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Transient receptor potential vanilloid type 4 (TRPV4) in urinary bladder structure and function

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Abstract

Bladder pain syndrome (BPS)/interstitial cystitis (IC) is a urologic, chronic pelvic pain syndrome characterized by pelvic pain, pressure, or discomfort with urinary symptoms. Symptom exacerbation (flare) is common with multiple, perceived triggers including stress. Multiple transient receptor potential (TRP) channels (TRPA1, TRPV1, TRPV4) expressed in the bladder have specific tissue distributions in the lower urinary tract (LUT) and are implicated in bladder disorders including overactive bladder (OAB) and BPS/IC. TRPV4 channels are strong candidates for mechanosensors in the urinary bladder and TRPV4 antagonists are promising therapeutic agents for OAB. In this perspective piece, we address the current knowledge of TRPV4 distribution and function in the LUT and its plasticity with injury or disease with an emphasis on BPS/IC. We review our studies that extend the knowledge of TRPV4 in urinary bladder function by focusing on (i) TRPV4 involvement in voiding dysfunction, pelvic pain, and non-voiding bladder contractions in NGF-OE mice; (ii) distention-induced luminal ATP release mechanisms and (iii) involvement of TRPV4 and vesicular release mechanisms. Finally, we review our lamina propria studies in postnatal rats studies that demonstrate: (i) the predominance of the TRPV4+ and PDGFRα+ lamina propria cellular network in early postnatal rats; (ii) the ability of exogenous mediators (i.e., ATP, TRPV4 agonist) to activate and increase the number of lamina propria cells exhibiting active Ca^{2+} events; and (iii) the ability of ATP and TRPV4 agonist to increase the rate of integrated Ca^{2+} activity corresponding to coupled lamina propria network events and the formation of propagating wavefronts.

Keywords

urothelium; lamina propria; Ca^{2+} events; painful bladder syndrome; syncytium

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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.

Introduction

Micturition relies on the components of the lower urinary tract (LUT) including the bladder and urethra, working together in a reciprocal manner to store and eliminate urine. In the storage phase, the bladder remains relaxed to act as a reservoir to collect and store urine produced by the kidneys, while the urethra remains contracted to prevent leakage. Upon elimination, the bladder contracts to expel urine through the relaxed urethra. The LUT consists of 3 main tissue layers: the mucosa (closest to the lumen), the muscularis propria, and the surrounding adventitia and serosa (Birder & Andersson, 2013).

LUT Tissues

Mucosa

The mucosa is composed of the urothelium, basement membrane, lamina propria and smooth muscle cells (i.e., muscularis mucosae) (Birder & Andersson, 2013).

Urothelium

The urothelium (lining the bladder lumen) consists of transitional epithelial cells organized in three layers including a basal, intermediate, and superficial cell layer. The basal cell layer adheres to the basement membrane. The intermediate and basal urothelial cell layers play important roles in proliferation and differentiation during injury. The umbrella cell, a specialized epithelial cell, forms the superficial urothelial layer. The bladder must be inherently pliable to properly store accumulating urine, expanding, and contracting, when necessary, while maintaining bladder function. Large (25–250 um) and hexagonally shaped, umbrella cells greatly contribute to this ability as they change shape in response to bladder filling, due to their unique protein expression profile and vesicular trafficking mechanisms. Umbrella cells express uroplakin, specialized lipid, and tight junction (e.g., occludins, claudins) proteins which aid in maintaining the urothelial barrier (Birder & Andersson, 2013). A polysaccharide glycosaminoglycan (GAG) layer lines the luminal surface of the umbrella cells and provides additional barrier support by preventing passage of microorganisms and toxic substances. The urothelial layers and properties remain relatively consistent throughout the bladder (i.e., detrusor, trigone regions); however, the urothelial layer of the bladder neck/proximal urethra and renal pelvis are notably different. The urethral urothelium exhibits stratified, columnar epithelium and lacks urothelial specific protein expression (i.e., uroplakin, occludins, claudins) (Birder & Andersson, 2013).

The urothelium was once thought to be a relatively passive component of the LUT, acting solely as a barrier, to prevent toxins, urinary metabolites, and microorganisms from penetrating the bladder wall. However, it is now well known that the urothelium is a biologically active layer. Numerous studies demonstrate that the urothelium plays critical modulatory roles in LUT sensory signaling (Birder & Andersson, 2013). Mechanical (i.e., stretch, pressure) or chemical (i.e., urine composition, heat, mediators released from nerves and surrounding cells) stimuli may induce the release of neuroactive chemicals (e.g., ATP, ACh, NO, growth factors) from urothelial cells. Additionally, urothelial cells express diverse channels and receptors (e.g., noradrenaline, ACh, ATP, UTP, ADP, adenosine, CRH,

neurotrophins, endothelins, estrogen, bradykinin, tachykinin, amiloride, ENaC, and TRP channels). Many of these channels and receptors are also expressed on afferent nerves. Compounds released from the urothelium may act by autocrine or paracrine mechanisms. Furthermore, they may modulate sensory information via interactions with urothelial cells, lamina propria cells, detrusor SMCs, blood vessels, inflammatory cells, and afferent nerves (Birder & Andersson, 2013).

Lamina Propria

The lamina propria lies between the most external layer of the bladder, muscularis detrusor, and the urothelial basement membrane. This tissue may also be referred to as the submucosa or suburothelium. The lamina propria is an extracellular matrix consisting of many fiber types (e.g., elastic, collagen), cell types (e.g., adipocytes, fibroblasts, interstitial cells), blood vessels, lymphatic vessels, smooth muscle fascicles (i.e., muscularis mucosae) and nerve terminal endings. The lamina propria is one continuous tissue; however, many investigators distinguish between a deep, more fibrous region near the detrusor and a superficial or upper region near the urothelium that is densely populated with LP cells (Gabella & Davis, 1998). A dense collection of afferent nerve fibers terminates within the lamina propria, with some endings extending into the urothelium (Birder & Andersson, 2013; Fowler et al., 2008). Afferent nerves primarily terminate in the lower region of the bladder body and neck (Birder & Andersson, 2013). Efferent nerve terminals are present but sparse in the LP and primarily terminate within smooth muscle (Andersson & Arner, 2004). Small, parasympathetic intramural ganglia are present in the LP and within detrusor smooth muscle bundles in the human bladder, but are absent in rats, cats, guinea pigs and rabbits (Birder & Andersson, 2013; Gosling & Dixon, 1974). Like the urothelium, investigators have recently begun to re-examine the involvement of the LP in afferent signaling as numerous studies suggest that urinary, LP interstitial cells may act as intermediaries or amplifiers of bladder sensations (Birder & Andersson, 2013; Koh et al., 2018; Neuhaus et al., 2020).

Muscularis Propria

The detrusor muscle is the muscularis propria component of the bladder, which primary function is to contract during voiding events to expel urine in the bladder, into urethra. The detrusor is made of smooth muscle cells (SMC) organized in three sheets. Two longitudinal smooth muscle sheets surround a middle, circular smooth muscle sheet. A urinary interstitial cell (IC) sub-population exists in the detrusor and the urethral muscularis that is separate and distinct from LP ICs (Koh et al., 2012; Sancho et al., 2017). These cells lie alongside and in between smooth muscle bundles and may contribute to regulating SMCs excitability (Koh et al., 2018). The detrusor muscle extends over much of the bladder body and base but is notably different than the smooth musculature of the ureter orifices/distal ureters, trigone, and urethra.

Urethra

Trigonal and detrusor smooth muscle both extend into the proximal urethra. Additional layers of urethral smooth muscle include a thick, inner longitudinal layer and a thin, outer circular layer (Mistry et al., 2020). An internal urethral sphincter does not exist in the true,

anatomical sense in males or females; however, the bladder base, neck and proximal urethral musculature all contribute to create sphincteric-like action (Elbadawi, 1996).

Neural Control of Micturition

Neural circuits in the brain and spinal cord coordinate the actions of the bladder and urethra to switch between the storage phase and the elimination phase (Figure 1). This coordination is mediated by three sets of peripheral nerves: (i) Hypogastric sympathetic nerves that inhibit the detrusor muscle and contract the bladder base and urethra for proper storage; (ii) Pelvic parasympathetic nerves that contract the detrusor muscle and relax the urethra for voiding; and (iii) Pudendal somatic nerves that excite the striated external urethral sphincter to close the bladder outlet (Yoshimura & Chancellor, 2003). All three peripheral nerves also contain afferent nerves that convey sensory information related to bladder filling (Andersson & Michel, 2011; Walsh, 1986). Finally, higher order brain areas provide input to the micturition circuit to support voluntary control of voiding.

Bladder storage (i.e., continence) is, in part, mediated by the sympathetic nervous system (Figure 1). Sympathetic preganglionic neurons that innervate the bladder originate in the intermediolateral nucleus of spinal cord levels T11-L2 in humans and L1-L2 in rodents (Nadelhaft et al., 1992). After leaving the spinal cord, these sympathetic fibers form lumbar splanchnic nerves and some synapse in the inferior mesenteric ganglion, while others travel farther to make their first synapse in the ganglia of the pelvic plexus. The postganglionic fibers then travel along the hypogastric nerve or pelvic nerve, respectively, to the bladder where they release norepinephrine to provide inhibitory input to the detrusor to promote bladder accommodation via β-adrenergic receptors, and excitatory input to the bladder base and smooth muscle of the urethra to facilitate storage via α-adrenergic receptors (Andersson & Arner, 2004; de Groat et al., 2015; Fowler et al., 2008).

Parasympathetic preganglionic neurons that innervate the bladder and urethra are located in the sacral parasympathetic nucleus in the lateral horn of the sacral spinal cord (S2-S4 in humans, L6-S1 in rodents) (Walsh, 1986). Axons from these neurons leave the spinal cord and immediately separate to become pelvic splanchnic nerves (i.e., pelvic nerve). Axons within this nerve either synapse with postganglionic neurons in the pelvic plexus or postganglionic neurons located in terminal ganglia within the bladder wall (e.g., intramural ganglia or major pelvic ganglia) (de Groat, 1990; de Groat et al., 2015; DeGroat & Saum, 1976; Elbadawi, 1996; Fowler et al., 2008; Girard et al., 2017). During bladder filling, the parasympathetic nervous system is inhibited; however, when the micturition threshold is exceeded and it a socially appropriate time to urinate, the parasympathetic nervous system is activated alone and leads to the contraction of the detrusor smooth muscle through the release of acetylcholine and ATP onto muscarinic (M3) and purinergic (P2X) receptors, respectively (Figure 1). Additionally parasympathetic fibers from the pelvic nerve relax the internal urethral sphincter through the release of nitric oxide (Bennett et al., 1995) (Figure 1).

