to the spinal cord and the brainstem (62, 63). Within the DRG, CGRP is found within small-diameter unmyelinated neurons and significantly less in the medium- to large-diameter neurons (62, 63). The neuropeptides is also prevalent in the primary afferent neurons (66, 67). There are two classes of CGRP receptors: CGRP1 and CGRP2 (62, 64, 65). The receptor is composed of three components: calcitonin receptor-like receptor (CRLR), receptor activity modifying protein 1 (RAMP1) and CGRP receptor component protein (RCP). All three proteins must be present in order to form a functional CGRP receptor (68).

CGRP plays a role in several areas including: involved in learning and memory (69), involved in opioid tolerance and addiction (70) and finally it is involved in the transmission and modulation of pain related information in PNS and CNS (71). Recently there has been increasing evidence that suggests that CGRP function and release are associated with inflammation (72). αCGRP KO mice failed to develop secondary hyperalgesia after inflammation (73)—thus indicating CGRP’s role in hypersensitivity following inflammatory diseases. There is a significant upregulation of CGRP in primary afferent neurons within the first new hours after application of the inflammatory stimulus (72).
1.6 Nerve Growth Factor in Inflammatory Pain Transmission

Nerve growth factor (NGF) is part of the neurotrophins family that plays a critical role in the development of the nervous system. It binds preferentially to the TrkA receptor (74). A null mutation of the NGF gene or the TrkA gene shows a loss of approximately 70% of primary sensory neurons (75). This indicates NGF plays an important role in the survival of the sensory neurons during development. However, other studies have shown that NGF plays an important role in maintaining and modulating the properties of sensory neurons during adult life as well (75). Once NGF binds to the TrkA receptor, there are several signaling pathways that are activated. Two examples are the PI3-K/Akt pathway and the MAPK pathway (10).

Several studies show that NGF levels increase in tissues during inflammation and modulates primary sensory neuron activity. Levels of NGF mRNA were increased in the inflamed bladder (76) and sequestration of this NGF prevented the expected increase in hyperreflexia in the bladder (16). Thus NGF proved to play a critical mediator in sensory disorders associated with inflammation (16). Not only can increases in NGF lead to hypersensitivity of the tissue producing the NGF, it has been shown that it can also contribute to the development of hypersensitivity in neighboring organs and cutaneous referral sites (18). During cystitis, NGF expression increased in the major pelvic ganglion of male rates (17). This increase may have been due to retrograde transport as there is evidence for increased retrograde transport of neurotrophins to DRG sensory neurons and spinal motor neurons after peripheral nerve injury (77). Thus NGF clearly plays a role in painful somatic and visceral inflammation pathways. It has been proposed this is through altered expression of TRPV1 or voltage-gated sodium channels that play a central role in inflammation or tissue injury-induced pain and hypersensitivity (78).
1.7 Signaling Pathways Mediated by Akt/PKB

Akt is a serine/threonine kinase, also called protein kinase B that is activated in the phosphoinositide-3 kinase (PI3-K) signaling cascade. The PI3-K pathway generates several second messengers (79) that activate various kinases, such as Akt and extracellular signal-regulated kinases (ERK). Although Akt is only one of the kinases activated in the PI3-K pathway, it has been postulated to mediate most of PI3-K’s effects (15). Because the PI3-K pathway activates several kinases, it has been proven to be involved in several signaling cascades and cellular processes (80).

Akt has several downstream effects including: cell survival, cell growth, cell proliferation, cellular metabolism and angiogenesis (15). Several different groups have shown that Akt plays a critical role in promoting cell survival downstream of growth factors, oncogenes and cell stress (81). The PI3-K pathway is also activated by several factors. Specifically it is activated by vascular endothelial growth factor (82), and the downstream effects contribute to endothelial cell growth, survival and proliferation via transcription of certain genes. Akt also activates endothelial nitric oxide (NO) synthase (eNOS), which releases the NO, and can stimulate vasodilation, vascular remodeling and angiogenesis (83). It may also be involved in cross-talk with other pathways. For example it has been reported that the PI3-K/Akt pathway activates NFκB survival signaling or inhibit the Jun N-terminal Kinase (JNK)/p38 apoptotic signaling (84, 85).

