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Urine Trouble: A Molecular and Anatomical Examination of Bladder Pain

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Urine Trouble: A Molecular and Anatomical Examination of Bladder Pain

by

Lara Wiley Crock

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ABSTRACT OF THE DISSERTATION

Urine Trouble: A Molecular and Anatomical Examination of Bladder Pain

by

Lara Wiley Crock

Doctor of Philosophy in Biology and Biomedical Sciences

Neurosciences

Washington University in St. Louis, 2013

Professor Robert W. Gereau, Chairperson

Interstitial cystitis or painful bladder syndrome (IC/PBS) is a chronic pain syndrome affecting 3-6% of women in the US. Some evidence suggests urothelial injury may cause some cases of IC/PBS. To examine how urothelial injury modulates bladder pain, three injury models were used: Infection with uropathogenic *Escherichia coli* (UPEC, inflammatory), Protamine sulfate (PS, non-inflammatory chemical), and lipopolysaccharide (LPS, non-infectious and inflammatory) treatment. Surprisingly, injury with PS decreased, while UPEC alone increased the pain response to bladder distention. These data suggest that changes in the urothelium may modulate some forms of IC/PBS. However, in most cases, patients with IC/PBS have no histological abnormalities. Therefore, other molecular mediators may modulate IC/PBS.

Glutamate, the major excitatory neurotransmitter in the CNS, modulates a host of physiological responses through the actions of ionotropic (iGluRs) and metabotropic receptors (mGluRs). Metabotropic glutamate receptor 5 (mGluR5) has previously been shown to have an important role in somatic inflammatory and neuropathic pain models. However, there is limited evidence that mGluR5 in the CNS has a role in bladder pain. To determine the function of mGluR5 in non-inflammatory bladder pain, mice with a targeted genetic deletion of mGluR5 (mGluR5 KO) were used. Both mGluR5 KO mice and mice given a specific mGluR5 antagonist (fenobam) had a reduced bladder pain response. Systemic treatment with fenobam in mice infected with UPEC also resulted in a reduced response to bladder distention. Thus suggesting that mGluR5 is necessary for the full expression of inflammatory and non-inflammatory bladder pain.

However, these techniques do not indicate the site of action because mGluR5 is expressed throughout the pain neuroaxis. Antagonism of mGuR5 in the right central nucleus of the amygdala (CeA) is analgesic in a somatic inflammatory injury model. To examine the role of mGluR5 in the CeA, pharmacological activation of Group I mGluRs (mGluR5 and 1) was examined. Activation of mGluR5/1 in the CeA was sufficient to increase the pain response to noxious bladder distention. Additionally, pharmacological inhibition and virally mediated conditional deletion of mGluR5 in the CeA reduced the evoked response to bladder distention. Finally, optogenetic activation of the CeA increases the pain response to bladder distention suggesting that mGluR5 activation increases neuronal excitability in the CeA, increasing sensitivity to bladder distention. Overall, these data suggest a role of urothelial injury and mGluR5 in bladder pain.

Chapter 1

<u>Introduction to the Role of Metabotropic Glutamate Receptor 5 (mGluR5) in Bladder Pain</u>

Pain serves as a vital warning system that protects one's body from harm. However, it serves no protective purpose if no injury or potential injury has occurred or if pain persists after an injury has healed. Chronic pain is often accompanied by secondary symptoms such as poor quality of life, anxiety and depression (Hunt and Mantyh, 2001). A type of chronic pain that affects 3-6% of women in the US is interstitial cystitis/painful bladder syndrome (IC/PBS) (Berry *et al.*, 2011). IC/PBS is characterized by: 1. Chronic pelvic pain (>6 months) that is perceived to be related to the urinary bladder, 2. at least one other urinary symptom, and 3. the absence of infection or other identifiable cause (Theoharides, 2007; Hanno *et al.*, 2010). Patients suffering from IC/PBS have a poor quality of life that is similar to patients in end-stage dialysis (Held PJ, 1990). In the U.S., the annual cost of IC/PBS symptom management exceeds $750 million (Clemens et al., 2008b; Hanno et al., 2011). Despite the prevalence and severity of IC/PBS, there have been no conclusive advances in understanding the pathogenesis or etiology of the disease (Sand, 2006; Clemens et al., 2008a; Hanno et al., 2010). Yet, several hypotheses exist (see etiological schema Figure 1.1) (Hanno *et al.*, 2010). These include, but are not limited to: infection (Domingue et al., 1995; Duncan and Schaeffer, 1997; Keay et al., 1998; Haarala et al., 1999; Nickel et al., 2010; Rudick et al., 2010; Rudick et al., 2011), autoimmunity (Altuntas *et al.*, 2011), alterations of the urothelium (Wilson et al., 1995; Hurst et al., 2007; Parsons, 2007; Keay, 2008; Birder, 2011) or neurochemical changes in the pain neuroaxis (Aaron and Buchwald, 2001; Aaron et al., 2001; Erickson et al., 2001; Brand et al., 2007). The symptom of bladder pain in IC/PBS has been the hypothesized to be the result of urothelial damage leading to neurochemical changes in the spinal cord or brain (Parsons, 2007; Keay, 2008; Hanno *et al.*, 2010) (see figure 1.1). Alternatively, it is hypothesized that IC/PBS is part of a larger group of chronic pain syndromes including fibromyalgia (Aaron and Buchwald, 2001; Aaron et al., 2001; Erickson et al., 2001; Brand et al., 2007). Regardless of the etiology, different types of chronic pain have been shown to produce distinct neurochemical changes in the spinal cord and brain (Hunt and Mantyh, 2001). Several recent studies have pointed to the amygdala as an important site in the brain for processing painful information(Neugebauer, 2007; Ji and Neugebauer, 2009; Kolber et al., 2010). Specifically, the central nucleus of the amygdala (CeA) has been identified as a key structure in

the modulation of somatic and visceral pain (Neugebauer et al., 2004; Carrasquillo and Gereau, 2007; Neugebauer, 2007; Kolber et al., 2010; Gustafsson and Greenwood-Van Meerveld, 2011; Myers et al., 2011). Decoding how injury to the bladder urothelium modulates bladder pain, as well as identification of molecular mediators in the pain neuroaxis that modulate bladder pain, could provide important molecular targets for new therapies. One possible molecular target in the pain neuroaxis is the metabotropic glutamate receptor 5 (mGluR5). Modulation of this G protein-coupled transmembrane receptor has shown promise in a variety of somatic inflammatory pain models (Varney and Gereau, 2002; Cruz and Cruz, 2007; Montana et al., 2009; Kolber et al., 2010). Furthermore, recently mGluR5 in the right CeA has been identified as a key modulator of somatic inflammatory pain(Ji and Neugebauer, 2009; Kolber et al., 2010). It is the goal of this thesis to examine how distinct urothelial injuries result in bladder pain. We use three distinct models of bladder injury to determine how urothelial injury modulates bladder pain. Additionally, it is the goal of this thesis to examine how an important regulator of other types of pain, mGluR5, modulates bladder pain. In chapter 3 of this thesis, we use genetic and pharmacologic methods to examine the role of mGluR5 in both inflammatory and non-inflammatory bladder pain. In chapter 4 of this thesis, we examine how mGluR5 in the CeA modulates bladder pain.
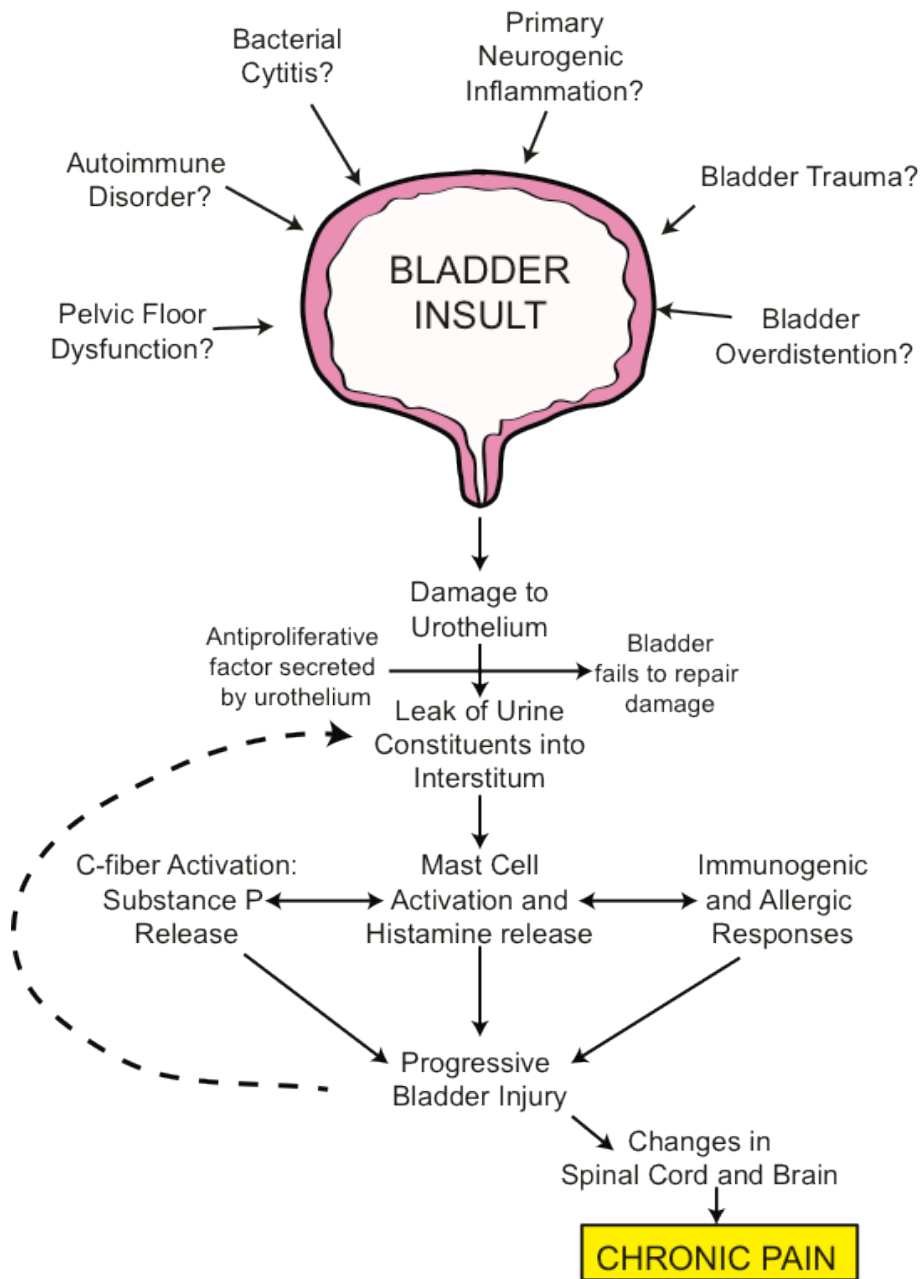
Figure 1.1. Proposed etiologic cascade leading to IC/PBS adapted from Figure 2 in (Hanno *et al.*, 2010)

Assessment of Bladder Pain In Rodents

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." While nociception is defined as "The neural process of encoding and processing noxious stimuli' (Loeser and Treede, 2008). Pain is emotional and subjective while

nociception is the sensory process that may or may not be associated with the experience of pain. Peripheral nociception can exist in the absence of pain and vice versa. For example peripheral anesthesia blocks the conscious experience of pain without affecting peripheral nociception. In contrast, a stroke in the area of the thalamus may lead to the severe and chronic experience of pain in the absence of peripheral nociception.

We can model pain and study nociception through the use of animal models. As Jeffery Mogil has pointed out "We might never be able to know what pain feels like in a rat, but we will never know what pain feels like in *you* either" (Mogil *et al.*, 2010). Pain is a subjective experience in both humans and animals, and we infer pain only by observing behavior. To infer the magnitude of bladder pain in both humans and animals, one commonly used technique is to measure a visceromotor response during the application of a noxious stimulus to the bladder.

VMR Recording in Response to Urinary Bladder Distention (UBD)

Visceromotor responses (VMRs) are the reflex contraction of abdominal muscles, and the magnitude of VMRs has been used as a tool to measure noxious visceral stimuli or analgesia (Ness and Gebhart, 1990). Three criteria are necessary for a valid measure of visceral pain: the measured responses must be (1) reliable and reproducible, (2) inhibited by known analgesics, (3) and they must not be inhibited by nonanalgesics (Ness, 1999). The measurement of VMR in response to urinary bladder distention (UBD) fits these criteria. In this model of visceral pain, 20 seconds of graded UBD (10 mm Hg to 80 mm Hg in 10 mm Hg steps) produced graded, reliable and reproducible VMR responses (increased abdominal muscle contractions) (Ness and Elhefni, 2004). The evoked response (VMR) to UBD was reversibly inhibited by morphine and intravesicular (injected into bladder) lidocaine, but not affected by the vehicle used to deliver them (Ness and Elhefni, 2004). In addition, intravesicular mustard oil (known to produce inflammation) results in a significant increase in evoked response to UBD (Ness and Elhefni, 2004). The evoked response (VMR) is a spinobulbospinal reflex to bladder distention, observed in decerebrate mice/rats but not in mice/rats with a transected spinal cord (Castroman and Ness, 2001; Ness et al., 2001; Ness and Elhefni, 2004). Bladder distention reliably produces pain

and/or discomfort in humans (Ness et al., 1998), and is frequently used in rats as a visceral pain model (Ness et al., 2001). Recently this visceral pain model was adapted for mice (Ness and Elhefni, 2004). We used this technique in thesis chapters 2-4 to measure distention-induced bladder pain in mice. See Figure 1.2 for a schematic of the VMR.
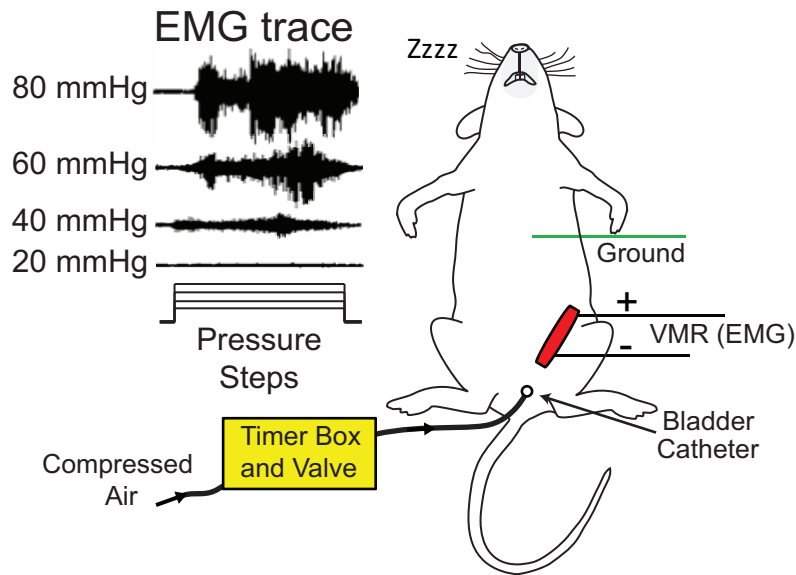
Figure 1.2 Illustration of *in vivo* urinary bladder distention visceromotor response (VMR) setup. Compressed air guided into the bladder with a catheter is used to distend the bladder. Distention induces a visceromotor response that is recorded as an electromyogram (EMG) from the abdominal muscles. Representative EMG traces show that the VMR response increases with increasing bladder distention pressure.

<u>Structure and Innervation of the Bladder</u>

Kidneys are essential to mammalian survival in part because they function to filter hazardous substances out of the bloodstream and excrete them as urine. The urinary bladder is a highly distensible organ that prevents the constant release of urine by storing it until disposal (micturition) is appropriate. The epithelial lining of the bladder, the urothelium or uroepithelium, is highly specialized watertight barrier designed to prevent toxic components of urine to diffuse across during prolonged storage (Hicks *et al.*, 1974). The urothelium of the mammalian bladder contains three cell layers: the superficial facet cell (SFC, also known as umbrella cells, facet cells or superficial cells) layer, intermediate cell layer and the basal cell layer (Hicks, 1975; Truschel *et al.*, 1999). SFCs form a single layer of highly differentiated and polarized cells on the apical

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surface of the bladder. They are either mono or multinucleate (species dependent), polyhedral, and between 25-250 micrometers in diameter (dependent on filling state of bladder) (Hicks, 1975; Truschel *et al.*, 1999). SFCs have distinct apical and basolateral membrane domains that are demarcated by tight junctions. The tight junctions of the SFCs create the impermeable barrier of the bladder wall (Khandelwal *et al.*, 2009).

The intermediate cell layer is found just below the SFCs. Intermediate cells rapidly differentiate when the overlying SFC is sloughed off to repopulate the SFC layer (Khandelwal *et al.*, 2009). In rodents, the intermediate cells are 1-2 layers thick while humans have up to five layers of intermediate cells (Jost *et al.*, 1989). However, through unknown mechanisms, the apparent number of intermediate cell layers is highly dependent on the filled status of the bladder (Jost *et al.*, 1989). The change in the number of intermediate cell layers may be one of the mechanisms by which the bladder is highly distensible. Furthermore, intermediate cells are mononucleate and ~10-15 micrometer in diameter (Hicks, 1975). Beneath the intermediate cell layer, is the basal cell layer. This layer consists of mononucleated cells ~10 micrometer in diameter, and the urothelium is continuously repopulated by stem cells within the basal layer (Kurzrock *et al.*, 2008). Normal turnover of the urothelium takes 3-6 months (Hicks, 1975; Jost, 1989), but rapid renewal of the urothelium can initiate within hours following injury such as bladder infection (Mulvey *et al.*, 1998; Mysorekar *et al.*, 2002)

Role of the Urothelium in IC/PBS

Abnormal urothelial turnover is common to multiple painful disease states including: recurrent urinary tract infections (UTIs), interstitial cystitis/PBS, and bladder cancer. It has been hypothesized that damage to the urothelium leads to the development of IC/PBS (Parsons, 2007; Hanno *et al.*, 2010). Disruption of urothelial integrity may be linked to expression of substances such as antiproliferative factor (see figure 1.1), which has been detected in the urine of patients with IC/PBS and may be related to the development of IC/PBS (Keay *et al.*, 2004a; Keay *et al.*, 2004b). Disruption of the impermeable urothelial barrier, leads to urinary solutes (such as potassium) causing tissue injury and pelvic nerve sensitization (Parsons, 2007). However, the

specific components of urothelial injury and the molecular signals generated by this injury that lead to bladder pain are not known. In chapter 2 of this thesis, we investigate how distinct urothelial injury models influence bladder pain. Additionally we aimed to tease out the unique molecular signals that are responsible for urothelial injury versus those that modulate pain.

Urothelium Injury Models

We used three models of bladder injury to induce proliferation and damage to distinct layers of the urothelium (Uropathogenic E. coli (UPEC), Protamine Sulfate (PS) and lipopolysaccharide (LPS)) (Mysorekar and Hultgren, 2006; Mysorekar et al., 2009; Shin et al., 2011). See Figure 1.3 for a summary of the injury models.

Phenotype	UPEC Infection	Protamine Sulfate	Lipopolysaccharide
Superficial cell exfoliation	Yes	Yes	No
Superficial cell regeneration	Yes	Yes	No
Proliferation of basal cells	Yes	No	Yes
Proliferation of intermediate cells	No	Yes	Yes
Immune reponse	Yes	No	Yes
Chronic barrier disruption	Yes	No	Yes

Figure 1.3: Summary of Bladder Injury Models.

UPEC Injury Model: Urinary tract infections are the most common community or hospital acquired bacterial infection, and the most common reason young women seek medical attention (Baerheim and Hunskar, 1997; Hooton and Stamm, 1997). Uropathogenic Escherichia coli (UPEC) account for over 80% UTI's (Ronald, 2003), and a murine model of bladder injury as the result of UPEC infection has been well characterized and established (Hung et al., 2009). 24 hours following Infection with UPEC induces urothelial barrier damage, inflammation as well as activation of cells in the basal cell layer (Mysorekar and Hultgren, 2006; Hung et al., 2009; Mysorekar et al., 2009).

UPEC infection induces a rapid recruitment of neutrophils into the bladder lumen and urothelial production of inflammatory chemokines and cytokines (Hang et al., 2000; Schilling et al., 2003). The rapid immune response is due to virulence factors on UPEC such as LPS, flagella, type I pili, and papa pili (Hedlund et al., 2001; Zhang et al., 2004). Recently, UPEC strain (NU14) was demonstrated to increase referred mechanical sensitivity, possibly due to LPS (Rudick et al., 2010). However, it is previously unknown if a bladder infection with UPEC increases sensitivity to bladder distention.

PS Injury Model: PS is a highly cationic peptide that is used clinically to reverse the anticoagulant effects of heparin. Intravesicular PS treatment results in exfoliation of the SFC barrier (Mysorekar and Hultgren, 2006) and increased ionic permeability of the urothelium (Tzan et al., 1993). Low dose intravesicular PS (10mg/ml) chemically exfoliates the SFCs within the first 12 hr (Mysorekar and Hultgren, 2006), resulting in a 'leaky urothelium' and proliferation of the intermediate cell layer (Mysorekar *et al.*, 2009). Low dose intravesicular treatment with PS does not induce bladder hyperactivity (Chuang *et al.*, 2003), or inflammation of the urothelium (Mysorekar and Hultgren, 2006; Mysorekar et al., 2009).

LPS Injury Model: LPS is a large molecule found on the outer membrane of Gram-negative bacteria. LPS binds Toll-Like receptor 4 (Poltorak et al., 1998), and is the most common bacterial product instilled into the bladder to cause cystitis in laboratory models. Intravesicular instillation of LPS induces inflammation of the urothelium without exfoliation of SFCs (barrier damage) (Saban *et al.*, 2002).

In chapter 2 of this thesis, we use these differential models of bladder injury to examine how distinct urothelial injury affects bladder pain. Furthermore, we can follow the expression of cytokines that are known to modulate both pain and injury. In this way, we can start to tease out the unique molecular signals that are responsible for urothelial injury versus pain.

Sensory Innervation of the Bladder

Although bladder submucosal granulations, hemmoragic tissue and mucosal denudation are frequent histological findings in IC/PBS, they are not pathognomonic. Indeed, 50% of patients

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with IC/PBS have histologically normal bladder biopsies (Rosamilia and Dwyer, 2000). In such cases, it is possible that molecular changes in nerves that innervate the bladder, the spinal cord or the brain may be the source of chronic bladder pain (a type of visceral pain).

The sensory innervation of the urinary bladder is associated with two principal pathways; the sacral pelvic (parasympathetic) and hypogastric/lumbar splanchnic (sympathetic) innervations (Xu and Gebhart, 2008). Despite the suggestive names, the afferent nerves are distinct from efferent, autonomic, pathways (Robinson and Gebhart, 2008). Axonal tracing studies in cats and rats have demonstrated that the pelvic afferent innervation (de Groat et al., 1990; Fall et al., 1990; Habler et al., 1990) of the bladder terminate at spinal cord level L6-S1 with their cell bodies located in L6 and S1 DRGs (de Groat, 1993). Furthermore, the hypogastric/lumbar splanchnic afferents from the bladder terminate at spinal cord levels L1-L2, and the cell bodies of the afferents are in the L1 and L2 DRGs (Moss et al., 1997; Nazif et al., 2007). Both the pelvic (L6-S1) and the hypogastric (L1-L2) nerves are thought to convey nociceptive signals (Xu and Gebhart, 2008; James I. Gillespie, 2009).

Nociceptors are defined as a high-threshold sensory receptor of the peripheral somatosensory nervous system that iscpable fo transducing and encoding noxious stimuli (IASP, 2011). The cell body of each nociceptor is located in the dorsal root ganglion (DRG) or trigeminal ganglion. Nociceptive information is transmitted from the periphery to the spinal cord, where nociceptors synapse on the second order neurons in the dorsal horn (central terminals). From the spinal cord, this nociceptive information is conveyed to rostral structures. Following injury or inflammation, increased sensitivity to pain (hyperalgesia) occurs by several mechanisms. First, nociceptors may become sensitized in a process termed peripheral sensitization, during which nociceptor activation thresholds are reduced and/or increased firing rates. One possible mechanism by which central sensitization may occur is by increased excitability of neurons within the CNS. Peripheral and/or central sensitization is one way that chronic pain has been hypothesized to be initiated and maintained. Chronic visceral pain (such as bladder pain) may also be the result of peripheral and/or central sensitization.

Much of what is known about nociception comes from studies of nociceptors from non-visceral structures such as the integument (Robinson and Gebhart, 2008). Clinically, visceral pain is often treated as a variant of somatic pain; under the assumption that the same neurological mechanisms may be responsible for both visceral and somatic pain (Gebhart, 2000a). However, conventional treatments for visceral pain are often ineffective, indicating significant differences between visceral and somatic pain (Robinson and Gebhart, 2008). This may be due to intrinsic differences between visceral nociceptors and non-visceral nociceptors (Gebhart, 2000b, 2004; Robinson and Gebhart, 2008).

Visceral and somatic afferents differ in several ways. First, in somatic structures there is a clear division between nociceptor afferents and low-threshold afferents (Sengupta and Gebhart, 1994; Cervero and Laird, 1996). Nociceptor afferents detect noxious or potentially damaging stimuli, while low-threshold afferents detect non-noxious stimuli. However, in the viscera this distinction is not as clear; for example, visceral low-threshold mechanosensory afferents encode organ distention over a wide range from innocuous to noxious pressures (Robinson and Gebhart, 2008). On the other hand, visceral high-threshold receptors are activated by stimuli only in the noxious range (Cervero and Laird, 1999). Furthermore, visceral organs have been reported to have 'silent' nociceptors that are only activated in the context of inflammation (Robinson and Gebhart, 2008). Second, cutaneous/somatic receptors are more specialized; different types of nerve ending (such as ruffini endings and pacinian corpusles) respond to distinct insults. In contrast, a high percentage of visceral afferents are sensitive to stimuli from multiple modalities (mechanical distention, chemical and thermal stimuli) (Robinson and Gebhart, 2008). Third, visceral organs, unlike somatic structures, are usually innervated by two additional sources; sympathetic and parasympathetic nerves (Christianson et al., 2009). Fourth, visceral afferents terminate in different lamina of the spinal cord when compared to non-visceral afferents. Visceral afferent fibers terminate in lamina X in addition to the superficial dorsal horn and lamina V, where non-visceral nociceptors terminate only in the superficial dorsal horn and lamina V (Christianson et al., 2009). Thus, there are major differences between visceral and somatic nociceptor anatomy and

physiology.

mGluR5 Regulates Inflammatory Pain Signaling

Glutamate is the predominant excitatory neurotransmitter in the mammalian nervous system. It mediates transmission of nociceptive information from the periphery to the spinal cord (Fundytus, 2001; Varney and Gereau, 2002). When glutamate is released from the sensory afferents onto the spinal cord dorsal horn neurons, it can activate two major classes of glutamate receptors; ligand-gated ionotropic glutamate receptors (iGluRs) and G-protein coupled metabotropic glutamate receptors (mGluRs). mGluR5 is found throughout the CNS and peripheral nervous system (PNS) (Lu et al., 1997; Varney and Gereau, 2002). Antagonists of mGluR5 such as MPEP and fenobam reversed or reduced thermal and mechanical hyperalgesia induced by cutaneously applied pro-inflammatory agents including complete Freund's adjuvant (CFA), formalin, glutamate, capsaicin, and carrageenan with no effect on baseline sensitivities (Fisher and Coderre, 1996; Zhou et al., 1996; Walker et al., 2001a). Furthermore, mGluR5 knockout mice and their WT littermates have similar baseline thermal and mechanical sensitivities (M. Montana, unpublished observations (Lu et al., 1997)). However, mGluR5 knockout mice display reduced nocifensive behavior in the formalin test (Montana et al., 2009). Suggesting that in somatic inflammatory pain, mGluR5 has a role in sensitization of peripheral afferents and/or the development of central sensitization (Hunskaar and Hole, 1987; Montana et al., 2009). Although much is known about the role of mGluR5 in somatic inflammatory nociception (Walker et al., 2001c; Walker et al., 2001a; Fisher et al., 2002; Hudson et al., 2002; Varney and Gereau, 2002), there is very limited data on the role/site of action of mGluR5 in visceral pain (Lindstrom et al., 2008).

In chapter 3 of this thesis, we examine the role of mGluR5 in inflammatory and non-inflammatory bladder pain. Using mGluR5 KO mice and a specific antagonist of mGluR5, we demonstrate that mGluR5 is necessary for the full expression of distention-induced bladder pain. Furthermore, through the use of UPEC-induced infectious cystitis and a specific antagonist to mGluR5, we demonstrate that mGluR5 is necessary for the full expression of inflammatory bladder pain.

However, in all of these experiments (mGluR5 KO and antagonist), activation of mGluR5 was blocked indiscriminately with respect to anatomical loci: mGluR5 KO mice lack mGluR5 throughout the PNS and CNS (global KO) and the specific antagonist of mGluR5 (fenobam) easily and quickly crosses the blood-brain-barrier (Montana et al., 2009). Therefore, these data do not distinguish where mGluR5 activation is important for regulating bladder pain.

mGluR5 and the Pain Neuroaxis:

Group I mGluRs (mGluR1 and 5) have been demonstrated to have a pro-nociceptive role in many parts of the nervous system including the spinal cord, periphery, brainstem, thalamus, cortex and amygdala (Bhave et al., 2001; Walker et al., 2001d; Zhou et al., 2001; Neugebauer and Carlton, 2002; Neugebauer and Li, 2002; Varney and Gereau, 2002) (see Figure 1.4). mGluR5 is expressed on peripheral terminals of unmyelinated nociceptors (Bhave et al., 2001), and intradermal administration of mGluR5 agonists induces mechanical (Walker et al., 2001c) and thermal hypersensitivity (Bhave et al., 2001; Walker et al., 2001b; Hu et al., 2002; Neugebauer and Carlton, 2002). Importantly, in somatic pain models, antagonists of mGluR5 had no effect on normal thermal or mechanical nociception (Bhave et al., 2001; Walker et al., 2001d; Kolber et al., 2010). mGluR5 expression has been detected in the small diameter DRG neurons of L1-2 and L6-S1 (Valerio et al., 1997; Ghosh et al., 2007). However, it is unknown if mGluR5 is expressed in the bladder. Recent work implies that central and not peripheral mGluR5 modulates distention-induced bladder pain (Hu et al., 2009). In that study, the authors used an in-vitro rat bladder-nerve preparation to measure pelvic nerve firing in response to bladder distention (Hu et al., 2009). The rate of nerve firing in response to bladder distention was unchanged by the presence of an mGluR5 antagonist (MPEP). These results suggest that the site of action of this mGluR5 antagonist is in the CNS and not in the bladder (Hu et al., 2009).
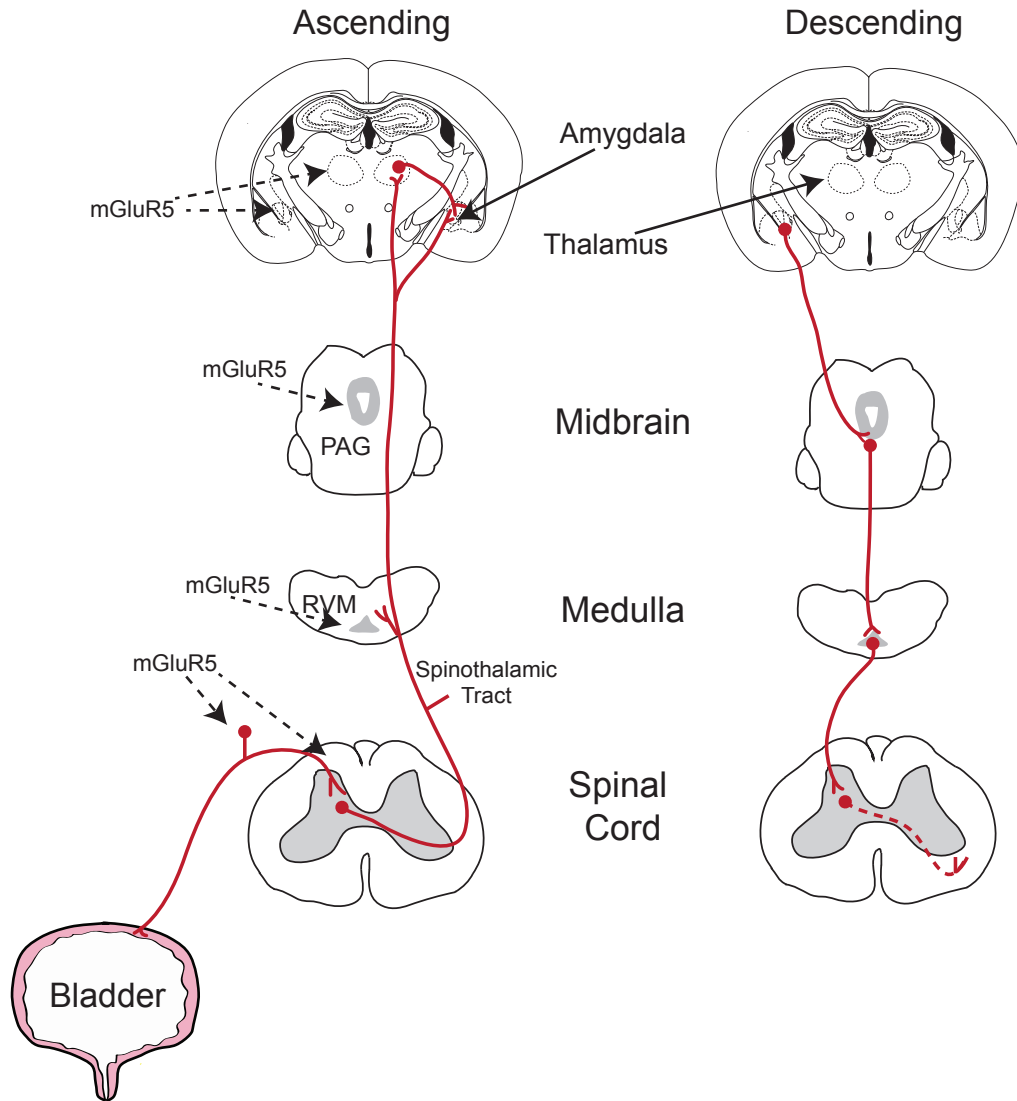
Figure 1.4 A simplified model of the pain neuroaxis and mGluR5 expression. Afferent nerves from the bladder (pelvic and hypogastric nerves) synapse in the spinal cord. The signal is then transmitted to the thalamus via the spinothalamic tract. The amygdala (central nucleus of the amygdala or CeA) receives nociceptive-specific information by way of the brainstem via the spino-parabrachio-amygdaloid pain pathway (simplified in Figure 1.3). Highly processed polymodal (including nociceptive) information reaches the amygdala (lateral and basolateral amygdala) by way of the thalamus. The CeA integrates polysensory and nociceptive-specific information (Neugebauer *et al.*, 2004). Descending information from the CeA travels to the spinal cord through circuits connecting the periaquiductal Grey (PAG) and the rostral ventromedial medulla (RVM) (Neugebauer *et al.*, 2004).

In the spinal cord, mGluR5 has been detected pre- and postsynaptically in the superficial dorsal horn (Neugebauer, 2002). Intrathecal injection of DHPG, a group I mGluR (mGluR1/5) agonist, results in spontaneous nociceptive behavior that includes licking of the hindpaw, tail and flanks. Pretreatment with selective mGluR1 and mGluR5 antagonists, significantly attenuated the DHPG-induced nociceptive behavior, indicating that DHPG does not produce the nociceptive effects via a nonspecific mechanism (Fisher and Coderre, 1996; Karim et al., 2001a).