The external urethral sphincter is striated muscle controlled by the somatic nervous system. Motor neurons responsible for innervating the external urethral sphincter reside in Onuf's

nucleus (humans) or the dorsolateral motor nucleus (rodents), both of which are a collection of motor neurons in the ventral horn of the sacral spinal cord. These neurons send projections to contract the external urethral sphincter via the pudendal nerve to maintain continence in synchronized fashion with the sympathetic nervous system (de Groat, 1990; Elbadawi, 1996; Fowler et al., 2008; Girard et al., 2017).

Afferent nerves containing sensory information related to bladder fullness travel to the spinal cord via the pelvic and hypogastric nerves, whereas sensory input from the bladder base and urethra travel to the spinal cord via the pudendal and hypogastric nerves (Fowler et al., 2008). During normal bladder filling, sensory information travels via myelinated Aδ-fibers that respond to mechanosensory channels that become activated during passive distension of the bladder. As the bladder nears capacity, tension increases and mechanosensitive channels that respond to pressure open and lead to increased firing of Aδ-fibers (Fowler et al., 2008). Under pathological conditions, small diameter, unmyelinated, C-fibers are recruited to carry sensory signals related to noxious stimuli from the LUT to the spinal cord. The cell bodies of both Aδ- and C-fibers are in dorsal root ganglia in spinal levels T11-L2 and S2-S4 in humans (de Groat & Yoshimura, 2010; Fowler et al., 2008). Bladder afferents enter the spinal cord through Lissauer's tract and terminate on neurons in the superficial dorsal horn, dorsal commissure, and sacral parasympathetic nucleus. Some neurons then project rostrally to supraspinal micturition integration centers in the brain.

Supraspinal neural substrates play a fundamental role in the initiation of voiding as well as maintaining continence. In humans, the pontine micturition center, also called Barrington's nucleus, is in the pontine medial tegmentum within the brainstem and is the most prominent efferent nucleus within the micturition reflex circuit (Andersson & Michel, 2011; de Groat, 1990; de Groat et al., 2015; Elbadawi, 1996; Fowler et al., 2008). Chemical or electrical activation of the PMC causes a contraction of the detrusor smooth muscle and a relaxation of the urethra that results in micturition (Hou et al., 2016; Sugaya et al., 1987). To do this, the PMC sends descending fibers to the sacral parasympathetic nucleus in the spinal cord to synapse on parasympathetic neurons that inhibit the detrusor (Andersson & Michel, 2011) as well as GABAergic interneurons that then inhibit alpha motor neurons in Onuf's nucleus to induce relaxation of the external urethral sphincter (Andersson & Michel, 2011). Though the PMC is considered the micturition control center, in that it switches the LUT from a state of storage to a state of elimination, it does not directly receive afferent input from the LUT in humans (Andersson & Michel, 2011) (Figure 1). After the Aδ- and C-fibers synapse in the spinal cord, their projections join ascending spinal projections to synapse in the periaqueductal gray (PAG), a region in the mesencephalon of the brainstem (Andersson & Michel, 2011). The PAG projects to the PMC and serves as an integration center of LUT sensory signals along with inputs from other higher order brain regions such as the hypothalamus, preoptic region, central nucleus of the amygdala, BNST, and prefrontal cortex (Beckel & Holstege, 2011). During bladder filling, input from these brain areas prevent the PAG from exciting neurons in the PMC to maintain conscious and voluntary control of micturition (Beckel & Holstege, 2011; Fowler et al., 2008).

Neurochemistry of LUT Afferent Neurons

Afferent fibers traveling in the pelvic, hypogastric (lumbar splanchnic), and pudendal nerves convey information from the LUT to the sacral spinal cord. The sensations transmitted vary from degree of bladder distension to bladder pain and the activation stimuli (e.g., stretch/volume/chemicals) of bladder afferents are varied (Andersson & Michel, 2011). To communicate this wide range of information, LUT afferent nerves, namely Aδ- and C-fibers have diverse properties. LUT afferent neurons in the lumbosacral DRG are not a focus of this perspectives piece but a thorough review of this topic can be found here (Andersson & Michel, 2011).

Urothelial Signaling

Although historically viewed as a passive barrier, research now suggests an integral role of the urothelial layer of the LUT as a sensory and signaling organ that receives, amplifies, and transmits both mechanical and chemical signals to cells deeper in the bladder wall. A variety of receptors and channels are expressed on afferent neurons in the bladder are also found on urothelial cells, including: bradykinin receptors (Chopra et al., 2005), neurotrophin receptors (TrkA, TrkB, and p75) (Murray et al., 2004), purinergic receptors (P_2X and P_2Y) (Birder et al., 2004; Chopra et al., 2008; Lee et al., 2000; Tempest et al., 2004), VEGF receptors (Kopparapu et al., 2013; Saban et al., 2008), adrenergic receptors (both α and β)(Birder et al., 1998; Birder, Nealen, et al., 2002; Kullmann et al., 2011; Limberg et al., 2010), muscarinic and nicotinic acetylcholine receptors (Beckel & Birder, 2012; Beckel et al., 2006; Birder et al., 1998; Kullmann et al., 2008; Kullmann et al., 2008), sodium channels (Araki et al., 2004; Smith et al., 1998; Wang et al., 2003), a variety of TRP channels (TRPV1, TRPV2, TRPV4, TRPM8, and TRPA1) (Birder et al., 2001; Birder, Nakamura, et al., 2002; Du et al., 2008; Gevaert et al., 2007; Girard et al., 2019; Kullmann et al., 2009; Mochizuki et al., 2009; Stein et al., 2004; Yamada et al., 2009), and Piezo1/2 channels (Dalghi et al., 2019; Liu et al., 2018; Marshall et al., 2020).

Activation of these channels and receptors occurs due to various stimuli such as increases in bladder wall tension from bladder filling, soluble chemical mediators in urine, and chemical mediators released from afferent neurons, inflammatory cells, and blood vessels (Birder & Andersson, 2013; Birder & de Groat, 2007; Hanna-Mitchell et al., 2007; LaBerge et al., 2006). In response to activation of these channels, urothelial cells release an array of signaling molecules including NO, ATP, NGF, and substance P onto both afferent and efferent nerves located in or near the urothelial cell layer (Birder & Andersson, 2013; Birder et al., 1998; Birder et al., 2003; Burnstock, 2001; Ferguson et al., 1997; Girard et al., 2019). ATP is one of the main urothelial signaling mediators and its release from urothelial cells and subsequent binding to P_2X receptors on afferent nerve terminals is essential for a proper functioning micturition reflex. As the bladder fills, the apical surface of the urothelial cells begins to stretch, causing an activation of stretch activated channels (SACs). This activation leads to an influx of calcium and ultimately results in a robust release of ATP from the urothelial cells (Ferguson et al., 1997). The precise mechanism of this release is still debated and is not limited to a singular mechanism (Birder & Andersson, 2013; Winder et al., 2014). Research supports a combination of both ATP release via vesicular

exocytosis and non-vesicular release through channels like pannexin and connexin, among other transporters (Lazarowski, 2012; Sui et al., 2014; Winder et al., 2014). For vesicular release, ATP is typically co-packaged into vesicles and released with other neurotransmitters like acetylcholine (Dahl, 2015; Unsworth & Johnson, 1990). For non-vesicular release in urothelial cells, ATP can leave the cell through non-junctional connexin or pannexin hemichannels and signal through both autocrine and paracrine mechanisms (Beckel et al., 2015).

Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS)

IC/BPS is a chronic pelvic pain condition in which patients experience pain, pressure or discomfort perceived to be related to the urinary bladder, in addition to LUT symptoms, particularly increased urinary frequency and/or urgency (Figure 2). IC/BPS presents with very diverse clinical phenotypes (Akiyama et al., 2021; Hanno, 2015). Despite this diversity, IC/BPS's hallmark feature, distinguishing the syndrome from related LUT conditions, is pain related to bladder filling. IC/BPS is diagnosable after symptoms have persisted for 6 weeks and other identifiable causes, such as infection, or related conditions have been excluded (Hanno, 2015) (Figure 2). There are currently few treatment options for IC/BPS patients. Most treatments that are available aim to manage symptoms, rather than targeting the underlying mechanisms of IC/BPS and many are used off-label. Additionally, treatment outcomes vary greatly among patients and even with short-term treatment success, long-term success may not be achieved. Pentosan polysulfate sodium, or Elmiron, is currently the only FDA-approved medication specifically for IC/BPS, yet with modest success (Hanno, 2015; Marcu et al., 2018).

Epidemiology

Prevalence estimates have increased over the past 50 years. This may be due to increased awareness of IC/BPS symptoms and recognition of IC/BPS as a condition amongst physicians, leading to fewer misdiagnoses. In a nationwide study conducted via telephone surveys with statistically modeled predictors for screening, Berry et al. (Berry et al., 2011) estimated 2.7–6.5% of US women (3.3–7.9 million) met IC/BPS diagnosis criteria, depending on a more or a less stringent definition of IC/BPS. Historically, it has been thought that IC/BPS has a higher prevalence in women compared to men, with a ratio of 5:1 (Akiyama et al., 2021; Berry et al., 2011). However, this notion has been challenged in more recent years with some studies suggesting the accepted prevalence amongst men is higher than originally estimated due to misdiagnoses and underreporting (Forrest & Schmidt, 2004; Suskind et al., 2013). Using a similar methodological approach to Berry et al. (Berry et al., 2011), Suskind et al. (Suskind et al., 2013) estimated the prevalence of IC/BPS in US men at 1.9–4.2% (2.1–4.6 million).

Pathophysiology

The etiology of IC/BPS is still not well understood. However, there are consistent pathological features associated with IC/BPS that may contribute to its development and/or persistence.