The PI3-K signaling cascade not only plays a role in cell survival and growth, but it has also been proven to be a key mediator of central sensitization in painful inflammatory conditions (14). The PI3-K pathway can be activated by growth factors such as NGF—specifically in DRG neurons (86) and Akt’s activation in the periphery can lead to pain behaviors induced by
capsaicin (87). Thus the PI3-K pathways involving Akt plays an important role in not only the sensitization of the spinal cord, but also pain behaviors.
OBJECTIVES & AIMS

Objectives
The objective of this study was to characterize the expression level of neuropeptides in sensory neurons during cystitis and determine the mechanism and signaling pathways that regulate their expression. Our hypothesis is that inflammatory mediators in the bladder cause sensory hypersensitivity by regulating transcription of different neuropeptides in sensory neurons in the dorsal root ganglia (DRG).

Aim 1: Characterize the Neuropeptides BDNF and CGRP in DRG during Cystitis

- Examine time and segment-dependent changes in BDNF and CGRP immunoreactivity in lumbosacral DRG during cystitis.
- Examine the transcription levels of BDNF and CGRP in DRG during cystitis.

Aim 2: Characterize the Signaling Pathways Involved in BDNF and CGRP Expression in DRG during Cystitis.

- Examine the level of Akt phosphorylation in DRG during cystitis.
- Characterize the correlation of phospho-Akt and BDNF/CGRP by examining their co-localization in the DRG.
Aim 3: Determine the Role of NGF Retrograde Signaling in BDNF and CGRP Expression in the DRG
RESEARCH DESIGN & PROTOCOLS

3.1 Research Design

3.1.1 CYP-induced Cystitis and Tissue Dissection

Animals were injected with CYP to induce cystitis. Following either 8 or 48 hours post injection, animals were sacrificed and tissue were removed and prepared for analysis with RT-PCR or immunohistochemistry (Figure 2A).

3.1.2 Nerve-Ganglia Retrograde Analysis

L6 DRG and ganglia were removed from control animals and placed into a two-compartmented chamber. The nerve terminal was treated with specific ligand and following incubation with the ligand, the DRG was removed and analyzed with immunohistochemistry to view changes in protein expression (Figure 2B, and 10).

3.2 Research Protocols

3.2.1 Experimental Animals

Adult male Sprague Dawley rats (100-120g) were purchased from Harlan Laboratories (Dublin, VA). All experimental protocols involving animal use were approved by the Institutional Animal Care and Use Committee at the Virginia Commonwealth University. Animal care was in accordance with the Association for assessment and Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. All efforts were made to
minimize the potential for animal pain, stress or distress, as well as to reduce the number of animals used.

### 3.2.2 Induction of Bladder Inflammation and Tissue Dissection

Cyclophosphamide (CYP) (Sigma Aldrich, St. Louis, MO) was used to induce cystitis in rat models. CYP-induced cystitis animals received single drug injections (150mg/kg, intraperitoneal) and were allowed to survive for 8 and 48 hours. Control rats received volume-matched injections of saline (0.9%, intraperitoneal). All injections were performed under isoflurane (2%) anesthesia. For real-time PCR (see below), the DRGs were removed from animals after sacrifice via thoracotomy. (Figure 1, Figure 2A) Some of the DRG were removed along with the attached spinal nerves for two-compartment incubation. (Figure 2B) For immunohistochemistry, the animals were first perfused with Krebs buffer followed by 4% paraformaldehyde. The L1 and L6 DRGs were removed for cryosectioning.

### 3.2.3 Real Time-Polymerase Chain Reaction (RT-PCR)

L1 and L6 DRGs were homogenized in lysis buffer included in the RNAqueous kit (Ambion, Carlsbad, CA). The total RNA then underwent reverse transcription using qScript (Quanta, Gaithersburg, MD) according to manufacturer’s instruction. The cDNAs were mixed with specific Taqman probes and PCR master mix (Applied Biosystems, Foster City, CA). The real-time PCR was performed with 7300 Systems (Applied Biosystems, Foster City, CA). The tested target genes include brain derived neurotrophin factor (BDNF), calcitonin gene-related peptide (CGRP) transient receptor potential vanilloid receptor 1 (TRPV1) and nerve growth factor (NGF). β-Actin or GAPDH was used as an internal control. The changes in the target
genes were normalized against the internal control and then were calculated using the ΔΔCt method.

