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Beyond Neurons: Involvement of Urothelial and Glial Cells in Bladder Function

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Abstract

Aim—The urothelium, or epithelial lining of the lower urinary tract (LUT), is likely to play an important role in bladder function by actively communicating with bladder nerves, smooth muscle, and cells of the immune and inflammatory systems. Recent evidence supports the importance of non-neuronal cells that may extend to both the peripheral and central processes of the neurons that transmit normal and nociceptive signals from the urinary bladder. Using cats diagnosed with a naturally occurring syndrome termed feline interstitial cystitis (FIC), we investigated whether changes in physiologic parameters occur within 3 cell types associated with sensory transduction in the urinary bladder: 1) the urothelium, 2) identified bladder dorsal root ganglion (DRG) neurons and 3) grey matter astrocytes in the lumbosacral (S1) spinal cord. As estrogen fluctuations may modulate the severity of many chronic pelvic pain syndromes, we also examined whether 17β-estradiol (E2) alters cell signaling in rat urothelial cells.

Results—We have identified an increase in nerve growth factor (NGF) and substance P (SP) in urothelium from FIC cats over that seen in urothelium from unaffected (control) bladders. The elevated NGF expression by FIC urothelium is a possible cause for the increased cell body size of DRG neurons from cats with FIC, reported in this study. At the level of the spinal cord, astrocytic GFAP immuno-intensity was significantly elevated and there was evidence for co-expression of the primitive intermediate filament, nestin (both indicative of a reactive state) in regions of the FIC S1 cord (superficial and deep dorsal horn, central canal and laminae V-VIl) that receive input from pelvic afferents. Finally, we find that E2 triggers an estrus-modifiable activation of p38 MAPK in rat urothelial cells. There were cyclic variations with E2-mediated elevation of p38 MAPK at both diestrus and estrus, and inhibition of p38 MAPK in proestrous urothelial cells.

Conclusion—Though urothelial cells are often viewed as bystanders in the processing of visceral sensation, these and other findings support the view that these cells function as primary transducers of some physical and chemical stimuli. In addition, the pronounced activation of spinal cord astrocytes in an animal model for bladder pain syndrome (BPS) may play an important role in the pain syndrome and open up new potential approaches for drug intervention.

Keywords

bladder epithelium; nerve growth hormone; sensor function; spinal cord astrocytes

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INTRODUCTION

Though the urinary bladder urothelium has classically been thought of as a passive barrier to ions/solutes, recent studies have revealed that these cells exhibit specialized sensory and signaling properties.[1] Evidence suggests that the urothelium exhibits both "sensor" (expressing receptors/ion channels capable of responding to thermal, mechanical, and chemical stimuli) as well as "transducer" (ability to release chemicals) properties. Modification of the urothelium in a number of pathologic conditions can result in altered release of urothelial-derived mediators, which can activate local sensory nerves. Additionally, our finding that 17-β-estradiol triggers an estrus-modifiable activation of p38 mitogen-activated protein kinase (MAPK) in bladder urothelial cells suggests that signaling by these cells can be altered by hormonal stimuli.

These functions also may be altered by diseases affecting the urinary bladder. For example, elevated levels of neurotrophin (NT) nerve growth factor (NGF) have been reported in a number of bladder pathologies, including bladder pain syndrome/interstitial cystitis (BPS/IC).[2–4] In cats diagnosed with a comparable disease (feline interstitial cystitis, FIC), we report increased urothelial expression of NGF and a trend toward neuronal dorsal root ganglion (DRG) hypertrophy. The importance of non-neuronal cells may extend to both the peripheral and central processes of the neurons that transmit normal and nociceptive signals from the urinary bladder. In this article, we report significant changes in the immunointensity and morphology of FIC spinal cord glial cells (astrocytes) in regions to which pelvic afferents project. These and other findings suggest that alterations in "non-neuronal cell" signaling may contribute to the sensory abnormalities in a number of pelvic disorders.

ANATOMY AND BARRIER FUNCTION OF THE UROTHELIUM

The urothelium is the epithelial lining of the lower urinary tract (LUT) between the renal pelvis and the urinary bladder (Fig. 1). It is composed of at least three layers: a basal cell layer attached to a basement membrane; an intermediate layer; and a superficial or apical layer composed of large hexagonal cells (25–250 μm in diameter) known as "umbrella cells."[5,6] The umbrella cells are covered on nearly 70–80% of their apical surface by crystalline proteins called uroplakins, which assemble into hexagonal plaques.[7,8] Uroplakins and other urothelial cellular differentiation markers, such as cytokeratin-20, are not expressed in the stratified epithelium of the urethra.[9] In some species, the umbrella cells, and perhaps also the intermediate cells, extend projections to the basement membrane.[7,10]

The ability of the bladder to maintain its barrier function despite large alterations in urine volume and increases in pressure during bladder filling and emptying is conferred by several unique features of the umbrella cells. These features include tight-junction complexes composed of multiple proteins, such as the claudins, which reduce the movement of ions and solutes between cells and specialized lipid molecules, and uroplakin proteins in the apical membrane, which reduce the permeability of the cells to small molecules (water, urea, protons).[7,8,11] A sulfated polysaccharide glycosaminoglycan (GAG) or mucin layer covers the apical surface. This layer is thought to act as a non-specific anti-adherence factor and as a defense mechanism against infection.[9,12]

During bladder filling, the umbrella cells become flat and squamous. This shape change is accompanied by vesicular traffic (i.e., exocytosis/endocytosis) that adds additional membrane to the apical surface. The resultant overall increase in surface area allows the bladder to accommodate increasing volumes of urine during filling without compromising its barrier function.[13–16] There is evidence that stretch-induced exocytosis depends on activation of the epidermal growth factor receptor (EGFR).[17] There is also interest in the

role of exocytosis/endocytosis (vesicular recycling) in modulating urothelial release of neurotransmitters/mediators, as well as a multitude of receptors, channels, and other proteins expressed by urothelial cells.

ROLES FOR UROTHELIAL CELLS IN VISCERAL SENSATION

Although urothelial cells are often viewed as bystanders in the process of visceral sensation, recent evidence has supported the view that these cells function as primary transducers of some physical and chemical stimuli and are able to communicate with underlying cells, including bladder nerves, smooth muscle, and inflammatory cells.