Urothelial Alterations

The GAG layer, specialized lipids, uroplakin proteins, and tight junction proteins create a high transepithelial resistance, allowing the urothelium to act as a fantastic barrier against irritative substances (Birder & Andersson, 2013). However, the urothelium exhibits drastic changes in patients with and without Hunner's ulcers, such as reduced and aberrant expression of urothelial barrier proteins and deficient urothelial repair mechanisms, leading to increased urothelial permeability, urothelial thinning, and denudation (Birder & Andersson, 2013; Hauser et al., 2008; Keay et al., 2004; Parsons et al., 1991) (Figure 3). Cationic substances (e.g., potassium), metabolites from diet, medication, or supplements, hormonal changes, and bacterial infections can exert cytotoxic effects on urothelial cells and impair barrier function (Akiyama et al., 2021). Furthermore, increased urothelial permeability allows irritative substances that would normally be contained and expelled, to permeate the urothelium, depolarize bladder wall constituents (e.g., interstitial cells, nerve terminal endings, SMCs) and trigger secondary inflammatory processes, in response to released endogenous pathogens from damaged tissue (Akiyama et al., 2021; Birder & Andersson, 2013). General inflammatory changes are also observed in IC/BPS patients including immune cell infiltration, MC degranulation, angiogenesis, edema, and stromal fibrosis (Akiyama et al., 2021; Arms et al., 2013; Arms et al., 2010; Gonzalez et al., 2013; Guo et al., 2018; Malley & Vizzard, 2002). B cell clonal expansion is specifically noted in IC/BPS patients with Hunner's ulcers (Moldwin et al., 2022).

When inflammation becomes chronic, the exposure and activation of nerve terminals to inflammatory mediators induces afferent nerve sensitization that, if left untreated, can lead to hyperalgesia and allodynia because of nerve hyperexcitability induced by neurogenic inflammation (Birder & Kullmann, 2018; Luke Grundy et al., 2018) (Figure 3). Neurogenic inflammation is a component of the positive feedback loop triggered when increased stimulation of afferent nerves causes them to release inflammatory peptides including Substance P, CGRP, and neurokinin A (Birder & Kullmann, 2018). This release of neuropeptides causes a cascade of events involving the immune system, the vasculature, and the nervous system in the following ways: (i) the vasculature becomes angiogenic which can increase edema thereby perpetuating the inflammatory response (Birder & Kullmann, 2018); (ii) immune system activation induces recruitment of additional pro-inflammatory mediators like mast cells and leukocytes to the site of inflammation (Birder & Kullmann, 2018); and (iii) underlying nerves become hyperexcitable due to increased nerve density (Pang et al., 1995), increased expression of receptors such as TRPs, and purinergic receptors (Birder & Kullmann, 2018; Brady, Apostolidis, Yiangou, et al., 2004; Brady et al., 2004), and increased activation and expression of free ions like K^+, Ca^{2+} that lower the action potential threshold in sensory neurons (de Groat & Yoshimura, 2006, 2010). This persistent activation of peripheral nociceptive signals creates an increased drive of C-fiber afferent input to the spinal cord which, over time, leads to aberrant functioning of CNS circuits. Specifically, nociceptive neurons in the brain and spinal cord will increase their firing given normal or sub-threshold stimuli (Baron et al., 2013). This phenomenon is called central sensitization and is the driver of the hyperalgesia (increased sensitivity to pain) and allodynia (pain response to non-noxious stimuli) symptoms in patients with IC/BPS (Reynolds et al., 2016) (Figure 3).

Stress-induced bladder dysfunction

Stress may also contribute to neural changes and upregulation in IC/BPS. Stress can exacerbate, and even induce, symptoms in IC/BPS patients (Nazif et al., 2007; Rothrock et al., 2001a; Rothrock et al., 2001b) and animal models (Birder & Andersson, 2018; Charrua et al., 2015; Girard et al., 2017; Matos et al., 2017; Merrill et al., 2013; Merrill & Vizzard, 2014; Mingin et al., 2015; Mingin et al., 2014; Nazif et al., 2007; Rothrock et al., 2001a; Rothrock et al., 2001b) (Figure 3). The hypothalamic-pituitary-adrenal (HPA) axis is a neuroendocrine feedback loop that acts to maintain physiological homeostasis. However, abnormal, or chronic HPA activation is associated with dysregulated responses to stress and inflammation and may result in aberrant sympathetic activation (Godoy et al., 2018; Nazif et al., 2007; Sheng et al., 2020). Symptom exacerbation due to stress is prevalent in many disease states, including functional disorders of the urinary bladder (e.g., overactive bladder (OAB), BPS/IC) (Nickel et al., 2016; Sutcliffe et al., 2014; Sutcliffe et al., 2018); however, the mechanisms underlying the effects of stress on the micturition reflex function are unclear. The repeated variate stress (RVS) model has been used previously to examine stress-induced changes in bladder function and pelvic sensation in rats (Girard et al., 2017; Gonzalez et al., 2016; Merrill et al., 2013; Merrill & Vizzard, 2014). The RVS paradigm (Hammack et al., 2009; King et al., 2017) has multiple advantages including: i) lack of habituation with novel stressor exposure; ii) reproducible and robust changes in urinary bladder function (Merrill et al., 2013; Merrill & Vizzard, 2014); and iii) reproducible decrease (~10–25%) in weight gain during RVS as demonstrated in rats and mice (Merrill et al., 2013; Merrill & Vizzard, 2014). We have previously used the RVS protocol to characterize effects on bladder function and somatic sensitivity (Merrill et al., 2013; Merrill & Vizzard, 2014) in rats. RVS in rats (Merrill et al., 2013; Merrill & Vizzard, 2014) produced similar changes in bladder function and somatic sensation observed in the present study, including increased urinary frequency, and somatic sensitivity of the hindpaw and pelvic region as well as increased NGF content in the urinary bladder. In addition, RVS in rats also caused additional changes in the inflammatory milieu of the urinary bladder including, changes in histamine, myeloperoxidase, and the chemokine, CXCL12 (Merrill et al., 2013; Merrill & Vizzard, 2014).

The contribution of other conditions and organ systems should be considered, as IC/BPS is commonly comorbid with psychological (e.g., depression, generalized anxiety), GI-related (e.g., irritable bowel syndrome, inflammatory bowel disease), auto-immune (e.g., Sjogren's syndrome, Lupus) or somatoform syndromes (e.g., fibromyalgia, chronic fatigue syndrome, vulvodynia) (Grundy & Brierley, 2018; Grundy et al., 2018; Yoshikawa et al., 2015). Non-ulcerative IC/BPS patients have higher rates of comorbidities than those with Hunner's ulcers (Akiyama et al., 2021). Patients report that certain drugs (e.g., alcohol, nicotine) and foods (e.g., caffeine, high potassium, high acid) aggravate IC/BPS symptoms; however, a direct link between diet and symptomology has not been established (Patnaik et al., 2017).

Multiple Contributing Factors

IC/BPS pathophysiology and etiology is likely a product of multiple factors mentioned above (Birder & Andersson, 2018). Immune cell infiltration, urothelial and GAG alterations, angiogenesis, afferent, and central sensitization work in concert to promote damaging

positive feedback cycles (Figure 3). Comorbid conditions and/or stress may further exacerbate or induce IC/BPS features. The combination of these factors and the feedback cycles they create influence IC/BPS progression and further complicates understanding, diagnosing, and managing/treating underlying IC/BPS pathophysiology (Figure 3).

IC/BPS Animal Models

There are multiple animal models of IC/BPS use to mimic the clinical signs and symptoms of IC/BPS. Birder and Andersson (Birder & Andersson, 2018) broadly group IC/BPS animal models into three categories: (i) bladder-centric models; (ii) models with complex mechanisms (i.e., CNS, immune system); and (iii) psychological and physical stressors, and natural models. For this perspectives piece, we focus on 2 bladder centric models and stress models. For additional information, please see (Birder & Andersson, 2018; Bjorling et al., 2011).

Cyclophosphamide (CYP)-induced cystitis

The most widely used bladder-centric model in the literature is IC/BPS induced by CYP. CYP is an anti-neoplastic alkylating agent used in chemotherapy to treat several types of cancer (Olivar & Laird, 1999). Though CYP itself is inert, it is metabolized in the liver to phosphamide mustard and acrolein, which are excreted into the urine and accumulate in the bladder (Olivar & Laird, 1999). This accumulation of toxic metabolites, especially acrolein, causes intense changes that to the urinary bladder that mimic changes seen in patients with IC/BPS (Olivar & Laird, 1999). CYP-induced cystitis can be used as an acute bladder inflammation model, induced with one intraperitoneal injection of CYP, or a chronic model, induced with a series of lower dose intraperitoneal CYP injections over the course of days (Birder & Andersson, 2018). Both models yield the hallmark symptoms of IC/BPS (pain and increased voiding frequency) while also inducing localized bladder specific pathological changes like mucosal edema, urothelium breakdown, inflammatory cell infiltration, petechial hemorrhaging, and afferent nerve hyperexcitability (Birder & Andersson, 2018; Olivar & Laird, 1999). Although CYP induces a greater inflammatory response than seen in some IC/BPS patients, it is an appealing model because it is a non-invasive and reproducible model that produces pathological and functional bladder-specific changes mimicking many of the signs/symptoms of the clinical syndrome (Bjorling et al., 2011; Olivar & Laird, 1999).

NGF-Overexpression (OE)

We have characterized a transgenic mouse model of chronic, urothelium-specific NGF-overexpression that represents a novel approach to exploring the role of NGF in urinary bladder inflammation and sensory function (Schnegelsberg et al., 2010). Functionally, NGF-OE mice exhibit frequent voiding, the presence of NVCs during the bladder filling phase and referred somatic pelvic hypersensitivity (Schnegelsberg et al., 2010). In addition, NGF-OE mice exhibit changes in the morphology and neurochemistry of the urinary bladder and increases in inflammatory cell infiltrates to the urinary bladder (Schnegelsberg et al., 2010). Neurochemical changes include increased expression of Sub P and CGRP in the suburothelial nerve plexus of the urinary bladder (Schnegelsberg et al., 2010). Our findings

support and extend many previous studies in rodents demonstrating involvement of NGF in altered bladder sensory function and the development of referred hyperalgesia in response to bladder inflammation (Dmitrieva & McMahon, 1996; Guerios et al., 2006; Guerios et al., 2008; Hu et al., 2005; Jaggar et al., 1999; Lamb et al., 2004; Zvara & Vizzard, 2007).

Stress-induced bladder dysfunction

Stress models have been shown to induce pain behaviors in rodents along with increased voiding frequency (Merrill & Vizzard, 2014; Smith et al., 2011; Wang et al., 2017). Experiments with the WAS model of stress have shown increased inflammatory cell infiltration, urothelial breakdown, evidence of peripheral and central sensitization, and increased blood flow to areas in the brain associated with voiding urgency (Birder & Andersson, 2018; Wang et al., 2017). Although underlying mechanisms are unknown, research suggests that it is a consequence of an aberrantly functioning sympathetic nervous system, more specifically the HPA axis. As evidence for this, chronic stress has been shown to increase sympathetic nerve activity which results in increased adrenergic stimulation due to dysfunctional HPA feedback mechanisms to various stress hormones (Birder & Andersson, 2018).