### 3.2.4 Immunohistochemistry

**BDNF, CGRP, VR-1 and phospho-Akt Immunoreactivity**

DRGs were sectioned at a thickness of 20µm and placed onto gelatin-coated slides for immunostaining. The slides were first incubated with antibodies against BDNF (1:500, Santa Cruz, CA), CGRP (1:1000, Abcam, Cambridge, MA), VR-1 (1:500, Chemicon, Billerica, MA) or phospho-Akt (1:400, Cell Signaling, Billerica, MA) for 48 hours at 4ºC in 5% donkey normal serum and 0.3% Triton buffer. After washing with 0.1M sodium phosphate buffer (pH7.4) for 3x 10 mins, the slides were then incubated with Cy3-conjugated species-specific secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 2 hours at room temperature. After another round of washing with 0.1M sodium phosphate buffer (3 x 10 mins), the slides were cover-slipped with Citiflour anti-fadent mounting medium (Electron microscopy Science, Hatfield, PA). Prepared slides were viewed with an Axiocam Carl Zeiss microscope. DRG cells exhibiting immunoreactivity that was greater than the observed background level were considered positively stained.

**Co-staining: BDNF, CGRP and phospho-Akt, TrkA, TrkB and phospho-Akt**

For double-staining procedures, DRG sections were processed as described for BDNF, CGRP, TrkA or TrkB. Next these same sections were processed for phospho-Akt. Photos were merged on Adobe Photoshop to determine whether or not the molecules were co-localized in sensory neurons.
3.2.5 Ganglia-Nerve Preparation for Two-compartmented Chamber

The DRG and attached spinal nerve were dissected out and placed into a two-compartment chamber with the ganglia in one compartment and the nerve terminal in the other compartment (Figure 10). The preparation was acutely incubated in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA) for 2 hours in a cell culture incubated with atmosphere of 37°C and 10% CO₂ (Napco Series 8000WJ; Thermo Scientific, Suwanee, GA). The nerve terminals were then stimulated with 50ng/ml nerve growth factor (NGF) (Alomone Labs, Jerusalem, Israel) for 3 hours. After stimulation, the DRG was removed and prepared for analysis with RT-PCR in the manner as described above.

3.2.6 Statistical Analysis

Data are presented as fold changes, unless otherwise stated. Data was assessed by repeated-measures ANOVA (one-way ANOVA), followed by Dunnett’s test for three or more groups. When only two groups were tested, a student’s t-test was used for analysis using GraphPad (San Diego, CA) Prism, version 4. Levels of statistical significance were set at *p<0.05.
RESULTS

4.1 Activation of DRG Neurons during Cystitis

To determine whether sensory neurons in the DRG were activated during cystitis, the mRNA expression level of TRPV-1, a pain receptor, was examined. The mRNA expression level of TRPV-1 was examined in L6 DRG of control animals and animals treated with CYP for 48 hours using RT-PCR. There was a significant 2.5 fold increase (p<0.05) in the level of TRPV-1 mRNA in animals 48 hours post-CYP injection (Figure 3A). TRPV-1 immunohistochemistry revealed that positively stained neurons were small-diameter, unmyelinated neurons (Figure 3B).

4.2 Time and Segment-dependent Changes in BDNF and CGRP Immunoreactivity in Lumbosacral DRG during Cystitis

BDNF and CGRP protein expression levels were characterized in the cystitis time course model using immunohistochemistry. Analysis revealed that the protein expression level of BDNF in L1 and L6 DRG significantly increased (p<0.05) at the 48-hour time course when compared to the control (Figure 4). Immunohistochemistry analysis of CGRP protein expression level revealed a decrease in CGRP in L1 DRG following 8 and 48 hours post-CYP injection. In L6 DRG, there was a significant increase in CGRP protein expression at the 48 hours post-CYP injection time point (Figure 5).
4.3 BDNF and CGRP were Transcribed by DRG Neurons

BDNF and CGRP mRNA expression levels were characterized in the cystitis time course model using RT-PCR. RT-PCR revealed no significant change in CGRP mRNA levels in L1 DRG 48 hours post-CYP injection when compared to the control. In L6 DRG however, there was a significant 5.0 fold increase (p<0.05) in CGRP mRNA levels 48 hours post-CYP injection when compared to control (Figure 6). RT-PCR analysis revealed that there was a significant 1.6-fold increase (p<0.05) of BDNF mRNA in L1 DRG following 48 hours post-CYP treatment. In L6 DRG, there was a significant 1.6-fold increase (p<0.05) in 48 hours post-CYP treatment compared to the control (Figure 7). These results mirrored the changes in protein expression levels examined by immunohistochemistry. This data showed that BDNF and CGRP protein transcription was by DRG neurons.