There are at least three lines of evidence suggesting that urothelial cells participate in the detection of both physical and chemical stimuli. First, recent studies have shown that bladder nerves (sensory afferent and autonomic efferent) are localized in close proximity to, and some within, the urothelium. Peptidergic, P2X-, and TRPV1-immunoreactive nerve fibers presumed to arise from afferent neurons in the lumbosacral DRG are distributed throughout the urinary bladder musculature as well as in a plexus beneath, and extending into, the urothelium.[1,18] In humans with neurogenic detrusor overactivity (NDO), intravesical administration of resiniferatoxin, a C-fiber afferent neurotoxin, reduces the density of TRPV1 and P2X3 immunoreactive suburothelial nerves, indicating that these are sensory nerves.[19,20] Studies have also revealed evidence that both adrenergic (tyrosine hydroxylase) and cholinergic (vesicular choline transporter, VAChT) nerves are in close proximity to the urothelium.[21,22]

A network of cells with morphologic characteristics similar to those of myofibroblasts or interstitial cells are also detected in the suburothelial space of the bladder in both humans and animals.[23,24] These cells, which are extensively linked by gap junctions and have close contacts with nerves, can respond to neurotransmitters such as adenosine triphosphate (ATP) released from nerves or urothelial cells, suggesting that they could act as intermediaries in urothelial–nerve interactions.[23–26] Thus, the anatomic substrates for bidirectional urothelial–neural communication exist within the urinary bladder.

A second line of evidence suggesting that urothelial cells play a role in sensory function is the expression of numerous receptors/ion channels that are linked to mechano- or nociceptive sensations. Examples of neuronal “sensor molecules” (receptors/ion channels) that have been identified in the urothelium include receptors for purines ($P2X_{1–7}$ and $P2Y_{1,2,4}$), adenosine (A_1, A_{2a}, A_{2b}, and A_3), norepinephrine (α and β), acetylcholine (muscarinic and nicotinic), protease-activated receptors (PARs), amiloride mechanosensitive channels (ENaC), bradykinin (B1 and B2), NTs (p75, trkA, EGF family ErbB1-3), corticotrophin-releasing factor (CRF2), estrogens (ERα and ERβ), endothelins, and various TRP channels (TRPV1, TRPV2, TRPV4, TRPM8, and TRPA1).[17,18,27–38] The expression of these various receptors enables the urothelium to respond to a number of “sensor inputs” from a variety of sources. These inputs include increased stretch during bladder filling; soluble factors (many found in the urine), such as EGF, or chemical mediators/peptides/transmitters, such as substance P (SP), calcitonin gene-related peptide (CGRP), CRF, acetylcholine, adenosine, or norepinephrine released from nerves; inflammatory cells; and even blood vessels.[1,7,14]

The third line of evidence suggesting a sensory role for urothelial cells is the secretion of a number of transmitters or mediators capable of modulating, activating, or inhibiting sensory neurons. These include NTs, peptides, ATP, acetylcholine, prostaglandins, prostacyclin, nitric oxide (NO), and cytokines.[1,14,37,39] For example, studies have shown that urothelial-derived NO can be released in response to mechanical and chemical stimulation and may either facilitate or inhibit the activity of bladder afferent nerves.[1,40] There are also studies

that demonstrate that the urothelium, via release of various soluble factors, can modulate the spontaneous activity of the underlying smooth muscle.[25,41] However, the mechanism underlying this release, including whether all sensory "inputs" stimulate membrane turnover (i.e., vesicular exocytosis) is not well understood. What little is known about the roles and dynamics of membrane-bound cytoplasmic vesicles in urothelial cell physiology is taken from measurements of membrane capacitance and microscopy of fixed tissues and cells. In this regard, there is evidence that once released, ATP can act as an important autocrine mediator that can induce membrane turnover and enhance both stretch-induced exocytosis and endocytosis.[42] Alterations in membrane turnover can not only increase apical surface area (as described above) but also influence the number and function of receptors and channels at the cell surface.

UROTHELIAL SENSOR TARGETS AND MEDIATORS: ROLE IN PATHOLOGY

ATP and the Urothelium

Since the first report of distension-evoked ATP release from the urothelium,[33] there is now abundant evidence supporting a role for urothelial-derived release of ATP in autocrine and paracrine signaling within the LUT. ATP is released from both the apical and basolateral urothelial surfaces in response to bladder stretch and can act via stimulation of $P2X_2$ and $P2X_3$ urothelial receptors to stimulate exocytosis.[42] The expression of both P2X and P2Y receptors in nerve fibers and myofibroblasts in close proximity to the bladder lumen, and the sensitivity of these cells to ATP, suggest that basolateral ATP release from the urothelium may also influence function of myofibroblasts and bladder nerves.[43,44] There is also evidence that the amiloride-sensitive apical sodium channel, ENaC, may be involved in mechanotransduction by controlling basolateral release of ATP.[45] In addition, intercellular communication mediated by gap junctions in myofibroblasts could provide a mechanism for long-distance spread of signals from the urothelium to the detrusor muscle.[25] Adenosine is also produced and released by the urothelium and may play an important role in modulating sensory afferent function and smooth muscle contraction.[37]

Pathology results in augmented release of ATP from the bladder urothelium, which can cause painful sensations via excitation of purinergic receptors on nearby sensory fibers.[31,46] This type of non-cholinergic mechanism is likely to play an important role in a number of bladder pathologies such as BPS/IC, which is chronic and characterized by urgency, frequency, and bladder pain upon filling.[47–50] Consistent with findings in patients with BPS/IC,[46] studies of FIC in cats also revealed an augmented stretch-evoked release of urothelium-derived ATP and changes in purinergic receptor profiles in urothelial cells,[31,51] suggesting that urothelial sensor molecules are altered by disease.