In addition to the spinal cord, mGluR5 is present throughout the pain neuroaxis, including within the amygdala. The amygdala is an important limbic region that is involved in processing emotional and motivational aspects of pain. The central nucleus of the amygdala (CeA) receives direct nociceptive information from the spinal cord and brainstem and indirect sensory (including nociceptive) information (via lateral and basolateral amygdala nuclei (BLA)) via the thalamus and cortex (Bernard and Besson, 1990; Burstein and Potrebic, 1993; Bernard et al., 1996; Bourgeais et al., 2001) (see Figure 1.4). The CeA is known to play an important role in processing of somatic and visceral pain. Noxious colorectal distention increases c-fos expression in the CeA (Traub *et al.*, 1996) and the excitability of CeA neurons increases after induction of zymogen-induced colitis in rats (Han and Neugebauer, 2004).

Outputs from the CeA to the hypothalamus (involved in autonomic regulation) and brainstem regions such as the periaqueductal gray (PAG; involved in descending pain modulation) make the amygdala well positioned to modulate behavioral responses to painful stimuli. Chronic activation of the CeA with corticosterone implants increases visceromotor responses to colorectal and noxious bladder distention in rats and sensitizes afferent visceral spinal neurons (Greenwood-Van Meerveld et al., 2001; Qin et al., 2003a, b; Qin et al., 2003c; Myers et al., 2005; Myers et al., 2007; Myers and Greenwood-Van Meerveld, 2010). The mechanism of these effects is unknown, but it likely involves transcriptional changes maintained by corticotropin-releasing factor signaling (Qin et al., 2003c; Myers and Greenwood-Van Meerveld, 2010). Nonetheless, it is undetermined if acute changes in excitability of CeA neurons modulate the response to noxious bladder stimulation.

The excitability of CeA neurons is modulated in part by metabotropic glutamate receptor 5 (mGluR5) signaling (Ji, 2004). Pharmacological mGluR5 activation in the CeA increases rectal distention-induced neuronal responses (Ji and Neugebauer, 2010) and behavioral vocalizations (Li *et al.*, 2011). Furthermore, our lab (Carrasquillo and Gereau, 2008; Kolber et al., 2010) and others (Ji and Neugebauer, 2009; Li et al., 2011) have shown a primary role for the right amygdala in somatic and visceral pain. Activation of mGluR5 in the right CeA is sufficient to induce peripheral hypersensitivity in the absence of injury (Kolber et al., 2010). Furthermore, injury-induced peripheral hypersensitivity is resolved by blocking mGluR5 activation in the right CeA.

In Chapter 4 of this thesis we use right CeA pharmacologic activation and inhibition of mGluR5 as well as conditional deletion of mGluR5 to determine the role of CeA-specific mGluR5 signaling in bladder pain. In addition, we used an optogenetic approach to stimulate CeA neurons to determine if increased activation of the CeA increases the VMR to noxious bladder distention. Overall, we found that either mGluR5 activation in the CeA or increased excitability of neurons in the right CeA is sufficient to induce increased responses to bladder distention. These results demonstrate a role for mGluR5 in the modulation of bladder pain in the absence of injury.

mGluR5 and ERK:

Rodent models of pain have demonstrated that mGluR5 signaling to extracellular regulated kinase 1/2 (ERK1/2) may play a key role in peripheral and central sensitization (Karim et al., 2001c; Adwanikar et al., 2004; Kawasaki et al., 2004; Hu et al., 2007). Intrathecal administration of an mGluR5 antagonist significantly attenuated formalin-induced phosphorylation of ERK1/2 in the spinal cord (Karim et al., 2001b). Furthermore, recent work in our lab demonstrates that noxious bladder distention results in activation (phosphorylation) of ERK1/2 in the lumbosacral spinal cord (Lai et al., 2011). In addition, intrathecal antagonism of an ERK1/2 inhibitor was analgesic in non-inflammatory and cyclophosphamide-induced inflammatory distention-induced bladder pain. mGluR5 signaling to ERK1/2 has been hypothesized to play a role in the

modulation of pain responses at the level of the spinal cord and amygdala (Ji, 2004), yet it is unknown if mGluR5 signals to ERK1/2 in bladder pain.

Intraplantar formalin results in mGluR5 mediated ERK activation in the CeA. However, the role of amygdala ERK1/2 in bladder pain is undetermined. In chapter 4 of this thesis, our results suggest that mGluR5 in the right CeA modulates distention-induced ERK1/2 activation in the lumbosacral spinal cord. We found that noxious bladder distention alone leads to ERK1/2 activation in the lumbosacral spinal cord, but not ERK1/2 activation in the amygdala. Surprisingly, conditional deletion of mGluR5 in the right CeA results in decreased ERK1/2 activation in the spinal cord following bladder distention. Furthermore, optogenetic stimulation of CeA neurons leads to both an increased response to bladder distention and increased ERK1/2 activation in the spinal cord following noxious bladder distention. However, optogenetic activation alone (no bladder distention) of the right CeA does not increase ERK activation in the lumbosacral spinal cord. Thus suggesting that bladder distention leads to ERK1/2 activation in the lumbosacral spinal cord and mGluR5 in the right CeA can modulate distention-induced ERK1/2 activation in the spinal cord

In chapters 2-4 of this thesis, we examine three aspects of bladder pain. First, in chapter 2, we examine if distinct urothelial injury leads to differential evoked responses to bladder distention. Second, in chapter 3, we investigate the role of mGluR5 on distention induced bladder pain. Third, in chapter 4, we investigate how mGluR5 in the right CeA modulates bladder pain and distention-induced ERK activation in the lumbosacral spinal cord. In summary, the work in this thesis examines modulation of bladder pain from the level of the bladder, spinal cord and brain.

REFERENCES

Aaron LA, Buchwald D (2001) A review of the evidence for overlap among unexplained clinical
conditions. Ann Intern Med 134:868-881.

Aaron LA, Herrell R, Ashton S, Belcourt M, Schmaling K, Goldberg J, Buchwald D (2001)
Comorbid clinical conditions in chronic fatigue: a co-twin control study. J Gen Intern Med
16:24-31.

Adwanikar H, Karim F, Gereau RW (2004) Inflammation persistently enhances nocifensive
behaviors mediated spinal group I mGluRs through sustained ERK activation. Pain
111:125-135.

Altuntas CZ, Daneshgari F, Sakalar C, Goksoy E, Gulen MF, Kavran M, Qin J, Li X, Tuohy VK
(2011) Autoimmunity to Uroplakin II Causes Cystitis in Mice: A Novel Model of Interstitial
Cystitis. European Urology In Press, Corrected Proof.

Baerheim A, Hunskar S (1997) [Current management of uncomplicated acute cystitis in general
practice]. Tidsskr Nor Laegeforen 117:1304-1307.

Bernard JF, Besson JM (1990) The spino(trigemino)pontoamygdaloid pathway:
electrophysiological evidence for an involvement in pain processes. Journal of
neurophysiology 63:473-490.

Bernard JF, Bester H, Besson JM (1996) Involvement of the spino-parabrachio -amygdaloid and -
hypothalamic pathways in the autonomic and affective emotional aspects of pain. Prog
Brain Res 107:243-255.

Berry SH, Elliott MN, Suttorp M, Bogart LM, Stoto MA, Eggers P, Nyberg L, Clemens JQ (2011)
Prevalence of Symptoms of Bladder Pain Syndrome/Interstitial Cystitis Among Adult
Females in the United States. The Journal of urology 186:540-544.

Bhave G, Karim F, Carlton SM, Gereau RW (2001) Peripheral group I metabotropic glutamate
receptors modulate nociception in mice. Nature neuroscience 4:417-423.

Birder LA (2011) Urothelial signaling. Handb Exp Pharmacol:207-231.

Bourgeais L, Gauriau C, Bernard JF (2001) Projections from the nociceptive area of the central nucleus of the amygdala to the forebrain: a PHA-L study in the rat. The European journal of neuroscience 14:229-255.

Brand K, Littlejohn G, Kristjanson L, Wisniewski S, Hassard T (2007) The fibromyalgia bladder index. Clin Rheumatol 26:2097-2103.

Burstein R, Potrebic S (1993) Retrograde labeling of neurons in the spinal cord that project directly to the amygdala or the orbital cortex in the rat. The Journal of comparative neurology 335:469-485.

Carrasquillo Y, Gereau RW (2007) Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. J Neurosci 27:1543-1551.

Carrasquillo Y, Gereau RW (2008) Hemispheric lateralization of a molecular signal for pain modulation in the amygdala. Molecular pain 4:24.

Castroman P, Ness TJ (2001) Vigor of visceromotor responses to urinary bladder distension in rats increases with repeated trials and stimulus intensity. Neurosci Lett 306:97-100.

Cervero F, Laird JM (1996) Mechanisms of allodynia: interactions between sensitive mechanoreceptors and nociceptors. Neuroreport 7:526-528.

Cervero F, Laird JMA (1999) Visceral pain. Lancet 353:2145-2148.

Christianson JA, Bielefeldt K, Altier C, Cenac N, Davis BM, Gebhart GF, High KW, Kollarik M, Randich A, Undem B, Vergnolle N (2009) Development, plasticity and modulation of visceral afferents. Brain Res Rev 60:171-186.

Chuang YC, Chancellor MB, Seki S, Yoshimura N, Tyagi P, Huang L, Lavelle JP, De Groat WC, Fraser MO (2003) Intravesical protamine sulfate and potassium chloride as a model for bladder hyperactivity. Urology 61:664-670.

Clemens JQ, Meenan RT, O'Keeffe Rosetti MC, Kimes TA, Calhoun EA (2008a) Case-control study of medical comorbidities in women with interstitial cystitis. The Journal of urology 179:2222-2225.

Clemens JQ, Meenan RT, Rosetti MC, Kimes T, Calhoun EA (2008b) Costs of interstitial cystitis in a managed care population. Urology 71:776-780; discussion 780-771.

Cruz CD, Cruz F (2007) The ERK 1 and 2 pathway in the nervous system: From basic aspects to possible clinical applications in pain and visceral dysfunction. Current Neuropharmacology 5:244-252.

de Groat WC (1993) Anatomy and physiology of the lower urinary tract. Urol Clin North Am 20:383-401.

de Groat WC, Kawatani M, Hisamitsu T, Cheng CL, Ma CP, Thor K, Steers W, Roppolo JR (1990) Mechanisms underlying the recovery of urinary bladder function following spinal cord injury. J Auton Nerv Syst 30 Suppl:S71-77.

Domingue GJ, Ghoniem GM, Bost KL, Fermin C, Human LG (1995) Dormant microbes in interstitial cystitis. The Journal of urology 153:1321-1326.

Duncan JL, Schaeffer AJ (1997) Do infectious agents cause interstitial cystitis? Urology 49:48-51.

Erickson DR, Morgan KC, Ordille S, Keay SK, Xie SX (2001) Nonbladder related symptoms in patients with interstitial cystitis. The Journal of urology 166:557-561; discussion 561-552.

Fall M, Lindstrom S, Mazieres L (1990) A bladder-to-bladder cooling reflex in the cat. J Physiol 427:281-300.

Fisher K, Coderre TJ (1996) Comparison of nociceptive effects produced by intrathecal administration of mGluR agonists. Neuroreport 7:2743-2747.

Fisher K, Lefebvre C, Coderre TJ (2002) Antinociceptive effects following intrathecal pretreatment with selective metabotropic glutamate receptor compounds in a rat model of neuropathic pain. Pharmacology Biochemistry and Behavior 73:411-418.

Fundytus ME (2001) Glutamate receptors and nociception - Implications for the drug treatment of pain. Cns Drugs 15:29-58.

Gebhart GF (2000a) Pathobiology of visceral pain: molecular mechanisms and therapeutic implications IV. Visceral afferent contributions to the pathobiology of visceral pain. Am J Physiol Gastrointest Liver Physiol 278:G834-838.

Gebhart GF (2000b) Visceral pain-peripheral sensitisation. Gut 47 Suppl 4:iv54-55; discussion iv58.

Gebhart GF (2004) Descending modulation of pain. Neurosci Biobehav Rev 27:729-737.

Ghosh C, Storey-Workley M, Usip S, Hafemeister J, Miller KE, Papka RE (2007) Glutamate and
metabotropic glutamate receptors associated with innervation of the uterine cervix during
pregnancy: receptor antagonism inhibits c-Fos expression in rat lumbosacral spinal cord
at parturition. J Neurosci Res 85:1318-1335.

Greenwood-Van Meerveld B, Gibson M, Gunter W, Shepard J, Foreman R, Myers D (2001)
Stereotaxic delivery of corticosterone to the amygdala modulates colonic sensitivity in
rats. Brain research 893:135-142.

Gustafsson JK, Greenwood-Van Meerveld B (2011) Amygdala activation by corticosterone alters
visceral and somatic pain in cycling female rats. Am J Physiol Gastrointest Liver Physiol
300:G1080-1085.

Haarala M, Kiilholma P, Lehtonen OP (1999) Urinary bacterial flora of women with urethral
syndrome and interstitial cystitis. Gynecol Obstet Invest 47:42-44.

Habler HJ, Janig W, Koltzenburg M (1990) Activation of unmyelinated afferent fibres by
mechanical stimuli and inflammation of the urinary bladder in the cat. J Physiol 425:545-
562.

Han JS, Neugebauer V (2004) Synaptic plasticity in the amygdala in a visceral pain model in rats.
Neuroscience letters 361:254-257.

Hang L, Frendeus B, Godaly G, Svanborg C (2000) Interleukin-8 receptor knockout mice have
subepithelial neutrophil entrapment and renal scarring following acute pyelonephritis. J
Infect Dis 182:1738-1748.

Hanno P, Lin A, Nordling J, Nyberg L, van Ophoven A, Ueda T, Wein A, Bladder Pain Syndrome
Committee of the International Consultation on I (2010) Bladder Pain Syndrome
Committee of the International Consultation on Incontinence. Neurourol Urodyn 29:191-
198.

Hanno PM, Burks DA, Clemens JQ, Dmochowski RR, Erickson D, Fitzgerald MP, Forrest JB,
Gordon B, Gray M, Mayer RD, Newman D, Nyberg L, Jr., Payne CK, Wesselmann U,
Faraday MM (2011) AUA guideline for the diagnosis and treatment of interstitial
cystitis/bladder pain syndrome. The Journal of urology 185:2162-2170.

Hedlund M, Frendeus B, Wachtler C, Hang L, Fischer H, Svanborg C (2001) Type 1 fimbriae
    deliver an LPS- and TLR4-dependent activation signal to CD14-negative cells. Mol
    Microbiol 39:542-552.

Held PJ HP, Wein AJ, Pauly MV, Cann MA. (1990) Epidemiology

of interstitial cystitis: 2. In: Interstitial cystitis (Hanno PM SD, Krane RJ WA, eds), pp 29-48.
    London: Springer.

Hicks RM (1975) The mammalian urinary bladder: an accommodating organ. Biological reviews
    of the Cambridge Philosophical Society 50:215-246.

Hicks RM, Ketterer B, Warren RC (1974) The ultrastructure and chemistry of the luminal plasma
    membrane of the mammalian urinary bladder: a structure with low permeability to water
    and ions. Philos Trans R Soc Lond B Biol Sci 268:23-38.

Hooton TM, Stamm WE (1997) Diagnosis and treatment of uncomplicated urinary tract infection.
    Infect Dis Clin North Am 11:551-581.

Hu HJ, Bhave G, Gereau RWt (2002) Prostaglandin and protein kinase A-dependent modulation
    of vanilloid receptor function by metabotropic glutamate receptor 5: potential mechanism
    for thermal hyperalgesia. J Neurosci 22:7444-7452.

Hu HJ, Alter BJ, Carrasquillo Y, Qiu CS, Gereau RWt (2007) Metabotropic glutamate receptor 5
    modulates nociceptive plasticity via extracellular signal-regulated kinase-Kv4.2 signaling
    in spinal cord dorsal horn neurons. J Neurosci 27:13181-13191.

Hu Y, Dong L, Sun B, Guillon MA, Burbach LR, Nunn PA, Liu X, Vilenski O, Ford AP, Zhong Y,
    Rong W (2009) The role of metabotropic glutamate receptor mGlu5 in control of
    micturition and bladder nociception. Neuroscience letters 450:12-17.

Hudson LJ, Bevan S, McNair K, Gentry C, Fox A, Kuhn R, Winter J (2002) Metabotropic
    glutamate receptor 5 upregulation in A-fibers after spinal nerve injury: 2-Methyl-6-
    (phenylethynyl)-pyridine (MPEP) reverses the induced thermal hyperalgesia. Journal of
    Neuroscience 22:2660-2668.

Hung CS, Dodson KW, Hultgren SJ (2009) A murine model of urinary tract infection. Nat Protoc
    4:1230-1243.

Hunskaar S, Hole K (1987) The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30:103-114.

Hunt SP, Mantyh PW (2001) The molecular dynamics of pain control. Nat Rev Neurosci 2:83-91.

Hurst RE, Moldwin RM, Mulholland SG (2007) Bladder defense molecules, urothelial differentiation, urinary biomarkers, and interstitial cystitis. Urology 69:17-23.

IASP (2011) IASP Taxonomy Update.

James I. Gillespie GAvK, Stefan G. de Wachter, Jan de Vente, (2009) On the origins of the sensory output from the bladder: the concept of afferent noise. Bju International 103:1324-1333.

Ji G, Neugebauer V (2009) Hemispheric lateralization of pain processing by amygdala neurons. Journal of neurophysiology 102:2253-2264.

Ji G, Neugebauer V (2010) Reactive oxygen species are involved in group I mGluR-mediated facilitation of nociceptive processing in amygdala neurons. Journal of neurophysiology 104:218-229.

Ji RR (2004) Peripheral and central mechanisms of inflammatory pain, with emphasis on MAP kinases. Current drug targets 3:299-303.

Jost SP (1989) Cell cycle of normal bladder urothelium in developing and adult mice. Virchows Arch B Cell Pathol Incl Mol Pathol 57:27-36.

Jost SP, Gosling JA, Dixon JS (1989) The morphology of normal human bladder urothelium. J Anat 167:103-115.

Karim F, Wang CC, Gereau RWt (2001a) Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. J Neurosci 21:3771-3779.

Karim F, Wang CC, Gereau RW (2001b) Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. J Neurosci 21:3771-3779.

Karim F, Wang CC, Gereau RW (2001c) Metabotropic glutamate receptor subtypes 1 and 5 are
    activators of extracellular signal-regulated kinase signaling required for inflammatory pain
    in mice. Journal of Neuroscience 21:3771-3779.

Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K, Woolf CJ, Ji
    RR (2004) Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and
    Src contribute to C-fiber-induced ERK activation and cAMP response element-binding
    protein phosphorylation in dorsal horn neurons, leading to central sensitization. J
    Neurosci 24:8310-8321.

Keay S (2008) Cell signaling in interstitial cystitis/painful bladder syndrome. Cell Signal 20:2174-
    2179.

Keay S, Zhang CO, Baldwin BR, Jacobs SC, Warren JW (1998) Polymerase chain reaction
    amplification of bacterial 16S rRNA genes in interstitial cystitis and control patient bladder
    biopsies. The Journal of urology 159:280-283.

Keay S, Zhang CO, Chai T, Warren J, Koch K, Grkovic D, Colville H, Alexander R (2004a)
    Antiproliferative factor, heparin-binding epidermal growth factor-like growth factor, and
    epidermal growth factor in men with interstitial cystitis versus chronic pelvic pain
    syndrome. Urology 63:22-26.

Keay SK, Szekely Z, Conrads TP, Veenstra TD, Barchi JJ, Jr., Zhang CO, Koch KR, Michejda CJ
    (2004b) An antiproliferative factor from interstitial cystitis patients is a frizzled 8 protein-
    related sialoglycopeptide. Proceedings of the National Academy of Sciences of the
    United States of America 101:11803-11808.

Khandelwal P, Abraham SN, Apodaca G (2009) Cell biology and physiology of the uroepithelium.
    Am J Physiol Renal Physiol 297:F1477-1501.

Kolber BJ, Montana MC, Carrasquillo Y, Xu J, Heinemann SF, Muglia LJ, Gereau RW (2010)
    Activation of metabotropic glutamate receptor 5 in the amygdala modulates pain-like
    behavior. J Neurosci 30:8203-8213.

Kurzrock EA, Lieu DK, Degraffenried LA, Chan CW, Isseroff RR (2008) Label-retaining cells of
    the bladder: candidate urothelial stem cells. Am J Physiol Renal Physiol 294:F1415-1421.

Lai HH, Qiu CS, Crock LW, Morales ME, Ness TJ, Gereau RW (2011) Activation of spinal
extracellular signal-regulated kinases (ERK) 1/2 is associated with the development of
visceral hyperalgesia of the bladder. Pain 152:2117-2124.

Li Z, Ji G, Neugebauer V (2011) Mitochondrial reactive oxygen species are activated by mGluR5
through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and
pain behavior. J Neurosci 31:1114-1127.

Lindstrom E, Brusberg M, Hughes PA, Martin CM, Brierley SM, Phillis BD, Martinsson R,
Abrahamsson C, Larsson H, Martinez V, Blackshaw LA (2008) Involvement of
metabotropic glutamate 5 receptor in visceral pain. Pain 137:295-305.

Loeser JD, Treede RD (2008) The Kyoto protocol of IASP Basic Pain Terminology. Pain 137:473-
477.

Lu YM, Jia Z, Janus C, Henderson JT, Gerlai R, Wojtowicz JM, Roder JC (1997) Mice lacking
metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term
potentiation (LTP) but normal CA3 LTP. J Neurosci 17:5196-5205.

Mogil JS, Davis KD, Derbyshire SW (2010) The necessity of animal models in pain research.
Pain 151:12-17.

Montana MC, Cavallone LF, Stubbert KK, Stefanescu AD, Kharasch ED, Gereau RW (2009) The
metabotropic glutamate receptor subtype 5 antagonist fenobam is analgesic and has
improved in vivo selectivity compared with the prototypical antagonist 2-methyl-6-
(phenylethynyl)-pyridine. The Journal of pharmacology and experimental therapeutics
330:834-843.

Moss NG, Harrington WW, Tucker MS (1997) Pressure, volume, and chemosensitivity in afferent
innervation of urinary bladder in rats. Am J Physiol 272:R695-703.

Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, Hultgren SJ (1998)
Induction and evasion of host defenses by type 1-piliated uropathogenic Escherichia coli.
Science (New York, NY 282:1494-1497.

Myers B, Greenwood-Van Meerveld B (2010) Elevated corticosterone in the amygdala leads to
persistent increases in anxiety-like behavior and pain sensitivity. Behavioural brain
research 214:465-469.

Myers B, Dittmeyer K, Greenwood-Van Meerveld B (2007) Involvement of amygdaloid
corticosterone in altered visceral and somatic sensation. Behavioural brain research
181:163-167.

Myers B, Schulkin J, Greenwood-Van Meerveld B (2011) Sex steroids localized to the amygdala
increase pain responses to visceral stimulation in rats. J Pain 12:486-494.

Myers DA, Gibson M, Schulkin J, Greenwood Van-Meerveld B (2005) Corticosterone implants to
the amygdala and type 1 CRH receptor regulation: effects on behavior and colonic
sensitivity. Behavioural brain research 161:39-44.

Mysorekar IU, Hultgren SJ (2006) Mechanisms of uropathogenic Escherichia coli persistence and
eradication from the urinary tract. Proceedings of the National Academy of Sciences of
the United States of America 103:14170-14175.

Mysorekar IU, Mulvey MA, Hultgren SJ, Gordon JI (2002) Molecular regulation of urothelial
renewal and host defenses during infection with uropathogenic Escherichia coli. The
Journal of biological chemistry 277:7412-7419.

Mysorekar IU, Isaacson-Schmid M, Walker JN, Mills JC, Hultgren SJ (2009) Bone morphogenetic
protein 4 signaling regulates epithelial renewal in the urinary tract in response to
uropathogenic infection. Cell Host Microbe 5:463-475.

Nazif O, Teichman JM, Gebhart GF (2007) Neural upregulation in interstitial cystitis. Urology
69:24-33.

Ness TJ (1999) Models of Visceral Nociception. ILAR J 40:119-128.

Ness TJ, Gebhart GF (1990) Visceral pain: a review of experimental studies. Pain 41:167-234.

Ness TJ, Elhefni H (2004) Reliable visceromotor responses are evoked by noxious bladder
distention in mice. J Urol 171:1704-1708.

Ness TJ, Lewis-Sides A, Castroman P (2001) Characterization of pressor and visceromotor reflex responses to bladder distention in rats: sources of variability and effect of analgesics. J Urol 165:968-974.

Ness TJ, Richter HE, Varner RE, Fillingim RB (1998) A psychophysical study of discomfort produced by repeated filling of the urinary bladder. Pain 76:61-69.

Neugebauer V (2002) Metabotropic glutamate receptors--important modulators of nociception and pain behavior. Pain 98:1-8.

Neugebauer V (2007) The amygdala: different pains, different mechanisms. Pain 127:1-2.

Neugebauer V, Carlton SM (2002) Peripheral metabotropic glutamate receptors as drug targets for pain relief. Expert Opin Ther Targets 6:349-361.

Neugebauer V, Li W (2002) Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. Journal of neurophysiology 87:103-112.

Neugebauer V, Li W, Bird GC, Han JS (2004) The amygdala and persistent pain. Neuroscientist 10:221-234.

Nickel JC, Shoskes DA, Irvine-Bird K (2010) Prevalence and impact of bacteriuria and/or urinary tract infection in interstitial cystitis/painful bladder syndrome. Urology 76:799-803.

Parsons CL (2007) The role of the urinary epithelium in the pathogenesis of interstitial cystitis/prostatitis/urethritis. Urology 69:9-16.

Poltorak A, He X, Smirnova I, Liu MY, Huffel CV, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science (New York, NY 282:2085-2088.

Qin C, Greenwood-Van Meerveld B, Foreman RD (2003a) Spinal neuronal responses to urinary bladder stimulation in rats with corticosterone or aldosterone onto the amygdala. Journal of neurophysiology 90:2180-2189.

Qin C, Greenwood-Van Meerveld B, Foreman RD (2003b) Visceromotor and spinal neuronal responses to colorectal distension in rats with aldosterone onto the amygdala. Journal of neurophysiology 90:2-11.

Qin C, Greenwood-Van Meerveld B, Myers DA, Foreman RD (2003c) Corticosterone acts directly
at the amygdala to alter spinal neuronal activity in response to colorectal distension.
Journal of neurophysiology 89:1343-1352.

Robinson DR, Gebhart GF (2008) Inside information: the unique features of visceral sensation.
Mol Interv 8:242-253.

Ronald A (2003) The etiology of urinary tract infection: traditional and emerging pathogens. Dis
Mon 49:71-82.

Rosamilia A, Dwyer PL (2000) Pathophysiology of interstitial cystitis. Curr Opin Obstet Gynecol
12:405-410.

Rudick CN, Billips BK, Pavlov VI, Yaggie RE, Schaeffer AJ, Klumpp DJ (2010) Host-pathogen
interactions mediating pain of urinary tract infection. J Infect Dis 201:1240-1249.

Rudick CN, Berry RE, Johnson JR, Johnston B, Klumpp DJ, Schaeffer AJ, Thumbikat P (2011)
Uropathogenic Escherichia coli induces chronic pelvic pain. Infect Immun 79:628-635.

Saban MR, Nguyen NB, Hammond TG, Saban R (2002) Gene expression profiling of mouse
bladder inflammatory responses to LPS, substance P, and antigen-stimulation. Am J
Pathol 160:2095-2110.

Sand PK (2006) Proposed pathogenesis of painful bladder syndrome/interstitial cystitis. The
Journal of reproductive medicine 51:234-240.

Schilling JD, Martin SM, Hung CS, Lorenz RG, Hultgren SJ (2003) Toll-like receptor 4 on stromal
and hematopoietic cells mediates innate resistance to uropathogenic Escherichiacoli.
Proceedings of the National Academy of Sciences of the United States of America
100:4203-4208.

Sengupta JN, Gebhart GF (1994) Mechanosensitive properties of pelvic nerve afferent fibers
innervating the urinary bladder of the rat. J Neurophysiol 72:2420-2430.

Shin K, Lee J, Guo N, Kim J, Lim A, Qu L, Mysorekar IU, Beachy PA (2011) Hedgehog/Wnt
feedback supports regenerative proliferation of epithelial stem cells in bladder. Nature
472:110-114.

Theoharides TC (2007) Treatment approaches for painful bladder syndrome/interstitial cystitis. Drugs 67:215-235.

Traub RJ, Silva E, Gebhart GF, Solodkin A (1996) Noxious colorectal distention induced-c-Fos protein in limbic brain structures in the rat. Neuroscience letters 215:165-168.

Truschel ST, Ruiz WG, Shulman T, Pilewski J, Sun TT, Zeidel ML, Apodaca G (1999) Primary uroepithelial cultures. A model system to analyze umbrella cell barrier function. The Journal of biological chemistry 274:15020-15029.

Tzan CJ, Berg J, Lewis SA (1993) Effect of protamine sulfate on the permeability properties of the mammalian urinary bladder. J Membr Biol 133:227-242.

Valerio A, Rizzonelli P, Paterlini M, Moretto G, Knopfel T, Kuhn R, Memo M, Spano P (1997) mGluR5 metabotropic glutamate receptor distribution in rat and human spinal cord: a developmental study. Neurosci Res 28:49-57.

Varney MA, Gereau RW (2002) Metabotropic glutamate receptor involvement in models of acute and persistent pain: prospects for the development of novel analgesics. Curr Drug Targets CNS Neurol Disord 1:283-296.

Walker K, Reeve A, Bowes M, Winter J, Wotherspoon G, Davis A, Schmid P, Gasparini F, Kuhn R, Urban L (2001a) mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurones mediate inflammatory hyperalgesia. Neuropharmacology 40:10-19.

Walker K, Reeve A, Bowes M, Winter J, Wotherspoon G, Davis A, Schmid P, Gasparini F, Kuhn R, Urban L (2001b) mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurones mediate inflammatory hyperalgesia. Neuropharmacology 40:10-19.

Walker K, Bowes M, Panesar M, Davis A, Gentry C, Kesingland A, Gasparini F, Spooren W, Stoehr N, Pagano A, Flor PJ, Vranesic I, Lingenhoehl K, Johnson EC, Varney M, Urban L, Kuhn R (2001c) Metabotropic glutamate receptor subtype 5 (mGlu5) and nociceptive function I. Selective blockade of mGlu5 receptors in models of acute, persistent and chronic pain. Neuropharmacology 40:1-9.

Walker K, Bowes M, Panesar M, Davis A, Gentry C, Kesingland A, Gasparini F, Spooren W, Stoehr N, Pagano A, Flor PJ, Vranesic I, Lingenhoehl K, Johnson EC, Varney M, Urban L, Kuhn R (2001d) Metabotropic glutamate receptor subtype 5 (mGlu5) and nociceptive function. I. Selective blockade of mGlu5 receptors in models of acute, persistent and chronic pain. Neuropharmacology 40:1-9.

Wilson CB, Leopard J, Nakamura RM, Cheresh DA, Stein PC, Parsons CL (1995) Selective type IV collagen defects in the urothelial basement membrane in interstitial cystitis. The Journal of urology 154:1222-1226.

Xu L, Gebhart GF (2008) Characterization of mouse lumbar splanchnic and pelvic nerve urinary bladder mechanosensory afferents. J Neurophysiol 99:244-253.

Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S (2004) A toll-like receptor that prevents infection by uropathogenic bacteria. Science (New York, NY 303:1522-1526.

Zhou S, Bonasera L, Carlton SM (1996) Peripheral administration of NMDA, AMPA or KA results in pain behaviors in rats. Neuroreport 7:895-900.

Zhou S, Komak S, Du J, Carlton SM (2001) Metabotropic glutamate 1alpha receptors on peripheral primary afferent fibers: their role in nociception. Brain research 913:18-26.

Chapter 2

<u>Protamine Sulfate Induced Bladder Injury Protects from Distention Induced Bladder Pain</u>

This chapter contains the manuscript:

Crock LW, Stemler KM, Lai HH, Mills JC, Gereau RW, Mysorekar IU (2013) Protamine sulfate induced bladder injury protects from distention induced bladder pain. J Urology 189(1):343-51.

ABSTRACT

Purpose: Bladder pain is a debilitating symptom of many urologic conditions, and there is no generally effective treatment. Abnormal urothelial turnover is common to multiple disease states, but the specific components of urothelial injury and the resulting molecular signals that lead to bladder pain are unknown. We examined mouse models of bladder injury induced by uropathogenic *E. coli* (UPEC), protamine sulfate (PS), and bacterial lipopolysaccharide (LPS) to identify cellular and molecular correlates underlying pain sensitization in response to the stimuli.

Materials and Methods: C57BL/6 female mice were given intravesicular PS, LPS or UPEC, and the impact of each on nociception was determined by measuring the evoked visceromotor response to bladder distention at 24 hours post inoculation. Levels of pyuria and tissue inflammation were examined by urinary cytology and tissue histology. Quantitative PCR and gene expression analysis were used to identify injury profiles associated with nociception.

Results: PS treatment was significantly analgesic upon bladder distention. PS-treated bladders did not exhibit pyuria or extensive tissue damage. PS injury was associated with a global decrease in expression of inflammation-associated genes. In contrast, UPEC injury significantly increased the nociceptive response to bladder distention. LPS treatment did not affect nociception. Finally, injury-induced expression of inflammation-associated genes correlated with nociceptive responses.

Conclusion: PS treatment of the bladder is analgesic, tissue protective, and suppresses inflammatory cytokine expression normally associated with nociception. Additionally, the injury modalities that result in differential tissue response patterns provide an innovative method for identification of mediators of visceral pain.

INTRODUCTION

The bladder is the site of several urologic conditions, and abnormal urothelial turnover is common to multiple painful disease states including interstitial cystitis/bladder pain syndrome (IC/BPS) and urinary tract infections (UTIs). IC/BPS and UTIs share the common clinical feature of increasing pelvic pain upon bladder filling (distention) leading to urinary frequency and urgency, with bladder pain being the most frequent reason for physician visits (Warren et al., 2008). IC/BPS affects 3-6% of women in the US (Berry et al., 2011). UTIs, caused primarily by uropathogenic *E. coli* (UPEC), affect 13 million women each year (Foxman, 2010).

Urothelial cell sloughing and defective urothelial barriers characterize patients with recurrent/chronic UTIs and IC/BPS. Disruption of the normally impermeable urothelial barrier leads to tissue injury and pain sensitization (Parsons, 2007). Although the urothelium is recognized as a nociceptive (pain)-sensing structure that modulates and transmits noxious stimuli through mediators such as cytokines and ATP (Keay, 2008; Birder et al., 2012), it is unclear how damage to each tissue layer of the bladder affects pain, and the specific molecular components generated by urothelial tissue injury that lead to bladder pain are unknown.