4.4 Akt was Activated in DRG Neurons during Cystitis

L1 and L6 DRG were dissected out of control animals and animals treated with CYP for 48 hours. To determine if phospho-Akt was involved in the signaling pathway, immunohistochemistry was performed. Immunohistochemistry analysis showed that in both L1 and L6 DRG, there was a significant increase (p<0.05) in the amount of phospho-Akt at 48 hours post-CYP injection (Figure 8).
4.5 NGF mRNA Expression in the Urinary Bladder of Control and CYP-induced Cystitis Animals

NGF mRNA expression levels in the urinary bladder were characterized in a CYP-induced cystitis time course model using RT-PCR. Analysis revealed that at both 8 hour and 48 hours post CYP-induced cystitis, the expression of NGF mRNA significantly increased when compared to the control (Figure 9). This increase in mRNA expression was constant with two internal controls: β-Actin and GAPDH.

4.6 Effects of Retrograde NGF on BDNF and CGRP Expression in DRG

NGF and its receptor TrkA both increased significantly in the urinary bladder after induction of cystitis. Thus to test whether it could possibly retrograde signal to the DRG, L6 ganglia and the spinal nerve were dissected out and only the nerve terminal was treated with 50ng/ml of NGF for 3 hours (Figure 10). Immunohistochemistry after incubation with NGF revealed that BDNF (Figure 11) and CGRP (Figure 12) protein expression levels both increased significantly (p<0.05).

4.7 Co-localization Study of p-Akt with BDNF or CGRP in DRG

Co-staining of CGRP and BDNF with phospho-Akt was performed to determine whether the Akt pathway was involved in the activation of the neuropeptides. The results showed that phospho-Akt was positively co-stained with BDNF, but not with CGRP (Figure 13). This indicated that there might be a relationship between Akt and BNDF. However, its relationship with CGRP may be indirect or via other signaling molecules and needs further investigation.
Figure 1. Rat spinal cord and dorsal root ganglia dissection. Lumbar dorsal root ganglia L1-L6 and sacral dorsal root ganglia labeled.
Figure 2. Animal models used for tissue dissection. A. CYP-induced cystitis animals sacrificed after designated time and tissues collected for immunohistochemistry and RT-PCR. B. Nerve-ganglia prep dissected out and nerve terminal treated with NGF.
A

CYP injected IP to induced cystitis

↓

L1 and L6 DRG dissected out after treatment time

RT-PCR IHC

B

L6 DRG and nerve prep dissected out

Placement into two-compartment chamber

Treatment with ligand
Figure 3. TRPV1 mRNA expression changes with cystitis in DRG. A. Immunohistochemistry of DRG, revealed positive staining of TRPV1 within small diameter neurons. Arrows indicated positively stained cells. B. TRPV1 mRNA levels in L6 DRG significantly increased following 48 hours post CYP-induced cystitis. Bar = 100µ. *, p<0.05. Results are from 4-5 animals for each experimental group.
A

B

L6 TRPV-1

TRPV1 mRNA (Fold Change)

control

48h CYP

*
**Figure 4.** BDNF immunohistochemistry in L1 and L6 DRG 8 and 48 hours post induction of cystitis with CYP.  A.  BDNF immunoreactivity in DRG from control animals and animals treated with CYP.  Arrows indicate positively stained cells.  B.  There were significant increases in the number of neurons positively stained for BDNF in L1 DRG at 48 hours post CYP-induced cystitis.  C.  There was also a significant increase in the number of neurons expressing BDNF following 48 hours post CYP-induction of cystitis in L6 DRG.  Bar = 100µ.  *, p<0.05.  Results are from 5 animals for each experimental group.
Figure 5. CGRP immunohistochemistry in L1 and L6 DRG 8 and 48 hours post induction of cystitis with CYP. A. CGRP immunoreactivity in DRG from control animals and animals treated with CYP. Arrows indicated positively stained cells. B. There was a significant decrease in the number of neurons positively stained for CGRP in L1 DRG at 48 hours post CYP-induced cystitis but not 8 hours. C. In L6 DRG, there was a significant increase in the number of neurons expressing CGRP following 48 hours post CYP-induction of cystitis. Bar = 100µ. *, p<0.05. Results are from 5 animals for each experimental group.
Control CGRP