Acetylcholine and the Urothelium

There is evidence that the urothelium expresses the full complement of muscarinic receptors, as well as the enzymes necessary for the synthesis and release of acetylcholine.[39,52] Further, the urothelium is able to release acetylcholine following both chemical and mechanical stimulation.[39] Once released, urothelial-derived acetylcholine is likely to exert effects via a number of sites including smooth muscle and nerves, as well as urothelial-associated muscarinic (or nicotinic) receptors; the latter could contribute to feedback mechanisms modifying urothelial function.[39,53] In addition, stimulation of urothelial cholinergic receptors elicits release of mediators such as NO and ATP, which could alter bladder sensation by stimulating nearby sensory afferent nerves.[54] Release of urothelial-derived mediators can also induce spread of Ca^{2+}-transients that begin near the urothelial–suburothelial interface and spread to the detrusor smooth muscle, raising the possibility that

the urothelium may initiate the generation of spontaneous, non-voiding contractions in the urinary bladder.[25,26] Thus, targeting muscarinic receptors and/or urothelial release mechanisms may play an important role in the treatment of a number of bladder disorders. Accordingly, recent evidence suggests that botulinum toxins prevent the release of transmitters from the urothelium, which may suggest that urothelial-released mediators contribute to sensory urgency.[55]

Estrogen and the Urothelium

The steroid hormone 17β-estradiol (E2) is a key regulator of growth, differentiation, and function in a wide array of target tissues. Its predominant biologic effects are mediated through two distinct estrogen receptors (ER); ERα and ERβ.[56] In addition to the well-established classical ("genomic") pathway, which involves interaction with an estrogen response element on the promoter region of the target gene, E2 can exert rapid "non-genomic" effects,[57] which involve putative estrogen-binding proteins in the cell membrane and cytoplasm.[58] Rapid effects of estrogen involve activation of distinct signal transduction cascades, such as the MAPK pathways. The p38 MAPK pathway can be activated in response to chemical and physical stress and has therefore been termed a "stress-activated kinase."[59]

To investigate the possibility that E2 may trigger an estrus-modifiable activation of p38 MAPK in the urothelium, which expresses both ERα and ERβ,[60] urothelial cells isolated from rats in diestrus, proestrus, and estrus phases[61] were treated with vehicle (control) or E2 (17-β estradiol; 10 nM; 60 min). As shown in Figure 2, we noted that treatment with E2 resulted in an increase in p38 MAPK phosphorylation that was significant in urothelial cells isolated from estrus rats ($P < 0.05$ compared to unstimulated cells). We also saw some increase in p38 MAPK phosphorylation in diestrus rats, but a slight decrease in proestus rats. During proestrus, there is a surge in estrogen followed by progesterone[61] that could desensitize the response to E2. We hypothesized that the inhibitory effect we saw in proestrus rats may be related to a recent finding of an inhibitory role for the G protein-coupled receptor 30 (GPR30) in mediating E2-induced effects in human urothelial cells.[62,63] Our finding of a phase-related p38 MAPK activation may suggest autocrine activation, possibly mediated by NGF, with a cyclical variation peaking in the proestrus phase. Further studies are needed to elucidate the full range of the influences of alterations in ovarian hormones on LUT structure and function, which potentially may be important in a number of bladder dysfunctions, such as urethra and pelvic floor weakness, detrusor instability, bladder pain syndrome, and even underactive detrusor.

UROTHELIAL RESPONSE TO INJURY

A variety of local and distant events can result in damage to the urothelium. For example, local factors, such as tissue pH, mechanical or chemical trauma, or bacterial infection can degrade the barrier function.[64,65] Basal cells, which are thought to be precursors for other cell types, normally exhibit a slow (3–6 month) turnover rate—in fact, the slowest turnover of any mammalian epithelial cells.[10,64] While neither urine-derived factors nor cyclic mechanical changes contribute to urothelial differentiation, injury readily accelerates proliferation. For example, protamine sulfate, which selectively damages the umbrella cell layer, rapidly induces both proliferation and differentiation to restore the barrier.[66a] The initiation of urothelial proliferation is also thought to involve up-regulation of growth factors. Besides NGF,[66b] fibroblast, epidermal, and transforming growth factors have been shown to initiate urothelial proliferation.[67,68]

BPS/IC, spinal cord injury, and even external environmental events can also change the urothelial barrier.[65,69–72] When the barrier is compromised, water, urea, and toxic

substances can pass into the underlying tissue (neural/muscle layers) resulting in urgency, frequency, and pain during bladder filling and voiding. In some pathologic conditions, the disruption of the urothelial barrier is associated with ultrastructural changes and alterations in the levels of chemical mediators, such as NO and ATP. Disruption of urothelial-barrier integrity has also been linked to the expression of substances such as anti-proliferative factor (APF), a frizzled-8 protein detected in the urine of patients with BPS/IC. Bladder epithelial cells obtained from these symptomatic patients also secrete APF.[73,74] Further, purified APF treatment of urothelial cells from normal patients decreases the expression of adhesion and tight junction proteins and slows urothelial growth.

Disruption of urothelial barrier function by more remote pathologic conditions may be mediated by neural and/or hormonal mechanisms. For example, spinal cord transection in rats leads to a rapid alteration in the urothelial barrier, including ultrastructural changes and increased permeability.[69] The changes were blocked by pretreatment with a ganglionic blocking agent, suggesting an involvement of efferent autonomic pathways on bladder urothelium in the acute effects of spinal cord injury. Based on recent reports that various stimuli induce urothelial cells to release chemical mediators that can in turn modulate the activity of afferent nerves, other types of urothelial–neural interactions are also likely.[1,7] This has raised the possibility that the urothelium may have a role in sensory mechanisms in the urinary tract.

NGF also influences bladder responses to injury. NGF belongs to the family of NTs, which in mammals include the NT-3, NT-4/5 and brain-derived neurotrophic factor (BDNF).[75] The biologic effects of NTs are mediated by a common pan-NT low-affinity receptor ($p75^{NTR}$) and three high-affinity tyrosine kinase-transducing receptors (trkA, trkB, trkC). NGF-induced signals are mediated by p75 and trkA receptors. Activation of $p75^{NTR}$ alone is reported to promote apoptosis, whereas NGF promotes cell survival by acting on trkA receptors.[76] NGF was the first NT to be characterized, based on its ability to stimulate growth, differentiation, survival, and maintenance of peripheral sensory and sympathetic neurons during development and after injury.[77] In adults, NGF modulates the sensitivity and plasticity of the nociceptive system.[78–80] It also plays a role in central sensitization in nociception, which is likely to involve the synthesis and release of nociceptive peptides including SP and CGRP.[81,82]

In the periphery, NGF is produced and utilized by several non-neuronal cell types including immune inflammatory cells, epithelial cells, and smooth muscle cells; because of these functions, NGF is better described as a pleiotropic factor.[83] A specific “epitheliotrophic”[84] role for NGF is seen in the epidermis, where it is endogenously synthesized and plays an important role in wound healing.[85,86] Numerous inflammatory cytokines, including interleukin (IL)-1, tumor necrosis factor (TNF)-α and IL-6, can induce NGF production in non-neuronal cell types, such as fibroblasts, endothelial cells, and glial cells.