The urothelium exhibits a remarkable ability to renew in response to environmental insults (e.g., pathogens and cytoinjurious factors). Intriguingly, the regenerative responses to injury modalities are distinct (Mysorekar et al., 2009). A murine model of bladder injury resulting from UPEC infection leads to urothelial barrier damage and inflammation within 24h (Mysorekar et al., 2009). Urothelial regeneration following infection is fueled by a rapid activation of stem and early progenitor cells (Mysorekar et al., 2009). In contrast, intravesicular treatment with protamine sulfate (PS), a highly cationic peptide that causes increased ionic permeability of the urothelium and chemically exfoliates urothelial barrier cells within 12h of instillation, does not induce inflammation (Tzan et al., 1993; Mysorekar et al., 2009). Rather than activating urothelial stem cells, PS-induced injury appears to activate epithelial repair via transiently amplifying cells (Mysorekar et al., 2009).

We used these two well-characterized models of urothelial damage to dissect the cellular

and molecular mechanisms underlying pain sensitization in response to injury and inflammation.

We report a striking protection from distention-induced bladder pain upon PS injury, which is in

contrast to the nociceptive response triggered by UPEC infection. Urine cytology and tissue

histology show that PS treatment protects against pyuria and distention-induced tissue damage.

Finally, we identified a molecular profile of globally down-regulated inflammatory cytokines with

an increase in transient amplifying cell signatures, thus providing novel insights into the

mechanisms of nociception and PS-induced analgesia.

MATERIALS AND METHODS

Mice

All animal experimental protocols were approved by the Washington University Institutional

Animal Care and Use Committee. Female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME),

9-13 weeks old were used for all experiments. Animals were housed on a 12-hour light/dark cycle

and allowed *ad libitum* access to food and water.

Intravesicular inoculations

UTI89, a pathogenic *Escherichia coli* strain, was grown statically in Luria-Bertani (LB) broth for 17

h at 37 $^{\circ}$C. 50 µl of PS (10 mg/ml in water; Sigma, St. Louis, MO) or 10^7 colony-forming units

(CFU)/mL bacterial suspensions in saline were intravesicularly administered.(Hung et al., 2009;

Mysorekar et al., 2009) Littermate controls received 50 µl of sterile 1x saline (Fisher, Waltham,

MA). Inflammatory damage was induced with lipopolysaccharide (LPS) (50 µl, 100 µg/ml LPS

from *E. coli* strain 055:B5, Sigma) by intravesicular inoculation once a day for four days.(Saban et

al., 2002) Littermate controls received a four-day administration of 50 µl of 1x sterile saline. After

intravesicular administration, the catheter was removed, and all animals were maintained under

isoflurane anesthesia for 10 minutes before spontaneous waking.

Visceromotor Response (VMR) analysis

At 24 hours post last inoculation (hpi), animals were lightly anesthetized under isoflurane and the

visceromotor response (VMR) of each animal was recorded. Visceral nociception was quantified

by an electromyographic recording of the abdominal muscle response to bladder distention as described previously (Ness and Elhefni, 2004; Lai et al., 2011; Crock et al., 2012). For each distention, the VMR signals were subtracted from the baseline, rectified, and integrated over 20 seconds to quantify the area under the curve.

Tissue preparation and inflammation scoring

Immediately following completion of VMR analysis, mice were sacrificed, and bladder and kidney tissues were aseptically removed. Tissues were fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid), embedded in paraffin, sectioned, and stained with hemotoxylin and eosin. Photomicrographs were taken using a Hamamatsu NanoZoomer HT (Hamamatsu Corporation) and observed in a blinded fashion to score the level of tissue damage and inflammation using a modified semi-quantitative scoring system (Hannan et al., 2010): 0, normal; 1A, subepithelial edema without cellular infiltrate; 1B, subepithelial inflammatory infiltration (focal and multifocal); 2, edema and subepithelial inflammatory infiltration (diffuse); 3, marked subepithelial inflammatory cells with necrosis and polymorphonuclear neutrophils (PMNs) in and on bladder mucosal epithelium; 4, grade 3 criteria plus inflammatory infiltrate extends into muscle; **5,** loss of surface epithelium (necrosis with full thickness inflammatory infiltration).

Urine collection, urine sediment, and bacterial titer analysis

Urines were collected prior to intravesicular treatment, at 6 hpi, 24 hpi, and twice during the VMR analysis. Urines collected prior to VMR were obtained as previously published (Hannan et al., 2010). During VMR rest periods, catheterized animals had gentle pressure applied to the skin just below the occiput, and voided urine was gathered by pipet and transferred to sterile tubes. "Non-noxious pressure" ($\leq$30 mmHg distention) and "noxious pressure" ($\geq$40 mmHg) urines were collected per mouse and pooled. Urine sediments were obtained as previously detailed (Hannan et al., 2010), fixed for 15 minutes in acetic acid/alcohol, and Papanicolaou stained following manufacturer's instructions (PROTOCOL brand, Fisher). Photomicrographs were taken using a Nikon Eclipse E800 microscope (Japan) and analyzed using Image J (NIH). Stained urine sediments were examined and scored in a blinded manner by light microscopy on a 0-4 scale, where 0 indicates <1 and 4 indicates >20 PMNs/high-powered field as previously described

(Hannan et al., 2010). Pre-treatment urines exhibited no evidence of superficial cell sloughing or pyuria prior to intravesicular instillations. Bladder infection was confirmed by spotting serial dilutions of urine on LB agar plates and quantifying CFUs after overnight growth at 37 ºC.

DNA Microarray inflammatory gene profiling

Total cellular RNA was isolated (RNeasy kit, Qiagen) from whole mouse bladders at 3 hpi, 6 hpi (for UPEC), and 3 hpi, 6 hpi, 24 hpi, and 48 hpi (for PS); (8-12 week old female mice, 10 animals/injury/time point, n=2 independent experiments). cRNAs were generated from pooled RNAs and used to interrogate U74 (UPEC) and 430 2.0 whole mouse genome GeneChips (Affymetrix) as previously published (Mysorekar et al., 2009). Genes and Gene Ontology (GO) terms that were enriched in each treatment were determined using dChip and GOurmet software (Doherty et al., 2006). To identify GO terms increased following PS treatment, enriched genes were associated with GO terms. GO terms that increased by $\geq$25%, and represented $\geq$3% of the genes expressed at one or more time points were determined. All such GO terms were plotted in Figures 2.5C-E.

Real time quantitative RT-PCR (qPCR)

Total cellular bladder RNA (TRIzol isolated following manufacturer's protocols) was pooled (5-7 animals, 6-11 week old females) from mice at 3.5 hpi, 6 hpi and 24 hpi. cDNAs were reverse transcribed, assayed in triplicate, and gene expression changes were determined using the $\Delta\Delta C_t$ method (normalized to 18s rRNA, and then to saline treated mice) as described (Mysorekar et al., 2002; Mysorekar et al., 2009). Primer sequences (listed in Table 2.1) were obtained from the qPCR PrimerBank public database (Spandidos et al., 2010).

Statistical analysis

VMR measurements (mean ±SEM) were analyzed by 2-way ANOVA with Bonferroni post-hoc analysis. Inflammation scores were analyzed using two-tailed Mann-Whitney U-tests (where appropriate) comparing injuries to their respective controls.

RESULTS

PS is analgesic in the distention VMR assay

We hypothesized that various injuries to the bladder architecture and the respective renewal responses may have differential effects on the pain-like response to bladder distention. We have previously used the visceromotor response (VMR) to reliably measure hypersensitivity in a chemically-induced bladder inflammation model, demonstrating that the VMR is potentiated by inflammation (mustard oil, cyclophosphamide, and zymosan) and inhibited by analgesics (morphine and intravesicular lidocaine) (Ness and Elhefni, 2004; Lai et al., 2011; Crock et al., 2012). The abdominal VMR is measured as the electromyographic signals of the external oblique muscle. Behaviorally, it corresponds to abdominal withdrawal and nocifensive guarding when an animal experiences pain while its bladder is distended. Therefore, the VMR is a surrogate measure of distension-evoked visceral nociception and represents a useful model for examining how different injuries to the bladder affect distention-induced bladder pain.

24 hours prior to VMR testing, mice were given intravesicular UPEC, PS or saline (control). As expected, UPEC infection significantly increased the evoked response to bladder distention when compared to controls (Figure 2.1A). In contrast, PS treatment resulted in a significantly decreased VMR when compared to controls (Figure 2.1A). To determine whether the differential effect was due to the absence of an inflammatory response in PS injury, we treated a separate cohort of mice with intravesicular LPS every 24 hours for 4 days, a regimen previously shown to elicit an inflammatory response (Saban et al., 2002). Multi-dose LPS injury did not significantly alter the VMR when compared to mice similarly treated with saline (Figure 2.1B).
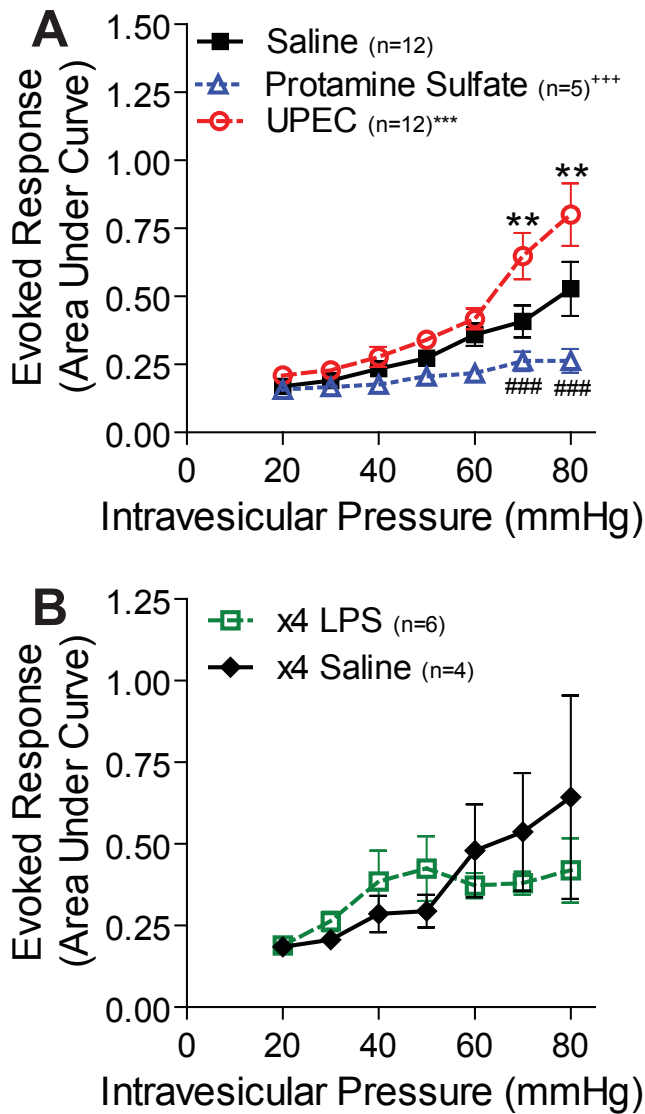
Figure 2.1. PS injury is analgesic to bladder distention. A. Female mice were administered saline, PS, or UPEC intravesicularly prior to VMR. PS-treated animals showed a significantly blunted VMR ([+++]p<0.0001) when compared to saline controls with 70 and 80mmHg pressures ([###]p<0.0001) eliciting statistically significantly decrease in the VMR. UPEC-infected animals had a significantly higher VMR (***p<0.0001) compared to saline controls, and sensitization was seen at the highest pressures of distention (70 and 80mmHg, **p<0.001). B. Female mice administered LPS or saline four times over four days had no significantly difference in their VMR. These data are presented in arbitrary units as mean ±SEM, and p-values were determined by 2-way ANOVA with Bonferroni's post-hoc test.

PS treatment does not induce pyuria

To track urothelial injury and the impact of distention on injury profiles, urine was collected for sediment analysis. Saline treatment did not induce injury as measured by cytology (Figure 2.2A-B, G-H). UPEC injury has been shown to result in formation of intracellular bacterial communities (IBCs) by 6 hpi. These are shed into the urine along with neutrophils (PMNs) as part of the host response (Hannan et al., 2010). Accordingly, we identified IBCs and PMNs in urines from UPEC-infected mice (Figures 2.2E-F). Analysis of urines from PS-treated mice showed minor cell

sloughing prior to distention (Figures 2.2C-D). LPS injury resulted in PMN recruitment by 6 hpi without associated loss of superficial cells (Figures 2.2I-J). Urine sediments scored for inflammation at time points post injury revealed that UPEC injury lead to sustained pyuria (urinary inflammation), whereas PS treatment did not (Figure 2.2K). LPS injury resulted in acute pyuria, which was resolved prior to VMR (Figure 2.2K).
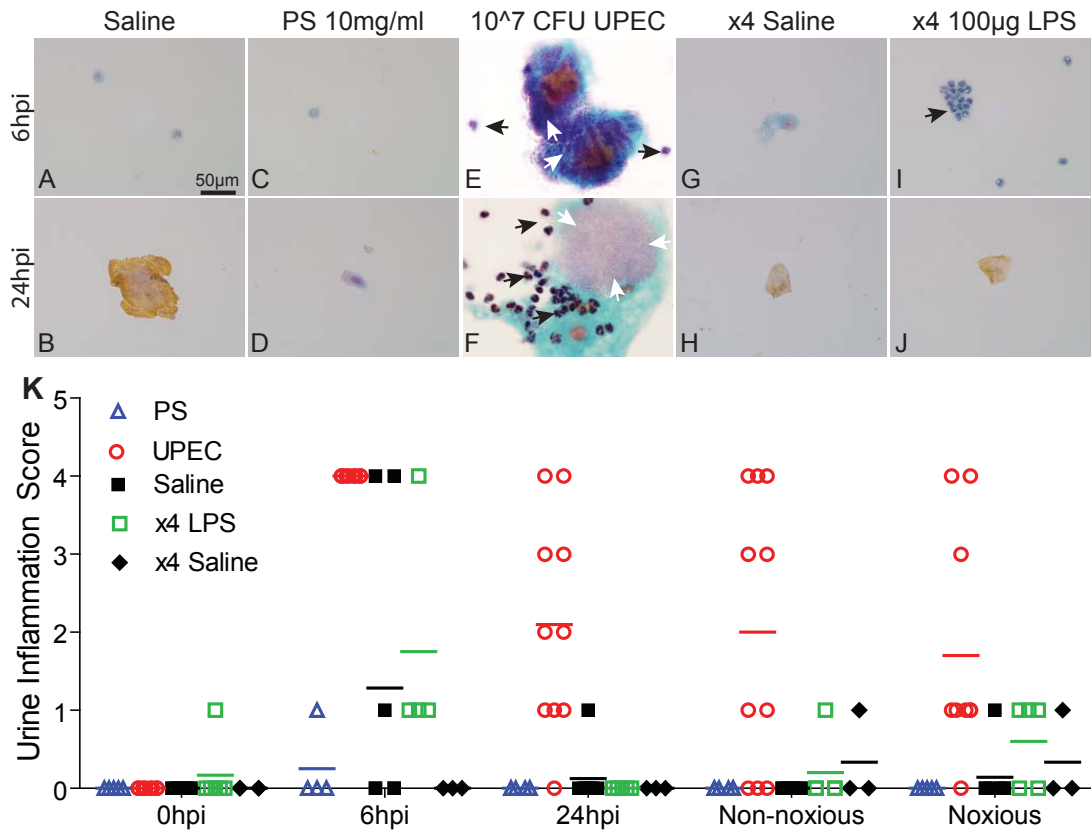
Figure 2.2. Pyuria is not induced by PS treatment. Representative urine cytology images of PAP-stained urine sediments from control and injured (PS, UPEC, LPS) animals. A-B & G-H. Saline-treated controls showed minimal urothelial cell sloughing (highly kertanized vaginal cells are brown). By 6 hpi, both UPEC (E) and LPS (I) injuries displayed pyuria whereas PS (C) did not. IBCs were found throughout UPEC urines (E&F). Black arrows depict PMNs and white arrows demark IBCs. K. Urinary inflammation scores from PS- (n=6), UPEC- (n=10), saline- (n=8), x4 LPS- (n=6), and x4 saline- (n=4) treated animals. Bars represent median score values.

PS treatment induces hematuria at noxious pressures

Hemorrhagic cystitis is frequently induced upon toxic chemical or infectious instillation **into** the bladder (Traxer et al., 2001). We observed gross and microhematuria in urines and sediments from the majority of PS-injured animals (Figures 2.3D, 4/5, 80%), which was not evident in urines prior to pathologic bladder distention (Figure 2.3C). The incidence of hematuria from infectious injury was far less prevalent (Figures 2.3E-F, 5/10, 50%). Saline treated animals exhibited hallmarks of distention-induced microhematuria (Figures 2.3A-B, 4/7, 57%). Moreover, we found that levels of hematuria and pyuria were inversely correlated in PS and UPEC injuries (Figures 2.3G).
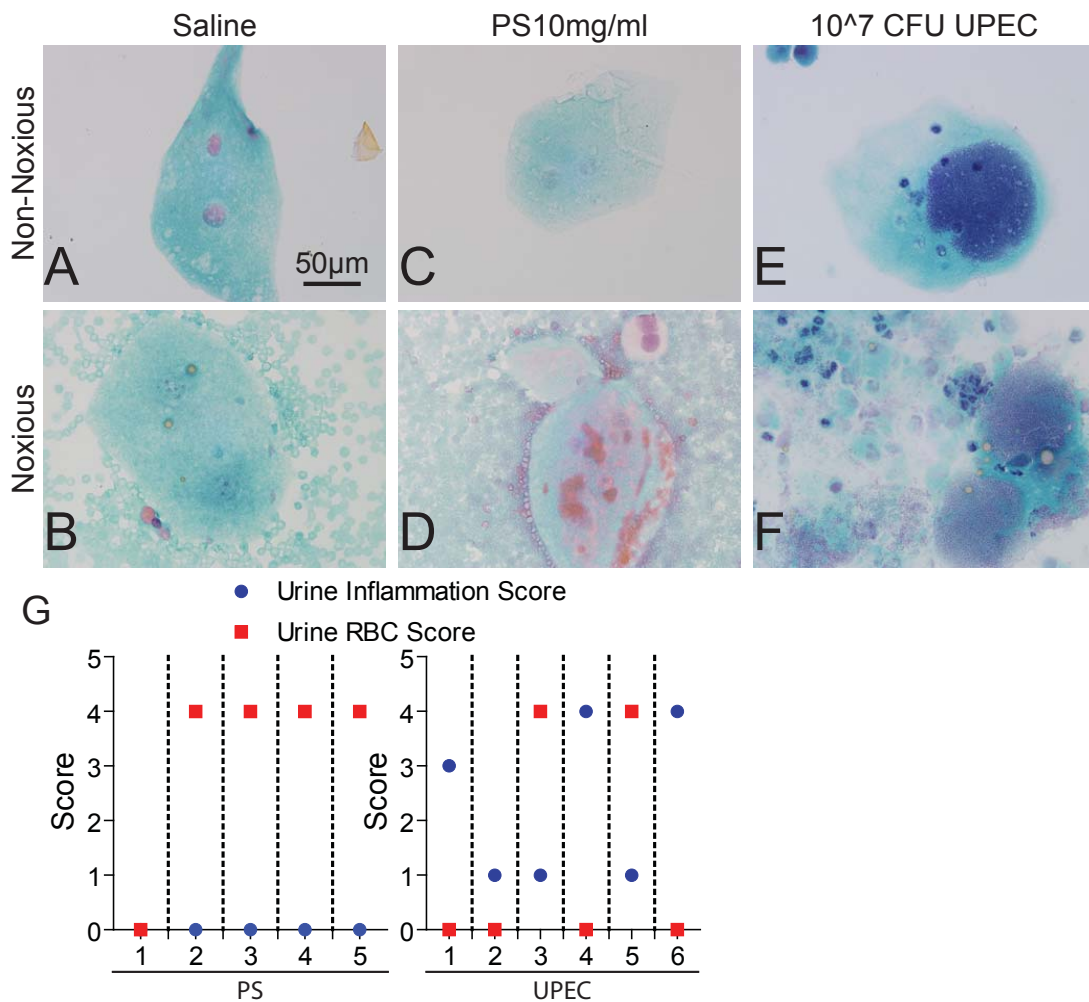
Figure 2.3. Noxious distention induces hematuria in PS-injured animals. Representative urine cytology images of PAP-stained urine sediments from control and injured animals. A, C, E.

Distention-induced sloughing of superficial cells from all animals. At noxious distention pressures (>40 mmHg), hematuria was induced in control (B) and PS (D) treated animals. G. Individual PS- (left) or UPEC- (right) treated animals showed an inverse correlation between the levels of pyuria (urine inflammation score) and hematuria (urine RBC score). Dotted lines partition individual animals' scores from one another.

PS treatment protects against distention-induced exacerbation of injury

To track architectural changes as a result of both distention and injury, bladders were collected immediately following completion of the VMR recordings. We found that control animals displayed superficial cell loss and edema of the lamina propria upon distention (Figures 2.4A-B). Interestingly, PS injury was not associated with extensive urothelial or stromal damage (Figure 2.4C) and displayed a relative lack of tissue inflammation (Figure 2.4E). In contrast, histological characterization of UPEC-injured bladders after distention revealed stromal edema, urothelial damage, and PMN influx (Figure 2.4D, inset) associated with significantly higher tissue inflammation scores (Figure 2.4E) when compared to saline-treated controls.
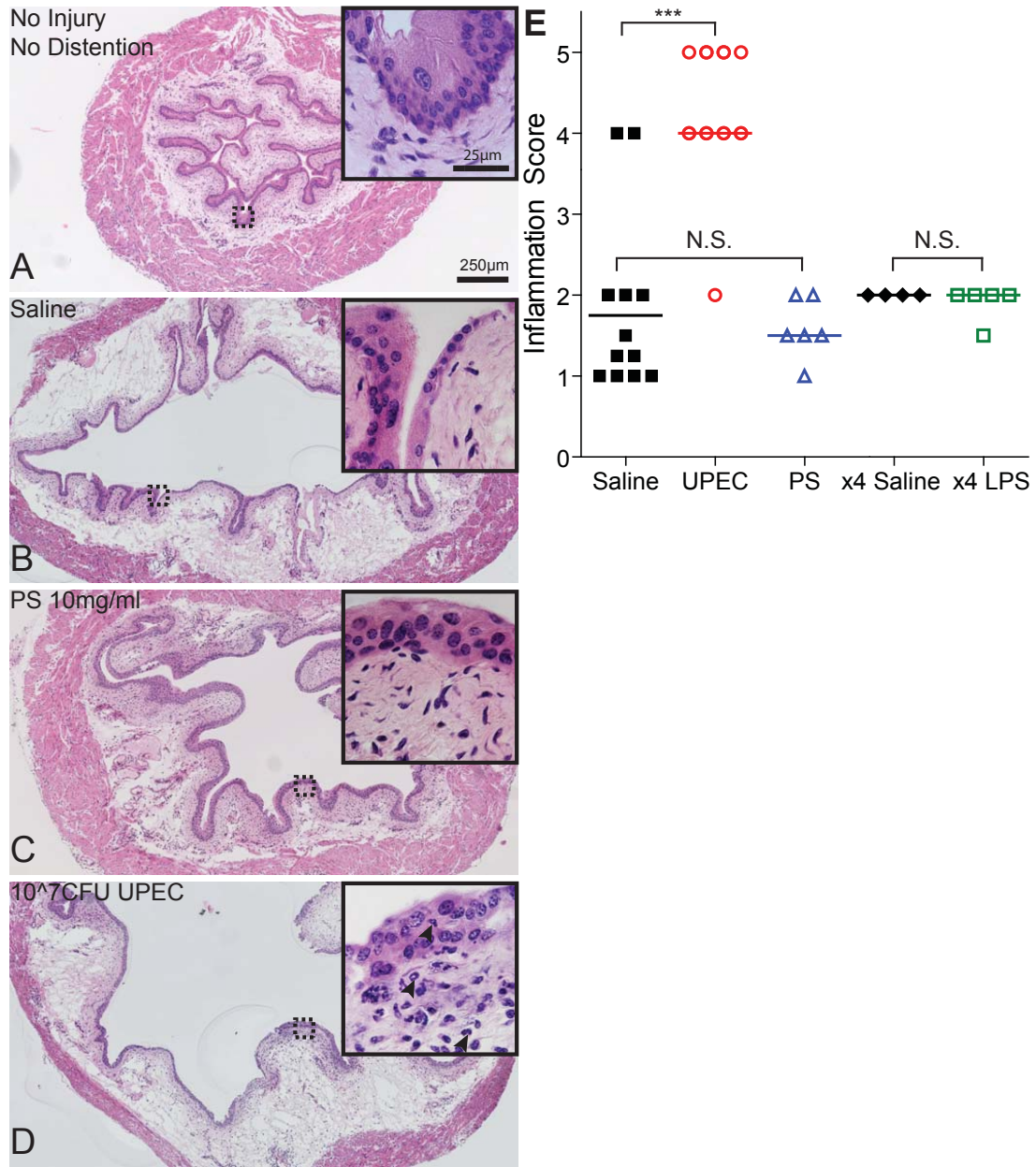
Figure 2.4. Histologic analysis shows PS injury protects against distention-induced damage. A. H&E of normal bladder histology without injury or distention. B. Saline treated bladder after distention showed edema and superficial cell disruption (inset). C. PS treatment prevented exacerbated superficial cell loss or edema due to distention. The urothelium and stromal compartment appeared almost normal, with no infiltration of immune cells (inset). D. Edema and major urothelial barrier disruption occurred in UPEC-infected bladders after distention. Immune cells were evident in the stromal and urothelial layers (black arrowheads,

inset). Dotted boxes denote area depicted in insets. E. Tissue inflammation scores from control (saline n=12; x4 saline n=4) and injured (PS n=6; UPEC n=9; x4 LPS n=5) animals after distention. Bars represent median score values. ***p<0.0001. Two-tailed Mann-Whitney U-test comparing to saline controls. N.S. = Not Significant.

PS treatment suppresses pro-inflammatory responses

The bladder and urothelium produce distinct pro-inflammatory cytokines and chemokines in response to inflammatory (e.g., CPX, LPS, substance P) and infectious injury (Saban et al., 2001; Saban et al., 2002; Hannan et al., 2010; Wood et al., 2011). Pro-inflammatory cytokines (e.g., IL-6, IL-1β) can also lead to pain hypersensitivity (Binshtok et al., 2008; Ren and Dubner, 2010). To determine how PS treatment regulates inflammation, we compared gene expression in bladders from mice treated with PS to mice infected with UPEC. UPEC infection lead to increased expression of genes with 'inflammation/immune response' GO terms, whereas PS down-regulated the expression of those genes (Figures 2.5A-B) (Doherty et al., 2006). Nearly 10 % of all genes enriched by UPEC at 6 hpi were characterized by the GO term 'inflammatory response', whereas only about 0.1% of those enriched in the PS at 48 hpi were characterized as having 'chemokine activity', indicating that the molecular signatures of bladder responses to PS were distinct from those induced by UPEC infection.

Microarray analysis following PS injury revealed an increase in expression of genes that function in tissue renewal mechanisms (Figures 2.5C-E). At early time points (6h and before, Figure 2.5C), genes related to signaling and cell adhesion were elevated, and by 12h, GO terms like 'DNA replication' and 'cell cycle', which characterize transient amplifying cells but not stem cells, were induced (Figure 2.5D).
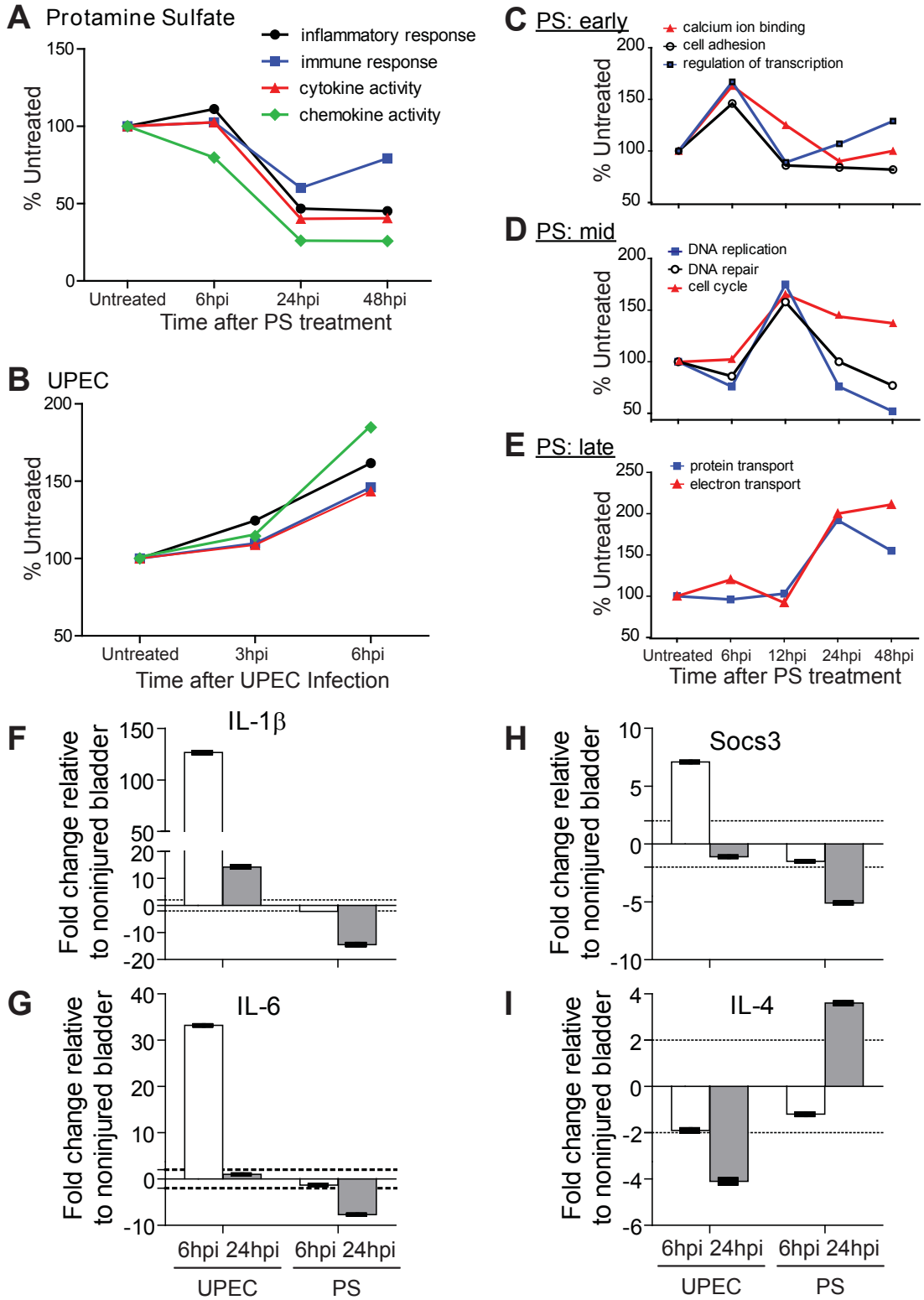
Figure 2.5. Global gene expression profiles after UPEC or PS injury reflect nociception trends. A. UPEC infection lead to a rapid global increase in gene expression associated with an

inflammatory state by 6 hpi. B. PS injury resulted in a global decrease in pro-inflammatory gene expression by 24 hpi maintained through 48 hpi. C-E. PS treatment induced increases in various GO terms that peaked at 6 h (C, early), 12 h (D, mid), or 24 h (E, late). The genes peaking at 6 h were associated with cell cycling, whereas those at 12 h had to do with transiently amplifying cell populations. F-I. qPCR analysis confirms inflammatory profiles of UPEC and PS injuries, where the pro-inflammatory, pro-nociceptive cytokines IL-1β (F) and IL-6 (G) were up-regulated by UPEC and down regulated by PS injury relative to untreated bladder. Socs3 was also up-regulated by UPEC and down-regulated by PS injury (H). Infection decreased mRNA expression levels of the anti-inflammatory cytokine, IL-4 (I) and PS injury up-regulated it by 24 hpi. Significant fold changes indicated by dotted line at ±2 fold.

We further confirmed these results by identifying multiple gene expression profiles that correlated between injury and its respective nociception response by qPCR (Figures 2.5F-I and Table 2.1). UPEC injury triggered an up-regulation of the early pro-inflammatory cytokines, IL-1β and IL-6, and of Socs3, a negative regulator of IL-6. Furthermore, infection with UPEC resulted in down-regulation of the anti-inflammatory cytokine, IL-4, but PS treatment correlated with an up-regulation of this cytokine. In contrast, PS injury resulted in down-regulation of IL-1β, IL-6, and Socs3 (Figures 2.5F-I). We have also identified molecular markers that were up-regulated by both UPEC and PS treatments or only regulated by UPEC or PS injury (Table 2.1).

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon length (bp)	Fold change in UPEC injury realtive to non-injured bladder	Fold change in PS injury relative to non-injured bladder
IL-1β	5'- GCAACTGTTCCTGAACTCAACT	5'- ATCTTTTGGGGTCCGTCAACT	89	126.5 (6hpi)	-14.5 (24hpi)
IL-6	5'- CCAGAAACCGCTATGAAGTTCCT	5'- CACCAGCATCAGTCCCAAGA	72	33.2 (6hpi)	-7.7 (24hpi)
Socs3	5'- CTTCCCATGCGCTCACA	5'- CCCAGCCCCATACCTGACTT	110	7.1 (6hpi)	-5.1 (24hpi)
IL-4	5'- GGTCTCAACCCCCAGCTAGT	5'- GCCGATGATCTCTCTCAAGTGAT	102	-4.1 (24hpi)	3.6 (24hpi)
KC	5'- ACCCAAACCGAAGTCATAGCC	5'- TTCAGGGTCAAGGCAAGCC	60	44.7 (6hpi)	N.S.
Cinc-1	5'- CTGGGATTCACCTCAAGAACATC	5'- CAGGGTCAAGGCAAGCCTC	117	26.4 (6hpi)	N.S.
Ccxl-10	5'- CCAAGTGCTGCCGTCATTTTC	5'- GGCTCGCAGGGATGATTTCAA	157	24.5 (6hpi)	N.S.
Tnf-α	5'- CCCTCACACTCAGATCATCTTCT	5'- GCTAC GACGTGGGCTACAG	61	7.9 (6hpi)	N.S.
Stat5b	5'- CACCCGCAATGATTACAGCG	5'- CTCTTGATTCGTTTCAGGGACA	117	-5.6 (6hpi)	N.S.
IL-10	5'- GCTCTTACTGACTGGCATGAG	5'- CGCAGCTCTAGGAGCATGTG	105	-5.3 (24hpi)	N.S.
IL-7	5'- TTCCTCCACTGATCCTTGTTCT	5'- AGCAGCTTCCTTTGTATCATCAC	200	-4.4 (6hpi)	N.S.
SOCS7	5'- TCAGTCGCCTGTTTCGCAC	5'- GTTTCCTCCCCGTATCCAGC	153	-3.6 (6hpi)	N.S.
Tgf-β1	5'- CTCCCGTGGCTTCTAGTGC	5'- GCCTTAGTTTGGACAGGATCTG	133	N.S.	5.4 (24hpi)
IL-1α	5'- GCACCTTACACCTACCAGAGT	5'- AAACTTCTGCCTGACGAGCTT	126	30.7 (6hpi)	2.6 (6hpi)
IL-1Ra	5'- GCTCATTGCTGGGTACTTACAA	5'- CCAGACTTGGCACAAGACAGG	132	5.7 (6hpi)	9.4 (24hpi)
18S	5'- CGGCTACCACATCCAAGGAA	5'- GCTGGAATTACCGCGGCT	187	-	-

Table 2.1. Inflammatory genes are differentially regulated by PS- or UPEC-mediated injuries.