A

48h CYP CGRP

B

L1 CGRP

C

L6 CGRP

*
Figure 6. Expression of CGRP mRNA in L1 and L6 DRG at 8 and 48 hours post CYP-inducted cystitis. A. There was no significant change in CGRP mRNA levels in L1 DRG post CYP treatment. B. Significant increase in the level of CGRP mRNA following 48 hours post CYP-induced cystitis in L6 DRG. *, p<0.05. Results are from 5-6 animals for each experimental group.
**A**

L1 CGRP

CGRP mRNA (fold change)

control 48h CYP

0.00 0.25 0.50 0.75 1.00

**B**

L6 CGRP

CGRP mRNA (fold change)

control 48h CYP

0 1 2 3 4 5 6 7 8

*
Figure 7. BDNF mRNA level changes in L1 and L6 DRG at 48 hours post CYP-induced cystitis. A. There was a significant change in BDNF mRNA levels in L1 DRG 48 hours following CYP-induced cystitis. B. Significant increase in the amount of BDNF mRNA following 48 hours post CYP-induced cystitis in L6 DRG. *, p<0.05. Results are from 5-6 animals for each experimental group.
A

L1 BDNF

BDNF mRNA (fold change)

control 48h CYP

0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75

B

L6 BDNF

BDNF mRNA (fold change)

control 48h CYP

0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75

*
Figure 8. Phospho-Akt immunoreactivity in L1 and L6 DRG in control animals and cystitis animals. Arrows indicate positively stained cells. A. Positively stained phospho-Akt neurons in DRG in control and CYP-induced cystitis animals. B. The number of neurons positively expressing phospho-Akt protein significantly increases at 48 hours post CYP-induced cystitis compared to control. Bar = 100µ. *, p<0.05. Results are from 3 animals for each experimental group.
A

B

L1 p-Akt

number of p-Akt-IR neurons/mm$^2$

control 48h CYP

C

L6 p-Akt

number of p-Akt-IR neurons/mm$^2$

control 48h CYP
Figure 9. NGF mRNA levels in the urinary bladder at 8 and 48 hours post CYP-induced cystitis. A. NGF mRNA significantly increased in the bladder, at both 8 and 48 hours post CYP-induced cystitis when β-actin was used as internal control. B. NGF mRNA significantly increased at both 8 and 48 hours post CYP-induced cystitis when GAPDH was used as internal control. *, p<0.05. Results are from 3 animals for each experimental group.
A

NGF mRNA
actin-control

NGF mRNA
(fold change)

control CYP 8h CYP 48h

B

NGF mRNA
GAPDH-control

NGF mRNA
(fold change)

control CYP 8h CYP 48h
**Figure 10.** Preparation of nerve-ganglion 2-compartmented chamber for retrograde signaling study. L6 and its attached spinal nerve was removed and placed into the 2-compartmented chamber with the nerve terminal in one compartment and the DRG into another. 50ng/ml of NGF was added only to the compartment containing the nerve terminal without contaminating the compartment containing the DRG. After stimulation, the DRG was collected for examination.
Figure 11. NGF retrograde effects on the protein expression level of BDNF in DRG. A. BDNF immunoreactivity of control and NGF treated BDNF. B. When 50ng/ml of NGF was applied to the nerve terminal, there was a significant increase in the protein level expression of BDNF in the DRG when compared to the non-treated DRG. Bar = 100µ. *, p<0.05. Results are from 3 animals for each experimental group.
A

Control BDNF

NGF BDNF

B

Retrograde BDNF

number of BDNF-IR neurons/mm²

control

+ NGF

*
Figure 12. NGF retrograde effects on the protein expression level of CGRP in DRG. CGRP immunoreactivity of control and NGF treated CGRP. B. When 50ng/ml of NGF was applied to the nerve terminal, there was a significant increase in the protein level expression of CGRP in the DRG when compared to non-treated DRG. Bar = 100µ. *, p<0.05. Results are from 3 animals for each experimental group.
Retrograde CGRP control + NGF

![Control CGRP](image1)

![NGF CGRP](image2)

**B**

Retrograde CGRP

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<th>control</th>
<th>+ NGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of CGRP-IR neurons/mm²</td>
<td></td>
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<tr>
<td></td>
<td>80 ± 10</td>
<td>120 ± 15</td>
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</tbody>
</table>