NGF levels are increased in the bladder smooth muscle and urothelium in animal models for bladder outlet obstruction, spinal cord injury, and cyclophosphamide-induced inflammation.[87,88] Increased NGF also has been identified in the urine of patients with overactivity and idiopathic sensory urgency, and in those diagnosed with BPS/IC.[89] In fact, NGF has been proposed as a potential biomarker for certain bladder disorders due to the possible link between elevated NGF levels in tissue and urine and overactivity and painful inflammatory conditions.[90] An antibody to NGF is currently in phase II clinical trials (ClinicalTrials.gov identifier NCT00601484) for BPS/IC-associated pain.[91]

As mentioned, cats diagnosed with FIC exhibit a number of similarities to BPS/IC in humans. We therefore compared NGF levels and associated receptors in urothelium from

healthy and affected cats. Our investigation revealed a significant ($P < 0.05$) between-group difference, with higher NGF levels in FIC urothelium than in control (normal) urothelium (Table I). In addition, we also found increased expression of the trkA receptor and, to a lesser extent, in the low affinity p75 receptor (Table I). The trend for a greater increase in trkA expression over p75 suggests that NGF autocrine activity may be involved in urothelial cells survival and possibly in repair of the urothelium. In animals, increased target NGF expression has been linked with increased urinary frequency and unstable bladder contractions, and intravesical NGF produces a hyperreflexia suggestive of inflammatory pain, likely by sensitizing bladder afferents.[92,93] Though the mechanism has not been established, our previous reports of increased spontaneous smooth muscle contractility and increased mechanical hypersensitivity in FIC support a possible role for augmented NGF.

Influence of Target-Organ Expression of NGF

NGF is internalized by the nerve endings of NGF-dependent neurons and is retrogradely transported to the neuronal soma.[94] The presence of NGF and its associated receptors in the neuronal somata of subpopulations of adult sensory neurons has been reported.[95] NGF has been associated with a number of features of neuroplasticity, including augmented cell size (following uptake from the target organ). Previous studies in cats with FIC have revealed an increased sensitivity to the TRPV1 agonist, capsaicin, and an increase in membrane capacitance (suggesting an increase in cell size).[96] To determine if augmented mucosal NGF is associated with hypertrophy of identified DRG neurons, we examined the cell body diameter profile in bladder DRG neurons from FIC and normal cats. As shown in Figure 3, there was a rightward-shift in the cell soma diameter profile in the pathologic state. NGF is reported to cause an enlargement in the perikarya of adult DRG cells both in vitro[94] and in vivo.[97] The physiologic significance of the observed increase in DRG cell-body size in our findings is unknown but might occur to accommodate a larger cytosolic environment necessary for increased metabolic processes driven by NGF and other trophic factors.

One effect of increased NGF expression in the FIC bladder could be to increase sensitivity of the urothelial cells and sensory afferents. For example, NGF is reported to increase the intracellular content of SP in sensory neurons,[98,99] and also can increase SP expression/release in non-neuronal cells. The expression of the neuropeptide SP was significantly higher in FIC urothelium (Table I), which may be as a consequence of the higher expression levels of NGF. We previously have reported increased excitability of low-threshold bladder afferents to distension and changes in electrical properties of neurons from FIC cats.[100] Taken together, these findings may explain, in part, changes in bladder afferent activity in chronic bladder disorders such as BPS/IC.

MODULATION OF NEURONAL SIGNALING BY SPINAL CORD GLIA

Recent evidence has shown that spinal cord activation of glial cells (astrocytes and microglia) may be involved in both the development and maintenance of central sensitization in various chronic pain conditions.[101] At the level of the spinal cord, the first relay site in the transmission of nociceptive information from the periphery to the brain,[102,103] dorsal horn glial cells may be activated by neuropeptides/neurotransmitters released from primary afferent nerve terminals, such as SP, CGRP, NO, purines, glutamate, opioid peptides, and the chemokines fractalkine or neuractin. Activation may result in altered cell morphology, changes in receptor expression, or release of factors by glial cells, which in turn can lead to changes in neuronal function and ultimately enhance nociceptive transmission.[101] There is evidence that microglia may mediate the activation of astrocytes seen in both somatic and visceral pain pathologies—the "neuropathic pain triad."[104] Generally, microglial activation is transient, while astrocytic activation is much longer lasting. However, activation of either of the two cell types promotes pain.[101,105] Although

most reports of the contribution of glia to nociception come from studies of somatic rather than visceral forms of chronic pain, there is now increasing evidence pointing to a role for glial cells as key modulators during visceral inflammatory pain.

Cats with FIC offer an opportunity to examine potential roles for glial cells in chronic bladder pathology. We compared the immune-intensity of glial fibrillary acidic protein (GFAP; an intermediate filament protein found in the astrocytes) of healthy versus FIC cats in lower lumbrosacral (S1) spinal cord regions, which receive sensory afferent input from pelvic viscera. As shown in Figures 4 and 5, there were significant ($P < 0.05$) increases in GFAP immuno-intensity (Fig. 4) and changes in morphology (Fig. 5a,b, representative images) in all regions of interest—the superficial and deep dorsal horn, near the central canal (lamina X), and laminae V–VII. This is an indicative of a reactive state of the astrocytes that is further supported by co-expression of GFAP and nestin, a more primitive intermediate filament[106] in FIC spinal cord astrocytes (Fig. 5c, representative image). Astrocytes contribute to the regulation of local ion (notably K^+) and pH homeostasis. In addition, these cells are involved in the clearance of synaptically released neurotransmitters, such as glutamate and GABA.[107] These multifunctional cells are key players in what is termed the tripartite synapse, which comprises pre- and post-synaptic neurons and extra-synaptic astrocytic contacts. These cells have the potential to modify synaptic transmission and plasticity.[108] Alterations in the physiology of astrocytes in response to inflammatory conditions, such as BPS/IC, may enhance synaptic transmission, laying the “physiologic” groundwork for the state of chronic pain. Pharmacologic interventions aimed at targeting neuronal–glial and glial–glial interactions may provide new targets for pharmacologic management of a number of bladder disorders.