DISCUSSION

Here we report that different bladder injuries result in distinct alterations in nociception and provide several lines of evidence that this is due to differential effects on inflammation. We find that although UPEC infection causes increases in distension-induced pain responses, PS treatment reduces these responses. Our urine and tissue inflammation assays and gene expression analyses all indicate that the pain modulation correlates with inflammation as a result of UPEC infection and suppression of inflammation by PS treatment.

Chronic bladder pain is a debilitating condition that is often unresponsive to conventional pain medications. Injury-induced sensitization can result in acute pain that resolves, but in some instances can transition to chronic pain that persists even in the absence of ongoing injury (Ren and Dubner, 2010). Renewal after injury typically requires barrier restoration and resolution of inflammation (Mysorekar et al., 2009). The pathophysiology of conditions such as IC/BPS remains poorly understood. Underlying mechanisms include occult UTI, non-infectious idiopathic inflammatory changes of the bladder, or urothelial cell sloughing and regeneration. Patients can be divided into subsets based on these underlying mechanisms. IC/BPS symptom exacerbation ("flare up") bears remarkable resemblance to recurrent UTI, with increasing bladder pain and urinary urgency, and in some cases, may be associated with subclinical UTI (Porru et al., 2004). IC urine contains an antiproliferative factor that inhibits the regeneration of bladder epithelial cells in culture (Keay, 2008). In IC/BPS and UTIs, disruption of the normally impermeable urothelial barrier by continued cell sloughing may further impede renewal and lead to exacerbated tissue injury and pain sensitization (Parsons, 2007; Keay, 2008).

The differential pain response to UPEC, LPS and PS stimuli could in part be explained by type of urothelial damage. Consistent with previous findings (Ness and Elhefni, 2004; Rudick et al., 2010; Lai et al., 2011; Crock et al., 2012), we find that UPEC infection results in visceral hyperalgesia and sensitization of nociceptive responses. UPEC infection appears to activate the nociceptive signals transmitted to the central nervous system; inhibition of a key receptor

(mGluR5) in this pathway results in a blunted VMR to UPEC infection (Crock et al., 2012). One explanation for this activation could be the potent inflammatory response induced upon infection.

To better study the effect of inflammation on VMR activation, we simplified the complex host-pathogen interaction in infection by focusing on inflammation induced by the surface antigen LPS. Although adjuvant pre-treatment has been used to increase levels of inflammation (Saban et al., 2001; Saban et al., 2002), we treated with LPS alone to mimic the naïve bladder's response to bacterial products. In contrast to adjuvant administration, we found that LPS treatment, although displaying acute pyuria, is insufficient to induce changes in VMR. Our findings suggest that acute inflammation alone is not critical to evoke bladder pain. Bladder distention is known to trigger ATP release from the urothelium, thus activating purinergic receptors on superficial cells and sensitizing the afferent neurons (Sun et al., 2001; Munoz et al., 2011; Birder et al., 2012). Within IC/BPS patients ATP release in response to distention is increased (Kumar et al., 2007). It is unknown how other injuries to the urothelium affect the release of ATP. Barrier cell sloughing due to PS could deplete the ATP-based sensitization mechanism on afferents by lowering ATP levels below an activation threshold (Sun et al., 2001; Munoz et al., 2011; Birder et al., 2012). Future work should focus on identifying how urothelial status affects the integration of signals important for nociception sensitization.

Although the presence of a foreign body within the bladder and other PS injury models have been shown to result in cystitis-like inflammation (Guiton et al.; Soler et al., 2008), our PS injury model uses short catheterization and incubation periods and does not result in inflammatory cystitis (Mysorekar et al., 2009). We found that PS injury was analgesic upon distention and had improved bladder histology when compared to distended infectious injury and controls. This is the first report of urothelial injury eliciting analgesia, but the mechanism by which PS treatment is protective is unknown.

Our microarray analysis revealed contrasting expression profiles in UPEC- and PS-treated bladders, consistent with the differential effects of UPEC and PS injury on VMR. UPEC infection resulted in increased pro-inflammatory gene expression changes within 3-6 hours, consistent with molecular changes preceding urothelial stem cell (USC) activation (Mysorekar et

al., 2002; Mysorekar et al., 2009). In contrast, we detected decreased expression of inflammatory-related genes 24 hours after PS injury. We confirmed the suppressed immune response after PS injury by qPCR, focusing on multiple pro-inflammatory and pro-nociceptive cytokines. Expression of IL-1β, a pro-inflammatory, pro-nociceptive cytokine that regulates the expression of other inflammatory cytokines, and is produced by the bladder in response to inflammatory injury (Binshtok et al., 2008; Wood et al., 2011), was decreased in PS injury, but increased following UPEC infection. Expression of the pro-inflammatory cytokine IL-6 was similarly affected (Hannan et al., 2010; Ren and Dubner, 2010; Blalock et al., 2012). Conversely, we observed decreased expression of IL-4, a prototypical anti-inflammatory cytokine, in UPEC-infected mice but increased expression following PS injury.

PS injury revealed an increase in renewal mechanisms initiating from transient amplifying regulation rather than USC activation (Mysorekar et al., 2009). It is possible that injuries, such as UPEC infection, that activate stem cell-mediated renewal might amplify a feed-forward loop that leads to enhanced nociception, whereas injuries like PS that do not activate stem cell-mediated renewal do not contribute to increased nociception. Therefore, this combination of suppressed immune response and transient amplifying renewal could be contributing to the mode of PS injury-induced analgesic action.

CONCLUSIONS

We report that various injuries to the bladder lead to differential effects on distention-induced nociception. PS injury leads to a decrease in global inflammatory profile expression, is analgesic in a model of bladder distention, and decreases the level of injury from distention as compared to controls. Our PS injury model provides a new avenue to understand analgesia and how pain is sensed in the bladder.

REFERENCES

Berry SH, Elliott MN, Suttorp M, Bogart LM, Stoto MA, Eggers P, Nyberg L, Clemens JQ (2011)
Prevalence of symptoms of bladder pain syndrome/interstitial cystitis among adult
females in the United States. J Urol 186:540-544.

Binshtok AM, Wang H, Zimmermann K, Amaya F, Vardeh D, Shi L, Brenner GJ, Ji RR, Bean BP,
Woolf CJ, Samad TA (2008) Nociceptors are interleukin-1beta sensors. J Neurosci
28:14062-14073.

Birder L, Ruggieri M, Takeda M, van Koeveringe G, Veltkamp S, Korstanje C, Parsons B, Fry C
(2012) How does the urothelium affect bladder function in health and disease?: ICI-RS
2011. Neurourol Urodyn 31:293-299.

Blalock EM, Korrect GS, Stromberg AJ, Erickson DR (2012) Gene expression analysis of urine
sediment: evaluation for potential noninvasive markers of interstitial cystitis/bladder pain
syndrome. J Urol 187:725-732.

Crock LW, Stemler KM, Song DG, Abbosh P, Vogt SK, Qiu C, Lai HH, Mysorekar IU, Gereau RW
(2012) Metabotropic glutamate receptor 5 (mGluR5) regulates bladder nociception.
Molecular pain 8.

Doherty JM, Carmichael LK, Mills JC (2006) GOurmet: a tool for quantitative comparison and
visualization of gene expression profiles based on gene ontology (GO) distributions. BMC
bioinformatics 7:151.

Foxman B (2010) The epidemiology of urinary tract infection. Nat Rev Urol 7:653-660.

Guiton PS, Hung CS, Hancock LE, Caparon MG, Hultgren SJ Enterococcal biofilm formation and
virulence in an optimized murine model of foreign body-associated urinary tract
infections. Infection and immunity 78:4166-4175.

Hannan TJ, Mysorekar IU, Hung CS, Isaacson-Schmid ML, Hultgren SJ (2010) Early severe
inflammatory responses to uropathogenic E. coli predispose to chronic and recurrent
urinary tract infection. PLoS pathogens 6:e1001042.

Hung CS, Dodson KW, Hultgren SJ (2009) A murine model of urinary tract infection. Nat Protoc 4:1230-1243.

Keay S (2008) Cell signaling in interstitial cystitis/painful bladder syndrome. Cell Signal 20:2174-2179.

Kumar V, Chapple CR, Surprenant AM, Chess-Williams R (2007) Enhanced adenosine triphosphate release from the urothelium of patients with painful bladder syndrome: a possible pathophysiological explanation. J Urol 178:1533-1536.

Lai H, Qiu CS, Crock LW, Morales MEP, Ness TJ, Gereau RW (2011) Activation of spinal extracellular signal-regulated kinases (ERK) 1/2 is associated with the development of visceral hyperalgesia of the bladder. Pain Epub.

Munoz A, Somogyi GT, Boone TB, Smith CP (2011) Lumbosacral sensory neuronal activity is enhanced by activation of urothelial purinergic receptors. Brain research bulletin 86:380-384.

Mysorekar IU, Mulvey MA, Hultgren SJ, Gordon JI (2002) Molecular regulation of urothelial renewal and host defenses during infection with uropathogenic Escherichia coli. J Biol Chem 277:7412-7419.

Mysorekar IU, Isaacson-Schmid M, Walker JN, Mills JC, Hultgren SJ (2009) Bone morphogenetic protein 4 signaling regulates epithelial renewal in the urinary tract in response to uropathogenic infection. Cell Host Microbe 5:463-475.

Ness TJ, Elhefni H (2004) Reliable visceromotor responses are evoked by noxious bladder distention in mice. J Urol 171:1704-1708.

Parsons CL (2007) The role of the urinary epithelium in the pathogenesis of interstitial cystitis/prostatitis/urethritis. Urology 69:9-16.

Porru D, Politano R, Gerardini M, Giliberto GL, Stancati S, Pasini L, Tinelli C, Rovereto B (2004) Different clinical presentation of interstitial cystitis syndrome. International urogynecology journal and pelvic floor dysfunction 15:198-202.

Ren K, Dubner R (2010) Interactions between the immune and nervous systems in pain. Nat Med 16:1267-1276.

Rudick CN, Billips BK, Pavlov VI, Yaggie RE, Schaeffer AJ, Klumpp DJ (2010) Host-pathogen interactions mediating pain of urinary tract infection. The Journal of infectious diseases 201:1240-1249.

Saban MR, Nguyen NB, Hammond TG, Saban R (2002) Gene expression profiling of mouse bladder inflammatory responses to LPS, substance P, and antigen-stimulation. Am J Pathol 160:2095-2110.

Saban MR, Hellmich H, Nguyen NB, Winston J, Hammond TG, Saban R (2001) Time course of LPS-induced gene expression in a mouse model of genitourinary inflammation. Physiol Genomics 5:147-160.

Soler R, Bruschini H, Freire MP, Alves MT, Srougi M, Ortiz V (2008) Urine is necessary to provoke bladder inflammation in protamine sulfate induced urothelial injury. J Urol 180:1527-1531.

Spandidos A, Wang X, Wang H, Seed B (2010) PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. Nucleic Acids Res 38:D792-799.

Sun Y, Keay S, De Deyne PG, Chai TC (2001) Augmented stretch activated adenosine triphosphate release from bladder uroepithelial cells in patients with interstitial cystitis. J Urol 166:1951-1956.

Traxer O, Desgrandchamps F, Sebe P, Haab F, Le Duc A, Gattegno B, Thibault P (2001) [Hemorrhagic cystitis: etiology and treatment]. Progres en urologie : journal de l'Association francaise d'urologie et de la Societe francaise d'urologie 11:591-601.

Tzan CJ, Berg J, Lewis SA (1993) Effect of protamine sulfate on the permeability properties of the mammalian urinary bladder. The Journal of membrane biology 133:227-242.

Warren JW, Brown J, Tracy JK, Langenberg P, Wesselmann U, Greenberg P (2008) Evidence-based criteria for pain of interstitial cystitis/painful bladder syndrome in women. Urology 71:444-448.

Wood MW, Breitschwerdt EB, Gookin JL (2011) Autocrine effects of interleukin-6 mediate acute-phase proinflammatory and tissue-reparative transcriptional responses of canine bladder mucosa. Infection and immunity 79:708-715.

Chapter 3

Metabotropic Glutamate Receptor 5 (mGluR5) Regulates Bladder Nociception

This chapter contains the manuscript:

Crock LW, Stemler KM, Song DG, Abbosh P, Vogt SK, Qiu CS, Lai HH, Mysorekar IU, and Gereau RW (2012) Metabotropic Glutamate Receptor 5 (mGluR5) Regulates Bladder Nociception. Mol Pain March 26; 8:20.

ABSTRACT

Background: Interstitial cystitis/painful bladder syndrome (IC/PBS), is a severely debilitating chronic condition that is frequently unresponsive to conventional pain medications. The etiology is unknown, however evidence suggests that nervous system sensitization contributes to enhanced pain in IC/PBS. In particular, central nervous system plasticity of glutamatergic signaling involving NMDA and metabotropic glutamate receptors (mGluRs) has been implicated in a variety of chronic pain conditions. Here, we test the hypothesis that mGluR5 mediates both non-inflammatory and inflammatory bladder pain or nociception in a mouse model by monitoring the visceromotor response (VMR) during graded bladder distention.

Results: Using a combination of genetic and pharmacologic approaches, we provide evidence indicating that mGluR5 is necessary for the full expression of VMR in response to bladder distention in the absence of inflammation. Furthermore, we observed that mice infected with a uropathogenic strain of Escherichia coli (UPEC) develop inflammatory hyperalgesia to bladder distention, and that the selective mGluR5 antagonist fenobam [N-(3-chlorophenyl)-N'-(4,5-dihydro-1-methyl-4-oxo-1H-imidazole-2-yl) urea], reduces the VMR to bladder distention in UPEC-infected mice.

Conclusions: Taken together, these data suggest that mGluR5 modulates both inflammatory and non-inflammatory bladder nociception, and highlight the therapeutic potential for mGluR5 antagonists in the alleviation of bladder pain.

BACKGROUND

Interstitial cystitis/painful bladder syndrome (IC/PBS) is a serious and painful condition of unknown etiology that affects 3-6% of women in the United States (Rosenberg et al., 2007; Berry et al., 2011). The major clinical symptom of IC/PBS is pain upon bladder filling (distention) leading to urinary frequency and urinary urgency (Warren et al., 2008). The current available treatments are often ineffective and do not treat the underlying pathology. Rodent bladder-injury models that induce some of the symptoms observed in IC/PBS have been used to evaluate potential treatments for IC/PBS (Maggi et al., 1993; Saban et al., 2002; Westropp and Buffington, 2002; Dang et al., 2008; Boudes et al., 2011; Lai et al.). One injury model, bacterial cystitis (urinary tract infection, UTI) is known to cause a similar constellation of symptoms as observed in IC/PBS (i.e. urinary frequency and urgency (Baerheim and Hunskar, 1997; Malterud and Baerheim, 1999; Bower et al., 2005)). In addition, bacterial cystitis can be modeled in rodents through bladder exposure to uropathogenic *Escherichia Coli* (UPEC) (Mulvey et al., 1998; Mysorekar and Hultgren, 2006). Bladder infections due to UPEC are responsible for approximately 80% of UTIs in otherwise healthy women (Foxman, 2002, 2010). Understanding the underlying molecular mechanisms of both non-inflammatory bladder pain and inflammatory bladder pain due to UPEC infection could lead to the development of novel treatments for painful bladder infections as well as for IC/PBS and possibly other visceral pain conditions.

Glutamate is the predominant excitatory neurotransmitter in the mammalian nervous system (Matsumoto et al.; Kakizaki et al.; Yoshiyama and de Groat). Glutamate mediates its effects through two major classes of glutamate receptors: ligand-gated ionotropic receptors (iGluRs) and G protein-coupled metabotropic glutamate receptors (mGluRs). Among the metabotropic glutamate receptors, one subtype, mGluR5, is of particular interest in the context of pain conditions. mGluR5 is expressed throughout the peripheral and central nervous system (Varney and Gereau, 2002) and has previously been shown to have a pro-nociceptive role in a variety of somatic pain models (Bhave et al., 2001; Karim et al., 2001a; Walker et al., 2001b; Hudson et al., 2002; Varney and Gereau, 2002; Hu et al., 2007) and some visceral pain models (Bianchi et al.,

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2003; Young et al., 2007; Lindstrom et al., 2008). Specific to visceral pain models, mGluR5 was found to modulate gastroesophogeal and colorectal afferent sensitivity (Page et al.; Young et al.; Lindstrom et al.). Based on this prior information, a previous study examined the ability of the mGluR5 antagonist, MPEP (2-methyl-6-(phenylethynyl)-pyridine), to reduce bladder pain responses in naïve (uninjured) rats (Hu et al., 2009). While this study suggests a potential role for mGluR5 in bladder pain, the evidence is based exclusively on the use of MPEP, which has recently been shown to act non-selectively *in vivo* (Montana et al., 2009). Thus, these intriguing initial findings are in need of validation. Furthermore, the role of mGluR5 in inflammatory bladder pain is unknown. Here, using a combination of genetic and pharmacological approaches we demonstrate that mGluR5 regulates both bladder nociception and normal bladder function in naïve mice. Furthermore, we observed an increased VMR to bladder distention in mice infected with UPEC. Finally, UPEC-induced hyperalgesia is reduced by treatment with the specific mGluR5 antagonist, fenobam. Together these data strongly support the hypothesis that mGluR5 is necessary for the full expression of inflammatory and non-inflammatory bladder nociception and may be a relevant target for the treatment of bladder pain arising from multiple pathologies, including IC/PBS.

RESULTS

mGluR5 is necessary for the full expression of non-inflammatory bladder nociception To assess bladder nociception in response to distension, we utilized the distension-evoked visceromotor response (VMR). The VMR is a spinobulbospinal reflex to bladder distention, increased in decerebrate mice/rats and absent in mice/rats with an acute mid thoracic spinal cord transection (Castroman and Ness, 2001; Ness et al., 2001; Ness and Elhefni, 2004). Bladder distention reliably produces pain and/or discomfort in humans (Ness et al., 1998), and is frequently used in rodents as a visceral pain model (Ness et al., 2001; Hu et al., 2009; Lai et al., 2011). To provide genetic evidence supporting a role for mGluR5 in bladder nociception, we tested the VMR to bladder distention in mGluR5 knock out mice (mGluR5 KO) compared to their WT littermates.

Stepwise increases in bladder distension resulted in progressively larger VMR in wild type mice, as shown in Figure 3.1B. Furthermore, mGluR5 KO mice showed a statistically significant decrease in the evoked response to bladder distention (VMR) in the noxious range of pressures when compared to the VMR of WT littermates (p< 0.0001).
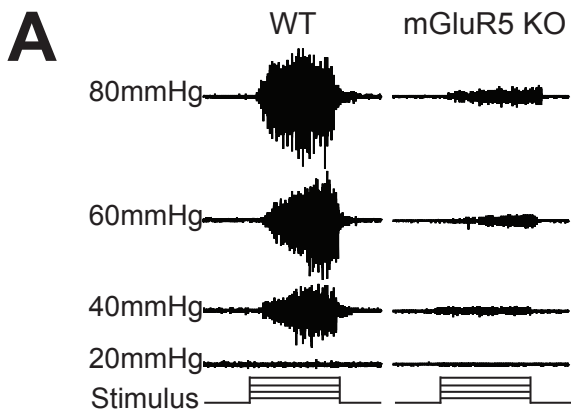
Figure 3.1: A. Representative VMR tracings from a WT and mGluR5 KO mice. As the intravesicular pressure is increased (20-80mmHg), the EMG activity of the abdominal muscle (VMR) is also increased. The total amount of activity (area under the curve) during the 20 second distention is calculated to determine the evoked response at each pressure. B. mGluR5 KO (n=18) mice have a significantly blunted VMR when compared to WT littermates (n=15) +/- SEM,* p<0.05, **p<0.01, ***p<0.001. 2-way ANOVA with Bonferroni post-hoc test. There were no obvious histological differences observed between mGluR5 KO mice (1D) and their WT littermates (1C). In both, the urothelium (above dashed line) has normal layers of superficial facet cells (arrows), intermediate cells (black arrowhead) and basal cells (white arrowhead).
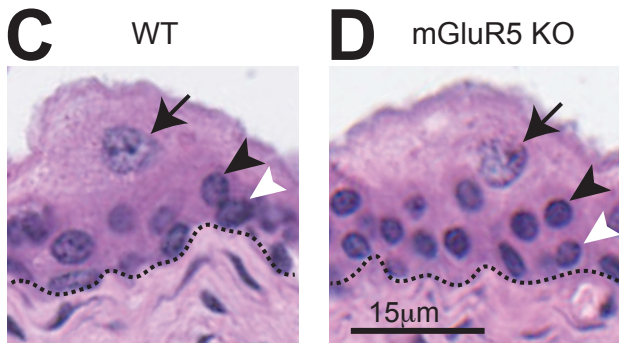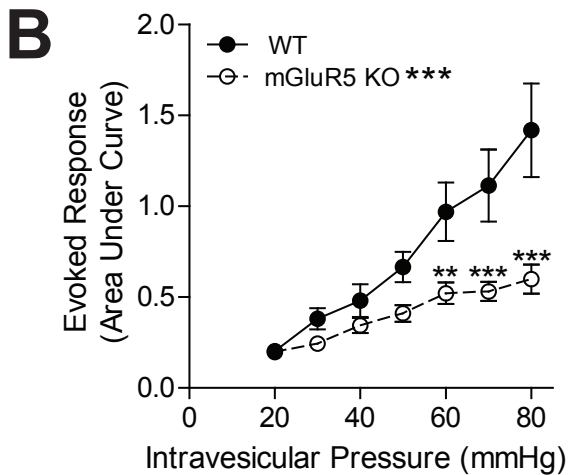
There are several possible reasons that genetic ablation of mGluR5 could lead to the observed suppression of the VMR. Recent work has implicated the urothelium in the sensation of physical

and chemical stimuli (Birder, 2010). One possibility is that loss of mGluR5 could lead to anatomical changes in the bladder lining, thus altering the response to distension. We therefore examined histological sections from mGluR5 KO mice and their WT littermates. The absence of mGluR5 does not appear to impact bladder architecture, as the bladders from mGluR5 KO mice are histologically indistinguishable from WT littermates. The superficial, intermediate and basal epithelial cells are present (see Figure 3.1C and D) and the underlying mesenchyme and muscle layers also remain intact. Furthermore, the barrier formed by the superficial cells is normal as evidenced by Uroplakin III staining (data not shown) in both the WT and mGluR5 KO mice. Therefore, the absence of mGluR5 affects the response to noxious bladder distention but does not alter gross urothelial architecture.

Compensatory changes in gene expression represent a potential confound to any experiment utilizing genetic manipulation. Thus, the robust phenotype observed in the mGluR5 KO mice could be the result of compensatory expression changes in other genes that have a role in bladder nociception. An acute pharmacological blockade of the receptor represents a potentially powerful approach to complement these findings from genetically modified mice. However, as mentioned above, the pharmacologic agent (MPEP) used in prior studies shows a clear lack of specificity in vivo (Montana et al., 2009). To ask whether mGluR5 activation is acutely involved in distention-induced bladder nociception, we tested whether specific pharmacologic inhibition of mGluR5 with the selective antagonist fenobam (Zhu et al., 2004; Porter et al., 2005; Montana et al., 2009), would suppress the VMR to urinary bladder distention. Systemic (intraperitoneal (IP)) administration of fenobam to WT mice resulted in a statistically significant reduction in the response to bladder distention compared to pretreatment baseline responses (Figure 3.2B, $p < 0.0001$), whereas treatment with vehicle had no statistically significant effect on VMR compared to baseline (Figure 3.2A). Thus, pretreatment with fenobam mimicked the reduced nociceptive response to bladder distention that was observed in mGluR5 KO mice relative to their WT littermates (compare Figure 3.1A and 3.2A). We next evaluated the effect of fenobam on the VMR of mGluR5 KO mice. Surprisingly, treatment with either vehicle (DMSO) or fenobam caused

a small but statistically significant decrease in the VMR evoked by bladder distention compared to

baseline responses in mGluR5 KO mice (Figure 3.2C and D, p<0.001 and p=0.007).
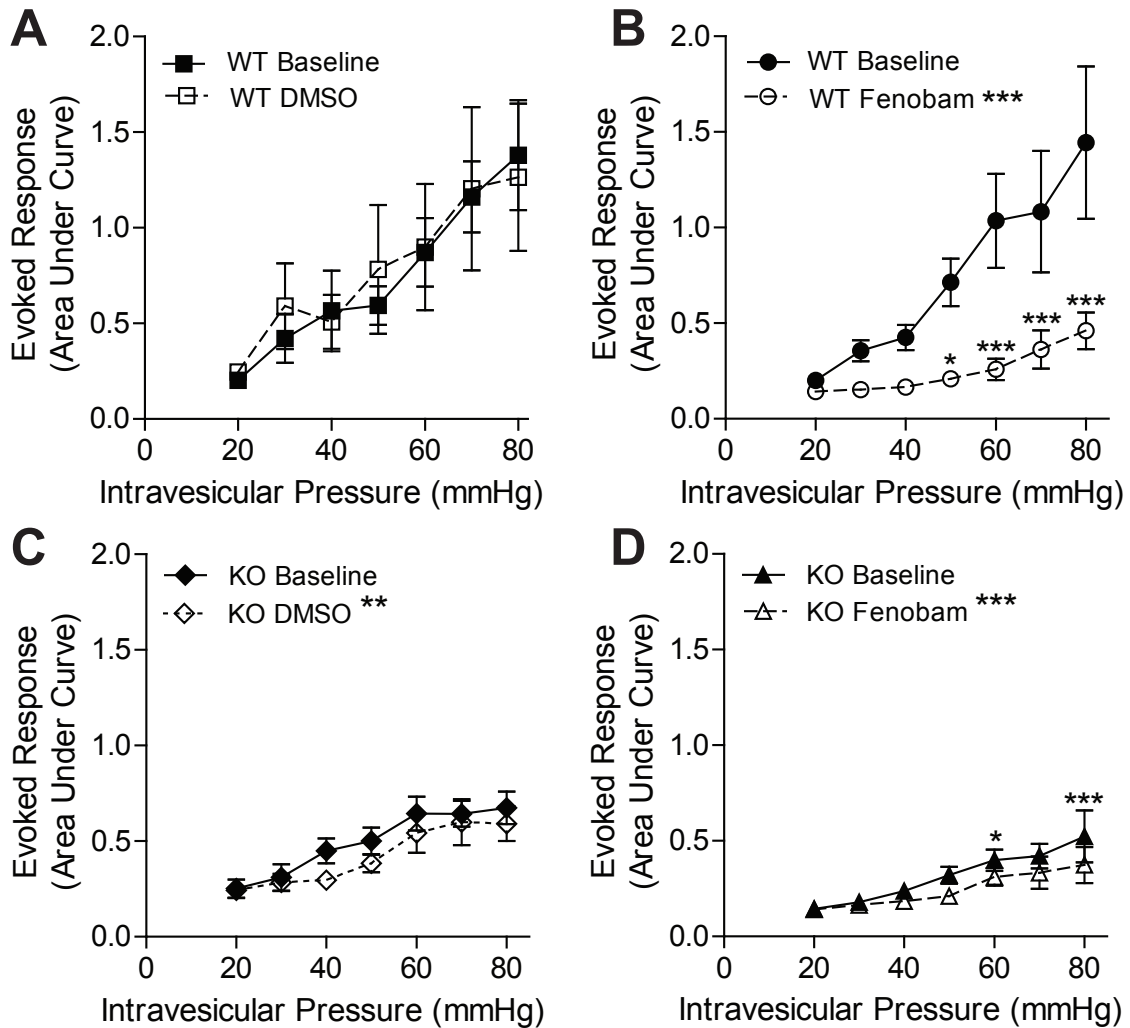
Figure 3.2: The selective mGluR5 antagonist, fenobam, is analgesic in distention-induced

bladder pain model. A. Treatment with the vehicle used to dissolve fenobam (100% DMSO) has

no effect on the response to bladder distention. B. Treatment with an mGluR5 antagonist,

fenobam, is analgesic in a bladder distention-evoked pain model. C&D. Fenobam and DMSO

reduce the evoked response in mGluR5 KO mice. +/- SEM,* p<0.05, **p<0.01, ***p<0.001. 2-way

ANOVA with Bonferroni post-hoc test.

<u>mGluR5 regulates intermicturition interval (IMI)</u> To test the role of mGluR5 in urodynamics, we compared the cystometry profile of mGluR5 KO mice and their WT littermates. mGluR5 KO mice had a significantly increased IMI (212.3 seconds *versus* 471.5 seconds, for WT and KO, respectively, p=0.0006) (Figure 3.3A-C). Despite the difference in IMI, the average amplitude of bladder contractions was not significantly different in mGluR5 KO mice relative to their WT littermates (Figure 3.3D, p=0.9215).
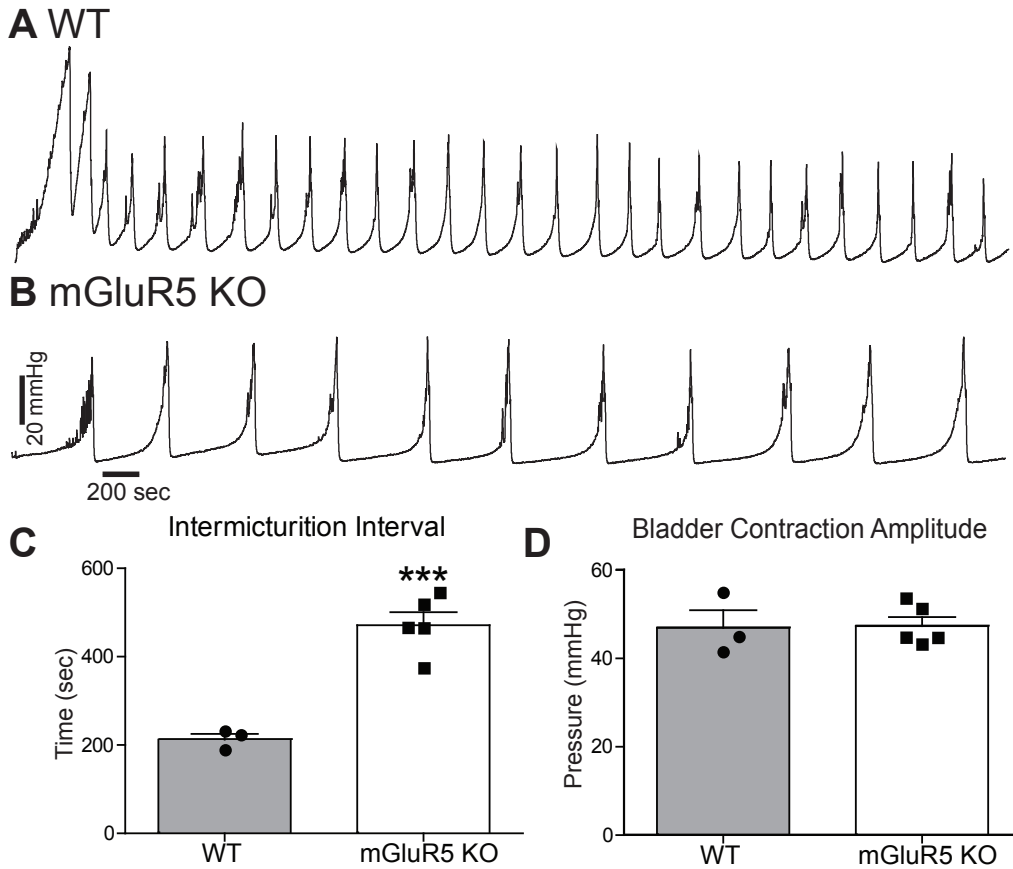
Figure 3.3: mGluR5 KO mice have an increased intermicturition interval. A. Representative urodynamic profile of a WT mouse. B. Representative urodynamic profile of a mGluR5 KO mouse. A-C. The IMI in WT mice was significantly smaller when compared to mGluR5 KO mice (WT baseline IMI 212.3 ± 12.94 N=3, mGluR5 KO IMI baseline 471.5 ± 29.14 N=5). D. However, there was no difference in the bladder contraction amplitude. ***P<0.001 unpaired Student's t-test compared to WT IMI.

To determine if the voiding behavior we observed in mGluR5 KO mice was the result of genetic ablation of mGluR5 or the result of compensatory changes, we acutely treated WT mice with fenobam or vehicle and measured the effect on urodynamics. While vehicle treatment had no effect on IMI (160.2 seconds versus 177.1 seconds, for baseline and vehicle (DMSO) respectively, p=0.66, Figure 3.4D), fenobam treatment increased the IMI when compared to the baseline IMI. The IMI before fenobam treatment was 182 seconds (n=8). After IP fenobam treatment, four of the eight mice stopped bladder cycling and micturition immediately (Figure 3.4C), while four of the mice had significantly increased IMI (481.3 seconds, p=0.031, Figure 3.4D and E). The mean time to resume bladder cycling and micturition after systemic fenobam administration was 18.6 minutes. This suggests a tight coupling between mGluR5 activation and micturition cycling. The acute effect of the mGluR5 antagonist also suggests that the increased IMI observed in mGluR5 KO mice is not likely due to developmental changes as a result of genetic ablation of mGluR5.
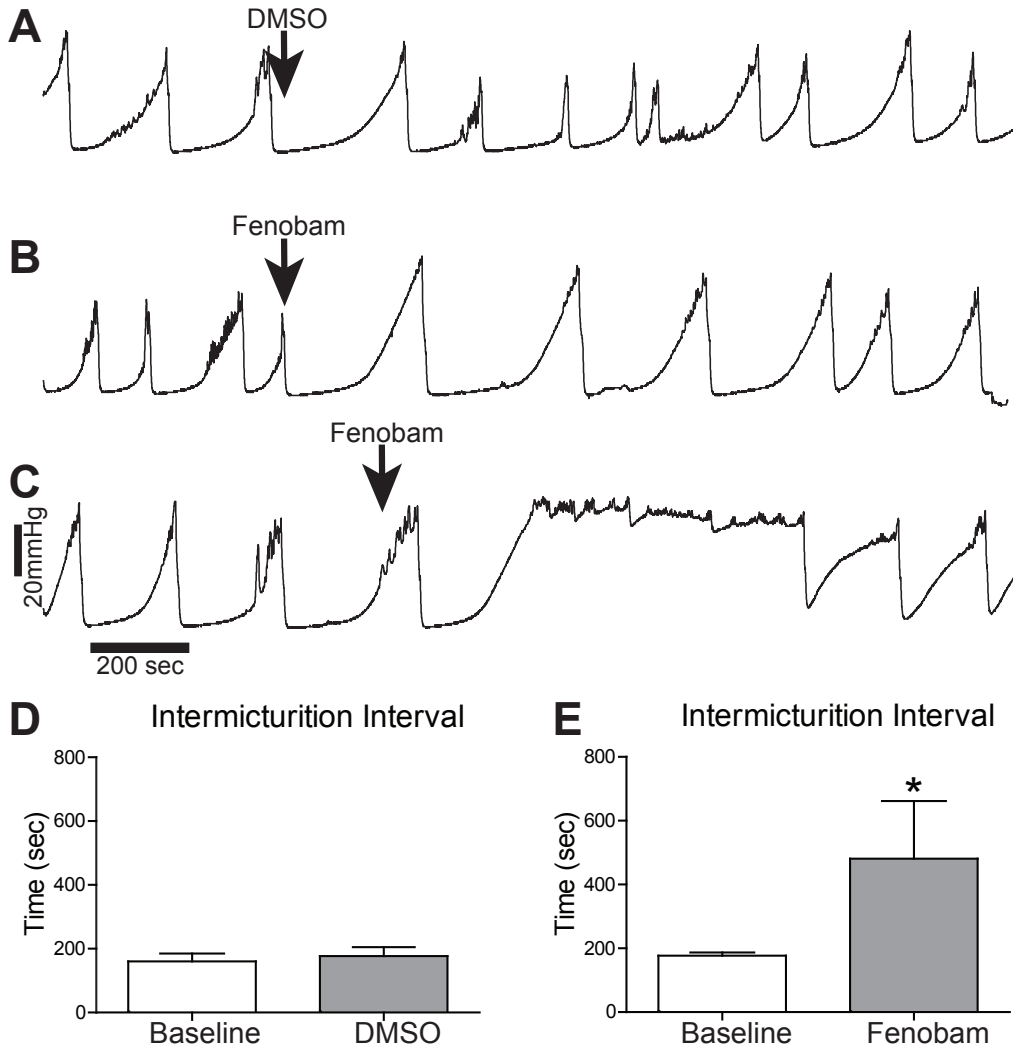
Figure 3.4: Fenobam treatment increases the intermicturition interval. A-C. Representative urodynamic profile of WT mice before and after treatment. An intraperitoneal (IP) injection with fenobam significantly increased the IMI in 4/8 mice treated (B,E), while 4/8 mice stopped cycling (C). In the mice that stopped cycling, the mean time to resume bladder cycling and micturition after IP fenobam administration was 18.6 ± 5.0 minutes. The IMI was not significantly affected by IP DMSO (n=7) (A,D). *P<0.05 paired Student's t-test compared to baseline.