Mechanotransduction

Mechanotransduction, the conversion of mechanical forces into biological signals, is an essential physiological process used throughout the body; however, understanding the mechanism of mechano-sensing is still largely unknown in multiple organ systems (Nilius, 2010). In the urinary bladder, mechanotransduction is thought to be the mechanism used to transmit signals related to bladder distention to sensory signals that relay the level of bladder fullness to the spinal cord and higher order brain centers that integrate that information to determine an appropriate time to void. Increasing evidence suggests mechano-sensing channels on urothelial cells are also activated by numerous diverse signals including bladder stretch, temperature changes, pH changes, and chemical irritation. Once activated, the urothelial cells transduce these signals to bladder afferent nerves by releasing chemical mediators, such as ATP (Birder & Andersson, 2013; Olsen et al., 2011).

Research is ongoing to determine which mechano-sensing channels are responsible for transducing this signal and the mechanism(s) by which they carry out this function. Calcium-permeable stretch-activated cation channels (SACs) were discovered over 20 years ago and research suggests they are the main drivers of mechanotransduction. SACs can be classified into channels that are directly gated or indirectly gated. An ion channel that has direct gating is the primary transducer of mechanical force, meaning that it alone senses the force and passes on a receptor current. A channel with indirect gating plays a necessary role in mechanical transduction but does so by second messenger signaling via G-proteins, phospholipases, and the activation of downstream kinases (Christensen & Corey, 2007; Nilius, 2010). For a channel to be considered directly mechano-sensitive, the candidate ion channel must meet multiple functional criteria as defined by (Christensen & Corey, 2007).

Research suggests the presence of several mechanosensors on urothelial cells that respond to mechanical stretch of the bladder, among other signals.

Transient Receptor Potential (TRP) Channels

The TRP channels are a novel superfamily of channels that contribute to changes in the intracellular concentration of calcium ($[Ca^{2+}]_i$) by either acting as a Ca^{2+} entry pathway in the plasma membrane or by changing membrane polarization and thus modulating the driving force for Ca^{2+} entry into the cell (Nilius et al., 2007). There are more than 50 TRP channels described in species from yeast to human and 28 known mammalian TRP channels (Everaerts et al., 2008). The TRP channel superfamily consists of 7 subfamilies: TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), TRPML (mucolipin), and TRPN (no mechanopotential). Each TRP channel consists of 4 subunits, and each subunit consists of 6 transmembrane spanning segments (S1-S6), a cation-permeable pore-forming loop between S5 and S6, and an intracellularly located NH_2 and COOH termini (Everaerts et al., 2008; Vriens et al., 2009).

The TRPV subfamily consists of six members (TRPV1–6). TRPV1–4 are all heat-activated and nonselective for cations. These channels are modestly permeable to Ca^{2+}, with a PCa: PNa of ~6:1 and are also chemosensors for many endogenous and synthetic ligands (Angelico & Testa, 2010; Nilius et al., 2007) TRPV5 and TRPV6 differ greatly; they are the only highly selective Ca^{2+} channels in the TRP superfamily (Angelico & Testa, 2010). TRPV channels can act as signal integrators, in that their stimuli have an additive effect on the gating of these channels, which becomes important for pathological states (Nilius et al., 2007).

The TRPV4 channel is a Ca^{2+}-permeable, stretch-activated, nonselective cation channel that is widely expressed throughout the body. In the brain, TRPV4 is found in neurons in the lamina terminalis, anterior hypothalamus, and choroid plexus where it acts as an osmosensor (Liedtke et al., 2000). The TRPV4 channel is also found in motor, spinal ventral root and DRG neurons, where it is thought to play a role in nociception (Nilius & Voets, 2013). TRPV4 is also expressed and has mainly a sensory role in auditory hair cells, skin keratinocytes, endothelial cells, cardiac fibroblasts, myocytes, skeletal muscle fibers, and renal and urinary bladder epithelium (Andersson et al., 2010; Angelico & Testa, 2010; Nilius & Voets, 2013; Vriens et al., 2009). It was originally identified as a channel activated by hypotonic cell swelling (Liedtke et al., 2000; Vriens et al., 2009), but has since been found to be activated by other physical and chemical stimuli, including mechanical or shear stress, moderate heat (>27°C), arachidonic acid, the endogenous ligand anandamide, and synthetic ligands such as 4alpha-phorbol 12,13-didecanoate (4α-PDD) and GSK1016790A (Andersson et al., 2010; Angelico & Testa, 2010; Nilius & Voets, 2013; Vriens et al., 2009).

TRPV4 Channels and the Urinary Bladder

Studies indicate that several TRP channels, including TRPV1/2/4, TRPM7/8, and TRPA1, are expressed in the urinary bladder and may act as sensors of stretch and/or chemical irritation in the lower urinary tract (Andersson et al., 2010; Yu et al., 2011). Most of these channels are also implicated in bladder diseases such as OAB and BPS/IC (Everaerts et al., 2008; Nilius et al., 2007). Therefore, it is important to understand the expression as well as

the mechanisms by which these channels act to create potential therapeutic targets for these diseases.

TRPV4 expression was first shown in the urinary bladder urothelium of mice and rats in 2007, where it was mainly found in bladder basal and intermediate urothelial cells (Gevaert et al., 2007). Since then, other groups have confirmed the expression of TRPV4 in urothelium (Birder, 2007; Kullmann et al., 2009; Mochizuki et al., 2009; Yamada et al., 2009; Yu et al., 2011). In addition, the TRPV4 agonist 4α-PDD increased $[Ca^{2+}]_i$ when administered to cultured urothelial cells, and this increase was blocked by the relatively nonselective TRP antagonist ruthenium red (RR), providing evidence that functional TRPV4 channels are in fact expressed in urothelium (Birder, 2007). Other groups found TRPV4 expression in the detrusor muscle in addition to the urothelium; however, expression levels were found to be approximately 20-fold higher (Thorneloe et al., 2008) to 36-fold higher (Xu et al., 2009) in the urothelium compared to detrusor smooth muscle.

TRPV4 and Bladder Function

The role of TRPV4 in bladder function was first reported in 2007 when an abnormal urine voiding pattern in the knockout (KO) mouse was observed (Angelico & Testa, 2010; Gevaert et al., 2007). Using cystometrograms (CMG), a decreased frequency of voiding contractions and an increased frequency of nonvoiding contractions, intermicturition interval (IMI), and total urine volume per micturition were observed in TRPV4 KO mice (Gevaert et al., 2007). Later studies replicated these results, finding a decrease in voiding frequency and an increase in bladder capacity and void volumes in the TRPV4 KO (Everaerts et al., 2010; Thorneloe et al., 2008). With the creation of a TRPV4 KO mouse model, the role of TRPV4 in bladder function has been further explored. TRPV4 KO mice are viable and fertile and have no obvious behavioral abnormalities (Everaerts et al., 2010). Several studies have reported various mild phenotypes including an abnormal response to osmotic and somatosensory mechanical stimuli, deficits in osmotic homeostasis, increased blood osmolarity, increased bone mass, aging-related hearing impairment, and they drink significantly less compared to their WT counterpart (Everaerts et al., 2010; Nilius et al., 2007).

In addition to the TRPV4 KO mouse model used to study bladder function, agonists and antagonists of this channel have been used to study its role. Agonists of the TRPV4 channel appear to increase bladder hyperactivity, while antagonists show a decrease, making it a promising target for OAB and possibly other bladder dysfunction disorders (Janssen et al., 2011). Administration of 4α-PDD to conscious rats caused an increase in amplitude of reflex bladder contractions during CMG (Birder, 2007). GSK1016790A, a highly selective and potent TRPV4 agonist that is approximately 300-fold more potent than 4α-PDD, induced hyperactivity *in vivo* by decreasing infused and voided volume in normal mice (Thorneloe et al., 2008) and rats (Aizawa et al., 2012). HC-067047, a potent and selective TRPV4 antagonist, provoked a decrease in micturition frequency and an increase in bladder capacity when administered systemically to mice and rats with CYP-induced cystitis (Everaerts et al., 2010). This TRPV4 blocker has advantages over other antagonists like

RR, which is relatively nonselective, as well as Gd^{3+} (RN-1734) and La^{3+} (RN-9893), of which little is known (Vriens et al., 2009).

Current research demonstrates that TRPV4 has an important role in normal bladder function due to the regulation of ATP release from the urothelium in response to increases in intravesical pressure (Everaerts et al., 2010; Gevaert et al., 2007). It is widely accepted that the urothelium participates in the initiation of the micturition reflex (Birder, 2007; Yamada et al., 2009). Evidence shows that mechanical stretch evokes the release of ATP from urothelial cells to then activate P2X3 receptors on sensory nerve terminals, leading to membrane depolarization, action potential generation, and signals to the spinal cord to initiate the micturition reflex (Birder, 2007). This process may occur, in part, to activation of TRPV4 channels (and possibly TRPV1 channels and other SACs) present on the urothelial cell surface. Administration of 4α-PDD to cultured urothelial cells resulted in an increase in ATP release, which was blocked by RR (Birder, 2007). Using calcium imaging in urothelial cells from WT and TRPV4 KO mice, 4α-PDD evoked an increase in $[Ca^{2+}]_i$ in WT cells that was absent in cells from KO mice. This increase in $[Ca^{2+}]_i$ was minimal in the absence of extracellular Ca^{2+}, indicating that the increase is due to Ca^{2+} influx through TRPV4 channels (Mochizuki et al., 2009). In an *in vitro* cell-stretch system, WT urothelial cells show a transient increase in $[Ca^{2+}]_i$ after stretching followed by a sustained increase, which is blocked by RR. Also, after stretch, there is a prominent increase in ATP release in these cells, which is significantly decreased in cells from TRPV4 KO mice and suppressed by RR and depletion of extracellular Ca^{2+} (Gevaert et al., 2007; Mochizuki et al., 2009). Furthermore, P2X3 receptor KO mice lack stretch-evoked reflex bladder contractions (Birder, 2007). Taken together, these results provide evidence that TRPV4 channels, located on urothelial cells in the urinary bladder, release ATP upon activation that binds to receptors on bladder sensory neurons, sending signals to the spinal cord to initiate micturition.