SUMMARY

Recent studies suggest that there may be a number of common or shared mechanisms in bladder pain syndrome associated with other functional pain syndromes.[47] For example, patients with bladder pain syndrome also suffer a variety of co-morbid symptoms and disorders, which can include irritable bowel syndrome (IBS), endometriosis, vulvodynia, fibromyalgia, rheumatoid arthritis, and even asthma. Though the etiology of these syndromes is incompletely understood, several factors may play an important role, such as changes in urothelial or epithelial sensor/barrier function, neurogenic inflammation, and even autoimmune involvement.[109,110] There is evidence that defects in urothelial sensor molecules and urothelial-cell signaling are likely to contribute to the pathogenesis of bladder diseases.

Changes in epithelial signaling/barrier function are not unique to the urinary bladder. For example, airway epithelia in asthmatic patients, as well as keratinocytes in certain types of skin diseases, also exhibit a number of similar abnormalities and compromise repair processes.[111] This is particularly relevant given the high incidence of associated diseases that can include both visceral and somatic conditions, many of which exhibit a shared loss of epithelial barrier function. In addition, glial cells (astrocytes and microglia) placed at the interface of communication between the periphery and the CNS may be major players in resetting and modulating these lines of communication with deleterious effects. Taken together, these non-neuronal cells can respond to a number of challenges, including environmental pollutants and mediators released from nerves or nearby inflammatory cells, resulting in altered expression and/or sensitivity of various receptors/channels, as well as changes in release of mediators, all of which could impact function.

METHODOLOGY

Animals

All procedures were conducted in accordance with Institutional Animal Care and Use Committee policies. Healthy and FIC adult cats were used for this study. All cats with FIC were obtained as donations from clients due to a history of chronic recurrent stranguria, hematuria, pollakiuria, and/or urination in inappropriate locations and were evaluated at The Ohio State University (OSU) Veterinary Teaching Hospital. Evaluation consisted of a complete physical examination (including body weight), complete blood count, serum biochemical analysis, urinalysis, urine bacteriological culture, and cystoscopy. Cystoscopy was performed using a 9-F rigid pediatric cystoscope (Karl Storz, Endoscopy America, Culver City, CA) in female cats and a 3-F flexible fiber optic cystoscope (Five Star Medical, San Jose, CA) in male cats. The diagnosis of FIC was based on compatible history and consideration of standard National Institutes of Health inclusion and exclusion criteria after the results of the above laboratory tests were obtained, including the presence of submucosal petechial hemorrhages (glomerulations) at cystoscopy. Healthy, age-matched cats obtained from commercial vendors and determined to be free of disease and signs referable to the LUT according to the same diagnostic criteria as cats with FIC were used as controls. All cats were housed in stainless steel cages in the OSU animal facilities and allowed to acclimate to their environment for at least 3 months before the study. For identification of bladder DRG somata, usual surgical aseptic precautions were employed to expose the bladder dome, which was injected with a retrogradely transported fluorescent dye (4% fast blue), in deeply anesthetized cats. Two to four weeks after injection, the animals were anesthetized and sacrificed via intracardiac perfusion first with Krebs buffer followed by 8% paraformaldehyde fixation. Serial frozen (10 μm) sections were mounted on slides (superfrost). The diameter of fast-blue labeled neurons in a ganglion was estimated from counts at moderate-to-high magnification of positively stained profiles in a minimum of 10 sections separated by 100 μm. All cell counts are presented as the mean number of identified neurons per 10 sections ± SEM from each ganglion. For the size distributions shown in Figure 3, the values reported are the percentage of identified neurons counted.

Tissue Preparation

Whole rat bladder and the bladder mucosa were homogenized separately in HBSS (5 mM KCl, 0.3 mM KH_2PO_4, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose, and 10 mM Hepes, pH 7.4) containing complete protease inhibitor cocktail (1 tablet/10 ml, Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma, 1:100). The homogenate was centrifuged (13,000*g*; 15 min). The membrane protein fraction was prepared by suspending the membrane pellets in lysis buffer containing 0.3 M NaCl, 50 mM Tris–HCl (pH 7.6), and 0.5% Triton X-100 and the same concentration of protease inhibitors as above. The suspensions were incubated on ice and centrifuged (13,000 rpm; 15 min at 4°C). The protein concentrations of the combined supernatants were determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL).

NGF ELISA

Cytosol lysates were diluted with 4 volumes of D-PBS (0.02% KCl, 0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, 0.0133% $CaCl_2$ $2H_2O$, and 0.01% $MgCL_2$ $6H_2O$, pH 7.35, Sigma). Following acidification (1 N HCl to pH 2.0–3.0) and neutralization (1 N NaOH to pH 7.5–8.0), the samples were assayed in duplicate by ELISA (Promega, Madison, WI) according to manufacturer instructions. Plates were read at 450 nm using a SpectraFluor Plus (Tecan, Maennedorf, Switzerland). The tissue NGF values were normalized against the protein concentrations of each sample and are expressed as pg/mg protein.

TrkA and p75 Immunoblotting

After denaturation (100°C for 5 min), lysate from each sample was separated on an SDS–PAGE gel using a standard Western protocol. Proteins were transferred to polyvinylidene fluoride membranes, blocked with 5% Milk TBS-T (1 hr), rinsed in TBS-T, and incubated (overnight at 4°C) with primary antibody [rabbit anti-TrkA or p75, Santa Cruz (Santa Cruz, CA) and Upstate (Waltham, MA), respectively] in 5% Milk TBS-T. The membranes were then incubated with secondary antibody (anti-rabbit IgG HRP, Santa Cruz) for 1 hr in 5% Milk TBS-T, developed with ECL Plus (Amersham, Piscataway, NJ), and exposed to film. The volume of each band was determined using a Personal Densitometer SI (Molecular Probes, Carlsbad, CA). The membranes were stripped (membrane recycling kit from Alpha Diagnostic International, San Antonio, TX) and reprobed overnight with rabbit anti-β-actin (Abcam, Cambridge, MA) as a loading control. Two immunoreactive bands (140 and 70 kDa) were observed by Western immunoblotting for TrkA. Both bands were blocked by the peptide; therefore, the volumes of both bands were totaled in our analysis. A single immunoreactive band was observed for p75 (75 kDa) and for β-actin (43 kDa).

p38 Immunoblotting

After denaturation at 100°C for 5 min, lysate from each sample was separated on an SDS–PAGE gel using a standard Western protocol. Western blots were probed with rabbit anti-phospho-p38 (Cell Signaling, Beverly, MA) diluted in 5% BSA TBS-T, followed by the secondary antibody, goat anti-rabbit IgG HRP (Santa Cruz) diluted in 5% Milk TBS-T. The membranes were stripped using a membrane recycling kit from Alpha Diagnostic International and reprobed using rabbit anti-p38 (in 5% BSA, Cell Signaling). Finally, the Western blots were stripped a second time and reprobed using rabbit anti-β-actin (Abcam Limited, Cambridgeshire, UK) and goat anti-rabbit IgG HRP (Santa Cruz). The volume of each band was determined using a Personal Densitometer SI (Molecular Probes, Eugene, OR) and a single immunoreactive band was observed for phospho-p38 at 38 kDa.