UPEC infection results in changes in bladder histology and an increased VMR Our results, together with previous studies (Guarneri et al.; Hu et al.; Larson et al.) suggest that mGluR5 is involved in the mediation of micturition cycling and controls nociceptive responses during noxious

bladder distention; however, the role of mGluR5 in inflammatory bladder nociception is unexplored. We therefore used the UPEC model of bladder inflammation to examine the role of mGluR5 in inflammatory bladder nociception. We found that UPEC infection resulted in an increased VMR to noxious bladder distention when compared to mock-infected littermate controls (Figure 3.5A, p< 0.0001). Furthermore, in mice whose bladders had been distended, UPEC infection produced an increased infiltration of polymorphonuclear leukocytes observed throughout the full thickness of the bladder tissue (Figure 3.5B, arrowheads), as well as an increased tissue thickness of both the urothelium and mesenchyme compared to distended mock-infected mice (Figure 3.5C). Noxious bladder distention combined with UPEC infection resulted in greater histological damage as measured by tissue inflammation scores when compared to mock-infected mice with noxious distention (data not shown) (Hopkins et al., 1998). UPEC infection (alone and with distention) resulted in barrier disruption through a loss of superficial facet cells, while noxious distention alone had no effect on these cells and the barrier remained intact (arrows, Figure 3.5C).
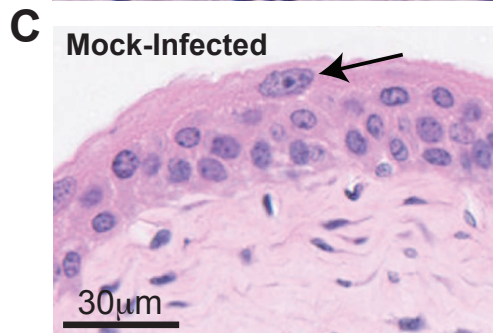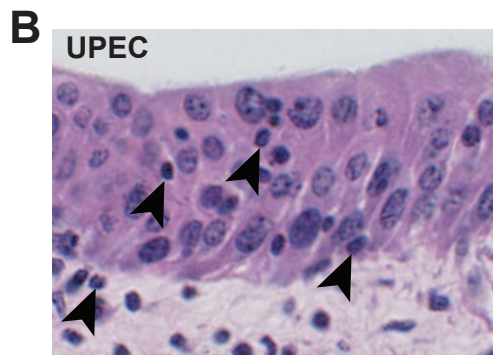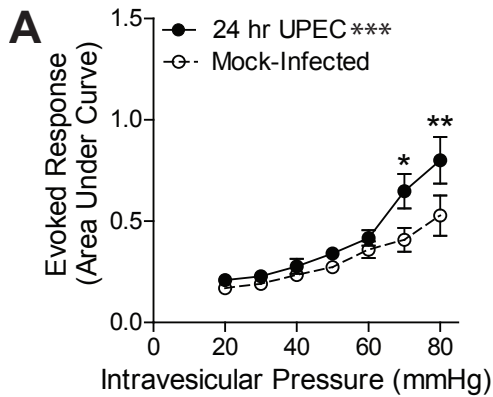
Figure 3.5: UPEC Infection results in histological changes and bladder hyperalgesia. A. 24 hour infection with uropathogenic E. coli (UPEC) increases the VMR to bladder distention when compared to mock-infected (PBS) littermates. B. Infection with UPEC results in an increased infiltration of polymorphonuclear leukocytes (arrowheads), and a sloughing of the superficial facet cells. C. Distention alone does not result in the loss of superficial facet cells (arrows,). +/- SEM *p<0.05, **p<0.01, ***p<0.001. Unpaired 2-way ANOVA with Bonferroni post-hoc test.

mGluR5 is necessary for the full expression of inflammatory bladder nociception In mice with a UPEC-induced UTI, mice treated with vehicle had an increased VMR when compared to baseline (Figure 3.6A, p=0.0007). While vehicle treatment increased the VMR, treatment with fenobam resulted in a significantly reduced VMR when compared to pre-fenobam measurements (Figure 3.6B, p=0.0006). In contrast, vehicle had no effect on the VMR in mice mock infected (with PBS instead of UPEC, Figure 3.6C). In these control mice (mock infected), treatment with fenobam significantly reduced the VMR when compared to pre-treatment VMR measurements (Figure

3.6D). These results are consistent with those observed in WT mice (not mock-infected, Figures 3.2A and 3.2B).
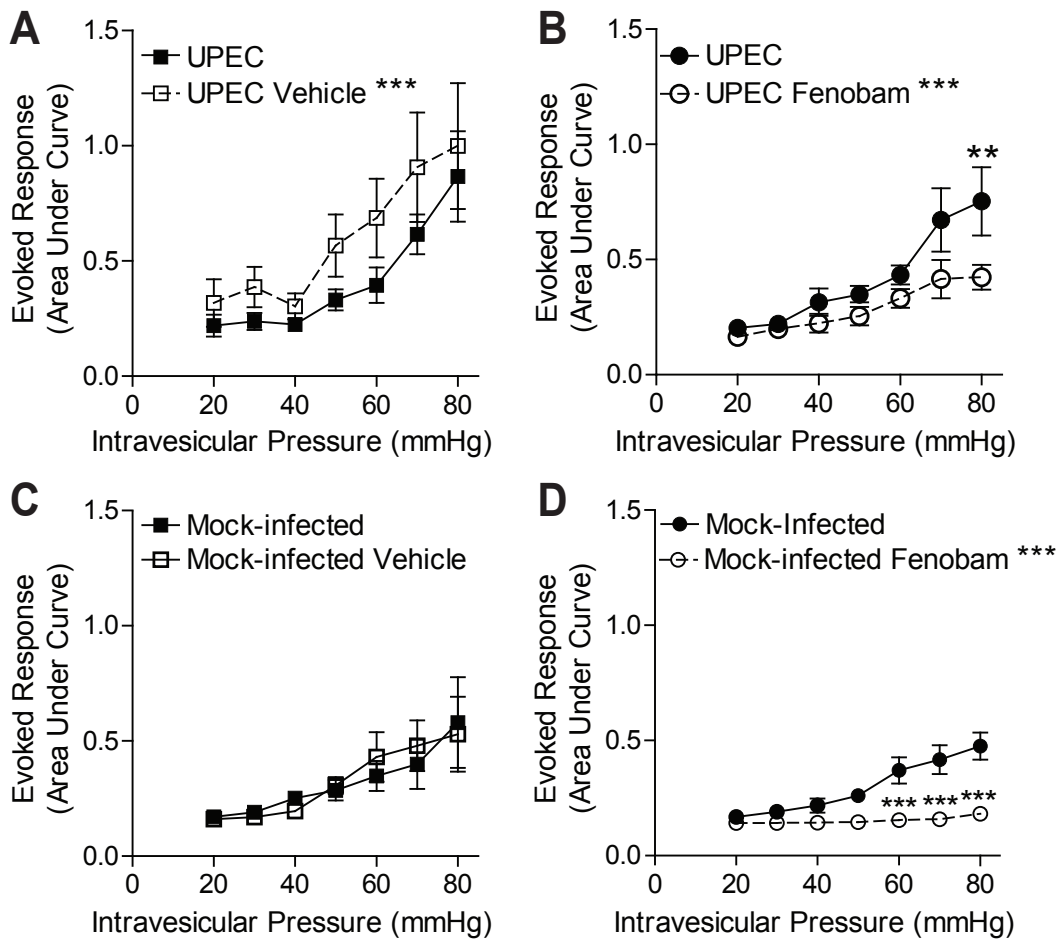
Figure 3.6: A. Treatment with an mGluR5 antagonist, fenobam, is analgesic in a UPEC infection-induced inflammatory bladder distention-evoked pain model (n=7). B. Treatment with the vehicle used to dissolve fenobam (100% DMSO) increases the evoked response to bladder distention (n=5). C. Fenobam is analgesic in mice given intravesicular PBS (control) 24 hr prior to VMR testing (n=6). D. Treatment with the vehicle used to dissolve fenobam (100% DMSO) had no effect on mice treated with intravesicular PBS (control, n=6), +/- SEM,* $p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Paired 2-way ANOVA with Bonferroni post-hoc test.

DISCUSSION

Clinically, visceral pain is often treated as a variant of somatic pain under the assumption that one neurological mechanism may be responsible for both visceral and somatic pain (Cervero and Laird, 1999). However, conventional treatments for somatic pain are often ineffective in treating visceral pain, indicating significant differences between visceral and somatic pain (Cervero and Laird, 1999; Robinson and Gebhart, 2008). This is likely due, in part, to intrinsic differences between visceral nociceptors and non-visceral nociceptors (Reviewed in: (Gebhart, 2000, 2004; Robinson and Gebhart, 2008)). Our results support this difference as we found that blocking mGluR5 activation resulted in a decreased VMR in the absence of injury. Whereas previous work has demonstrated that blocking mGluR5 activation has no effect on somatic pain in the absence of injury (Montana et al.; Kolber et al.).

Although the role of mGluR5 in inflammatory bladder pain is unclear, a few reports have shown that mGluR5 antagonism can lead to analgesia in visceral pain models (acetic acid writhing and colonic distention) (Walker et al., 2001b; Walker et al., 2001a; Fisher et al., 2002; Hudson et al., 2002; Varner, 2002; Zhu et al., 2004; Lindstrom et al., 2008; Hu et al., 2009). A recent publication reported that an mGluR5 antagonist, MPEP, effectively reduced the nociceptive VMR to noxious distention of non-inflamed bladders in rats (Hu et al., 2009). However, MPEP, has since been shown to have off-target effects, as it retains analgesic efficacy in mGluR5 KO mice (Montana et al., 2009). Thus, the precise role of mGluR5 in bladder distention-induced nociception remains unclear. Further, it is unknown whether mGluR5 activation has a role in the more clinically relevant condition of inflammatory bladder pain. In the present study, we demonstrate that mGluR5 is necessary for the full expression of distention-induced bladder nociception in naïve as well as UPEC-infected mice.

Here, we demonstrate the importance of mGluR5 in non-inflammatory bladder nociception using both genetic and pharmacologic techniques. First, mice lacking mGluR5 have a significantly reduced response to noxious bladder distention when compared to their WT littermates. Despite

this dramatic effect, mGluR5 KO mice have no baseline motor deficiencies (Lu et al., 1997). However, genetic deletion of mGluR5 could result in unpredictable upregulation or compensation of other genes involved in bladder function. Acute systemic treatment with a selective mGluR5 antagonist (fenobam) mimics the blunted VMR observed in the mGluR5 KO mice, suggesting that the absence of mGluR5 activity accounts for the differences in VMR as well as the cystometric profile observed in mGluR5 KO mice. Together these data suggest that mGluR5 is necessary for the full expression of distention-induced bladder pain in naïve mice and highlight the therapeutic potential for mGluR5 antagonists in the treatment of bladder pain.

Surprisingly, both fenobam and vehicle (DMSO) induced a small but statistically significant reduction in the distention induced VMR in mGluR5 KO mice. Fenobam at the dose used in this study was previously demonstrated to be specific to mGluR5 *in vivo* (Montana et al., 2009), and we do not believe that these results challenge that finding because fenobam but not DMSO, had an effect on the VMR of WT mice. Because both fenobam and vehicle significantly reduced the VMR in mGluR5 KO mice, we conclude that the vehicle is having effects in the KO that are not observed in WT mice. A possible explanation for the effects of the DMSO in KO but not in WT mice is that mGluR5 KO mice are significantly smaller than their WT littermates (Bradbury et al., 2005). DMSO has been shown to block peripheral C-fiber nerve conduction (Evans et al., 1993). It is possible that the volume (20µl) delivered in relation to the weight of the mGluR5 KO mice leads to such a conduction block.

The dramatic phenotype of mGluR5 KO mice as well as the significantly reduced pain response in fenobam-treated WT mice suggests that mGluR5 regulates bladder nociception. It is also possible that mGluR5 could regulate normal bladder function such as micturition. To examine the role of mGluR5 in normal bladder function, we examined the cystometric profiles of mGluR5 KO mice compared to their WT littermates. Genetic disruption of mGluR5 significantly increased the IMI, but had no effect on the bladder contraction amplitude (pressure at which voiding occurred). An increased IMI suggests that mice lacking mGluR5 are able to urinate, but they tolerate higher

bladder volumes (*i.e.* higher bladder distension) before voiding. Our results are consistent with previous work demonstrating a role for mGluR5 in the micturition reflex (Guarneri et al., 2008; Hu et al., 2009; Larson et al., 2011). Importantly, mGluR5 does not appear to be involved in bladder development, as bladders of mGluR5 KO mice were indistinguishable from their WT littermates upon gross inspection. These results suggest that mGluR5 has a role in bladder sensation in naïve mice.

Bladder infections due to UPEC are the most common reason that women see a doctor (Baerheim and Hunskar, 1997; Hooton and Stamm, 1997). This is the first evidence that infection with UPEC sensitizes mice to noxious bladder distention. WT mice infected with UPEC had a greater VMR to bladder distention when compared to mock-infected littermate controls. These data are consistent with the finding that mice with a UTI have increased referred abdominal pain (Rudick et al.). Furthermore, our results indicate that pharmacologic blockade of mGluR5 activation can reduce the VMR to bladder distention in UPEC-infected mice. The reduction in the distention-evoked VMR after fenobam treatment in mice infected with UPEC may be even larger than is apparent in Figure 6B, as the VMR is actually increased in vehicle-treated mice (see Fig 3.6A). Because distention in combination with UPEC infection was more damaging to bladder histology than distention in mock infected mice, the increased VMR after vehicle is likely the result of injury induced by bladder infection in combination with repeated noxious bladder distention. Another, yet unlikely, explanation is that systemic DMSO might lead to bladder sensitization in mice with UPEC infection.

Models of somatic inflammatory pain have observed an increase in mGluR5 CNS expression (specifically the central nucleus of the amygdala and the spinal cord) following peripheral inflammation (Dolan et al.; Neugebauer et al.). However, it remains undetermined if visceral inflammation leads to increased expression of mGluR5.

CONCLUSIONS

Up to 85% of community-acquired UTIs are due to infection with UPEC (Foxman and Brown, 2003; Zhang and Foxman, 2003). Standard-of-care for a UTI is antibiotic treatment. However, in women with normal urological anatomy (uncomplicated UTI), UTIs are self-limiting (Foxman, 2010). Therefore, uncomplicated UTIs will resolve without antibiotic treatment. Despite this, the pain due to a UTI necessitates antibiotic treatment for symptomatic relief. Antibiotic use can lead to UTI recurrence by selecting for antibiotic resistant bacteria (Foxman, 2010). By treating the pain of a UTI rather than the infection, it may be possible to allow the UTI to take its natural course and become self-limited and reduce antibiotic-resistant reoccurrence. Based on our findings, we suggest that mGluR5 antagonists could be a useful for therapy for symptomology in UTI that could reduce antibiotic use.

Although less common, bladder pain in the form of IC/PBS is a serious and debilitating medical condition. Unfortunately for patients, no reliably effective treatment options exist. Previous work has suggested a role for mGluR5 in the CNS in distention-induced bladder pain (Hu et al.). However, the mGluR5 antagonist used in this study (MPEP) is analgesic in mGluR5 KO mice, suggesting off-target effects (Montana et al.). Here, using a combination of genetics and pharmacology, we demonstrate that mGluR5 has a crucial role in the modulation of distention induced bladder nociceptive responses. Because in these studies mGluR5 was blocked systemically and knocked out globally, the anatomical localization where mGluR5 modulates bladder pain is undefined. In addition, mGluR5 is expressed throughout the peripheral and central nervous system (Valerio et al.; Jia et al.; Karim et al.; Varney and Gereau; Li and Neugebauer; Ferraguti and Shigemoto; Kolber et al.). However, it should be noted that the mGluR5 antagonist, fenobam, readily and rapidly crosses the blood brain barrier (Montana et al.). If the chronic pain experienced due to IC/PBS is also mediated by mGluR5, then antagonists of mGluR5 such as fenobam may be useful in treating chronic bladder pain. Antagonists of mGluR5 may have clinical potential for the treatment of both acute and chronic bladder pain. Previous work using non-visceral pain models has demonstrated that mGluR5 activity modulates the activation of ERK1/2 (extracellular regulated kinase 1/2) in both the spinal cord (Karim et al.; Hu

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et al.) and/or central nucleus of the amygdala (CeA) (Kolber et al.; Li et al., 2011). Inhibition of

mGluR5 in the CeA is analgesic in multiple models of somatic inflammatory pain (Li and

Neugebauer; Kolber et al.). Furthermore, recent work from our lab has demonstrated that

intrathecal inhibition of ERK is analgesic in both an inflammatory and non-inflammatory bladder

pain model (Lai et al.). Future work can help determine the anatomical and molecular

mechanism by which mGluR5 modulates bladder nociceptive responses.

METHODS

1. Subjects and ethical approval

All animal experiments were performed in accordance with the guidelines of the Committee for

research and Ethical Issues of International Association for the Study of Pain (Zimmermann,

1983). The experimental protocol was approved by the Washington University Institutional Animal

Care and Use Committee (St. Louis, MO). All experiments were performed on female mice aged

10-13 weeks. All mice were humanely euthanized at the end of the experiments by decapitation

under deep isoflurane (5%) anesthesia. Female C57BL/6 mice used in UPEC experiments were

purchased from The Jackson Laboratory (Bar Harbor, ME). For experiments involving mice

lacking mGluR5 (mGluR5 KO), animals were bred in-house on a C57BL/6 background and

compared with wildtype (WT) littermates (Lu et al., 1997). Unless otherwise indicated, all mice

were group housed in cages of 3-5. All mice were kept on a 12:12-h light/dark schedule with ad

libitum access to food and water.

2. Drugs and other agents

Fenobam was purchased from Tocris Bioscience (Ellisville, MO), and is a specific negative

allosteric modulator of mGluR5 (Porter et al., 2005; Montana et al., 2009). The dosage (30mg/kg,

intraperitoneal) of fenobam was chosen because it was the lowest effective dose to induce

analgesia in two mouse models of inflammatory pain, and had no effect on mGluR5 KO mice

(Montana et al., 2009). Fenobam was dissolved in 100% dimethyl sulfoxide (DMSO, Sigma-

Aldrich, St. Louis, MO) on the day of the experiment. All intraperitoneal injections were 20μl.

Throughout all experiments and analysis, the investigator performing the VMR was blinded to

pharmacological treatment.

3. Phasic bladder distentions and VMR measurements

Visceral nociception was quantified using VMR, an electromyographic (EMG) recording of the

abdominal muscle response to bladder distention that has previously been validated as a

measure of nociception (Ness and Gebhart, 1991; Ness and Elhefni, 2004). An example of the

bladder-distention induced VMR of WT mice can be seen in Figure 1. Abdominal VMRs to urinary

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bladder distention were performed as described previously (Lai et al., 2011). The mice were anesthetized with 2% isoflurane, and chlorinated silver wire electrodes were placed on the superior oblique abdominal muscle. One was placed subcutaneously across the abdominal wall (as a ground) to allow differential amplification of the abdominal VMR signals. A lubricated 24G angiocatheter was inserted into the bladder via the urethra for bladder distention. After completion of the surgical preparation, isoflurane anesthesia was reduced to approximately 0.9% until a flexion reflex response was present (evoked by pinching the paw) but spontaneous escape behavior and righting reflex were absent. Once a stable depth of anesthesia was obtained, the level was not changed for the duration of the experiment. The animals were not restrained in any fashion. Body temperature was monitored throughout the experiment and maintained using an overhead radiant light. Phasic bladder distention with compressed air was then used to evoke a VMR. The air pressure was controlled by an automated distention control device custom made in the Washington University School of Medicine Electronics Shop. The distention stimulus applied 20 to 80 mm Hg pressure (10mmHg steps) for 20 seconds every 2 minutes. The VMR signal was relayed in real time using a Grass CP511 preamplifier (Grass Technologies, West Warwick, RI) to a PC via WinDaq DI-720 module (Dataq Instruments, Arkon, OH). These data were exported to Igor Pro 6.05 software (Wavemetrics, Portland, OR). Using a custom script, the VMR signals were subtracted from the baseline, rectified, and integrated over 20 seconds to quantify the area under the curve (see Figure 1). The VMR is presented in arbitrary units.

After baseline responses to distensions 20mmHg-80mmHG (10mmHg steps) were recorded, the mouse was allowed to recover for at least 30 min (level of anesthesia remained unchanged). Following baseline recordings, the mice were given a 20ul IP injection of either fenobam (30mg/kg, dissolved in 100% DMSO) or 100% DMSO (vehicle). A second set of bladder distentions (20mmHg-80mmHg, 10mmHg steps) was started 5 minutes following administration of fenobam or vehicle and completed within 45 minutes. The investigator who tested, processed and quantified the VMR was blinded to the drug treatment and genotype.

4. *Infection with UPEC*

Infection with a clinical UPEC isolate, UTI89, was performed as previously described (Hung et al., 2009). Briefly, mice were anesthetized with isoflurane and inoculated via transurethral catheterization with 50 µl of 2-8x10^7 colony-forming units/mL of bacteria in phosphate buffered saline (PBS). Control mice (mock-infection) received 50ul of intravesicular PBS. Following intravesicular instillation of either PBS or UPEC, the mice were individually housed. Urine was collected at 6 and 24 hours, and infection was confirmed. At 24 hours post infection, the VMR response to bladder distention was recorded as previously described (Lai et al., 2011). Following the VMR, isoflurane anesthesia was increased and the mice were euthanized by cervical dislocation. Bladder tissue was quickly removed and fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) (Mysorekar and Hultgren, 2006). The bladder tissue was then embedded in paraffin, sectioned and stained with hemotoxylin and eosin. Photomicrographs were taken using a Hamamatsu NanoZoomer HT (Hamamatsu Corporation).

5. Urodynamics

Urodynamic recordings were performed as described previously (Lai et al., 2004). Briefly, mice were anesthetized with subcutaneous urethane (1.2g/kg). A midline laparotomy incision was made, and the dome of the urinary bladder was exposed. The bladder dome was punctured with a 25-gauge needle. Intravesicular pressure was monitored continuously *in vivo* while the bladder was filled with room temperature saline at a rate of 0.04 mL per minute using a syringe pump (KD Scientific, New Hope, PA). Voiding was allowed to occur spontaneously via the urethra. The intravesicular pressure was recorded in real time using WINDAQ data acquisition program (DataQ Instruments, Akron, OH) at a sampling rate of 20 Hz. The intermicturition interval (IMI) was calculated as the average time (seconds) between peaks. The amplitude of contraction was the value (cmHg) of the peak.

LIST OF ABBREVIATIONS

DMSO**,** dimethyl sulfoxide; IC/PBS, Interstitial cystitis/painful bladder syndrome; IMI,

intermicturition interval; IP, intraperitoneal; KO, knockout; mGluRs, metabotropic glutamate

receptors; mGluR5, metabotropic glutamate receptor 5; MPEP, 2-methyl-6-(phenylethynyl)-

pyridine; PBS, phosphate-buffered saline; UPEC, uropathogenic *Escherichia Coli*; UTI, urinary

tract infection; VMR, visceromotor response; WT, wildtype

REFERENCES

Baerheim A, Hunskar S (1997) [Current management of uncomplicated acute cystitis in general
        practice]. Tidsskr Nor Laegeforen 117:1304-1307.

Berry SH, Elliott MN, Suttorp M, Bogart LM, Stoto MA, Eggers P, Nyberg L, Clemens JQ (2011)
        Prevalence of Symptoms of Bladder Pain Syndrome/Interstitial Cystitis Among Adult
        Females in the United States. The Journal of urology 186:540-544.

Bhave G, Karim F, Carlton SM, Gereau RW (2001) Peripheral group I metabotropic glutamate
        receptors modulate nociception in mice. Nature neuroscience 4:417-423.

Bianchi R, Rezzani R, Borsani E, Rodella L (2003) mGlu5 receptor antagonist decreases Fos
        expression in spinal neurons after noxious visceral stimulation. Brain research 960:263-
        266.

Birder LA (2010) Urothelial signaling. Auton Neurosci 153:33-40.

Boudes M, Uvin P, Kerselaers S, Vennekens R, Voets T, De Ridder D (2011) Functional
        characterization of a chronic cyclophosphamide-induced overactive bladder model in
        mice. Neurourol Urodyn 30:1659-1665.

Bower JM, Eto DS, Mulvey MA (2005) Covert operations of uropathogenic Escherichia coli within
        the urinary tract. Traffic 6:18-31.

Bradbury MJ, Campbell U, Giracello D, Chapman D, King C, Tehrani L, Cosford ND, Anderson J,
        Varney MA, Strack AM (2005) Metabotropic glutamate receptor mGlu5 is a mediator of

appetite and energy balance in rats and mice. The Journal of pharmacology and experimental therapeutics 313:395-402.

Castroman P, Ness TJ (2001) Vigor of visceromotor responses to urinary bladder distension in rats increases with repeated trials and stimulus intensity. Neuroscience letters 306:97-100.

Cervero F, Laird JM (1999) Visceral pain. Lancet 353:2145-2148.

Dang K, Lamb K, Cohen M, Bielefeldt K, Gebhart GF (2008) Cyclophosphamide-induced bladder inflammation sensitizes and enhances P2X receptor function in rat bladder sensory neurons. Journal of neurophysiology 99:49-59.

Dolan S, Kelly JG, Monteiro AM, Nolan AM (2003) Up-regulation of metabotropic glutamate receptor subtypes 3 and 5 in spinal cord in a clinical model of persistent inflammation and hyperalgesia. Pain 106:501-512.

Evans MS, Reid KH, Sharp JB, Jr. (1993) Dimethylsulfoxide (DMSO) blocks conduction in peripheral nerve C fibers: a possible mechanism of analgesia. Neuroscience letters 150:145-148.

Ferraguti F, Shigemoto R (2006) Metabotropic glutamate receptors. Cell and tissue research 326:483-504.

Fisher K, Lefebvre C, Coderre TJ (2002) Antinociceptive effects following intrathecal pretreatment with selective metabotropic glutamate receptor compounds in a rat model of neuropathic pain. Pharmacology, biochemistry, and behavior 73:411-418.

Foxman B (2002) Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Am J Med 113 Suppl 1A:5S-13S.

Foxman B (2010) The epidemiology of urinary tract infection. Nat Rev Urol 7:653-660.

Foxman B, Brown P (2003) Epidemiology of urinary tract infections: transmission and risk factors, incidence, and costs. Infect Dis Clin North Am 17:227-241.

Gebhart GF (2000) Pathobiology of visceral pain: molecular mechanisms and therapeutic implications IV. Visceral afferent contributions to the pathobiology of visceral pain. Am J Physiol Gastrointest Liver Physiol 278:G834-838.

Gebhart GF (2004) Descending modulation of pain. Neuroscience and biobehavioral reviews 27:729-737.

Guarneri L, Poggesi E, Angelico P, Farina P, Leonardi A, Clarke DE, Testa R (2008) Effect of selective antagonists of group I metabotropic glutamate receptors on the micturition reflex in rats. BJU Int 102:890-898.

Hooton TM, Stamm WE (1997) Diagnosis and treatment of uncomplicated urinary tract infection. Infect Dis Clin North Am 11:551-581.

Hopkins WJ, Gendron-Fitzpatrick A, Balish E, Uehling DT (1998) Time course and host responses to Escherichia coli urinary tract infection in genetically distinct mouse strains. Infect Immun 66:2798-2802.

Hu HJ, Alter BJ, Carrasquillo Y, Qiu CS, Gereau RW (2007) Metabotropic glutamate receptor 5 modulates nociceptive plasticity via extracellular signal-regulated kinase-Kv4.2 signaling in spinal cord dorsal horn neurons. J Neurosci 27:13181-13191.

Hu Y, Dong L, Sun B, Guillon MA, Burbach LR, Nunn PA, Liu X, Vilenski O, Ford AP, Zhong Y, Rong W (2009) The role of metabotropic glutamate receptor mGlu5 in control of micturition and bladder nociception. Neuroscience letters 450:12-17.

Hudson LJ, Bevan S, McNair K, Gentry C, Fox A, Kuhn R, Winter J (2002) Metabotropic glutamate receptor 5 upregulation in A-fibers after spinal nerve injury: 2-methyl-6-(phenylethynyl)-pyridine (MPEP) reverses the induced thermal hyperalgesia. J Neurosci 22:2660-2668.

Hung CS, Dodson KW, Hultgren SJ (2009) A murine model of urinary tract infection. Nat Protoc 4:1230-1243.

Jia H, Rustioni A, Valtschanoff JG (1999) Metabotropic glutamate receptors in superficial laminae of the rat dorsal horn. The Journal of comparative neurology 410:627-642.

Kakizaki H, Yoshiyama M, Roppolo JR, Booth AM, De Groat WC (1998) Role of spinal glutamatergic transmission in the ascending limb of the micturition reflex pathway in the rat. The Journal of pharmacology and experimental therapeutics 285:22-27.

Karim F, Bhave G, Gereau RW (2001a) Metabotropic glutamate receptors on peripheral sensory neuron terminals as targets for the development of novel analgesics. Molecular psychiatry 6:615-617.

Karim F, Wang CC, Gereau RW (2001b) Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. J Neurosci 21:3771-3779.

Kolber BJ, Montana MC, Carrasquillo Y, Xu J, Heinemann SF, Muglia LJ, Gereau RW (2010) Activation of metabotropic glutamate receptor 5 in the amygdala modulates pain-like behavior. J Neurosci 30:8203-8213.

Lai HH, Qiu CS, Crock LW, Morales ME, Ness TJ, Gereau RW (2011) Activation of spinal extracellular signal-regulated kinases (ERK) 1/2 is associated with the development of visceral hyperalgesia of the bladder. Pain 152:2117-2124.

Lai HH, Boone TB, Yang G, Smith CP, Kiss S, Thompson TC, Somogyi GT (2004) Loss of caveolin-1 expression is associated with disruption of muscarinic cholinergic activities in the urinary bladder. Neurochem Int 45:1185-1193.

Larson JA, Ogagan PD, Chen G, Shen B, Wang J, Roppolo JR, de Groat WC, Tai C (2011) Involvement of Metabotropic Glutamate Receptor 5 in Pudendal Inhibition of Nociceptive Bladder Activity in Cats. J Physiol.

Li W, Neugebauer V (2004) Differential roles of mGluR1 and mGluR5 in brief and prolonged nociceptive processing in central amygdala neurons. Journal of neurophysiology 91:13-24.

Li Z, Ji G, Neugebauer V (2011) Mitochondrial reactive oxygen species are activated by mGluR5 through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and pain behavior. J Neurosci 31:1114-1127.

Lindstrom E, Brusberg M, Hughes PA, Martin CM, Brierley SM, Phillis BD, Martinsson R, Abrahamsson C, Larsson H, Martinez V, Blackshaw LA (2008) Involvement of metabotropic glutamate 5 receptor in visceral pain. Pain 137:295-305.

Lu YM, Jia Z, Janus C, Henderson JT, Gerlai R, Wojtowicz JM, Roder JC (1997) Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. J Neurosci 17:5196-5205.

Maggi CA, Lecci A, Santicioli P, Del Bianco E, Giuliani S (1993) Cyclophosphamide-induced cystitis in rats: involvement of capsaicin-sensitive primary afferents. Agents Actions 38 Spec No:C28-30.

Malterud K, Baerheim A (1999) Peeing barbed wire. Symptom experiences in women with lower urinary tract infection. Scand J Prim Health Care 17:49-53.

Matsumoto G, Hisamitsu T, de Groat WC (1995) Role of glutamate and NMDA receptors in the descending limb of the spinobulbospinal micturition reflex pathway of the rat. Neuroscience letters 183:58-61.

Montana MC, Cavallone LF, Stubbert KK, Stefanescu AD, Kharasch ED, Gereau RW (2009) The metabotropic glutamate receptor subtype 5 antagonist fenobam is analgesic and has improved in vivo selectivity compared with the prototypical antagonist 2-methyl-6-(phenylethynyl)-pyridine. The Journal of pharmacology and experimental therapeutics 330:834-843.

Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, Hultgren SJ (1998) Induction and evasion of host defenses by type 1-piliated uropathogenic Escherichia coli. Science (New York, NY 282:1494-1497.

Mysorekar IU, Hultgren SJ (2006) Mechanisms of uropathogenic Escherichia coli persistence and eradication from the urinary tract. Proceedings of the National Academy of Sciences of the United States of America 103:14170-14175.

Ness TJ, Gebhart GF (1991) Interactions between visceral and cutaneous nociception in the rat. I. Noxious cutaneous stimuli inhibit visceral nociceptive neurons and reflexes. Journal of neurophysiology 66:20-28.

Ness TJ, Elhefni H (2004) Reliable visceromotor responses are evoked by noxious bladder distention in mice. The Journal of urology 171:1704-1708.

Ness TJ, Lewis-Sides A, Castroman P (2001) Characterization of pressor and visceromotor reflex responses to bladder distention in rats: sources of variability and effect of analgesics. The Journal of urology 165:968-974.

Ness TJ, Richter HE, Varner RE, Fillingim RB (1998) A psychophysical study of discomfort produced by repeated filling of the urinary bladder. Pain 76:61-69.

Neugebauer V, Li W, Bird GC, Bhave G, Gereau RW (2003) Synaptic plasticity in the amygdala in a model of arthritic pain: differential roles of metabotropic glutamate receptors 1 and 5. J Neurosci 23:52-63.

Page AJ, Young RL, Martin CM, Umaerus M, O'Donnell TA, Cooper NJ, Coldwell JR, Hulander M, Mattsson JP, Lehmann A, Blackshaw LA (2005) Metabotropic glutamate receptors inhibit mechanosensitivity in vagal sensory neurons. Gastroenterology 128:402-410.