TRPV Channels and Pain

Evidence indicates that the TRPV channel subfamily is involved in the detection of acute noxious thermal, mechanical, and chemical stimuli. Hypersensitivity and pain in various pathological conditions are often due to upregulated expression and/or increases in sensitivity of TRPV channels (Nilius et al., 2007). TRPV1 is expressed in >50% of DRG neurons and is activated by heat, acid, and vanilloids, indicating an important role in the detection of noxious stimuli. In fact, TRPV1 KO mice show a decreased response to noxious thermal stimuli and are defective in developing inflammatory thermal hyperalgesia (Nilius et al., 2007). In addition, TRPV4 KO mice have a decreased sensitivity to tail pressure, show deficits in mechanically evoked paw withdrawal responses (Birder, 2007), and thermal hyperalgesia is also absent in these mice. Therefore, TRPV1 and TRPV4 are implicated in the etiology of thermal allodynia and hyperalgesia. Further, taxol-induced painful neuropathy can be treated by gene silencing of TRPV4 (Alessandri-Haber et al., 2004).

Our major contributions to understanding the role(s) of TRPV4 in micturition have focused on (i) TRPV4 function in the NGF-OE mouse that exhibits increased voiding frequency and somatic sensitivity; (ii) TRPV4 expression and function in platelet-derived growth factor

receptor-α (PDGFRα)$^{+}$ ICs in the lamina propria of the postnatal urinary bladder; and (iii) the role of TRPV4 in ATP release via vesicular exocytosis. The following sections will summarize these contributions and provide suggestions for additional areas of study.

TRPV4 function in NGF-OE mice that exhibit increased voiding frequency and somatic sensitivity

We previously demonstrated (Girard et al., 2013) increased TRPV4 transcript and protein expression in the LUT tissues (e.g., urothelium, suburothelium, suburothelial nerve plexus, lumbosacral DRG) in NGF-OE consistent with TRP channel function and modulation being dependent upon target tissue expression of growth factors (e.g., NGF, glial-derived neurotrophic factor (GDNF) (Ernsberger, 2009; Homma et al., 2013; Malin et al., 2011; Shimizu et al., 2018)). Interestingly, NGF-OE mice display opposite phenotypic changes in bladder function compared to TRPV4 KO (Gevaert et al., 2007) mice including reduced bladder capacity, increased frequency of voiding and somatic pelvic hypersensitivity (Schnegelsberg et al., 2010). The role of NGF in bladder sensory function and inflammation-induced referred hyperalgesia and in IC/BPS is well documented. We used three complementary approaches (i.e., conscious cystometry, natural voiding assays and void spot assays) to characterize the effects of intravesical TRPV4 blockade on urinary bladder function in NGF-OE and wildtype (WT) mice.

We demonstrated that intravesical TRPV4 blockade with HC-067047 significantly reduced voiding frequency and NVCs using conscious cystometry with continuous saline instillation in NGF-OE mice with an open outlet. Intravesical instillation of HC-067047 in littermate WT mice also significantly reduced voiding frequency but the reduction was less than that observed in NGF-OE mice. Using void spot assays, we showed that intravesical HC-067047 in NGF-OE mice reduced the number void spots (small and large) but no effects were observed in WT mice. Natural voiding assessments using the Urovoid approach demonstrated that intravesical instillation of HC-067047 significantly increased the void mass and IMI in NGF-OE mice studied over multiple 12 hr light: dark cycles. In addition, pelvic sensitivity determination, using von Frey filament testing, was significantly reduced in NGF-OE mice following intravesical infusion of HC-067047. These studies continue to suggest that TRPV4 blockade at the level of the urinary bladder may be a useful target to reduce urinary frequency (Everaerts et al., 2010; Gevaert et al., 2007; Girard et al., 2013; Merrill & Vizzard, 2014) and somatic sensitivity.

TRPV4 has a functional role in ATP release via vesicular exocytosis

ATP release from urothelial cells and subsequent binding to purinergic receptors regulate a variety of actions in the urinary bladder and micturition reflexes including sensory nerve activity, detrusor smooth muscle contraction, synaptic transmission, and nociception (Chen et al., 1995; Sui et al., 2014; Wang et al., 2005). Intravesical instillation of ATP enhanced spinal bladder neuron excitability (Munoz et al., 2011) and increased bladder activity (Pandita & Andersson, 2002). In contrast, attenuation of luminal ATP release (Beckel et al., 2015; Smith et al., 2005; Timoteo et al., 2014) reduced NVCs and voiding frequency. Altered ATP signaling may play a significant role in several functional LUT

pathologies (e.g., OAB, IC/BPS) (Ruggieri, 2006; Silva-Ramos et al., 2013; Sun & Chai, 2006). Pathological conditions of the bladder that elevate luminal ATP presumably activate mucosal purinoceptors to increase nerve excitability (Beckel et al., 2015; Gonzalez et al., 2016; Vlaskovska et al., 2001; Yu & de Groat, 2008). One mechanism that may account for this effect is autocrine purinoceptor activation on the urothelium stimulating continual ATP release (Sun & Chai, 2006). This aberrant signaling of ATP could be limited by controlling extracellular release from the urothelium and/or its binding to purinergic receptors within deeper layers of the bladder wall. Previous studies suggested a functional role for TRPV4 in ATP release such that TRPV4 activation (e.g., stretch or agonist application) leads to an increase in Ca^{2+} and ATP that is attenuated in TRPV4 KO mice or by TRPV4 antagonist (Gevaert et al., 2007; Mochizuki et al., 2009). Several mechanisms of urothelial ATP release including vesicular exocytosis (Gonzalez et al., 2016; McLatchie & Fry, 2015; Sui et al., 2014; Wang et al., 2005), pannexin and/or connexin ion channels (Beckel et al., 2015; McLatchie & Fry, 2015; Sui et al., 2014), and nucleoside transporters (Lazarowski et al., 2003; Wang et al., 2005) have been demonstrated. Our studies focused on the pharmacological manipulation of vesicular exocytosis and pannexin-1 channels (Figure 4). We intravesically instilled the general secretory inhibitor, BFA, to inhibit ATP vesicular release (Figure 4). BFA has previously been shown to decrease mechanical- and stretch-evoked ATP release from the urothelium (Gonzalez et al., 2016; McLatchie & Fry, 2015; Sui et al., 2014; E. C. Wang et al., 2005). We also intravesically instilled the inhibitory peptide, 10Panx, to block pannexin-1 channels, implicated in distention-evoked ATP release from the urothelium (Beckel et al., 2015; Gonzalez et al., 2016; Negoro et al., 2014; Timoteo et al., 2014) (Figure 4).

Our studies with BFA, 10Panx or co-administration of both in NGF-OE and WT mice with urinary bladder distention to 25 cm H_20 demonstrated that vesicular exocytosis contributed to most of the distention-evoked luminal ATP release consistent with previous studies (Gonzalez et al., 2016) (Figure 4). We quantified luminal ATP after functionally inhibiting known mechanisms of ATP release via vesicular exocytosis and pannexin channels as well as through TRPV4 (Gevaert et al., 2007; Mochizuki et al., 2009) and P2X blockade (Sui et al., 2014; Sun et al., 2009; Vlaskovska et al., 2001) (Figure 4). Upon intravesical inhibition of TRPV4 or inhibition of vesicular release in the urinary bladder, distention-induced luminal ATP release was decreased in both WT and NGF-OE mice although the magnitude of reduction was greater in NGF-OE mice. Inhibition of pannexin channels locally in the urinary bladder did not decrease the luminal release of ATP in WT or NGF-OE mice. These data suggest ATP is released by vesicular exocytosis in the urinary bladder and TRPV4 plays a functional role (Figure 4). Although these results suggest a vesicular secretory mechanism, the effects of BFA may also generalize to inhibit the transport of cell surface proteins, like hemichannels, to attenuate release (Wang et al., 2005). Future studies are needed to differentiate the effects of vesicle release and hemichannel inhibition.

ATP and TRPV4 activate cells in the laminar propria network, leading to the appearance of organized, propagating wavefronts

As discussed above, the lamina propria is located between the urothelium and the detrusor and is composed of loose connective tissue, ICs, vasculature, lymphatic vessels, nerve fibers and nerve terminals and may serve to integrate epithelial and smooth muscle input to maintain normal bladder function (Andersson, 2002, 2004; Birder & Andersson, 2013). The physical proximity of the structural components of the urinary bladder suggests that reciprocal communication among urothelial cells, the lamina propria and detrusor smooth muscle may be possible (Birder & Andersson, 2013).

Lamina propria ICs may interact with an extensive network of nerve fibers (subepithelial plexus), including afferents, that course through the lamina propria (Gabella & Davis, 1998). Immunohistochemistry as well as electron microscopy imaging reveal close physical proximity between lamina propria cells and nerve fibers (Andersson & McCloskey, 2014; Davidson & McCloskey, 2005; Wiseman et al., 2003). A dense network of kit+ lamina propria cells that expressed platelet-derived growth factor receptor-α (PDGFRα) and exhibited stellate or spindle-shaped morphology were also found in murine bladder (Koh et al., 2012). Although the lamina propria is heterogeneous, consisting of diverse cell types, certain cells may be important modulators of neural activity and form a communication link between the urothelium and detrusor; the importance of which may depend upon age or presence of pathology.

We demonstrated that the lamina propria cell network in early postnatal rat pups (P≤21) exhibited a predominance of PDGFRα- and TRPV4-IR. Application of exogenous mediators (i.e., ATP, TRPV4 agonist) activated and increased the number of lamina propria cells that exhibited active Ca^{2+} events. Further, lamina propria cell activity was not random as demonstrated with spatio-temporal maps and calcium transient particle analyses that demonstrated varying degrees of coupling (e.g., tight, partial, or loose organization). This coupling of cellular Ca^{2+} activity of cells could be modified to generate organized, propagating bands of activity (wavefronts) with ATP in the lamina propria network. These findings suggest that lamina propria Ca^{2+} signaling may facilitate communication through this syncytial network to other cell types or tissue layers of the urinary bladder. Spatio-temporal patterning in the lamina propria network may be a means to affect change in sensory processing and/or detrusor contractility.

Using lamina propria wholemounts from postnatal rats, we identified Ca^{2+} events that had long durations with slow upstroke and downstroke phases. These Ca^{2+} events in the lamina propria network were largely dependent on Ca^{2+} release from internal stores because CPA significantly reduced the (i) overall duration of cell activation and (ii) the number of cells exhibiting Ca^{2+} events. In lamina propria wholemounts from postnatal rats, we demonstrated Ca^{2+} events spreading along processes between cells. The lamina propria network exhibited a range of Ca^{2+} behaviors from ongoing, loosely coupled individual cellular activity to near synchronous cell activation in a particular direction to form a Ca^{2+} wavefront that propagated substantial distances throughout the lamina propria. The range of Ca^{2+} behaviors is indicative of flexible coupling between lamina propria cells (Neuhaus et al., 2020).

Although the mechanism underlying the formation of a Ca^{2+} wavefront is unclear, the lamina propria exhibits substantial Cx43 expression, consistent with the presence of gap junctions (Ikeda et al., 2007).