Substance P

The mucosa was stripped from underlying smooth muscle in both FIC and normal urinary bladder. Tissues were placed in 0.5 M boiling acetic acid for 10 min and homogenized with a Tissue Tearor (BioSpec Products, Brattlesville, OK). After centrifugation (14,000*g*, 15 min) the SP was extracted from the supernatants using C-18 SPE cartridges following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). SP was measured in a SP EIA kit from Cayman Chemical and is expressed as pg/mg wet tissue weight.

Immunohistochemistry

The lower lumbosacral spinal cord (S1) was dissected from deeply anesthetized (induction with 2% halothane; maintained with α-chloralose 60–70 mg/kg) cats diagnosed with FIC (n = 4) and healthy cats (n = 4). After removal of tissue, animals were humanely sacrificed and the spinal cord was post-fixed, placed in 30% sucrose solution, and subsequently frozen. Transverse cryo-sections (6 μm) were prepared and mounted on glass slides (Fisher Scientific, Pittsburgh, PA) in preparation for immunohistochemistry. GFAP (1:200, Sigma) antibody was used to identify astrocytes. Reactive astrocytes were identified by positive staining for Nestin (1:100, Abcam). FITC- and CY3-conjugated secondary antibodies were used to visualize binding. DAPI fluorescent stain (1:5,000, Molecular Probes) was used to detect cell nuclei. Non-specific staining was assessed in the absence of primary antibodies. Tissue was viewed on a Zeiss Axioplan microscope with an attached Leica DC 200 digital camera. Images were visualized, saved, and subsequently analyzed using C. Imaging software (Hamamatsu, PA).

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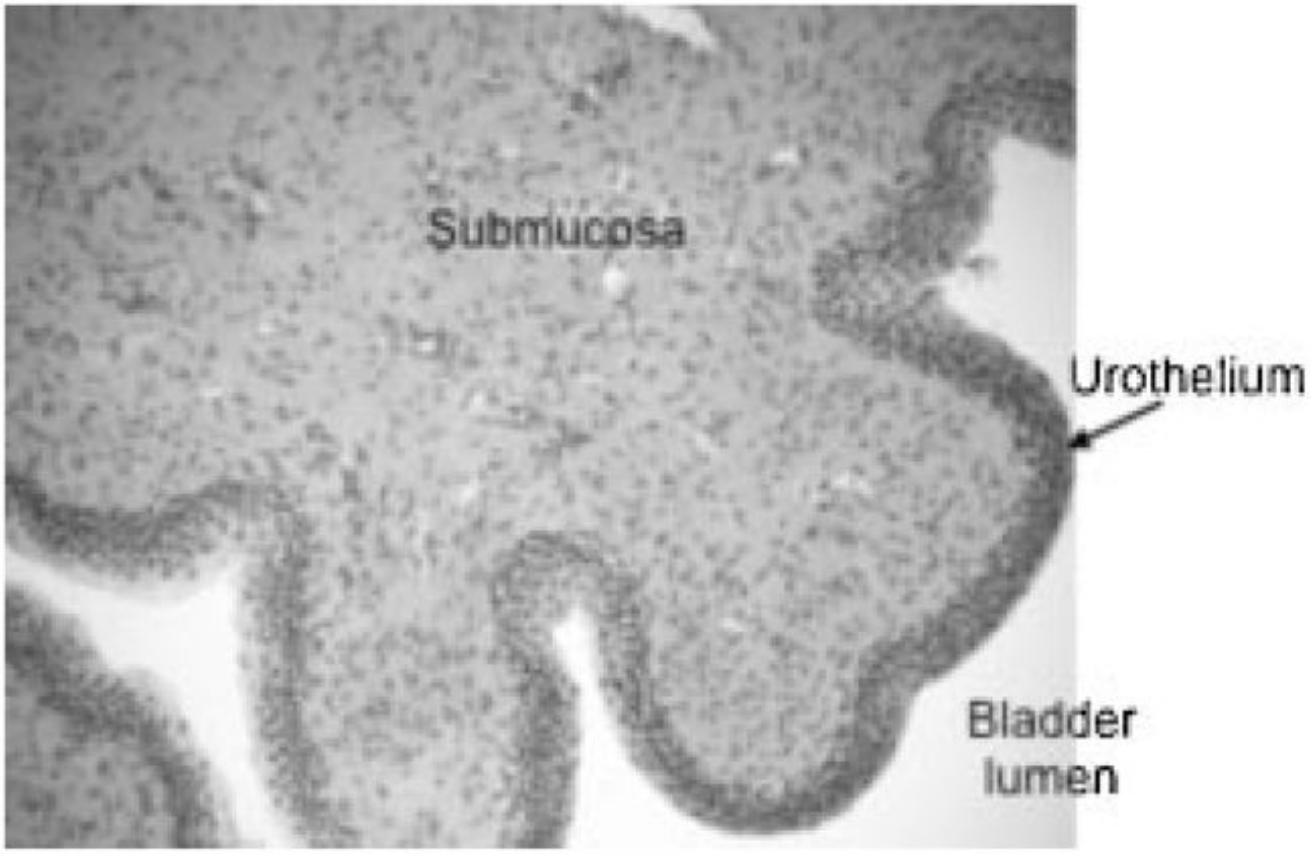

Fig. 1.
Cross-section of urinary bladder wall. H&E stained cross-section of the cat urinary bladder wall.