Porter RH, Jaeschke G, Spooren W, Ballard TM, Buttelmann B, Kolczewski S, Peters JU, Prinssen E, Wichmann J, Vieira E, Muhlemann A, Gatti S, Mutel V, Malherbe P (2005) Fenobam: a clinically validated nonbenzodiazepine anxiolytic is a potent, selective, and noncompetitive mGlu5 receptor antagonist with inverse agonist activity. The Journal of pharmacology and experimental therapeutics 315:711-721.

Robinson DR, Gebhart GF (2008) Inside information: the unique features of visceral sensation. Mol Interv 8:242-253.

Rosenberg MT, Page S, Hazzard MA (2007) Prevalence of interstitial cystitis in a primary care setting. Urology 69:48-52.

Rudick CN, Billips BK, Pavlov VI, Yaggie RE, Schaeffer AJ, Klumpp DJ (2010) Host-pathogen interactions mediating pain of urinary tract infection. J Infect Dis 201:1240-1249.

Saban MR, Saban R, Hammond TG, Haak-Frendscho M, Steinberg H, Tengowski MW, Bjorling DE (2002) LPS-sensory peptide communication in experimental cystitis. Am J Physiol Renal Physiol 282:F202-210.

Valerio A, Rizzonelli P, Paterlini M, Moretto G, Knopfel T, Kuhn R, Memo M, Spano P (1997) mGluR5 metabotropic glutamate receptor distribution in rat and human spinal cord: a developmental study. Neurosci Res 28:49-57.

Varner AE (2002) Beneficial effect of nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors in patients with asthma during viral infection. J Infect Dis 186:723; author reply 723-724.

Varney MA, Gereau RW (2002) Metabotropic glutamate receptor involvement in models of acute and persistent pain: prospects for the development of novel analgesics. Curr Drug Targets CNS Neurol Disord 1:283-296.

Walker K, Reeve A, Bowes M, Winter J, Wotherspoon G, Davis A, Schmid P, Gasparini F, Kuhn R, Urban L (2001a) mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurones mediate inflammatory hyperalgesia. Neuropharmacology 40:10-19.

Walker K, Bowes M, Panesar M, Davis A, Gentry C, Kesingland A, Gasparini F, Spooren W, Stoehr N, Pagano A, Flor PJ, Vranesic I, Lingenhoehl K, Johnson EC, Varney M, Urban L, Kuhn R (2001b) Metabotropic glutamate receptor subtype 5 (mGlu5) and nociceptive function. I. Selective blockade of mGlu5 receptors in models of acute, persistent and chronic pain. Neuropharmacology 40:1-9.

Warren JW, Brown J, Tracy JK, Langenberg P, Wesselmann U, Greenberg P (2008) Evidence-based criteria for pain of interstitial cystitis/painful bladder syndrome in women. Urology 71:444-448.

Westropp JL, Buffington CA (2002) In vivo models of interstitial cystitis. The Journal of urology 167:694-702.

Yoshiyama M, de Groat WC (2005) Supraspinal and spinal alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid and N-methyl-D-aspartate glutamatergic control of the micturition reflex in the urethane-anesthetized rat. Neuroscience 132:1017-1026.

Young RL, Page AJ, O'Donnell TA, Cooper NJ, Blackshaw LA (2007) Peripheral versus central modulation of gastric vagal pathways by metabotropic glutamate receptor 5. Am J Physiol Gastrointest Liver Physiol 292:G501-511.

Zhang L, Foxman B (2003) Molecular epidemiology of Escherichia coli mediated urinary tract infections. Front Biosci 8:e235-244.

Zhu CZ, Wilson SG, Mikusa JP, Wismer CT, Gauvin DM, Lynch JJ, 3rd, Wade CL, Decker MW, Honore P (2004) Assessing the role of metabotropic glutamate receptor 5 in multiple nociceptive modalities. European journal of pharmacology 506:107-118.

Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16:109-110.

Chapter 4

<u>Amygdala mGluR5 in the modulation of visceral pain</u>

This chapter contains the manuscript:

Crock LW, Kolber BK, Morgan CD, Sadler KE, Vogt SK, Bruchas MR, and Gereau RW (2012)

Central amygdala metabotropic glutamate receptor 5 in the modulation of visceral pain. J

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ABSTRACT

Painful bladder syndrome is a debilitating condition that affects 3-6% of women in the United States. Multiple lines of evidence suggest that changes in central nervous system processing are key to the development of chronic bladder pain conditions, but little is known regarding the underlying cellular, molecular, and neuronal mechanisms. Using a mouse model of distension-induced bladder pain, we found that the central nucleus of the amygdala (CeA) is a critical site of neuromodulation for processing of bladder nociception. Furthermore, we demonstrate that metabotropic glutamate receptor 5 (mGluR5) activation in the CeA induces bladder pain sensitization by increasing CeA output. Thus, pharmacological activation of mGluR5 in the CeA is sufficient to increase the response to bladder distension. Additionally, pharmacological blockade or virally-mediated conditional deletion of mGluR5 in the CeA reduced responses to bladder distention suggesting that mGluR5 in the CeA is also necessary for these responses. Finally, we used optogenetic activation of the CeA and demonstrated that this caused a robust increase in the visceral pain response. The CeA localized effects on responses to bladder distention are associated with changes in extracellular signal regulated kinases 1/2 phosphorylation in the spinal cord. Overall, these data demonstrate that mGluR5 activation leads to increased CeA output that drives bladder pain sensitization.

INTRODUCTION

Visceral pain is the most common reason that patients seek medical attention and the most common form of pain produced by disease (Cervero and Laird, 1999). Visceral pain associated with interstitial cystitis or painful bladder syndrome (PBS/IC) affects 3-8 million women in the US (Berry et al., 2011), yet PBS/IC is poorly understood and treated (Dimitrakov et al., 2007). Up to 91% of these patients carry a diagnosis of another chronic disorder such as chronic fatigue, migraine, fibromyalgia, anxiety, and/or depression (Warren et al., 2009). Factors such as stress and depression (Macaulay et al., 1987; Baldoni et al., 1995) increase PBS/IC pain, and chronic pain is associated with increases in both stress and depression. Activity in the amygdala, a primary limbic structure, is positively correlated with stress, anxiety, and pain behavior (Neugebauer et al., 2004; Carrasquillo and Gereau, 2007; Ikeda et al., 2007; Ji et al., 2007; Neugebauer, 2007). The fact that emotion and stress modulate visceral pain and that the amygdala processes stress and nociceptive signals suggests that the amygdala is involved in the pathogenesis of chronic visceral pain.

The central nucleus of the amygdala (CeA) receives both indirect and direct nociceptive information (Bernard and Besson, 1990; Burstein and Potrebic, 1993; Bernard et al., 1996; Bourgeais et al., 2001). Noxious colorectal distension increases c-fos expression in the CeA (Traub et al., 1996) and the excitability of CeA neurons increases after induction of colitis in rats (Han and Neugebauer, 2004). Afferent outputs from the CeA to the hypothalamus and brainstem regions such as the periaqueductal gray (PAG) also make the amygdala well positioned to modulate responses to painful stimuli. Activation of the CeA with chronic corticosterone implants increases visceromotor responses to distension in rats (Greenwood-Van Meerveld et al., 2001; Myers and Greenwood-Van Meerveld, 2010). However, it is undetermined if acute changes in excitability of CeA neurons modulate the response to noxious bladder stimulation.

The excitability of neurons in the CeA during visceral stimulation is modulated in part by metabotropic glutamate receptor 5 (mGluR5) (Li and Neugebauer, 2004). mGluR5 activation of extracellular signal regulated kinases 1/2 (ERK1/2) has been hypothesized to play a role in the

modulation of pain responses (Ji, 2004; Kolber et al., 2010). Pharmacological activation of mGluR5 in the CeA increases rectal distension-induced neuronal responses (Ji and Neugebauer, 2010) and behavioral vocalizations (Li et al., 2011). However, it is unknown whether mGluR5 in the CeA plays a key role in bladder pain.

Here, we used right amygdala-specific pharmacological activation and inhibition of mGluR5 as well as conditional deletion of mGluR5 to determine the role of CeA-specific mGluR5 signaling in bladder pain. Next, we used optogenetic approaches to stimulate CeA neurons to determine if increased activation of the CeA neurons increases the visceromotor response to noxious bladder distention. Overall, we demonstrate that either mGluR5 activation in the CeA or optogenetic activation of the CeA is sufficient to sensitize responses to painful bladder distention; we have identified a novel role for mGluR5 in the ongoing control of acute visceral pain.

MATERIALS AND METHODS

Animals.

All mouse protocols were in accordance with National Institutes of Health guidelines and were approved by the Animal Care and Use Committee of Washington University (St. Louis, MO) and Duquesne University (Pittsburgh, PA). Female mice (all C57Bl/6J background) age 10-13 weeks old were housed on a 12/12 h light/dark schedule with *ad libitum* access to rodent chow and water. Unless otherwise noted, wildtype (WT) mice were used for all experiments.

Visceromotor response (VMR) to urinary bladder distention.

The VMR is a spinobulbospinal reflex to bladder distention that has been validated as a measure of pain, as the response is suppressed by analgesics and potentiated by bladder inflammation. The VMR is observed in decerebrate rodents and not in rodents with a transected spinal cord (Castroman and Ness, 2001; Ness et al., 2001; Ness and Elhefni, 2004) but can also be modulated by higher brain centers (Qin et al., 2003). Bladder distention reliably produces pain and/or discomfort in humans (Ness et al., 1998) and is frequently used in rodents as a model of visceral pain. Procedures for bladder distention and visceromotor response (VMR) recording were similar to those previously described (Lai et al., 2011) (see Fig. 4.1*A* for illustration of setup). Experimenter was blinded to treatment and/or genotype in all experiments. Briefly, on the day of VMR recording, the mice were anesthetized with 2% isoflurane. Chlorinated silver wire electrodes were placed on the external oblique abdominal muscle and subcutaneously across the abdominal wall (as a ground) to allow differential amplification of the abdominal VMR signals. A lubricated angiocatheter was inserted into the bladder via the urethra for bladder distention. After surgical preparation, isoflurane was reduced to approximately 1.0% until a flexion reflex response was present (evoked by pinching the paw) but spontaneous escape behavior and righting reflex were absent. The use of low levels of isoflurane during UBD has been validated previously (Ness and Elhefni, 2004). After the desired level of anesthesia was achieved, the level of isoflurane was not changed for the duration of the experiment. The animals were not restrained in any

fashion and body temperature was maintained using an overhead radiant light and monitored throughout the experiment.   Phasic bladder distention with compressed air was then used to evoke bladder nociception. The air pressure was controlled by an automated distention control device custom made in the Washington University School of Medicine Electronic Shop (St. Louis, MO). The distention stimulus applied was 20 to 80 mmHg pressure for 20s with 2min inter-trial interval (ITI). The precise pressure gradient used varied depending on the specific experiment (see Results).   The VMR signal was relayed in real time using a Grass CP511 preamplifier (Grass Technologies, West Warwic, RI) to a PC via a WinDaq DI-720 module (Dataq Instruments, Akron, OH). Data were exported to Igor Pro 6.05 software (Wavemetrics, Portland, OR). Using a custom script, the VMR signals were subtracted from the baseline, rectified, and integrated over 20s to quantify the evoked response.

Surgical procedure for cannula implantation.

WT mice were cannulated as previously described (Kolber et al., 2010). Briefly, mice were deeply anesthetized with a combination ketamine/xylazine anesthetic and mounted in a stereotaxic frame. An 8mm stainless steel guide cannula was implanted above the right CeA (coordinates: 1.25mm anterior to Bregma; 2.70mm lateral to midline; 4.2mm ventral to skull). The guide cannula was affixed to the skull with two bone screws and dental cement. An 8mm stylet was inserted in the guide cannula to prevent clogging. Mice recovered for 3d (for HSV optogenetic experiments) or 6d (for pharmacological experiments) before further testing. At the end of experiments, brains were sectioned to verify cannula position and injection site.   Briefly, for all experiments, the cannula end was verified by histological or visual analysis (under 4x magnification). This included first using a mouse stereotaxic atlas (Paxinos and Franklin, 2001) and the hippocampus as a rostral-caudal guide to identify the brain section corresponding to the tip of the cannula. Next, a determination of correct CeA targeting was done by using the internal branch of the external capsule and the optic tract as markers for the lateral and medial aspects of the CeA. The center of the cannula target was verified by looking at serial sections across the extent of the cannulated area. For Western experiments in which only 1mm sections were

analyzed, the section was cut directly over the cannula tract and punches were made on either side of the cannula tip after visual verification of CeA targeting. For experiments described in this manuscript, we have included insets in figures that show cannula tip placements.   In all experiments, the right amygdala only was targeted for manipulation because previous results from both mice (Carrasquillo and Gereau, 2008; Kolber et al., 2010) and rats (Ji and Neugebauer, 2009; Li et al., 2011) have shown a specific lateralization of the right amygdala in somatic and visceral pain.

Intra-amygdala drug infusion

Mice were cannulated targeting the right amygdala as described above. 6d after cannulation surgery, drugs were infused. Microinjections were performed as previously described (Carrasquillo and Gereau, 2007). Briefly, injections were performed using a 32-gauge injection cannula that extended 0.5mm beyond the tip of the guide cannula. The injection cannula was attached to flexible plastic tubing and a microliter syringe (Hamilton, Reno, NV) was used to deliver drug or vehicle. A total volume of 0.3mL was infused over a period of 3min and the injection cannula was kept in place for an additional 1min to allow for drug diffusion.

Drugs Injected. (RS)-3,5-Dihydroxyphenylglycine (DHPG) (Tocris, Bristol, UK) is an agonist that activates both mGluR5 and mGluR1. On the day of the experiment, DHPG was dissolved in artificial cerebrospinal fluid (aCSF; 25mM NaHCO3, 122mM NaCl, 1.3mM CaCl2, 1.2mM MgSO4, 3mM KCl, and 0.4mM KH2PO4, pH 7.35) as a 5mM stock solution, which was diluted in aCSF to 0.1nmol (final injected volume 0.3ml). Mice were treated with DHPG 30min following baseline distention and then distended again 30min after treatment. 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP, Tocris) is a selective non-competitive antagonist of mGluR5. On the day of the experiment, MPEP was dissolved in aCSF a stock 5mM solution, which was diluted in aCSF to a final concentration of 0.01 nmol (final injected volume 0.3ml). For bladder distention experiments with baseline distention, mice were treated with MPEP (or vehicle) immediately following baseline distention and then distended again 60min after treatment. For bladder distention experiments with no baseline distention, mice were treated with MPEP (or

vehicle) 60min prior to bladder distention at 80 mmHg. Dosing and timing for DHPG and MPEP

was derived from our previous studies examining amygdala-induced somatic hypersensitivity

(Kolber et al., 2010).

Preparation of CeA specific mGluR5 knockout mice (mGluR5^{CeAKO}).

Mice homozygous for the mGluR5loxP allele (Xu et al., 2009) or WT littermates were used. All

animals were injected in the right amygdala with virus containing recombinant lentiviral vector LV-

Ef1α-Cre (LV-Cre) as previously described (Kolber et al., 2008; Kolber et al., 2010). Briefly, mice

were anesthetized with a combination ketamine/xylazine anesthetic and mounted in a stereotaxic

frame. A small hole was drilled over the right CeA (coordinates: 1.25mm anterior to Bregma;

2.7mm lateral to midline; 4.7mm ventral to skull). A 32-Gauge needle was lowered into the hole

and 4×10^5 infectious viral particles were injected over 5min. The injection needle was kept in

place after injection for 4min to allow for stable diffusion. All testing in these mice occurred 2

weeks after recovery from surgery. After testing, brains were sectioned to determine needle

targeting and 1mm diameter x 1mm high punches were made of the amygdala in two adjacent

sections to determine amount of mGluR5 protein disrupted in mGluR5^{CeAKO} mice compared to WT

mice injected with LV-Cre. In addition, tissue from the lumbosacral spinal cord was harvested for

analysis of ERK phosphorylation. See below for a description of processing of tissue samples.

Optogenetic vectors, infusion, and stimulation parameters.

   Vectors. Herpes simplex virus (HSV) vectors were obtained from McGovern Institute

(MIT, Boston, MA). Briefly, HSV vectors driven by the CMV promoter (channelrhodopsin

hChR2(H134R)-EYFP or EYFP only) were prepared as previously described (Neve et al., 2005;

Covington et al., 2010).   All experiments were performed on day 4 after postinfection because

maximal expression of HSV vectors occurs on days 3-4 (Covington et al., 2010).

   Vector infusion.   Mice were cannulated in the right amygdala as described above. 3d

after cannulation surgery, HSV vectors were infused. Microinjections were performed similarly to

that described above for drugs. A total volume of 1mL was infused over a period of 5min and the injection cannula was kept in place for an additional 1min to allow for viral infusion.

Optogenetic CeA stimulation during bladder distention. On the day of the experiment, an 8.00mm fiber optic fiber (0.2 mm diameter; Thor Labs, Newton, New Jersey) was placed in the animal's cannula. Optic stimulation was performed similar to previously published protocols (Covington et al., 2010; Zhang et al., 2010). Briefly, the fiber optic was connected via a FC/PC adaptor to a 473nm blue laser diode (OEM East Lansing, MI) connected to a power supply. Laser pulses were controlled using a Techtronix pulse generator. During all stimulations, a 5ms 20Hz (18ms spike width) pulse train was delivered for 30min. The stimulation protocol was modeled to mimic the firing pattern of CeA neurons that occurs after painful injury in rodents (Ji and Neugebauer, 2009). The intensity of the fiber optic light was verified before and after each experiment use using a luxometer (Thor Labs). For the distention experiment, we applied a bladder stimulus of 30 mmHg (20s x3 with 2min ITI) then 60 mmHg (20s x3 with 2min ITI) during baseline testing, 20 min after the laser was turned on, and 15 min after the laser was turned off. For Western blot and immunohistochemistry experiments, mice were stimulated with the laser as above for 30min and then killed 15min after laser off with no distention. Mice were killed after the experiment and analyzed by immunohistochemistry and western blotting for targeting, ERK phosphorylation, and/or eYFP expression (see below).

Western Blotting.

Protein expression in mGluR5^{CeAKO} mice. After bladder distention (see above), spinal cords were removed by hydraulic extrusion from mGluR5^{CeAKO} mice (compared to LV-Cre injected WT mice). The lumbosacral portion of the spinal cord that is innervated by bladder afferent input (L6-S1) was isolated by (1) identifying the thickest portion of the lumbar enlargement as L4/L5, (2) measuring 2mm caudal to this portion as the start of L6, and then (3) isolating a section of cord that started at this L6 point and continuing 7mm caudal. This method of identification has been validated by our immunohistochemical analysis of pERK1/2 in the dorsal horn after bladder distention (Lai et al., 2011). That is, the Western "lumbosacral" isolates used in this experiment

correspond to the immunohistochemical sections that stain positive for phosphorylated ERK1/2 after bladder distention. After the lumbosacral portion of the spinal cord was isolated, the tissue was frozen on dry ice. Brains were removed, sectioned into 1-mm-thick coronal sections using an acrylic brain matrix (Stoelting) and amygdala punches (2 per side per animal) were obtained using a custom-made 1mm punch tool and frozen on dry ice. All tissue samples were homogenized with ice-cold homogenization buffer (20mM Tris-HCl, pH7.4, 1mM EDTA, 1mM sodium pyrophosphate, 25mg/mL aprotinin, 25mg/mL leupeptin, and 100mM phenylmethylsulphonyl fluoride). The levels of mGluR5 in the left or right amygdala were measured by Western blotting using antibodies specific for mGluR5 and the loading control protein b-Tubulin. Total protein (7.5mg) for amygdala homogenates was separated using 4-12% SDS-PAGE and transferred to nitrocellulose membranes. For mGluR5 Western blotting, membranes were blocked in Odyssey blocking buffer for 1h and then co-incubated in mouse anti-b-Tubulin (1:10,000, Sigma) and rabbit anti-mGluR5 (1:1,000, Millipore, Billerica, MA) primary antibodies in Odyssey buffer with 0.1% Tween-20 for 1h at RT. After washing in TBS with 0.1% Tween-20, blots were incubated in goat anti-mouse IR800 (1:20,000, Sigma) and goat anti-rabbit Alexa 680 (1:20,000, Sigma) for 1h at RT. Blots were washed and scanned using an Odyssey Infrared scanner. Densitometry of bands corresponding to mGluR5 and b-Tubulin was performed using Odyssey software (V3.0). Each mGluR5 band was divided by the pixel value for that sample's b-Tubulin value and then normalized to the left amygdala because our previous results showed that mGluR5 protein is increased in the right amygdala compared to the left amygdala (Kolber et al., 2010). The levels of ERK1/2 phosphorylation in the spinal cord were measured by Western blotting using antibodies specific for phosphorylated ERK and for total ERK. Total protein (5mg) for spinal cord homogenates was run as described above. Blots were blocked as above and then co-incubated in mouse anti-p44/42 ERK (1:1000, Cell Signaling, Beverly, MA) and rabbit antiphospho-p44/42 ERK (1:1000, Cell Signaling) primary antibodies in Odyssey buffer with 0.1% Tween-20 (Sigma) for 1h at RT. Final processing was done as above. Densitometry of bands corresponding to ERK1 (p44) and ERK2 (p42) was performed using Odyssey v3.0 software (Licor).

Bladder distention induced ERK1/2 activation. All mice (sham undistended and distended) were anesthetized and catheterized as described above for urinary bladder distention testing. Distended animals received 20s 80 mmHg bladder distention stimulation (x5 with 2min ITI). Mice were killed 5min after last bladder distention (or equivalent amount of time for sham mice). Spinal cords and brains were processed as above. Westerns were done as above on spinal cord and amygdala tissue for phosphorylated ERK and for total ERK.

Optogenetically-induced spinal cord ERK1/2 phosphorylation. Mice were anesthetized and catheterized as described above for urinary bladder distention testing. All mice (HSV-EYFP and HSV-hChR2(H134R)-EYFP) received 30min of stimulation using the optical stimulation protocol described above. 15min following termination of optical stimulation, mice were killed, and spinal cord was processed as above. Western blotting was completed using spinal cord tissue and assessing levels of phosphorylated ERK1/2 and total ERK1/2.

Immunohistochemistry.

YFP CeA expression. Following the optogenetic bladder stimulation experiment (described above), mice were deeply anesthetized with pentobarbital, perfused transcardially with PBS (37°C), followed by 25mL of ice-cold 4% paraformaldehyde (PFA) solution in PBS. The brain was dissected and postfixed in PFA overnight at 4°C. After cryoprotection in 30% sucrose, coronal sections (30mm) were obtained on a cryostat and stored in PBS at 4°C until immunostaining. Sections were rinsed in TBS and blocked in 1% BSA/0.2% milk/0.1% triton X for 1h at RT. Sections were incubated overnight at 4°C in rabbit anti-GFP antibody (1:2000, Invitrogen) in blocking solution. Sections were rinsed in TBS with 0.1% triton X-100 and incubated in donkey anti-rabbit Alexa 555 secondary antibody (1:250, Invitrogen) in blocking solution for 1h at RT. Sections were rinsed in TBS with 0.2% triton X-100, TBS, and then wet mounted and visualized using a Nikon Eclipse80i fluorescent microscope.

Optogenetically-induced spinal cord ERK1/2 phosphorylation. Following optogenetic CeA stimulation (described above), mice were deeply anesthetized with pentobarbital then perfused

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with PBS (37°C) followed by ice cold 4% PFA. The spinal cord was removed and the L4-S2 region was isolated. Samples were fixed in 4% PFA for 4h, followed by 30% sucrose for 48h, both at 4°C. 30µm transverse sections were cut on a cryostat then immediately placed in PBS. Representative sections from L6-S1 of the spinal cord were rinsed in PBS then placed in 1.0% sodium borohydride for 10 minutes. Following additional rinses, sections were washed in PBS with 0.1% Triton X-100 then rinsed again. Sections were blocked in 5% normal goat serum with PBS containing 0.1% Triton X-100, 1% BSA, and 0.1% fish gelatin at RT for 1h. Exposure to rabbit anti-pERK (1:200, Cell Signaling) occurred overnight at 4°C in 1.5% NGS blocking solution. Sections were rinsed in PBS then incubated with goat anti-rabbit Alexa Fluor488 antibody (1:200, Life Technologies) in 1.5% NGS blocking solution at RT for 1h. After a final rinse, sections were wet mounted and visualized using a Nikon Microphot-SA microscope. pERK was quantified in 4 L6-S1 sections for each animal. For cell counts, each spinal cord section was divided into 4 main regions as previously established (Lai et al., 2011); deep dorsal commissure (DCM), spinal parasympathetic nuclei (SPN), lateral dorsal horn (LDH), and medial dorsal horn (MDH). The total number of cells in each region was counted for each section. For each animal, an average cell count was determined using the 4 sections so that presented data represent the average number of positive cells per section per mouse. All spinal cords were removed and processed on the same day. Sections from all animals were stained at the same time. The researcher who performed the cell counts was blinded to the treatment groups.

Data Analysis.

Results are expressed as means $\pm$ SEM. Student's t-tests were used to compare pairs of means. In cases of two independent variables (e.g. genotype and pressure), 2-way ANOVA with repeated measures was used followed by Bonferroni post-hoc tests when significant main effects were found. A *P* value < 0.05 was considered statistically significant for all statistical comparisons. All statistical comparisons were performed with Prism 5 software (GraphPad, La Jolla, CA).

RESULTS

Amygdala mGluR5 activation induces bladder hypersensitivity

In order to study pain-like behavior originating from the bladder as directly as possible, we chose to study the response to urinary bladder distention (Figure 4.1*A*). The behavioral response to bladder distension is quantified using the visceromotor response (VMR), which is an electromyographic (EMG) recording of the abdominal muscle contraction in response to bladder distension (Ness and Gebhart, 1988, 1991; Ness and Elhefni, 2004). Previous studies have demonstrated a critical role for mGluR5 activation in the right amygdala in the development of somatic pain sensitization (Ji and Neugebauer, 2009; Kolber et al., 2010) but it is not known whether mGlu5 also regulates bladder nociception. To address this question, the selective mGluR1/5 agonist, DHPG, was infused to the right amygdala via an indwelling cannula (Figure 4.1*B*) and the effects of this infusion on the VMR to bladder distension was measured. Bladder distention (20-80 mmHg distension in 10 mmHg steps) reliably produces an evoked VMR that increases with increasing distension pressure (Figure 4.1*A*) (Figure 4.1*C*, 2-way ANOVA main effect of pressure $F_{6,42}$=8.2 P<0.0001; Figure 4.1*D* $F_{6,42}$=26.3 P=0.0019). When a control vehicle is infused, and the bladder distension protocol is repeated, no changes in the evoked response are observed (Figure 4.1*D*). In contrast, infusion of DHPG (0.1nmol) significantly increased the evoked responses to bladder distension compared to pre-treatment baseline values (Figure 4.1*C*, 2-way ANOVA significant main effect of treatment, $F_{1,42}$=26.3 P<0.0001; before DHPG versus after DHPG Bonferroni post-hoc test at 50 mmHg t=2.9 P<0.05). Thus, activation of mGluR1/5 in the right CeA results in significant physiological sensitization to bladder distension.
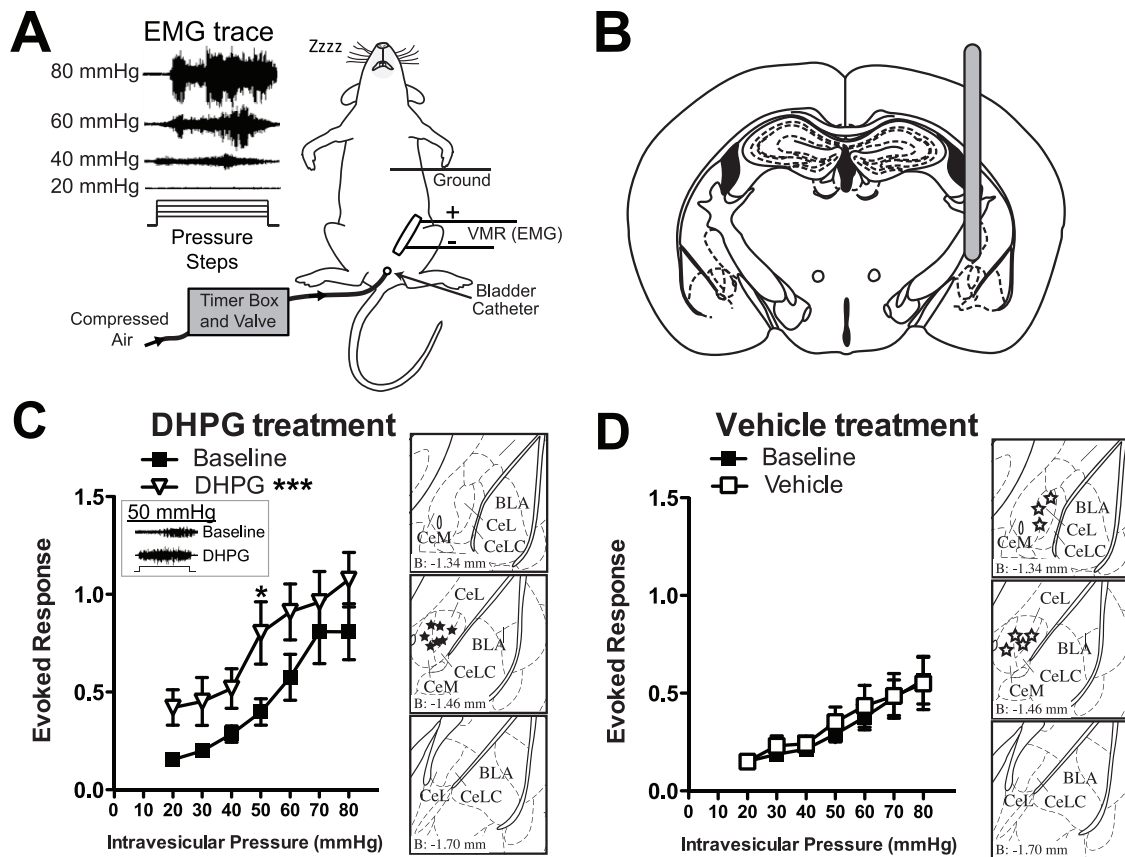
Figure 4.1. Intra-Amygdala DHPG results in hyperalgesia to bladder distention. *A*,

Illustration of *in vivo* urinary bladder distention visceromotor response (VMR) setup.

Compressed air guided into the bladder with a catheter is used to distend the bladder. Distention

induces a visceromotor response that is recorded as an electromyogram (EMG) from the

abdominal muscles. Representative EMG traces show that the VMR response increases with

increasing bladder distention pressure. *B,* Mice are cannulated in the right central nucleus of the

amygdala (CeA) and drug is delivered via the cannula. *C,* Infusion of an mGluR1/5 agonist,

DHPG (0.1 nmol), into the right CeA (n=7) induces a hyperalgesic response to graded bladder

distention compared to baseline (pre-treatment) values. EMG inset shows representative EMG

trace to 50 mmHg distention at baseline and following DHPG treatment (step represents 20 sec

distention stimulus). Brain atlas insets show cannula targeting with black stars indicative of

cannula tip. *D,* Treatment with intra-amygdala vehicle (aCSF) (n=7) had no effect on the evoked

response to graded bladder distention. Brain atlas insets show cannula targeting with white stars indicative of cannula tip. (***$P<0.001$ 2-way ANOVA main effect of treatment; *$P<0.05$ Bonferroni post test compared to baseline value)

Intra-amygdala mGluR5 antagonism decreases evoked responses to bladder distention

Prior evidence suggested that mGluR5 in the amygdala is involved in the modulation of somatic pain only after injury and that mGluR5 signaling does not affect acute responses to noxious stimulation (Kolber et al., 2010; Li et al., 2011). Whether mGluR5 modulates acute responses to bladder stimulation is not known, however. To address the role of amygdala mGluR5 in the modulation of bladder pain, the mGluR5 antagonist MPEP (or vehicle) was delivered locally to the right amygdala between two rounds of bladder distensions (from 20 to 80 mmHg in 10 mmHg increments) and the effects on the evoked VMR were determined. We found that MPEP infusion to the CeA induced a significant decrease in bladder distention-evoked responses (2-way ANOVA significant main effects of pressure $F_{6,35}$=5.9 P=0.0003 and treatment $F_{1,35}$=22.45 $P<0.0001$) that was particularly noticeable at the higher noxious pressures (Figure 4.2*A*, before MPEP versus after MPEP Bonferonni post-hoc test at 70 mmHg t=4.5 $P<0.001$ and at 80 mmHg t=6.1 $P<0.001$) compared to baseline VMRs.   These results were corroborated in a second experiment where we measured the effect of pretreatment with MPEP on the VMR to a single round of noxious distension of the bladder with no baseline distention. We found that mice that had received infusion of MPEP into the right CeA had significantly decreased VMR to high noxious (80 mmHg) bladder distention when compared to responses in vehicle-infused mice (Figure 4.2*C*, Student's t-test t_{11}=2.3 P=0.04). These findings provide further support for the notion that mGluR5 activation in the amygdala is required for acute regulation of bladder nociception.
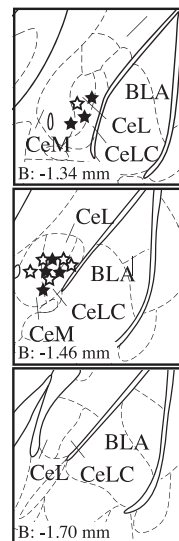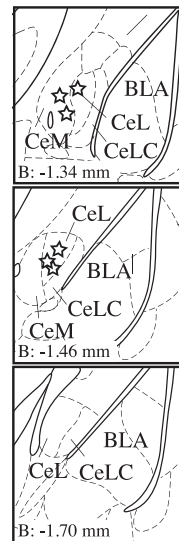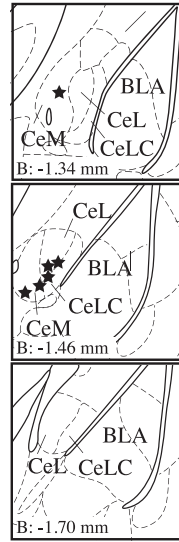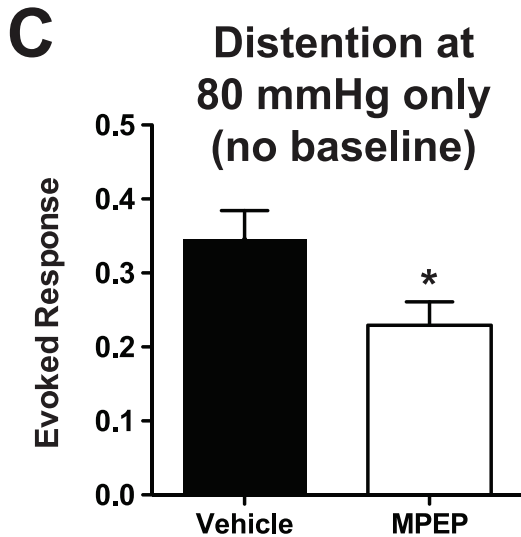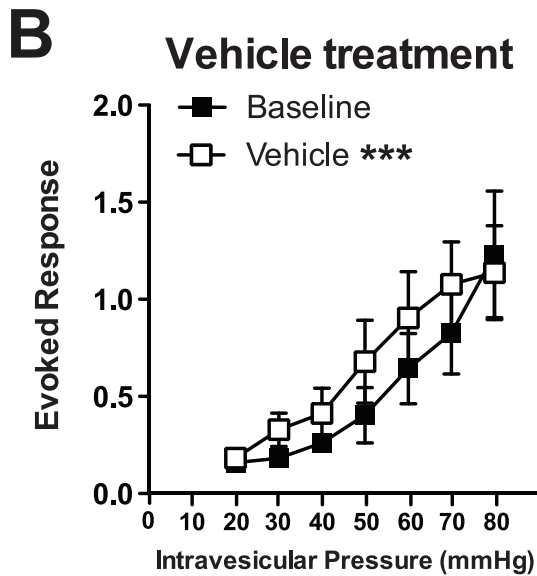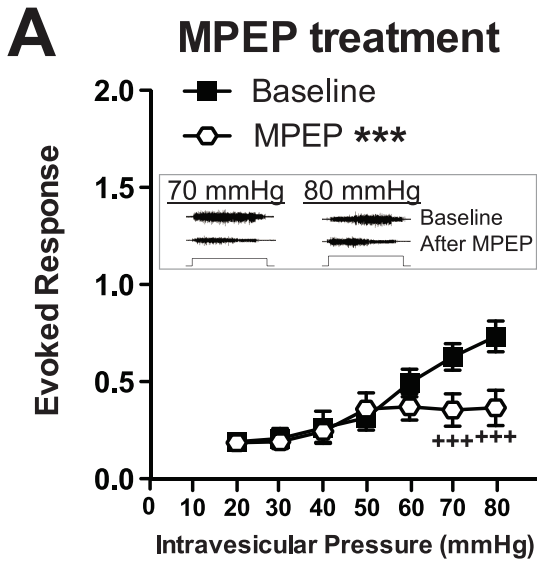
Figure 4.2. Intra-amygdala MPEP is analgesic during bladder distention. *A*, Infusion of a mGluR5 antagonist, MPEP (0.01nmol), into the right CeA (n=6) induced an analgesic response to graded bladder distention in the noxious range compared to baseline (pre-treatment) values. Inset shows representative EMG traces to 70 and 80 mmHg distention at baseline and following MPEP treatment (step represents 20 sec distention stimulus). Brain atlas insets show cannula targeting with black stars indicative of cannula tip. *B*, Treatment with intra-amygdala injection of vehicle (aCSF) (n=6) induced a significant increase in evoked response during bladder distention. However, this change was in the opposite direction of treatment with MPEP and likely represents a small non-specific effect possibly due to repeated bladder distention. Brain atlas insets show cannula targeting

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with white stars indicative of cannula tip.   **C**, In a separate cohort, pre-treatment with MPEP (n=7) before a single round of higher noxious pressure bladder distention (i.e. no baseline distention) induced a significant decrease in the evoked response compared to vehicle-treated mice (n=6). Brain atlas insets show cannula targeting with solid black stars indicative of cannula tip of MPEP-treated mice and white stars indicative of cannula tip of vehicle-treated mice. (***P<0.001 2-way ANOVA main effect of treatment; +++P<0.001 Bonferroni post test compared to indicated baseline value; *P<0.05 Student's t-test compared to vehicle-treated group)

Surprisingly, when vehicle was infused between two rounds of graded bladder distension, the evoked responses were significantly increased relative to pre-infusion baseline measurements (Figure 4.2B, 2-way ANOVA significant main effects of pressure $F_{6,35}$=5.0 P=0.0009 and treatment $F_{1,35}$=15.4 P=0.0004).   These results might suggest that repeated distension to noxious pressures can induce mild sensitization of the bladder.   To address this issue, we examined the VMR in control mice that received only a single round (4-5 distentions) 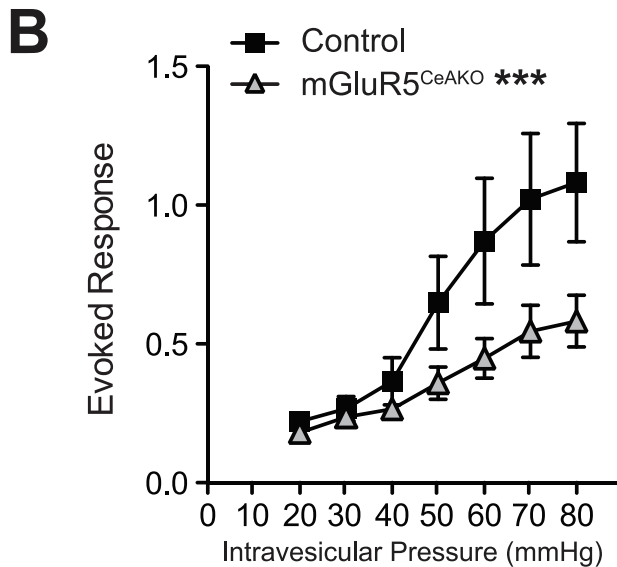of 80mmHg distention (with no baseline UBD). Comparing the VMR after the first distention at 80mmHg in these mice to the last distention, we found no significant sensitization of the VMR to repeated distentions (1st distention = 0.54 $\pm$ 0.10 evoked response; last distention = 0.30 $\pm$ 0.05 evoked response; paired t-test t_5=1.9 P=0.11). These data suggest that repeated bladder distention does not sensitize the bladder.