Purinergic signaling in the LUT involves multiple tissues and cell types, including: suburothelial nerve plexus, smooth muscle cells, lamina propria cells including ICs as well as inflammatory cells (Birder & Andersson, 2013; Birder, 2005; Li et al., 2013). ICs express P2X and P2Y (i.e., P2X3 P2Y2, P2Y4, P2Y6) receptors and are proposed to form a functional syncytium with smooth muscle cells (Drumm et al., 2014; Sui et al., 2006). Although the mechanism coupling ICs to sensory activity is unknown, the location of ICs and the responsiveness to ATP suggest they may have a regulatory role in the afferent limb of the micturition reflex (Wu et al., 2004). We demonstrated that exogenous ATP increased Ca^{2+} transient activity in numerous lamina propria cells that resulted in multiple Ca^{2+} waves propagating through the lamina propria (Heppner et al., 2017) (Figures 5, 6). In addition, we showed that exogenous ATP converted lamina propria cells from a loosely coupled pattern to a highly organized pattern with the formation of a Ca^{2+} wavefront (Heppner et al., 2017) (Figures 5, 6). This finding is consistent with the hypothesis that ATP released from urothelium or other tissues (i.e., suburothelial nerves) increases lamina propria Ca^{2+} signaling and facilitates communication through this syncytial network to other cell types or tissue layers (Andersson & McCloskey, 2014) (Heppner et al., 2017) (Figure 5, 6). A large area of the lamina propria network undergoing near-synchronous activity may provide a much more potent signal/stimulus to other cell types compared to loosely coupled patterns of activation of lamina propria cells.

As described above, multiple TRP channels from different subfamilies are expressed in the urinary bladder and have specific LUT tissue distributions. We demonstrated functional TRPV4 expression in lamina propria cells in postnatal rat pups (Heppner et al., 2017). In wholemount tissue preparations from postnatal rats (≤P21), TRPV4-IR was observed in lamina propria cells that exhibited similar morphology to those expressing PDGFRα-IR. Application of the TRPV4 agonist, GSK1016790, increased the number of cells that exhibited active Ca^{2+} events and time active as shown by the rate of integrated Ca^{2+} activity (Heppner et al., 2017). The TRPV4 antagonist, GSK2193874, did not affect the number of lamina propria cells exhibiting Ca^{2+} transients or the duration of Ca^{2+} transients suggesting that TRPV4 channels are not consistently active in lamina propria cells. Activation of TRPV4 channels via proteolytic activation of protease-activated receptor 2 under certain condition including inflammation (Sostegni et al., 2015) has been demonstrated, but the background level of TRPV4 activation in normal bladder function is apparently quite minimal.

In wholemount preparations from rat pups (≤P21), we consistently identified a dense network of PDGFRα- or TRPV4-IR cells in the lamina propria; however, few such cells were present in adult preparations (Heppner et al., 2017). Differences between preparations from adult and postnatal rats may reflect the continuing maturation of the micturition reflexes during the early postnatal period. The functional syncytium between ICs and the detrusor smooth muscle may have greater importance for coordinating bladder emptying in the early postnatal period due to the absence of mature and functional neural input

needed to coordinate the activities of the CNS and PNS including the urinary bladder (Kanai et al., 2007). The current studies in rat pups (≤P21) are consistent with this suggestion as demonstrated by: (i) the predominance of the lamina propria cellular network in early postnatal rat pups; (ii) the ability of exogenous mediators (i.e., ATP, TRPV4 agonist) to activate and increase the number of lamina propria cells that exhibited active Ca^{2+} events; and (iii) the ability of ATP and TRPV4 agonist to increase the rate of integrated Ca^{2+} activity corresponding to coupled lamina propria network events and the formation of propagating wavefronts (Heppner et al., 2017) (Figures 5, 6).

In future studies, it would be of interest to examine the lamina propria network in wholemount preparations from preclinical animal models of bladder dysfunction or SCI to determine if the lamina propria network at the urothelial-lamina propria junction is changed and/or if spontaneous and evoked Ca^{2+} transients and network activity is altered. A change in the number of cells exhibiting Ca^{2+} events, their pattern of activation, the duration of Ca^{2+} events and/or the ability of exogenous mediators (e.g., ATP, TRPV4 agonists) to increase the rate of integrated Ca^{2+} activity corresponding to coupled lamina propria network events could contribute to altered sensory processing (e.g., mechanosensation, pain) and altered urinary bladder function (i.e., hyperactivity). Plasticity in lamina propria cellular network has been previously demonstrated. For example, ICs in the detrusor are decreased 5 weeks following SCI, target organ innervation is reduced, and the smooth muscle is hypertrophied (Johnston et al., 2012). However, there are variable and inconsistent changes in ICs from individuals with bladder dysfunction (e.g., neurogenic and idiopathic detrusor overactivity) (McCloskey, 2010). Thus, future studies should examine the lamina propria network at the urothelial-lamina propria junction following neural injury, disease, or urinary bladder dysfunction to fully understand its functional significance.

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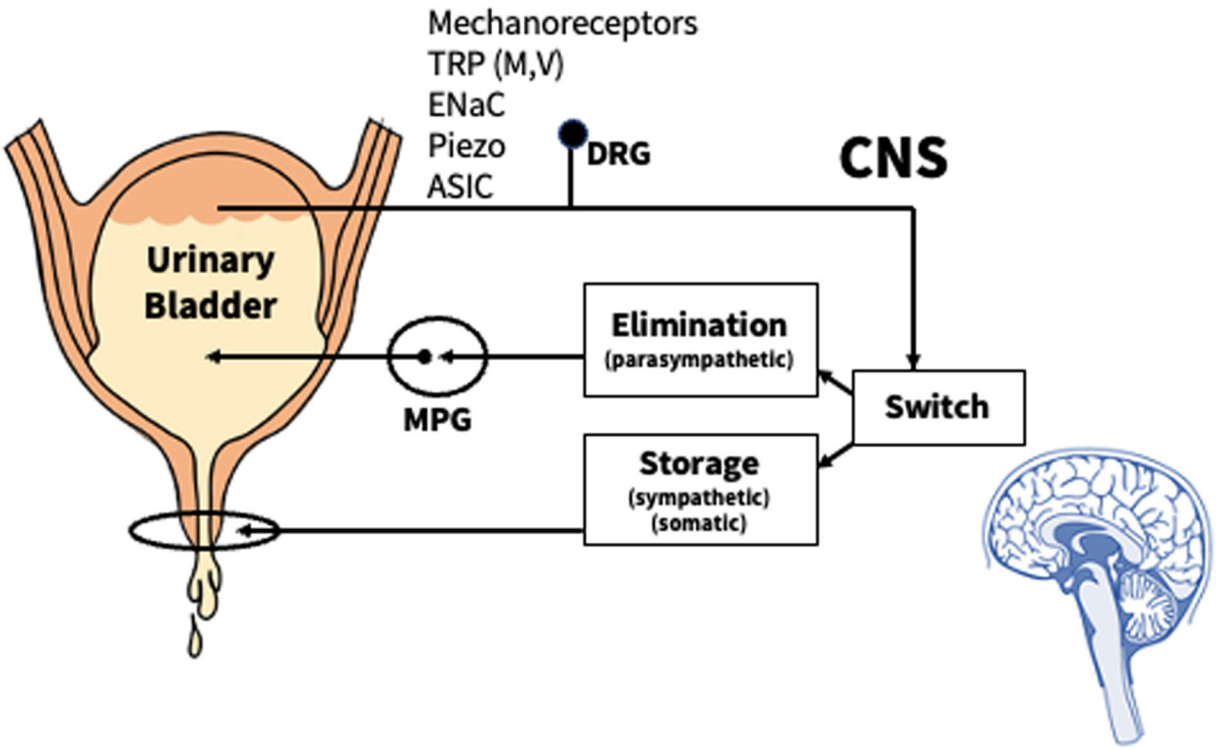

Figure 1. Storage and elimination (voiding) of urine.

LUT function is controlled by neural pathways that maintain a reciprocal relationship between the urinary bladder and the urethral outlet. During bladder filling, storage reflexes, organized primarily in the spinal cord, are activated whereas voiding is mediated by reflex mechanisms that are organized in the brain. During bladder filling and storage, the parasympathetic innervation of the detrusor is inhibited, and the urethral sphincter is activated, preventing involuntary urine release. During bladder filling, the parasympathetic efferent pathway to the bladder, including a population of CNS (e.g., pontine micturition center) neurons, is turned off. As bladder filling continues and a threshold level of bladder distension (i.e., stretch) is achieved, the afferent activity from urinary bladder mechanoreceptors switches the pathway to the elimination mode. During elimination (voiding), parasympathetic activity is activated resulting in urinary bladder contraction, whereas sympathetic activity and somatomotor activity is withdrawn. DRG, dorsal root ganglion; MPG, major pelvic ganglion. TRP, transient receptor potential channel; M, melastatin; V, vanilloid; ENaC, epithelial sodium channel; ASIC, acid-sensing ion channels. Modified from (Vizzard, 2006).