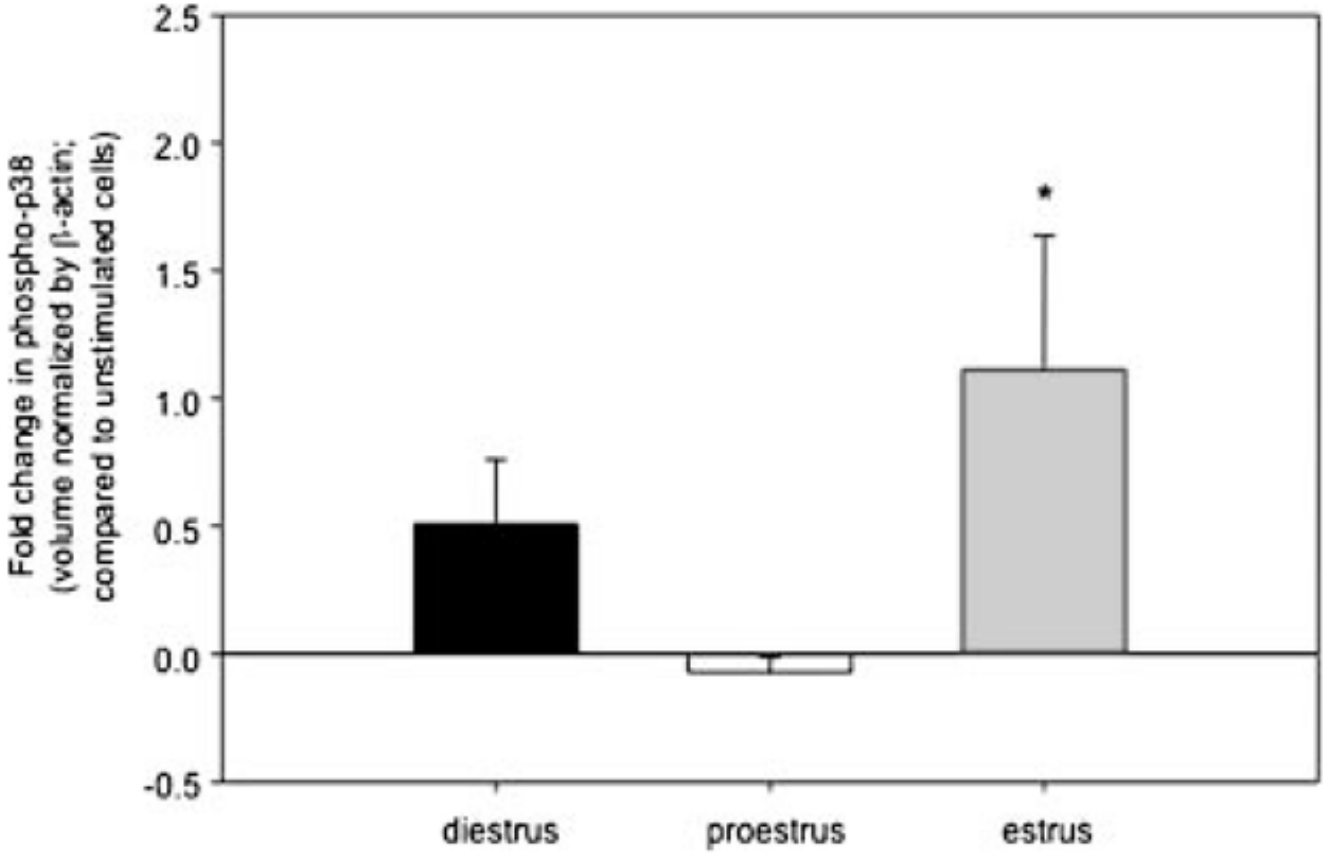

Fig. 2.

E2 triggers an estrus-modifiable activation of p38 MAP kinase in rat urothelial cells. Urothelial cells isolated from rats in diestrus, proestrus, and estrus phases were treated with 10 nM of β-estradiol (or vehicle, saline) for 60 min. Levels of phospho-p38 in cellular whole cell lysates were determined after Western blotting. **P* < 0.05; Mann–Whitney rank sum test; n = 2–4.