Disruption of mGluR5 in the right amygdala reduces responses to bladder distension

The results described above demonstrate that pharmacological activation of mGluR5 in the right CeA enhances the evoked response to bladder distension whereas inhibition of mGluR5 in the CeA inhibits these responses. These data are consistent with the hypothesis that mGluR5 in the right CeA plays a central role in the endogenous modulation of visceral bladder pain. However, pharmacological manipulations *in vivo* can always have the potential for off-target effects. To compliment our pharmacological studies, we took a genetic approach to test the

hypothesis that endogenous mGluR5 activation is required for bladder pain. A conditional

knockout approach was used to specifically disrupt mGluR5 expression in the right CeA using

recombinant lentivirus-mediated expression of Cre recombinase (LV-Cre) in floxed mGluR5 mice.

Floxed mGluR5 mice injected with LV-Cre (mGluR5^{CeAKO}) exhibited a significant decrease in

mGluR5 protein levels when compared with mGluR5 expression in the right amygdala of LV-Cre

injected WT (control) mice, thus confirming deletion of mGluR5 (Figure 4.3*A*, Student's t-test

t_9=3.8 *P*=0.0045).

Figure 4.3. Lentivirus-mediated conditional disruption of mGluR5 in the central amygdala is analgesic. ***A***, Representative Western blot for mGluR5 (top) and b-tubulin (bottom) in mGluR5[CeAKO] and WT LV-Cre-injected (control) mice. Graph of mGluR5/b-tubulin shows reduced mGluR5 expression in the right amygdala of mGluR5[CeAKO] mice (n=8) compared to the right amygdala of control mice (n=8). ***B***, Disruption of mGluR5 in the right amygdala (n=8) induced a significant decrease in evoked response during bladder distention compared to control mice (n=9). Brain atlas insets show cannula targeting with black squares indicative of cannula tips of control mice and gray triangles indicative of cannula tips of mGluR5[CeAKO] mice. (***$P<0.001$ 2-Way ANOVA main effect of genotype; **$P<0.01$ Student's t-test)

To measure the impact of CeA mGluR5 disruption on bladder pain-related behavior, we compared the responses of mGluR5^{CeAKO} to control mice during graded bladder distention (distention from 20-80 mmHg in 10 mmHg steps). We found a significant decrease in the evoked response to bladder distension in the mGluR5^{CeAKO} mice compared to control mice (Figure 4.3*B*, 2-way ANOVA significant main effects of pressure $F_{6,105}$=7.8 *P*<0.0001 and genotype $F_{1,105}$=14.4 *P*=0.0003). Overall, these data suggest that mGluR5 activity in the CeA modulates ongoing sensitivity to visceral stimulation.

Amygdala control of bladder hyperalgesia is associated with ERK activation in the spinal cord

Next, we sought to evaluate the mechanisms by which activation of the CeA might modulate evoked responses to bladder distention. We hypothesized that mGluR5 activation in the CeA could modulate bladder nociception by regulating ERK1/2 activity in the CeA or spinal cord (through modulation of descending pathways). We first tested whether bladder distention in naive mice would induce ERK1/2 phosphorylation. We compared the protein collected from the lumbosacral spinal cord and the CeA in mice distended 5 times at 80 mmHg to protein from undistended sham mice. We found that bladder distension induced a significant increase in phosphorylated ERK1 and ERK2 in the spinal cord (Figures 4.4*A-B*, Student's t-test pERK1 t_{12}=2.6 *P*=0.022; pERK2 t_{12}=3.3 *P*=0.0068). In contrast, we found no significant change in activation of ERK1 or ERK2 in the CeA following distention compared to sham mice (Figures 4.4*C-D*).

Figure 4.4. Bladder distention induces spinal cord ERK phosphorylation. *A*, Representative Western blot showing phosphorylated ERK1 & ERK2 bands (top) and total ERK1 & ERK2 (bottom) from spinal cord tissue of undistended control (sham) mice (n=7) or 80 mmHg bladder distended mice (n=7). *B*, Quantification of Western blots showing increased ERK1 and ERK2 phosphorylation in distended mice compared to sham mice. *C*, Representative Western blot showing phosphorylated ERK1 & ERK2 bands (top) and total ERK1 & ERK2 (bottom) from left and right amygdala of sham mice or 80 mmHg bladder distended mice. *D*, Quantification of Western blots showing no significant change ERK1 and ERK2 phosphorylation from right amygdala tissue samples of distended mice compared to sham mice. (*P<0.05, **P<0.01 unpaired Student's t-test compared to sham group)

Since bladder distension induced an increase in activated ERK1/2 in the spinal cord and we found a decrease in the evoked response to bladder distention in mGluR5^{CeAKO} mice, we reasoned that mGluR5^{CeAKO} mice might show a decrease in spinal ERK1/2 activation following

bladder distention. Indeed, we found that disruption of mGluR5 in the right CeA caused a

significant decrease in the phosphorylation of ERK1 and ERK2 in the spinal cord after bladder

distention compared to distended control mice (Figures 4.5A-B, Student's t-test pERK1 t_{14}=2.3

P=0.038; pERK2 t_{14}=2.3 P=0.035). These data suggest the presence of tonic modulation of

spinal circuitry driven by mGluR5 activation in the CeA.   In contrast, when we compared the

phosphorylation of ERK1 and ERK2 by Western analysis in the spinal cord after CeA treatment

with MPEP or vehicle followed by a single round of noxious distention, we failed to find any

significant differences between groups (data not shown).
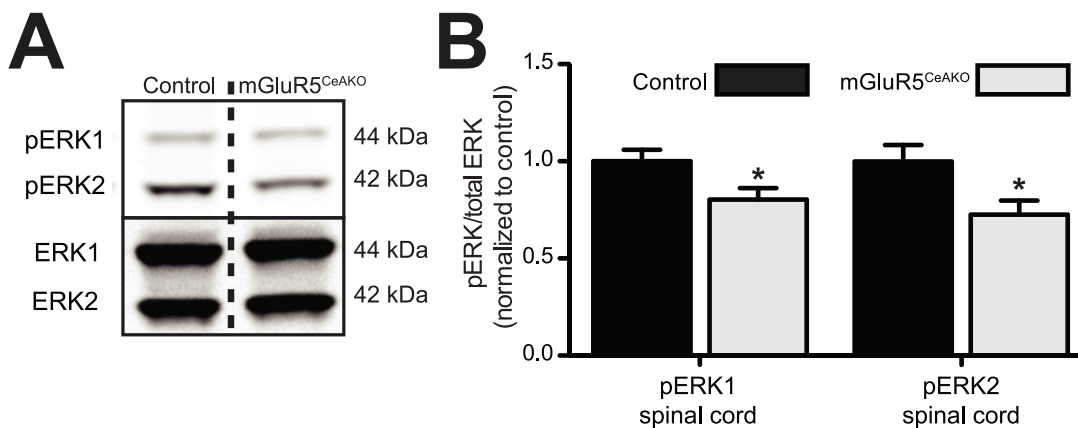
**Figure 4.5. Genetic disruption of mGluR5 in the right amygdala reduces bladder
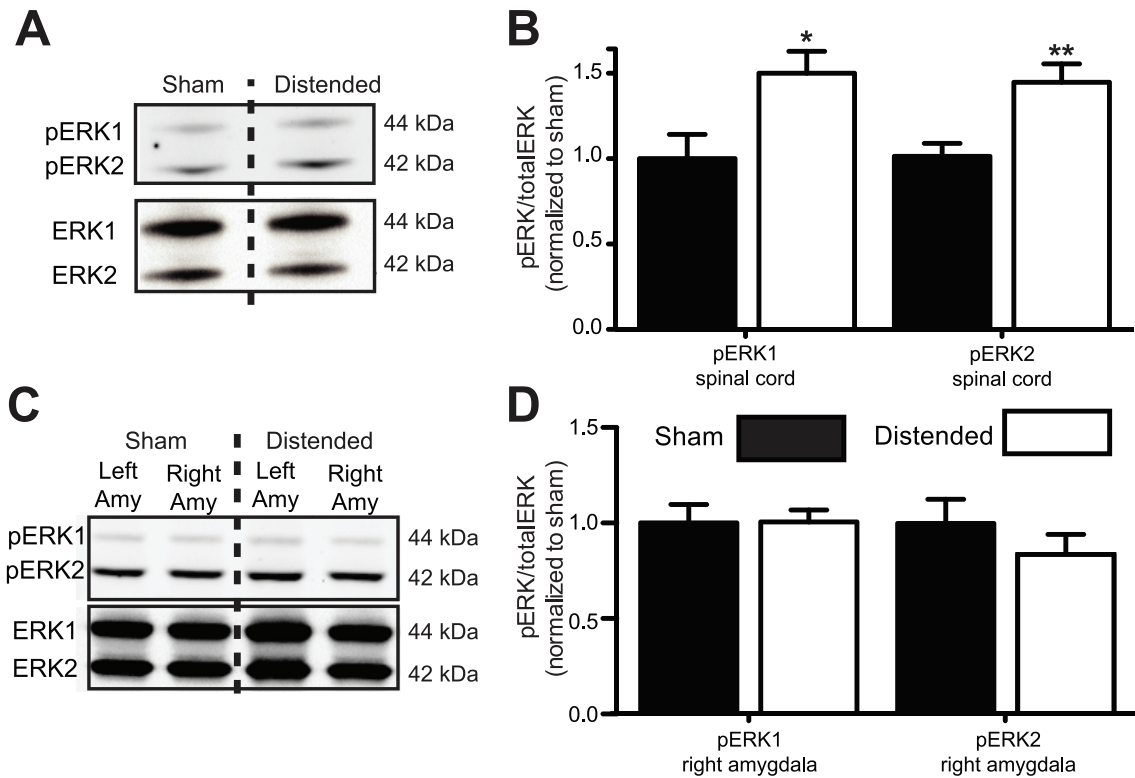
distention-induced ERK phosphorylation.   *A*,** Representative Western blot showing

phosphorylated ERK1 & ERK2 bands (top) and total ERK1 & ERK2 (bottom) from spinal cord

tissue from LV-Cre injected control mice (n=9) or mGluR5[CeAKO] mice (n=7) following graded

bladder distention. ***B***, Quantification of Western blots showing decreased ERK1 and ERK2

phosphorylation in mGluR5[CeAKO] mice compared to control mice. (*P<0.05 unpaired Student's t-

test compared to control group)

**Optogenetic activation of the central amygdala is sufficient to induce pain hypersensitivity

with no changes in ERK1/2 phosphorylation**

The mechanisms through which mGluR5 in the CeA might modulate responses to bladder distention are unknown. For example, mGluR5 activation in the CeA could suppress a tonically active descending inhibitory pathway to the spinal cord, or conversely could drive increased descending excitatory circuitry. To address the hypothesis that an overall increase in the activity of CeA neurons may be sufficient to induce bladder hyperalgesia, we utilized an optogenetic approach to drive excitation of CeA neurons and asked whether this increased CeA activity could modulate the VMR elicited by innocuous and noxious bladder distention.

To accomplish this goal, channelrhodopsin-2 or control protein expression was driven in the CeA by targeted injection in the right CeA (Figure 4.6*A*) of HSV vectors expressing YFP only (HSV-YFP control; Figure 4.6*B*) or channelrhodopsin-2 fused to YFP (HSV-ChR2-YFP; Figure 4.6*C*). Four days after viral injection, mice were stimulated with low (30 mmHg) or with higher noxious (60 mmHg) bladder distention at baseline, during, and after laser stimulation (5ms, 20Hz pulse with 18ms spike width). The optical stimulation protocol chosen for these experiments was modeled to mimic the CeA neuronal firing pattern that occurs after injury in rodents (Ji and Neugebauer, 2009). In HSV-YFP control mice, optical stimulation did not alter evoked responses to bladder distention at 30 mmHg (Figures 4.6*D,F*) or 60 mmHg (Figures 4.6*G,I*). In contrast, 30 minutes of optical stimulation of HSV-ChR2-YFP treated mice caused a significant increase in bladder distention-evoked responses compared to baseline measurements (Figures 4.6*E-F*, *H-I,* Bonferroni post-hoc test at 30 mmHg t=2.5 P<0.05 and at 60 mmHg t=4.3 P<0.001) and compared to HSV-YFP control mice (Figure 4.6*I*, Bonferonni post-hoc test at 60 mmHg t=3.0 P<0.05). Interestingly, CeA stimulation induced a lasting hypersensitivity to bladder distention in HSV-ChR2-YFP treated mice. That is, 15 min after the laser stimulation was terminated, HSV-ChR2-YFP treated mice still showed increased responses to bladder distention (Figures 4.6*E-F*, Bonferonni post-hoc test at 30 mmHg compared to baseline t=2.8 P<0.05; Figures 4.6*H-I,* at 60 mmHg compared to baseline t=4.6 P<0.001 and at 60 mmHg compared to HSV-YFP control mice t=3.1 P<0.05).

A

B HSV-YFP (Control)

CeA

BLA

C HSV-ChR2-YFP

CeA

BLA

D 30 mmHg Distention
HSV-YFP

Baseline

Laser on

Post-laser

E 30 mmHg Distention
HSV-ChR2-YFP

Baseline

Laser on

Post-laser

F 30 mmHg Distention

G 60 mmHg Distention
HSV-YFP

Baseline

Laser on

Post-laser

H 60 mmHg Distention
HSV-ChR2-YFP

Baseline

Laser on

Post-laser

I 60 mmHg Distention
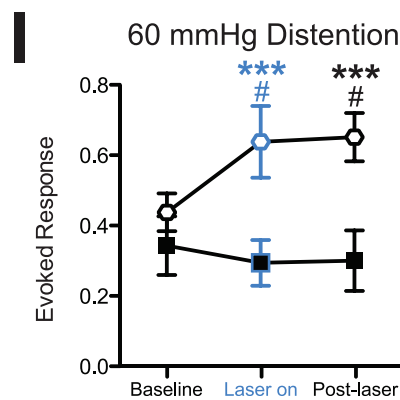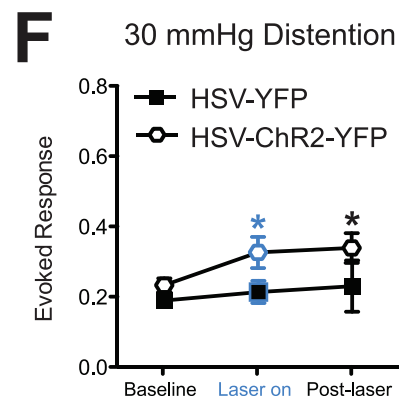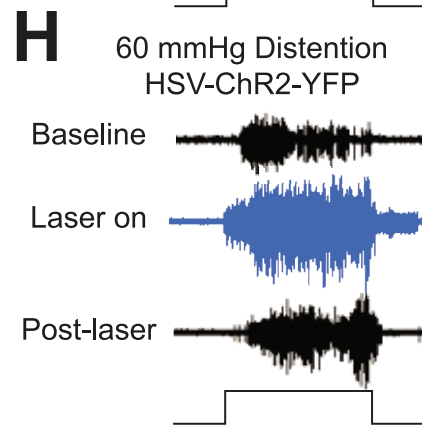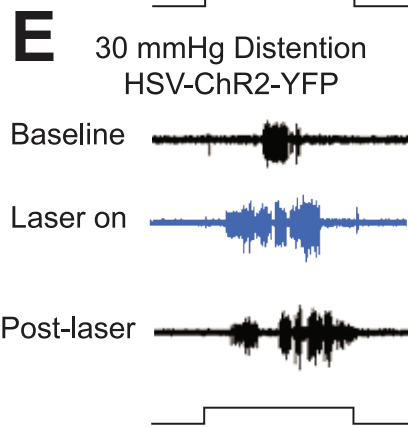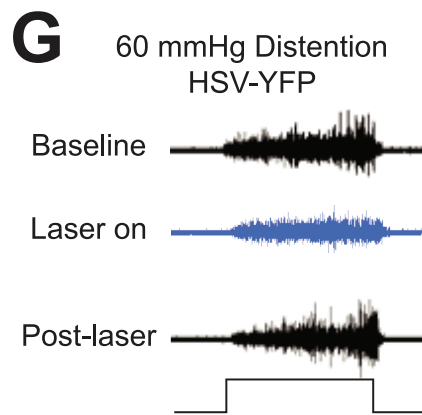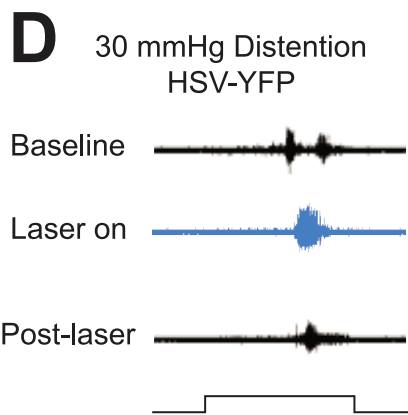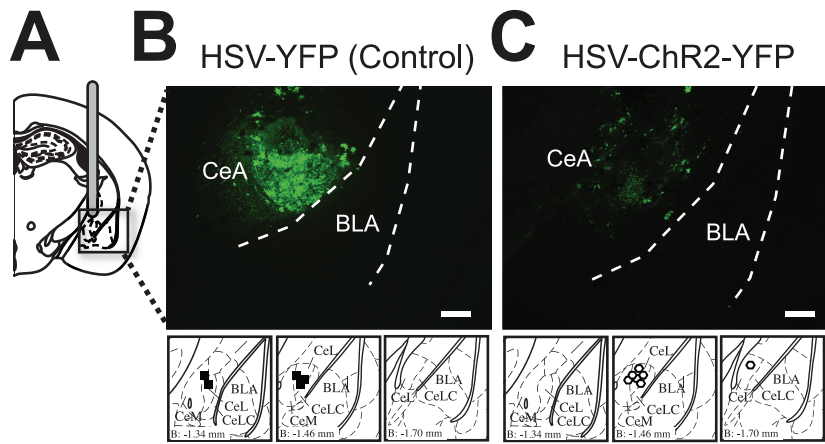
Figure 4.6. Optogenetic stimulation of right central amygdala induces bladder hyperalgesia. *A*, Mice are cannulated in the right central nucleus of the amygdala (CeA) and HSV vectors are delivered via the cannula. *B-C*, YFP fluorescence (via immunohistochemistry) is seen in the CeA of both (*B*) HSV-YFP (control vector) and (*C*) HSV-ChR2-YFP (optogenetic vector) injected mice (scale bars = 0.2mm). Differences in staining pattern for HSV-YFP versus HSV-ChR2-YFP-treated mice are likely due to the fact that there is probably higher expression of cytoplasmic YFP (i.e. HSV-YFP) compared to the ChR2-YFP fusion protein, which is membrane bound. Brain atlas insets show cannula targeting with black squares indicative of cannula tips of HSV-YFP mice and white hexagons indicative of cannula tips of HSV-ChR2-YFP mice. Representative images of raw EMG traces from HSV-YFP or HSV-ChR2-YFP injected mice during (*D-E*) 30 mmHg and (*G-H*) 60 mmHg bladder distention. Optic stimulation of HSV-ChR2-YFP treated mice (n=7) increases the evoked response to both 30 and 60mmHg bladder distention compared to (*E,H*) baseline (pre-stimulation) responses and increases the evoked response to 60 mmHg bladder distention compared to (*I*) HSV-YFP injected mice (n=5). Laser light alone does not increase the evoked response to bladder distention in HSV-YFP mice (*D, F, G, I*). Black steps in traces show 20sec bladder distention stimuli. (*$P<0.05$; ***$P<0.001$ Bonferroni post test compared to baseline for HSV-ChR2-YFP mice; #$P<0.05$ Bonferroni post test compared to HSV-YFP control mice at indicated time points)

To test one possible downstream effect of optogenetic activation of the amygdala, we evaluated whether optogenetically driven activation of the amygdala (5ms, 20Hz pulse with 18ms spike width) in the absence of bladder distention would induce spinal cord ERK1/2 phosphorylation. Mice received local infusions in the CeA of HSV-ChR2-YFP or HSV-YFP as described above. Four days after viral infusion, mice were anesthetized and stimulated with the laser on for 30 min and were killed after the laser had been off for 15 min. Comparing spinal cord protein samples analyzed by Western or spinal cord sections analyzed for pERK1/2 immunofluorescence, we found that optogenetic stimulation of the CeA failed to induce an

increase in ERK1/2 phosphorylation compared to HSV-YFP control mice (Western: Student's t-test pERK1 HSV-YFP n=12 1.0 $\pm$ 0.08 vs HSV-ChR2-YFP n=10 1.0 $\pm$ 0.09 t=0.1 P=0.93; pERK2 HSV-YFP 1.0 $\pm$ 0.07 vs HSV-ChR2-YFP 1.1 $\pm$ 0.07 t=0.7 P=0.51)(IHC: Student's t-test deep dorsal commissure HSV-YFP n=3 40.0 $\pm$ 10.6 vs HSV-ChR2-YFP n=3 38.9 $\pm$ 5.5 pERK1/2 positive cells t=0.10 P=0.92; spinal parasympathetic nuclei HSV-YFP 23.0 $\pm$ 8.9 vs HSV-ChR2-YFP 29.5 $\pm$ 2.3 pERK1/2 positive cells t=0.82 P=0.45; lateral dorsal horn HSV-YFP 12.6 $\pm$ 2.4 vs HSV-ChR2-YFP 13.2 $\pm$ 4.1 pERK1/2 positive cells t=0.11 P=0.91; medial dorsal horn HSV-YFP 15.3 $\pm$ 6.9 vs HSV-ChR2-YFP 16.6 $\pm$ 3.8 pERK1/2 positive cells t=0.18 P=0.86; total dorsal horn HSV-YFP 90.8 $\pm$ 25.6 vs HSV-ChR2-YFP 98.1 $\pm$ 12.7 pERK1/2 positive cells t=0.28 P=0.79). Similar to our results with optogenetic activation, treatment of the CeA with DHPG in the absence of bladder distention did not alter ERK1/2 phosphorylation in the spinal cord compared to vehicle-treated mice (data not shown).

DISCUSSION

Our results demonstrate that activation of group I mGluRs in the right amygdala was sufficient to induce an increased pain response to bladder distention. Furthermore, pharmacological inhibition and genetic deletion of mGluR5 in the right amygdala resulted in a reduced response to bladder distention. Optogenetic activation of the right amygdala was sufficient to increase the pain response to bladder distention. Together, these results suggest that mGluR5 in the right CeA modulates bladder pain responses via increased excitation of CeA neurons. Our results are consistent with previous work demonstrating that noxious bladder distention in the absence of injury results in ERK1/2 activation in the lumbosacral spinal cord (Lai et al., 2011). Furthermore, we found that the level of activated ERK1/2 is dependent on the presence of mGluR5 in the right CeA. Our results suggest that distension-evoked ERK1/2 activation in the lumbosacral spinal cord is modulated in part by mGluR5 activity in the right amygdala.

Here, we show for the first time that mGluR5 in the right CeA is necessary for the visceral bladder pain response to noxious stimuli. Our results are consistent with previous work indicating that activation of Group I mGluRs in the right amygdala leads to increased somatic pain (Kolber et al., 2010). Specifically, activation of mGluR5 in the right amygdala results in bilateral mechanical hypersensitivity (Kolber et al., 2010). Our results extend this phenomenon to visceral bladder pain and suggest that pharmacological activation of group I mGluRs (mGluR1 and 5) in the right CeA is sufficient to increase the nociceptive responses to bladder distention in naïve (non-injured) mice.

Given that previous studies have shown the mGluR1/5 agonist DHPG potentiates the excitatory response of the CeA during visceral colorectal distention (Ji and Neugebauer, 2010), we hypothesized that activation of Group I mGluRs in the amygdala could lead to increased excitability as has been reported previously (Li and Neugebauer, 2004), thus resulting in the observed increased response to bladder distention. We demonstrate that optogenetic stimulation of CeA neurons in the right amygdala is sufficient to increase the VMR to urinary bladder

distention. Furthermore, this increased VMR is maintained 15 minutes after the optogenetic

activation is turned off.   Injury in a colitis model increases the excitability of evoked responses in

the CeA (Han and Neugebauer, 2004) and arthritis-induced somatic injury increases the

background and evoked responses of CeA neurons (Li and Neugebauer, 2004). The sustained

behavioral response we observe after optical excitation suggests that 30 min activation of the

CeA may, in effect, model the changes seen after either visceral or somatic injury. Future studies

are required to test this hypothesis.

Our previous studies have shown that inhibition of mGluR5 in the right amygdala reduces

persistent injury-induced hypersensitivity of the paw, but that inhibition of mGluR5 does not alter

acute somatic sensitivity or spontaneous somatic nociceptive responses (Kolber et al., 2010).

Here, we find that pharmacological inhibition of mGluR5 following a round of bladder distension is

analgesic when compared to the baseline bladder distention. These results could represent

either a novel role of mGluR5 in modulating acute visceral sensation or provide additional

evidence that mGluR5 modulates sensation after mild injury caused by repeated bladder

distensions. We believe that the significant increase in the evoked response in the vehicle-treated

mice (Figure 4.2B) is likely a statistical aberration as no such effect was seen in a similar

experiment (Figure 4.1D), and we found that the first VMR is not significantly different from the

last VMR during a round of noxious bladder distention with no baseline testing. Nonetheless, to

unequivocally determine whether mGluR5 in the CeA modulates baseline (uninjured) responses

to bladder distension, we inhibited mGluR5 before noxious stimulation with no baseline distention

trials. Providing evidence for the hypothesis that mGluR5 modulates acute bladder nociception,

mice pretreated with MPEP have a smaller VMR in response to a single round of 80 mmHg

distension when compared to vehicle-treated mice. This hypothesis is further supported by the

reduced VMR observed in mice with conditional disruption of mGluR5 in the right amygdala,

because these animals receive only a single series of distensions. Interestingly, these data are

contrary to previous reports showing that inhibition of mGluR5 in the right amygdala has no effect

on vocalizations induced by visceral colorectal distention (Li et al., 2011). One possible

explanation for this apparent discrepancy is that there are inherent differences in the responses

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measured (e.g. vocalizations (Li et al., 2011) versus reflexive abdominal contractions). Our novel results demonstrate that ongoing mGluR5 activity in the right CeA modulates the visceromotor response to noxious bladder distention in naive (uninjured) mice.

The mechanism of the modulation of distension-evoked bladder pain by CeA-localized mGluR5 seems to be related in part to a modulation of ERK1/2 in the spinal cord. Our previous experiments have demonstrated that ERK1/2 in both the spinal cord and amygdala is involved in the modulation of acute spontaneous somatic pain and behavioral changes after injury (Carrasquillo and Gereau, 2007; Hu et al., 2007; Carrasquillo and Gereau, 2008). Furthermore, we know from previous work that ERK1/2 is activated in the dorsal horn neurons of the lumbosacral spinal cord (lamina I, II, V, X) following bladder distention in mice with and without bladder inflammation (Lai et al., 2011). Our results suggest that noxious bladder distention in lightly anesthetized mice immediately activates ERK1/2 in the lumbosacral spinal cord, but not in the amygdala. Furthermore, genetic deletion of mGluR5 in the right CeA reduced activation of ERK1/2 in the lumbosacral spinal cord following bladder distention. In contrast, pharmacologic inhibition of mGluR5 with MPEP did not change the activation of ERK1/2 following noxious distention. This difference may be related to the long-term disruption that exists in the knockout mice compared to the acute inhibition in the MPEP experiment, or a difference in the extent of mGluR5 inhibition achieved by the two approaches. Nonetheless, these data suggest the presence of a functional connection between the amygdala and the spinal cord nociceptive neurons.

To further test this connection, we activated the amygdala (via optogenetic or pharmacologic techniques) in the absence of bladder distention and measured ERK1/2 phosphorylation in the spinal cord. Finding no effect of amygdala activation on pERK1/2 in the spinal cord, these results suggest either that ERK1/2 modulation occurs only in the context of nociceptive input (e.g. bladder distention) and/or that there are other changes that occur in the spinal cord during amygdala activation.   Nociceptive neurons in the CeA project to the PAG and then to the rostral ventromedial medulla (RVM) (Neugebauer et al., 2004). Descending inputs

from the RVM exert facilitatory and inhibitory influence on nociceptive transmission in the spinal cord dorsal horn (Millan, 2002; Porreca et al., 2002; Ren and Dubner, 2002). Modulation of visceral nociception between the amygdala and the spinal cord could occur at the sensory fiber/spinal cord synapse (in the spinal cord dorsal horn), on modulatory interneurons, or potentially on motor neurons that mediate abdominal contractions during bladder distention. Future studies will be necessary to determine the precise location and mechanism of this interaction.

Overall, our results suggest that increased CeA output, driven by mGluR5 activation, in combination with bladder distention leads to modulation of ERK activation in the spinal dorsal horn. These results highlight the important role that limbic brain structures play in the regulation of peripheral responses to noxious stimuli. Furthermore, our results showing that mGluR5 inhibition modulates acute visceral nociception provide evidence for a novel role of the amygdala in the modulation of on-going nociception in the absence of injury or external stress.

REFERENCES

Baldoni F, Ercolani M, Baldaro B, Trombini G (1995) Stressful events and psychological
symptoms in patients with functional urinary disorders. Percept Mot Skills 80:605-606.

Bernard JF, Besson JM (1990) The spino(trigemino)pontoamygdaloid pathway:
electrophysiological evidence for an involvement in pain processes. Journal of
neurophysiology 63:473-490.

Bernard JF, Bester H, Besson JM (1996) Involvement of the spino-parabrachio -amygdaloid and -
hypothalamic pathways in the autonomic and affective emotional aspects of pain. Prog
Brain Res 107:243-255.

Berry SH, Elliott MN, Suttorp M, Bogart LM, Stoto MA, Eggers P, Nyberg L, Clemens JQ (2011)
Prevalence of Symptoms of Bladder Pain Syndrome/Interstitial Cystitis Among Adult
Females in the United States. The Journal of urology 186:540-544.

Bourgeais L, Gauriau C, Bernard JF (2001) Projections from the nociceptive area of the central
nucleus of the amygdala to the forebrain: a PHA-L study in the rat. The European journal
of neuroscience 14:229-255.

Burstein R, Potrebic S (1993) Retrograde labeling of neurons in the spinal cord that project
directly to the amygdala or the orbital cortex in the rat. The Journal of comparative
neurology 335:469-485.

Carrasquillo Y, Gereau RW (2007) Activation of the extracellular signal-regulated kinase in the
amygdala modulates pain perception. J Neurosci 27:1543-1551.