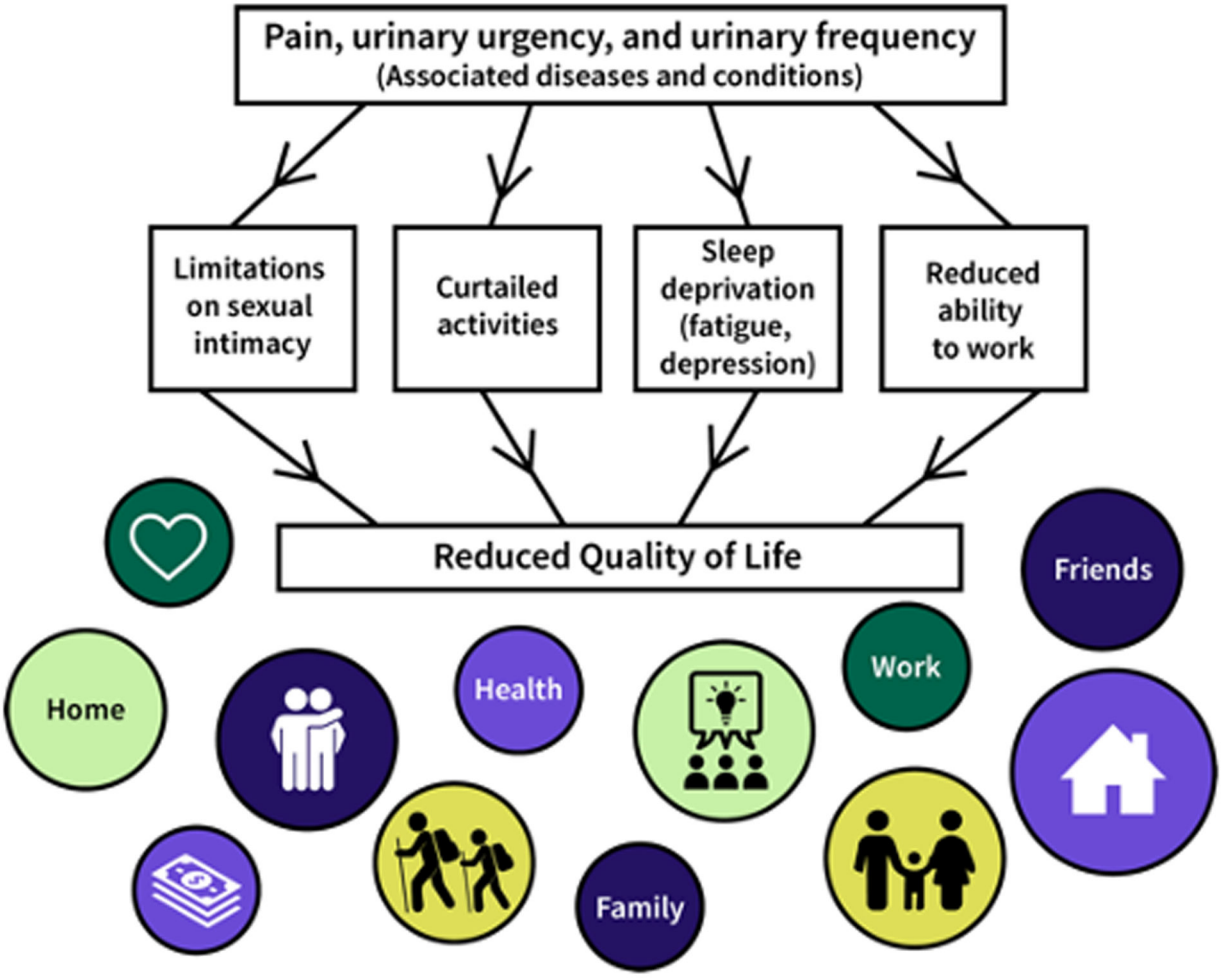

Figure 2. IC/BPS reduces quality of life (QoL).

Symptoms of IC/BPS can negatively affect sexual relationships and relationships in general and limit one's ability to work and to participate in other activities, resulting in sleep deprivation, fatigue and depression which collectively reduce QoL.

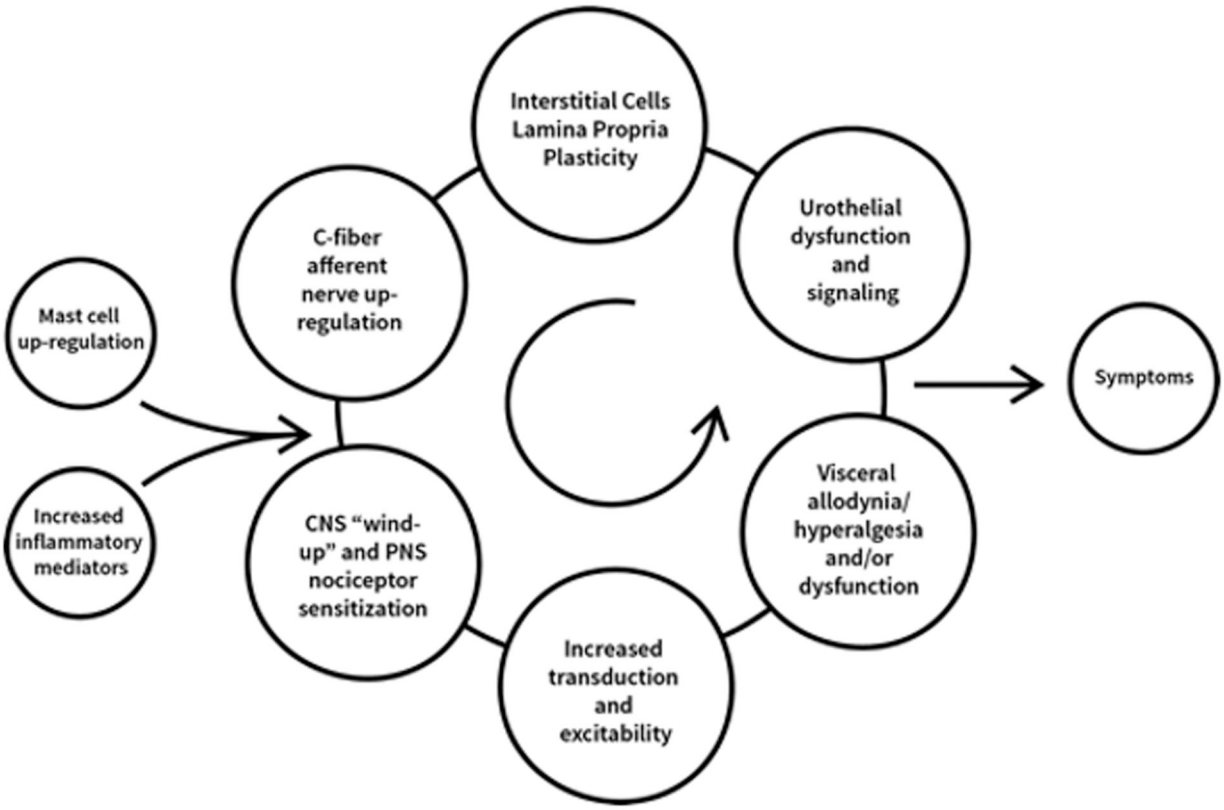

Figure 3. Potential etiologic cascade and pathogenesis underlying painful bladder syndrome (BPS)/interstitial cystitis (IC).

BPS/IC likely has a multifactorial etiology. There is no consensus regarding the etiology or pathogenesis of BPS/IC, but a number of proposals include: a "leaky epithelium," mast cell activation and release of neuroactive compounds at the level of the urinary bladder, "awakening" of C-fiber bladder afferents, and upregulation of inflammatory mediators (e.g., cytokines and chemokines). Inflammatory mediators can produce CNS "wind-up" and peripheral nociceptor sensitization resulting in chronic bladder pain and voiding dysfunction. Figure adapted from (Gonzalez et al., 2014; Sant et al., 2007).

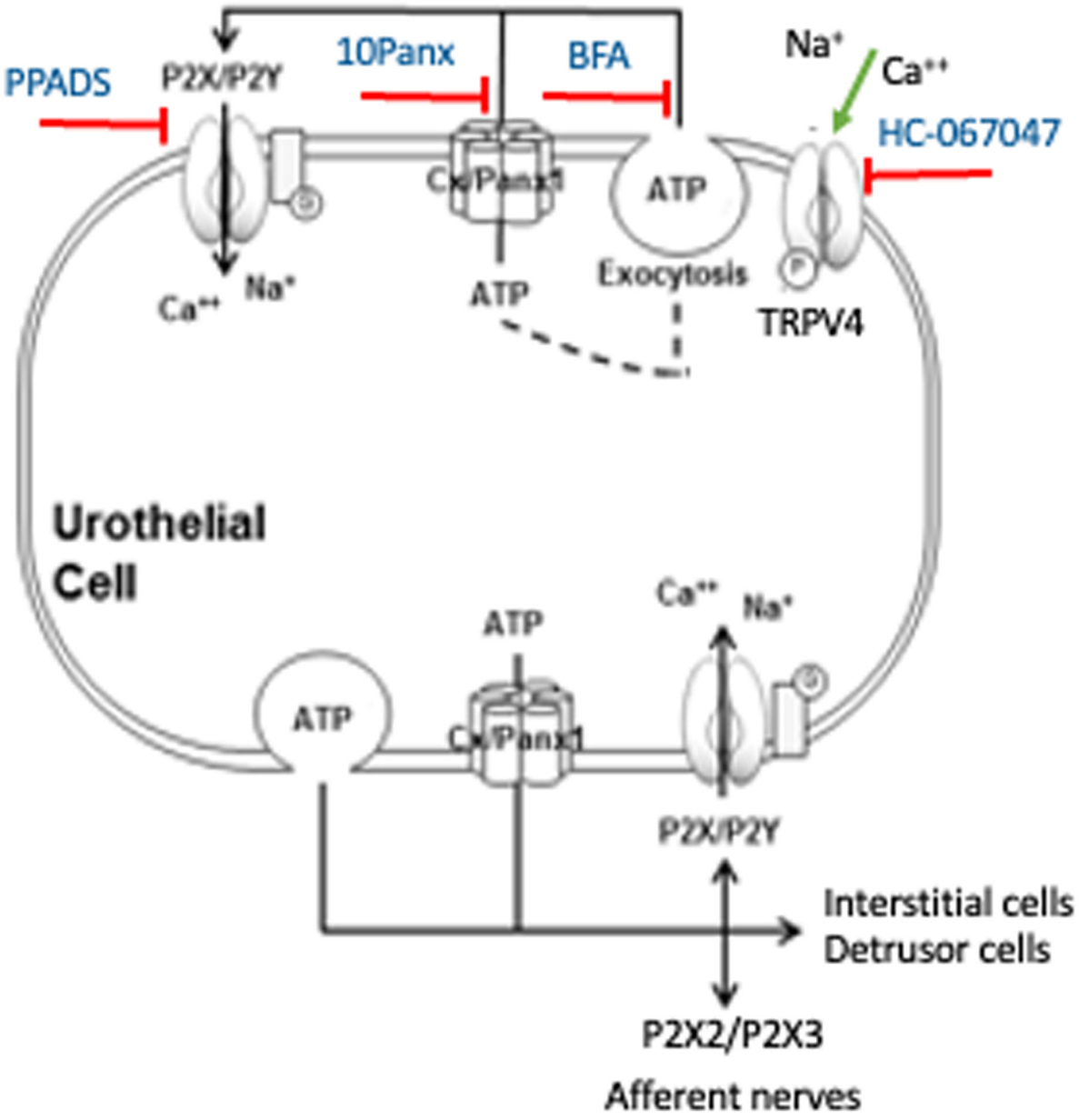

Figure 4. ATP is released by vesicular exocytosis in the urinary bladder and TRPV4 plays a functional role in the urothelium.