*p < 0.05*
Figure 13. Co-localization immunohistochemistry of BDNF and CGRP with phospho-Akt in L6 DRG following 48 hours post CYP-induced cystitis. BDNF (A1) and CGRP (A2) positively stained cells in DRG following cystitis. In the DRG following cystitis, there was also p-Akt positively stained cells (B1 & B2). Co-localization revealed that BDNF co-localized with p-Akt (C1), but not with CGRP (C2).
DISCUSSION

Interstitial cystitis is a chronic debilitating disease that causes pain and increased frequency of micturition, amongst other symptoms, without any identifiable cause. Within the United States alone, it has been estimated that there are approximately 52-67 per 100,000 cases (5). Thus it is important to characterize this disease in order to provide better treatment and quality of life to patients. The present study aimed to characterize BDNF and CGRP—two neuropeptides that have both been proven to play an important role in the transmission of pain (57, 58) as well as in hypersensitivity (55). The signaling pathways regulating the expression of the two neuropeptides were also examined.

Previous studies have shown that interstitial cystitis (IC) is a hypersensitive state where bladder fullness occurs at lower volumes than normal (30). Two ways to sensitize afferent fibers include alteration of channel properties or increase the expression of the pain receptors (35). To determine whether or not sensory afferent fibers were activated, the expression level of TRPV1 was examined in L6 DRG in animals 48 hours after CYP-induced cystitis. RT-PCR results revealed 2.5-fold increase (p<0.05) in TRPV1 mRNA following cystitis induction. Immunohistochemistry results also revealed that the expression of the channel was in small diameter unmyelinated neurons. These small diameter neurons were most likely C-fibers relaying nociceptive information (28). Activation of these nociceptive fibers indicated that bladder inflammation induced increased pain sensation. Further examination of the changes in
the sensory neurons in the DRG revealed that two neuropeptides, BDNF and CGRP, were also involved in the bladder inflammation-induced increased sensory plasticity.

Immunohistochemistry showed significant increases in BDNF in small to medium diameter neurons in both L1 and L6 DRG at 8 hour and 48 hour following CYP treatment. BDNF and its receptor have both proven to be important in the transmission of nociception (56). Specifically in inflammatory pain, BDNF has proven to be an important modulator of hypersensitivity (57, 58). Thus increases in BDNF in the lumbosacral DRG following cystitis, illustrates that BDNF plays an important role in the inflammatory pain transmission during cystitis. In addition, cystitis also changed the expression level of the excitatory neurotransmitter CGRP in the DRG during cystitis. Immunostaining of CGRP revealed that in L6 DRG of animals following 8 and 48 hours post CYP-induction of cystitis; the CGRP protein level was significantly increased, while in L1 DRG CGRP protein expression was decreased. One explanation could be that cystitis induced CGRP release from these neurons because there was increased density of CGRP nerve fibers in the DRG during cystitis. Another possibility could be due to degradation of CGRP.

In order to determine whether BDNF and CGRP were being transcribed by the DRG or if they were being transported to the DRG, RT-PCR was done to determine mRNA levels of each neuropeptide. RT-PCR results showed no significant change in CGRP mRNA levels in L1 DRG 48 hours post-CYP injection when compared to the control. Together with the findings that CGRP immunoreactivity was decreased in L1 DRG during cystitis. It suggests that CGRP might be transported out of the DRG to nerve terminals. During cystitis, there were an increased number and density of CGRP nerve fibers in the spinal cord. CGRP could also be internalized and then degraded upon binding to its receptor in the neurons. In L6 DRG however, there was a
significant 5.0 fold increase (p<0.05) in CGRP mRNA levels 48 hours post-CYP injection when compared to control. BDNF mRNA levels were also increased significantly by 1.6-fold (p<0.05) in L1 DRG and L6 DRG following 48 hours post-CYP treatment. This data reveals that BDNF and CGRP were both generated by the sensory neurons in DRG.