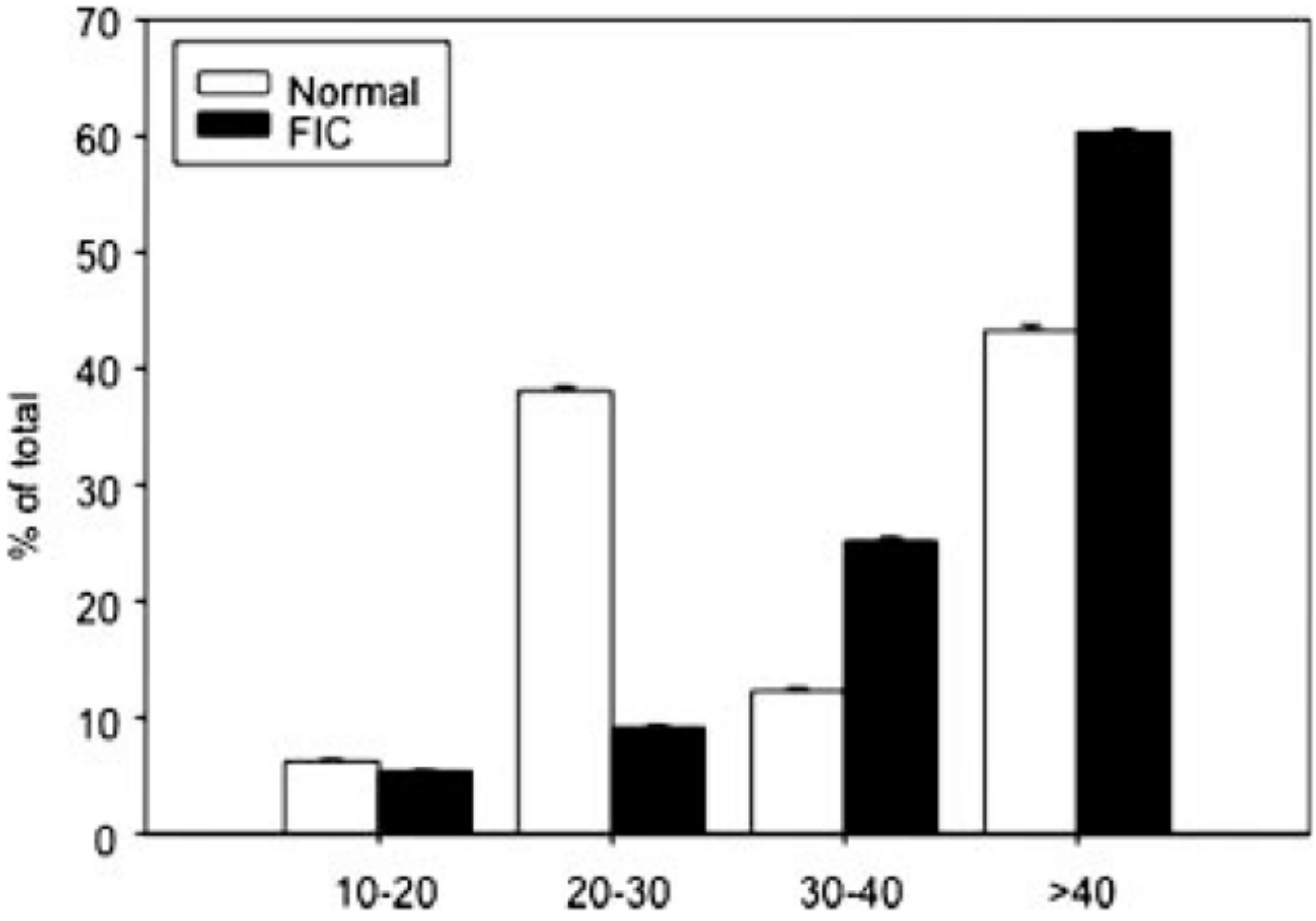

Fig. 3.

Diameter distribution of identified DRG neurons in FIC and normal cats. Mean number (±SEM) of identified (fast-blue dye labeled) DRG neurons in a minimum of 10 sections of the S1 DRG from FIC (n = 4) and normal (n = 4) animals.

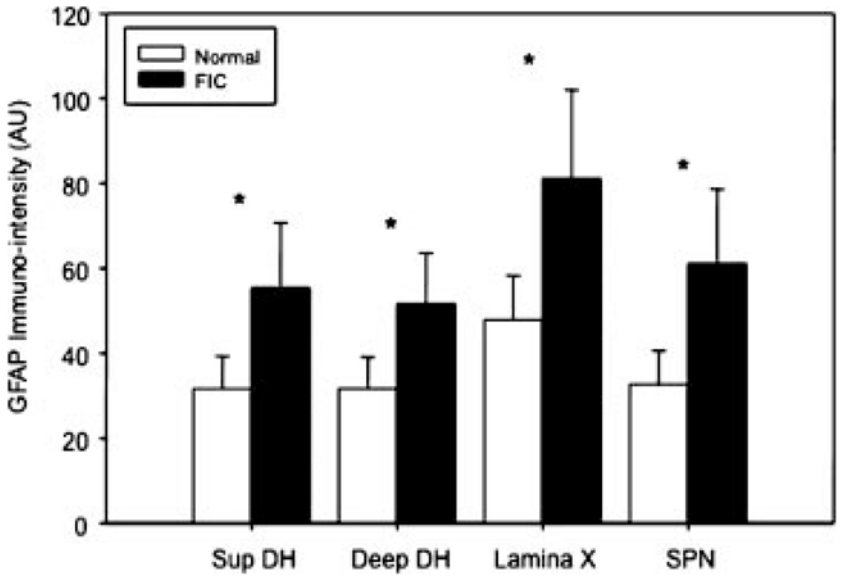

Fig. 4.

Increased GFAP immuno-intensity from FIC as compared to unaffected cat spinal cord. GFAP immuno-intensity levels in the S1 spinal cord superficial and deep dorsal horn; lamina × (surrounding the central canal) and the sacral parasympathetic nucleus (SPN region) from cats with FIC are higher compared with similar regions from unaffected cats (unpaired *t*-test; n = 4 each, FIC and unaffected).

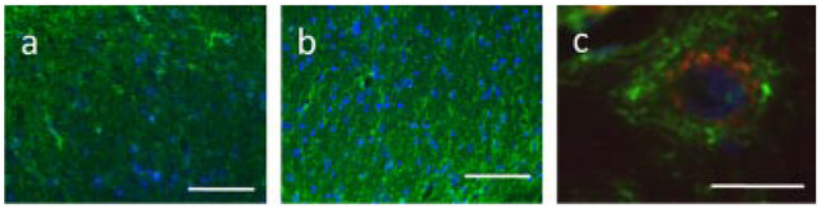

Fig. 5.

GFAP immuno-intensity in FIC and normal cat spinal cord. The immunointensity of glial fibrillary acidic protein (GFAP), an intermediate filament protein found in astrocytes, is significantly elevated in lower lumbosacral (S1) FIC spinal cord. **a**: Representative image of astrocytes (green—GFAP astrocytic marker; blue-4′,6-diamidino-2-phenylindole (DAPI)—used to stain cell nuclei) in S1 spinal cord dorsal horn (DH) region from a healthy cat as compared with similar S1 DH spinal cord region from an FIC cat (in **b**). **c**: FIC astrocytes (GFAP, green) co-express the intermediate filament, nestin (red), indicating a reactive state (blue—DAPI, nuclear marker). Scale bars: (a,b) 100 μm; (c), 10 μm.

TABLE I

Levels of NGF (and Corresponding Receptors), Substance P, and Phospho p38 in Normal and FIC Cat Urothelium

	Normal	FIC
NGF (pg/μg protein)	5.814 ± 0.989	13.814 ± 1.510*
trkA (volume normalized by β-actin)	941.870 ± 358.172	1,461.788 ± 470.851
p75 (volume normalized by β-actin)	49.737 ± 13.774	63.562 ± 14.156
SP (pg/mg wet tissue weight)	1.495 ± 0.726	2.827 ± 0.512*
Phospho-p38 (volume normalized by β-actin)	32.438 ± 18.009	69.217 ± 18.194

Protein (mucosal) lysates from normal and FIC cat urinary bladders (n = 3–7 for each) were analyzed for NGF and SP by ELISA; trkA, p75, and phospho-p38 by Western immunoblotting.

Values are presented as units ± SEM. Statistical significances (*$P < 0.05$) between the groups were explored using the Student's *t*-test.