Diagram illustrating the possible mechanisms and underlying key components contributing to ATP release in urothelial cells with TRPV4 playing a functional role. Our studies focused on the pharmacological manipulation of vesicular exocytosis and pannexin-1 channels using the general secretory inhibitor, brefeldin A (10 μM) (BFA), to inhibit ATP vesicular release and/or the inhibitory peptide, 10Panx (50 μM), to block pannexin-1 channels. We quantified luminal ATP after functionally inhibiting known mechanisms of ATP release via vesicular exocytosis and pannexin channels as well as through TRPV4 (HC-067047) (1μM) and P2X (PPADS)(300 μM) blockade. With intravesical inhibition of TRPV4 or inhibition of vesicular release in the urinary bladder, distention-induced luminal ATP release was decreased in both WT and NGF-OE mice although the magnitude of reduction was greater

in NGF-OE mice. Inhibition of pannexin channels locally in the urinary bladder did not decrease the luminal release of ATP. Sites of inhibition are indicated with red lines. PPADS, pyridoxalphosphate-6-azophenyl-2’,4’-disulfonic acid.

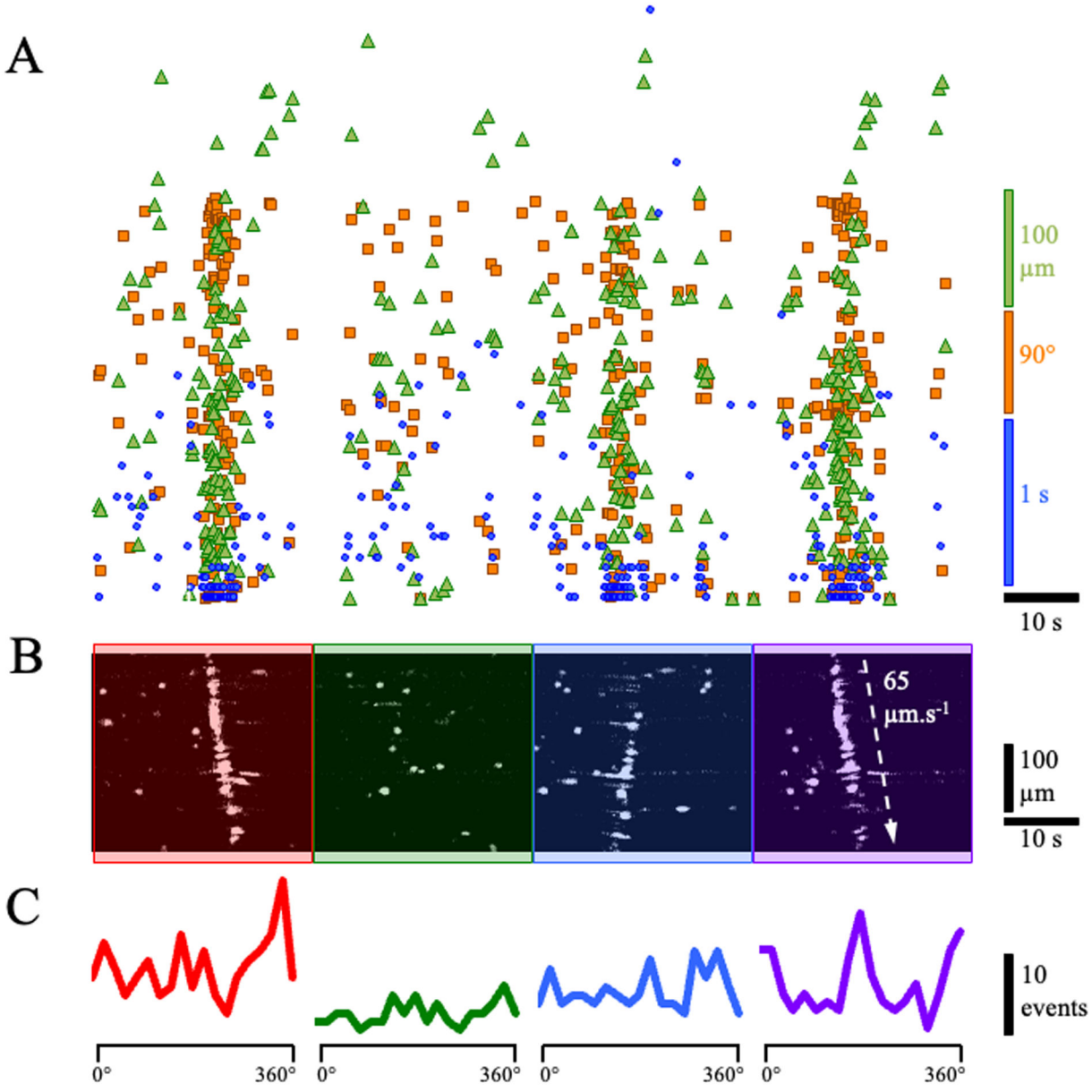

Figure 5. Tightly and loosely coupled Ca^{2+} network activity in the lamina propria syncytium.
(**A**) Plot of the firing characteristics of lamina propria cells showing (i) the distance (green triangles), (ii) the angle (orange squares) and (iii) the time delay (blue dots) between lamina propria cells in the forward firing sequence. (**B**) Spatio-temporal map of lamina propria cells firing showing 4 network firing events denoted by red, green, blue and purple overlays. (**C**) Frequency of angles histograms between next-to-fire cells (forward sequence) during the 4 network firing events. The first and last network firing events show a high degree organization (**B: red and purple overlays**) with a cluster of small delays (**A: blue dots**) between firing of cells and a strong bias for next-to-fire cells to occur at specific angles (**C: red & purple** lines; 160 & 340°) corresponding to the direction of the wavefront (90° to the propagation direction). The second network event (**B: green overlay**) does not show a

tightly organized network firing sequence, with variable delays (**A: blue dots**) and little bias in the angle between next-to-fire cells (**C: green line**). The third event (**B: blue overlay**) in which the wavefront propagates in the opposite direction, shows partially coupled network activity with some bias in the angle between next-to-fire cells but does not involve all the activatable cells in the field of view. Figure modified from (Heppner et al., 2017).

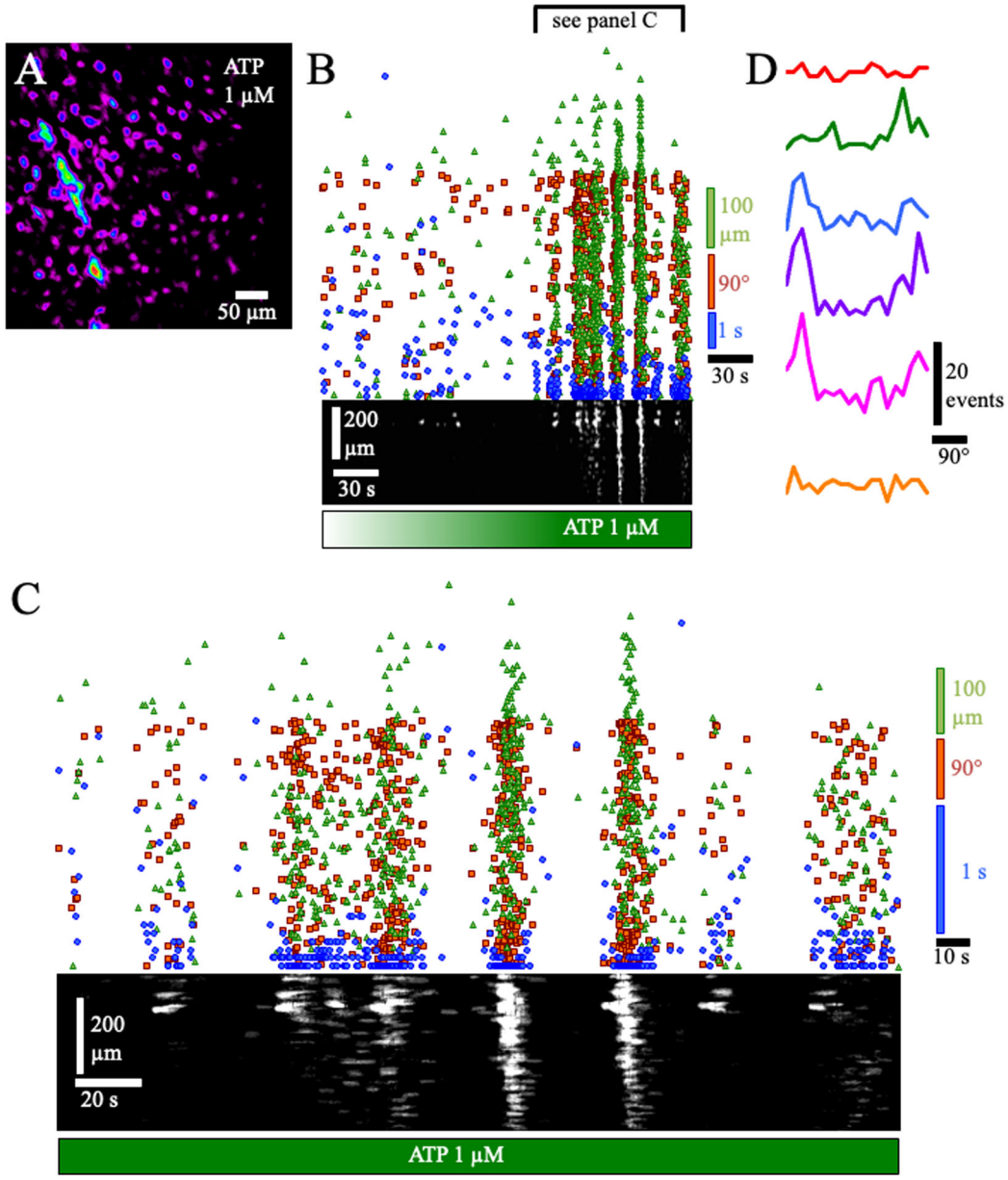

Figure 6. Lamina propria cell network response to ATP.

(**A**) Prevalence map of lamina propria syncytium. (**B**) Gradual perfusion of ATP to 1 µM (green bar) resulted in increasing number of cells firing, eventually leading to the development of propagating wavefronts. (**C**) Zoomed-in area from (**B**) showing greater detail of altered activity resulting in wavefront development. (**D**) Angle histograms showing the progression from unorganized activity to well-defined wavefronts based on angle bias. In preparations with the presence of propagating network Ca^{2+} events, the velocity (60–70 µm/s) of propagation was consistent, even though the direction of propagation was often variable. The bias in angle (10.65 ± 1.63 (n=8)) was determined to evaluate the overall degree of coupling between active lamina propria cells and indicated that lamina propria

cell activity was not random and had a defined direction (loosely/tightly coupled wavefront). Figure modified from (Heppner et al., 2017).