The PI3-K/Akt pathway is important for cellular growth and survival (15). However, recent studies also indicated that this pathway has a role in central sensitization in painful inflammatory conditions (14). The up-regulation of PI3-K/Akt pathway has been seen in the spinal cord during peripheral inflammation and inhibition of this pathway attenuated inflammatory pain in rats (14). In cystitis, it is not clear if the PI3-K/Akt pathway has a role in sensory activation. The current study revealed that the level of phospho-Akt was increased in lumbosacral DRG of animals following CYP-induced cystitis. Immunohistochemistry results revealed a 1.5-fold increase in the level of phospho-Akt in both L1 and L6 following 8 and 48 hours post CYP-induced cystitis. These results confirmed that indeed the PI3K/Akt pathway was activated during cystitis. To determine the association of this signaling pathway with the transcription of BDNF and CGRP, co-localization staining was performed. The results revealed that BDNF, but not CGRP was co-localized with phospho-Akt. This suggests that the activation of the PI3K/Akt pathway was specifically correlated with BDNF expression but not CGRP expression in the DRG during cystitis. There were two possibilities: 1) Transcription of BDNF was regulated by PI3-K/Akt activation, and 2) BDNF activates PI3K/Akt pathway via an autocrine mechanism. The former requires activation of PI3-K/Akt by factors regulated by cystitis, possibly retrograde NGF signaling originating from the bladder; the latter requires the expression of TrkB in the phospho-Akt/BDNF co-expressing neurons. The finding that CGRP did not co-localize with phospho-Akt suggests that CGRP transcription may be activated via
another signaling pathway in the CGRP neurons or the PI3-K/Akt pathway is not directly upstream of CGRP activation.

To further investigate the mechanism underlying BDNF and CGRP expression in the DRG during cystitis, the L6 ganglia/nerve two-compartment preparation was made to study the effects of retrograde NGF signaling in this system. NGF was chosen based on several studies that showed that NGF levels increase in tissues during inflammation and modulates primary sensory neuron activity. During bladder inflammation, the levels of NGF mRNA were also increased in the inflamed bladder (76) and sequestration of this NGF prevented the expected increase in hyper-reflexia in the bladder (16). In patients, the level of NGF also significantly increased following idiopathic sensory urgency and interstitial cystitis (89). Nerve-ganglia preparations were carried out and 50ng/ml NGF was applied solely to the nerve terminal compartment. The changes in BDNF and CGRP expression were examined in the DRG using immunohistochemistry. Results revealed that both CGRP and BDNF were significantly increased in the DRG when the terminal was incubated in 50ng/ml of NGF. Previous studies show that NGF retrograde signaling is activated following peripheral nerve injury (77). NGF increases in the inflamed bladder binds to its receptor, TrkA and activates bladder afferent neurons via retrograde transport. In the in-vitro cell culture studies, it was revealed that two pathways were involved in NGF retrograde signaling: the MEK/ERK5 pathway and the PI3-K/Akt pathway. Given that PI3-K/Akt was co-localized with BDNF in the DRG during cystitis, it is possible that the excess level of NGF up-regulated in the inflamed urinary bladder could retrograde transport to DRG and then activate PI3-K/Akt pathways, leading to BDNF expression in these neurons. CGRP up-regulation in the DRG by cystitis could be due to pathways other than PI3-K/Akt, such as the ERK5 pathway in the DRG.

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In summary, the present study characterized two neuropeptides—BDNF and CGRP and found that protein expression level increased in L1 and L6 DRG following CYP-induction of cystitis, except no significant change in CGRP in L1. Furthermore, we determined that both neuropeptides were being actively transcribed by observing increases in mRNA levels. One possible signaling pathway involved is the PI3K/Akt pathway as we observed increases in phospho-Akt levels following CYP-induced cystitis. Other signaling pathways were also activated during cystitis such as the ERK5 pathway (88). Retrograde studies revealed that application of NGF to the nerve terminal was able to increase protein expression levels of both BDNF and CGRP. These results underlie a possible mechanism that excess NGF arising in the urinary bladder may have a significant role in bladder afferent activation, thus leading to bladder sensory hypersensitivity. Up-regulation of TRPV1, BDNF and CGRP in the DRG during cystitis further supports the notion that bladder inflammation causes sensory hyperactivity, and results in painful bladder sensation seen in IC.
Literature Cited


Sharon J. Yu was born on August 20, 1985 in San Francisco, CA. She graduated from Lucy Ragsdale High School in 2003. She received her Bachelor of Science in Biology with a minor in Chemistry and Religious Studies from The University of North Carolina at Chapel Hill in 2007. Subsequently she worked at Central Dermatology Center in Chapel Hill for two years.