Carrasquillo Y, Gereau RW (2008) Hemispheric lateralization of a molecular signal for pain
modulation in the amygdala. Molecular pain 4:24.

Castroman P, Ness TJ (2001) Vigor of visceromotor responses to urinary bladder distension in
rats increases with repeated trials and stimulus intensity. Neuroscience letters 306:97-
100.

Cervero F, Laird JM (1999) Visceral pain. Lancet 353:2145-2148.

Covington HE, 3rd, Lobo MK, Maze I, Vialou V, Hyman JM, Zaman S, LaPlant Q, Mouzon E,
Ghose S, Tamminga CA, Neve RL, Deisseroth K, Nestler EJ (2010) Antidepressant effect
of optogenetic stimulation of the medial prefrontal cortex. J Neurosci 30:16082-16090.

Dimitrakov J, Kroenke K, Steers WD, Berde C, Zurakowski D, Freeman MR, Jackson JL (2007)
Pharmacologic Management of Painful Bladder Syndrome/Interstitial Cystitis: A
Systematic Review. Arch Intern Med 167:1922-1929.

Greenwood-Van Meerveld B, Gibson M, Gunter W, Shepard J, Foreman R, Myers D (2001)
Stereotaxic delivery of corticosterone to the amygdala modulates colonic sensitivity in
rats. Brain research 893:135-142.

Han JS, Neugebauer V (2004) Synaptic plasticity in the amygdala in a visceral pain model in rats.
Neuroscience letters 361:254-257.

Hu HJ, Alter BJ, Carrasquillo Y, Qiu CS, Gereau RW (2007) Metabotropic glutamate receptor 5
modulates nociceptive plasticity via extracellular signal-regulated kinase-Kv4.2 signaling
in spinal cord dorsal horn neurons. J Neurosci 27:13181-13191.

Ikeda R, Takahashi Y, Inoue K, Kato F (2007) NMDA receptor-independent synaptic plasticity in
the central amygdala in the rat model of neuropathic pain. Pain 127:161-172.

Ji G, Neugebauer V (2009) Hemispheric lateralization of pain processing by amygdala neurons.
Journal of neurophysiology 102:2253-2264.

Ji G, Neugebauer V (2010) Reactive oxygen species are involved in group I mGluR-mediated
facilitation of nociceptive processing in amygdala neurons. Journal of neurophysiology
104:218-229.

Ji G, Fu Y, Ruppert KA, Neugebauer V (2007) Pain-related anxiety-like behavior requires CRF1
receptors in the amygdala. Molecular pain 3:13.

Ji RR (2004) Peripheral and central mechanisms of inflammatory pain, with emphasis on MAP
kinases. Current drug targets 3:299-303.

Kolber BJ, Roberts MS, Howell MP, Wozniak DF, Sands MS, Muglia LJ (2008) Central amygdala
glucocorticoid receptor action promotes fear-associated CRH activation and conditioning.

Proceedings of the National Academy of Sciences of the United States of America 105:12004-12009.

Kolber BJ, Montana MC, Carrasquillo Y, Xu J, Heinemann SF, Muglia LJ, Gereau RW (2010) Activation of metabotropic glutamate receptor 5 in the amygdala modulates pain-like behavior. J Neurosci 30:8203-8213.

Lai HH, Qiu CS, Crock LW, Morales ME, Ness TJ, Gereau RW (2011) Activation of spinal extracellular signal-regulated kinases (ERK) 1/2 is associated with the development of visceral hyperalgesia of the bladder. Pain 152:2117-2124.

Li W, Neugebauer V (2004) Differential roles of mGluR1 and mGluR5 in brief and prolonged nociceptive processing in central amygdala neurons. Journal of neurophysiology 91:13-24.

Li Z, Ji G, Neugebauer V (2011) Mitochondrial reactive oxygen species are activated by mGluR5 through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and pain behavior. J Neurosci 31:1114-1127.

Macaulay AJ, Stern RS, Holmes DM, Stanton SL (1987) Micturition and the mind: psychological factors in the aetiology and treatment of urinary symptoms in women. Br Med J (Clin Res Ed) 294:540-543.

Millan MJ (2002) Descending control of pain. Progress in neurobiology 66:355-474.

Myers B, Greenwood-Van Meerveld B (2010) Elevated corticosterone in the amygdala leads to persistent increases in anxiety-like behavior and pain sensitivity. Behavioural brain research 214:465-469.

Ness TJ, Gebhart GF (1988) Colorectal distension as a noxious visceral stimulus: physiologic and pharmacologic characterization of pseudaffective reflexes in the rat. Brain research 450:153-169.

Ness TJ, Gebhart GF (1991) Interactions between visceral and cutaneous nociception in the rat. I. Noxious cutaneous stimuli inhibit visceral nociceptive neurons and reflexes. Journal of neurophysiology 66:20-28.

Ness TJ, Elhefni H (2004) Reliable visceromotor responses are evoked by noxious bladder distention in mice. The Journal of urology 171:1704-1708.

Ness TJ, Lewis-Sides A, Castroman P (2001) Characterization of pressor and visceromotor reflex responses to bladder distention in rats: sources of variability and effect of analgesics. The Journal of urology 165:968-974.

Ness TJ, Richter HE, Varner RE, Fillingim RB (1998) A psychophysical study of discomfort produced by repeated filling of the urinary bladder. Pain 76:61-69.

Neugebauer V (2007) The amygdala: different pains, different mechanisms. Pain 127:1-2.

Neugebauer V, Li W, Bird GC, Han JS (2004) The amygdala and persistent pain. Neuroscientist 10:221-234.

Neve RL, Neve KA, Nestler EJ, Carlezon WA, Jr. (2005) Use of herpes virus amplicon vectors to study brain disorders. BioTechniques 39:381-391.

Paxinos G, Franklin KBJ (2001) The mouse brain in stereotaxic coordinates, 2nd Edition. San Diego: Academic Press.

Porreca F, Ossipov MH, Gebhart GF (2002) Chronic pain and medullary descending facilitation. Trends Neurosci 25:319-325.

Qin C, Greenwood-Van Meerveld B, Foreman RD (2003) Spinal neuronal responses to urinary bladder stimulation in rats with corticosterone or aldosterone onto the amygdala. Journal of neurophysiology 90:2180-2189.

Ren K, Dubner R (2002) Descending modulation in persistent pain: an update. Pain 100:1-6.

Traub RJ, Silva E, Gebhart GF, Solodkin A (1996) Noxious colorectal distention induced-c-Fos protein in limbic brain structures in the rat. Neuroscience letters 215:165-168.

Warren JW, Howard FM, Cross RK, Good JL, Weissman MM, Wesselmann U, Langenberg P, Greenberg P, Clauw DJ (2009) Antecedent nonbladder syndromes in case-control study of interstitial cystitis/painful bladder syndrome. Urology 73:52-57.

Xu J, Zhu Y, Contractor A, Heinemann SF (2009) mGluR5 has a critical role in inhibitory learning. J Neurosci 29:3676-3684.

Zhang F, Gradinaru V, Adamantidis AR, Durand R, Airan RD, de Lecea L, Deisseroth K (2010) Optogenetic interrogation of neural circuits: technology for probing mammalian brain structures. Nature protocols 5:439-456.

Chapter 5

<u>Conclusions and Future Directions</u>

Overview on the Anatomical and Molecular Examination of Bladder Pain

Although the etiology of IC/PBS is unknown, there are many hypotheses on how IC/PBS develops (See chapter 1, Figure 1.1) (Keay, 2008; Hanno et al., 2010; Birder, 2011). Understanding the molecular modulation of bladder pain is vital to the development of therapeutic targets. The overall goal of this thesis is to increase understanding of how bladder pain is modulated in two anatomical areas; the bladder and in the amygdala. In chapter 2 of this thesis, we test the hypothesis that distinct bladder injuries differentially affect bladder pain. Using three distinct models of bladder injury, our results demonstrate that not all bladder injuries result in increased sensitivity to bladder distention. Previous work has suggested that mGluR5 in the CNS may modulate non-inflammatory bladder pain (Hu et al.). In chapter 3, we test the hypothesis that mGluR5 is necessary for the full expression of inflammatory and non-inflammatory bladder pain. We found that systemic deletion or inhibition of mGluR5 significantly reduced the evoked response to bladder distention. Furthermore, systemic inhibition of mGluR5 was analgesic to distention-induced inflammatory and non-inflammatory bladder pain. However, mGluR5 is expressed throughout the pain neuroaxis. In chapter 4 of this thesis, we test the hypothesis that amygdala mGluR5 is necessary for the modulation of bladder pain. These data suggest that mGluR5 in the central nucleus of the amygdala is necessary for the full expression of distention-induced bladder pain. Furthermore, these data suggest that mGluR5 in the amygdala has a role in descending modulation of nociception in the spinal cord.

Differential Injury to the Bladder Results in Distinct Evoked-Responses to Bladder Distention

Bladder pain has been extensively modeled through the use of noxious inflammation-inducing chemical agents such as hydrochloric acid, turpentine, mustard oil, LPS, and CYP (Westropp and Buffington, 2002). However, the nature of the injury to the urothelium that results in increased pain after bladder distention was undetermined. To accomplish this goal, we examine how three different bladder injury models affect the nocifensive response to bladder distention. Using these distinct injury models we discovered that not all bladder injuries lead to increased pain-like responses to bladder distention. Furthermore, the results from chapter 2 highlight that careful

correlation of urine cytology and bladder histology with VMR measurements are key in determining how differential injuries affect the pain response to bladder distention.

Previous studies have shown that UPEC infection causes inflammation and activation of the basal stem cells and barrier disruption (Mysorekar et al., 2009). As described in chapter 2, our results show that infection with UPEC strain UTI89 also causes increased evoked-responses to bladder distention. Taken together, these data suggest that the combination of inflammation, activation of the basal cell layer and loss of barrier cells (SFCs) results in an increased sensitivity to bladder distention.

The increased sensitivity to bladder distention in UPEC-infected mice may be related to the binding of the bacterial product LPS, as demonstrated in a recent publication (Rudick et al., 2010). Therefore, we examined how chronic treatment with the bacterial endotoxin LPS (produced by E. coli O55:B5) modulates the response to bladder distention. As reported in chapter 2, chronic (4 day) treatment with the bacterial endotoxin LPS had no effect on bladder inflammation or the evoked response to bladder distention when compared to similarly treated controls. It was surprising to discover that chronic treatment with a highly immunogenic compound did not result in increased sensitivity to bladder distention or chronic inflammatory changes. The urine cytology from these mice suggests that the peak window of inflammation occurs on days 1 and 2 after LPS treatment, indicating that we had not measured the response to bladder distention during peak inflammation. Although this protocol has previously shown chronic inflammatory changes (Saban et al., 2002) using a much larger volume (200ul versus 50ul of 100mg/ml LPS), intravesicular volumes greater than 50ul increase the vesicoureteral reflux (Schaeffer et al., 1987; Hung et al., 2009) and cause pain that does not originate from the bladder. Increased pain and inflammatory changes from LPS in the ureters would complicate our hypothesis examining how injury to the bladder results in distention-induced bladder pain. For this reason, we used a more appropriate intravesicular volume of 50ul to induce chronic bladder inflammation. However, in our hands, 4 days of LPS treatment did not induce chronic inflammatory changes (Saban et al., 2002). This failure could be due to a reduced volume

decreasing the total amount of LPS instilled into the bladder. To test the hypothesis that chronic

LPS-induced inflammation is dose-dependent, we could repeat the urine cytology and histology

studies with an increased concentration of LPS (4x the concentration used previously 400mg/ml)

delivered in the 50ul volume. If this replicates the chronic inflammatory changes seen by Saban

et al (Saban et al., 2002), it would support the hypothesis that the chronic inflammatory changes

are dependent on the total LPS dosage. If we did not observe inflammatory changes at this

higher dose of LPS, then the chronic inflammatory changes previously reported might be the

result of LPS entering the ureters. Alternatively, the failure to develop inflammation after chronic

LPS treatment could be due to the type of LPS used. Recent work has demonstrated that

different strains of E. coli result in distinct levels of bladder pain, largely due to differences in LPS

composition (Rudick et al., 2010). Even though we used the same type of LPS used in the

previous study (Saban et al., 2002), it is possible that there are batch-dependent differences in

the LPS we obtained and the LPS used previously in 2002. To test the hypothesis that the lack of

observed chronic inflammation in LPS (strain O55:B5) treated mice was an LPS-dependent

effect, we could use LPS purified from UPEC (UTI89). Although infection with UPEC strain UTI89

is capable of inducing chronic bladder infection (Cusumano et al., 2011; Schwartz et al., 2011), it

is not certain that chronic treatment with LPS from UPEC UTI89 would necessarily result in

chronic inflammation. If we were unsuccessful at inducing chronic inflammation, with LPS-

derived from UPEC, we could still examine the differences in bladder pain responses to acute

LPS treatment and acute UPEC infection. Acute application of UPEC-derived LPS would

elucidate if acute UPEC-induced inflammation is due to LPS or another (LPS-independent)

mechanism.

In surprising contrast with UPEC treatment, treatment with protamine sulfate (PS) was analgesic

when compared to similarly treated PBS controls. PS treatment, at the dose used, is known to

induce exfoliation of the superficial cell layer of the urothelium without inflammation or activation

of the stem cell niche (Mysorekar et al., 2009). Beyond the important barrier function, there is

increasing evidence to suggest that the urothelium plays a key role in the detection and

transmission of physiological and nociceptive stimuli (Birder, 2011). It is possible that the

exfoliated superficial cells have a key role in the sensory function of the urothelium. Therefore, PS-induced exfoliation of SFCs may result in the bladder temporarily losing the ability to 'sense' distention-induced pain. This may be regulated by decreases in molecules such as IL-1β which known to directly activate nociceptors. We found that in mice treated with PS, the level of IL-1β in the bladder tissue was significantly reduced following PS-treatment. It has been demonstrated that IL-1β is produced by the bladder urothelium (Wood et al., 2011) and smooth muscle cells (Bouchelouche et al., 2006) in response to inflammatory injury. However, the specific IL-1β producing cells in the urothelium are not known. We could determine if SFCs are responsible to the production of IL-1β by quantifying the level of IL-1β in the SFCs that are exfoliated in the urine following PS treatment. If the exfoliated SFCs in PS express high amounts of IL-1β it could explain the dramatic decrease in cytokine expression. However, we would need to examine where in the urothelium IL-1β was being produced in UPEC treated mice since both UPEC and PS treated mice have SFC exfoliation.

Interestingly, in addition to a decreased sensitivity to bladder distention, PS treatment was protective against distention-induced damage to the bladder. The mechanism by which exfoliation of the superficial cells could protect the bladder against stretch-induced injury is unknown. While the bladder is uniquely tolerant of stretch, the pressures we used to elicit pain were supra-physiologic. One explanation for this is that the tight junctions formed by the superficial cell layer prevent stretching at very high pressures. Therefore, the chemically-induced exfoliation of the SFCs would allow the bladder to stretch past the point at which the tight-junctions would cause damage as a result of cell shearing. We could test this hypothesis using scanning electron microscopy to could compare the structure of a highly stretched urothelium with and without the presence of SFCs. If equal stretch of the urothelium induces the SFCs to shear at their tight junctions but causes no damage in samples that experienced shearing, it would support this hypothesis.

An additional observation reported in chapter 2 is that PS treated mice had frank hematuria following noxious distention despite less pain. In all mice with distention-induced hematuria,

pyuria (urine inflammation) was inversely correlated with the presence of hematuria (see supplementary figure 2.1). Scanning-electron microscopy of the stretched bladder urothelium might indicate if the hematuria in PS-treated mice is the result of shearing of the capillaries in the bladder wall.

The results presented in chapter 2 suggest that activation of the stem cell niche or inflammation has a role in the development of increased pain to bladder distention. UPEC treatment results in both stem cell activation and increased inflammation while PS treatment does not (Mysorekar et al., 2009). Furthermore, in our hands, LPS treatment did not cause increased inflammation or stem cell activation (data not shown). To test the hypothesis that increased bladder pain is the result activation of the stem cell niche and not inflammation; we could use a double dose treatment of PS (ddPS). This technique was recently established in the Mysorekar lab, and they demonstrate that ddPS induces both barrier damage and activation of the basal cell layer (stem cells) without inflammation (unpublished observation by KMS and IUM). If mice treated with ddPS had an increased VMR to bladder distention when compared to PBS-treated controls, then this would suggest that stem cell activation are, and inflammation is not, important for the development of distention-induced bladder pain. If ddPS treated mice had a reduced response to bladder distention, it might suggest that barrier disruption in the absence of inflammation is analgesic in this mouse model of bladder pain.

Overall, the results from chapter 2 demonstrate that UPEC-induced injury results in a significantly enhanced nociceptive response to bladder distention whereas chronic LPS treatment did not. Furthermore, UPEC infection but not chronic LPS treatment resulted in sustained bladder tissue inflammation and inflammatory efflux into the urines. Most intriguingly however, chemical injury with PS results in a reduced pain response to bladder distention and protection from distention-induced damage to the bladder architecture. This is the first report, to our knowledge, of an injury resulting in an analgesic response to bladder distention. Through the use of three injury models and analysis of the impact of these on nociceptive responses, we have differentiated unique molecular signals that may modulate bladder pain from those that modulate injury, including IL-6,

IL-1β, IL-4 and SOCS3. Taken together, our data suggest that 1) differential injury to the urothelium results in distinct nociceptive responses, with not all injuries leading to increased pain and 2) that detailed histological and urinary characterization in combination with analysis of pain responses is an innovative method for identification of novel mediators of visceral pain.

mGluR5 is Necessary for The Full Expression of Bladder Pain

A large body of evidence implicates mGluR5 in the development of hyperalgesia following a somatic inflammatory injury (Varney and Gereau, 2002; Cruz and Cruz, 2007; Montana et al., 2009; Kolber et al., 2010). Additionally, there is limited evidence suggesting a role for mGluR5 in visceral pain (Bianchi et al., 2003; Blackshaw et al., 2011), and in the modulation of non-inflammatory bladder pain (Hu et al., 2009). However, the role of mGluR5 in inflammatory bladder pain was undetermined. In chapter 3 of this thesis, we hypothesized that mGluR5 is an important modulator of inflammatory and non-inflammatory bladder pain. To test this hypothesis, we compared the VMR of mGluR5 KO mice to their WT littermates. mGluR5 KO mice had a significantly smaller evoked response to bladder distention when compared to WT littermate controls. Furthermore, systemic treatment with a specific antagonist of mGluR5 also the reduced evoked-response to bladder distention in WT mice. These results indicate that mGluR5 has an important role in the modulation of distention-induced (non-inflammatory) bladder pain. Furthermore, these results support the previous finding that a less-specific antagonist of mGluR5 is analgesic in distention-induced bladder pain (Hu et al., 2009). We then used an infectious inflammatory model (UPEC) of bladder injury to investigate if mGluR5 has a role in inflammatory bladder pain. We measured the VMR in mice with an UPEC-induced urinary tract infection, and then measured the VMR again following acute systemic treatment of with a specific mGluR5 antagonist (fenobam). UPEC-infected mice treated with fenobam has a reduced VMR when compared to the first round of bladder distention, suggesting that fenobam is analgesic. These data presented in chapter 3 suggest that mGluR5 is necessary for the full expression of inflammatory and non-inflammatory bladder pain.

To determine if mGluR5 modulates micturition reflexes, we examined the urodynamic profile of mGluR5 KO mice compared to WT mice. The intermicturition interval is the rate at which the mouse voids while the bladder is being filled at a constant rate and an increased IMI indicates that the mouse allows the bladder to fill longer before voiding. mGluR5 KO mice had a significantly increased intermicturition interval (IMI) when compared to their WT littermates. Furthermore, acute systemic administration of the mGluR5 antagonist fenobam significantly increased the IMI in WT mice. To continue this line of investigation, we would examine the IMI in mice with and without a UPEC bladder infection. Since bladder infection is known to increase the voiding frequency in humans (Foxman, 2010) we could examine cystometry in mice with and without UPEC-induced infection to determine if a urinary tract infection results in an increased voiding frequency. If UPEC infection increased voiding frequency and fenobam reduced the IMI in mice infected with UPEC, this may indicate that fenobam would be useful for treating overactive bladder. The data presented in chapter 3 suggests that fenobam may be a useful drug for the treatment of bladder pain and overactive bladder.

Together the results from chapter 3 support the hypothesis that mGluR5 has an important role in modulation of both inflammatory and non-inflammatory bladder pain. Previous work using an in-vitro bladder-nerve preparation demonstrated that an mGluR5 antagonist had no effect on distention-induced pelvic nerve firing (Hu et al., 2009). These results suggest that mGluR5 in the bladder proper is not the site of modulation of bladder pain (Hu et al., 2009). In addition, our preliminary rtPCR results of bladder mRNA suggesting that mGluR5 is not expressed in the bladder. Taken together, these data imply that mGluR5 in the CNS may be important for the modulation of bladder pain. We hypothesized, in chapter 4, that mGluR5 in the central nucleus of the amygdala (CeA) modulates distention-induced bladder pain.

mGluR5 in the CeA Modulates Distention-Induced Bladder Pain

mGluR5 has a pro-nociceptive role at multiple levels of the pain neuroaxis (Bhave et al., 2001; Walker et al., 2001; Neugebauer and Carlton, 2002; Neugebauer and Li, 2002; Varney and Gereau, 2002; Kolber et al., 2010). Recent work has demonstrated that mGluR5 in the central

nucleus of the right amygdala is important in the modulation of somatic inflammatory pain (Ji and Neugebauer, 2009; Kolber et al., 2010). In addition, recent work has implicated the CeA in visceral pain (Ji and Neugebauer, 2009, 2010; Li et al., 2011). In chapter 4 of this thesis, we hypothesized that mGluR5 in the CeA modulates bladder pain. Pharmacologic inhibition and genetic deletion of mGluR5 in the right CeA was analgesic in the distention-induced bladder pain model, supporting our hypothesis. However, we were surprised that inhibition of CeA mGluR5 reduced the VMR in naïve (uninjured) mice. Previous work has demonstrated that inhibition of mGluR5 in the CeA is only analgesic in the presence of an injury (Kolber et al., 2010; Li et al., 2011). Although multiple rounds of bladder distention results in urothelial injury, we have not observed an increased VMR when compared to baseline responses. We hypothesized that the injury to the bladder due to multiple rounds of distention may be allowing antagonism of mGluR5 in the CeA to have analgesic effects. We tested this hypothesis by injecting MPEP, an mGluR5 antagonist, into the CeA prior to bladder distention. We found that mice pretreated with MPEP had a reduced VMR when compared to mice pretreated with vehicle alone (figure 4.2). These data suggest that mGluR5 modulates bladder pain in the absence of injury, which is a novel finding. The apparent difference between these data and previously published work may highlight a distinction in the modulation of visceral and somatic pain or it may represent differences in the sensitivity of the pain tests used. Any difference in sensitivity may be attributable to the fact that the VMR measures an evoked response to bladder distention, while previous work measured the mechanical threshold necessary to induce a withdrawal response or ultrasonic vocalizations in response to knee-joint compression or colon distention. A second explanation for this apparent difference may be that mGluR5 modulation of bladder pain may be different than mGluR5 modulation of distention-induced colon pain. In support of this, previous work has demonstrated that mGluR5 has a direct effect on the firing rate of peripheral nerves in the colon and not the bladder (Hu et al., 2009).

To further support the hypothesis that mGluR5 in the CeA is necessary for the full expression of bladder pain, we examined how activation of mGluR5 would affect the VMR. We pharmacologically activated group I mGluRs (mGluR5 and mGluR5) in the right CeA, and found

that this increased the VMR to bladder distention. Activation of mGluR5 in the CeA increased the VMR, while inhibition or conditional deletion of mGluR5 in the CeA lead to a decrease in the VMR. Together, these data support the hypothesis that mGluR5 in the right CeA is necessary for the full expression of distention-induced bladder pain. Furthermore, these data suggest that activation of mGluR5 in the right CeA is sufficient to increase sensitivity to bladder distention. We only examined the role of mGluR5 in the right CeA because of previous work demonstrating right hemispheric lateralization of pain modulation (Carrasquillo and Gereau, 2008; Ji and Neugebauer, 2009), and specifically of mGluR5 modulation of amygdala nociceptive processing (Kolber et al., 2010). However, it is unknown if mGluR5 in the left amygdala has an important role in modulating bladder pain. Future work could examine if pharmacologic or genetic inhibition of mGluR5 in the left CeA is sufficient to reduce the VMR to bladder distention. If inhibition of mGluR5 in the left CeA resulted in a similar reduction in the VMR to bladder distention, this may suggest that the processing/modulation of bladder pain does not undergo hemispheric lateralization. Interestingly, a recent publication found no lateralization in corticosterone-induced visceral hypersensitivity to colorectal distention (Tran and Greenwood-Van Meerveld, 2012).

Our results suggest that activation of mGluR5 in the right CeA is sufficient to increase the evoked response to bladder distention. However, it is unknown how mGluR5 CeA activation leads to changes the spinal-evoked bladder pain reflex. Possible mechanisms include increasing synaptic transmission or increased neuronal excitability. We hypothesized that mGluR5 activation may lead to increased excitability of CeA neurons and thus an increase in output from the CeA. To test this hypothesis, we optogenetically activated the right CeA and examined its effect on the VMR. We found that an increase in CeA activity leads to an increased VMR in response to bladder distention. These data support the hypothesis that activation of mGluR5 in the CeA modulates the VMR through increased activity of CeA neurons.

In addition to a role for CeA mGluR5 in the modulation of bladder pain, we hypothesized that activation of mGluR5 in the CeA leads to descending modulation of neurons in the spinal cord. Previous work has demonstrated that chronic corticosterone implants in the CeA lead to both an

increased VMR to noxious bladder distention and increased excitability of visceral afferent neurons in the spinal cord (Qin et al.; Myers and Greenwood-Van Meerveld). Furthermore, recent work by our lab found ERK activation in the lumbosacral spinal cord following noxious bladder distention (Lai et al., 2011). Furthermore, inhibition of ERK activation at the level of the spinal cord was analgesic in both inflammatory (cyclophosphamide-induced) and non-inflammatory distention-induced bladder pain (Lai et al., 2011). However, it was unknown if mGluR5 in the right CeA modulates activation ERK in the lumbosacral spinal cord. We found that noxious bladder distention results in ERK activation in the lumbosacral spinal cord, but not in the amygdala. Mice lacking mGluR5 expression in the right CeA (mGluR5CKO) had less distention-induced ERK activation in the spinal cord when compared to mice with normal mGluR5 expression. This suggests a relationship between descending activity from the CeA and distention-induced ERK activation. We hypothesized if mGluR5 in the CeA modulates distention-induced ERK activation in the spinal cord through increased CeA activity, then optogenetic activation of the CeA alone would not affect ERK activation in the lumbosacral spinal cord. Our results support this hypothesis. We found that optogenetic activation alone of the right CeA had no effect on ERK activation in the spinal cord, suggesting that increased CeA activity only modulates distention-induced ERK activation in the spinal cord. Future work could focus on understanding how mGluR5 in the amygdala modulates ERK activation in the spinal cord. To confirm this link, we could activate mGluR5 in the CeA with and without noxious bladder distention and examine ERK activation in the spinal cord. If activation of mGluR5 in the CeA coupled to noxious bladder distention resulted in higher levels of ERK activation in the spinal cord when compared to noxious bladder distention alone, this would support the hypothesis that mGluR5 in the CeA modulates distention-induced ERK activation in the spinal cord.

The descending arm of the pain neuroaxis may follow the PAG to the RVM, and is connected to the spinal cord via the dorsolateral funiculus (DLF) (Basbaum et al., 1978; Basbaum and Fields, 1979). Descending modulation of visceral pain has been demonstrated to descend the spinal cord via the DLF (Zhuo and Gebhart; Gebhart). To determine if the CeA descending modulation of ERK activation travels through the DLF, we could lesion the DLF and examine how this lesion

affected CeA mGluR5 modulation of bladder pain. If a lesion of the DLF prevented CeA mGluR5 modulation of ERK activation in the spinal cord, then this would support our hypothesis that mGluR5 in the CeA modulates distention-induced ERK activation in the spinal cord. Additionally, we could lesion the DLF and assess whether inhibition or activation of mGluR5 in the CeA affected VMR responses. If mGluR5 in the CeA modulates bladder pain by way of the DLF, then activation or inhibition of mGluR5 in the CeA would have no effect on the VMR in mice with a DLF lesion. Future work could also examine which cell types in both the amygdala and spinal cord are involved in this pathway. A first step in identifying the relevant cell types would be staining for cell-specific markers and the co-localization of mGluR5 in the CeA. Additionally, we could look for co-localization of cell-specific markers and distention-induced phosphorylated (activated) ERK in the lumbosacral spinal cord. This information would be helpful in determine which cell types may be important in regulating the downstream action of mGluR5.

An additional area of future study could examine the secondary messengers downstream of mGluR5 in the CeA that have a role in the modulation of bladder pain. Previous work has shown that mGluR5 activation couples through $G_{q/11}$, leading to activation of phospholipase C (PLC), phosphoinositide (PI) hydrolysis, and protein kinase C (PKC) activation (Gereau and Heinemann, 1998; Ferguson et al., 2008).   However, recent work in the CeA demonstrated that reactive oxygen species (ROS) link mGluR5 to ERK and PKA through an IP_3-dependent and PKC-independent mechanism (Li et al., 2011). We could determine if this PKC-independent pathway exists in bladder pain by determining if ROS scavengers block the increased VMR induced by an mGluR5 agonist. We could also block PKA, PKC or IP_3 in the CeA and see how they modulate the VMR. To determine the downstream signaling from mGluR5 in the CeA that modulates ERK activation in the spinal cord, we could examine how intra-amygdala ROS scavengers, PKA or IP_3 inhibitors modulate distention-induced ERK activation in the spinal cord.

Targeting mGluR5 to treat IC/PBS?

The results of this thesis suggest that bladder pain is modulated at distinct anatomical levels. Furthermore, these data suggest that distention-induced bladder pain is modulated by mGluR5

activation. The specific mGluR5 antagonist, fenobam, reduced the VMR to bladder distention in mice with and without bladder inflammation. In addition, fenobam increased the IMI (see chapter 3). These results suggest that mGluR5 might be a good molecular target for treatment of IC/PBS or overactive bladder. Furthermore, the results of chapter 4 strongly suggest that mGluR5 in the CeA has a key role in bladder pain modulation. Inhibition of mGluR5 in the right CeA reduced the VMR to a similar extent as systemic administration of fenobam. Overall, these data suggest that mGluR5, including activation in the CeA, is an important modulator of bladder pain.

REFERENCES

Basbaum AI, Fields HL (1979) The origin of descending pathways in the dorsolateral funiculus of
the spinal cord of the cat and rat: further studies on the anatomy of pain modulation. The
Journal of comparative neurology 187:513-531.

Basbaum AI, Clanton CH, Fields HL (1978) Three bulbospinal pathways from the rostral medulla
of the cat: an autoradiographic study of pain modulating systems. The Journal of
comparative neurology 178:209-224.

Bhave G, Karim F, Carlton SM, Gereau RW (2001) Peripheral group I metabotropic glutamate
receptors modulate nociception in mice. Nature neuroscience 4:417-423.

Bianchi R, Rezzani R, Borsani E, Rodella L (2003) mGlu5 receptor antagonist decreases Fos
expression in spinal neurons after noxious visceral stimulation. Brain research 960:263-
266.

Birder LA (2011) Urothelial signaling. Handb Exp Pharmacol:207-231.

Blackshaw LA, Page AJ, Young RL (2011) Metabotropic glutamate receptors as novel therapeutic
targets on visceral sensory pathways. Front Neurosci 5:40.

Bouchelouche K, Alvarez S, Horn T, Nordling J, Bouchelouche P (2006) Human detrusor smooth
muscle cells release interleukin-6, interleukin-8, and RANTES in response to
proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha. Urology
67:214-219.

Carrasquillo Y, Gereau RW (2008) Hemispheric lateralization of a molecular signal for pain
modulation in the amygdala. Molecular pain 4:24.

Cruz CD, Cruz F (2007) The ERK 1 and 2 pathway in the nervous system: From basic aspects to
possible clinical applications in pain and visceral dysfunction. Current
Neuropharmacology 5:244-252.

Cusumano CK, Pinkner JS, Han Z, Greene SE, Ford BA, Crowley JR, Henderson JP, Janetka
JW, Hultgren SJ (2011) Treatment and prevention of urinary tract infection with orally
active FimH inhibitors. Sci Transl Med 3:109ra115.

Ferguson AR, Bolding KA, Huie JR, Hook MA, Santillano DR, Miranda RC, Grau JW (2008)
Group I metabotropic glutamate receptors control metaplasticity of spinal cord learning
through a protein kinase C-dependent mechanism. J Neurosci 28:11939-11949.

Foxman B (2010) The epidemiology of urinary tract infection. Nat Rev Urol 7:653-660.

Gebhart GF (2004) Descending modulation of pain. Neuroscience and biobehavioral reviews
27:729-737.

Gereau RWt, Heinemann SF (1998) Role of protein kinase C phosphorylation in rapid
desensitization of metabotropic glutamate receptor 5. Neuron 20:143-151.

Hanno P, Lin A, Nordling J, Nyberg L, van Ophoven A, Ueda T, Wein A, Bladder Pain Syndrome
Committee of the International Consultation on I (2010) Bladder Pain Syndrome
Committee of the International Consultation on Incontinence. Neurourol Urodyn 29:191-
198.

Hu Y, Dong L, Sun B, Guillon MA, Burbach LR, Nunn PA, Liu X, Vilenski O, Ford AP, Zhong Y,
Rong W (2009) The role of metabotropic glutamate receptor mGlu5 in control of
micturition and bladder nociception. Neuroscience letters 450:12-17.

Hung CS, Dodson KW, Hultgren SJ (2009) A murine model of urinary tract infection. Nat Protoc
4:1230-1243.

Ji G, Neugebauer V (2009) Hemispheric lateralization of pain processing by amygdala neurons.
Journal of neurophysiology 102:2253-2264.

Ji G, Neugebauer V (2010) Reactive oxygen species are involved in group I mGluR-mediated
facilitation of nociceptive processing in amygdala neurons. Journal of neurophysiology
104:218-229.

Keay S (2008) Cell signaling in interstitial cystitis/painful bladder syndrome. Cell Signal 20:2174-
2179.

Kolber BJ, Montana MC, Carrasquillo Y, Xu J, Heinemann SF, Muglia LJ, Gereau RW (2010)
Activation of metabotropic glutamate receptor 5 in the amygdala modulates pain-like
behavior. J Neurosci 30:8203-8213.

Lai HH, Qiu CS, Crock LW, Morales ME, Ness TJ, Gereau RW (2011) Activation of spinal

extracellular signal-regulated kinases (ERK) 1/2 is associated with the development of

visceral hyperalgesia of the bladder. Pain 152:2117-2124.

Li Z, Ji G, Neugebauer V (2011) Mitochondrial reactive oxygen species are activated by mGluR5

through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and

pain behavior. J Neurosci 31:1114-1127.

Montana MC, Cavallone LF, Stubbert KK, Stefanescu AD, Kharasch ED, Gereau RW (2009) The

metabotropic glutamate receptor subtype 5 antagonist fenobam is analgesic and has

improved in vivo selectivity compared with the prototypical antagonist 2-methyl-6-

(phenylethynyl)-pyridine. The Journal of pharmacology and experimental therapeutics

330:834-843.

Myers B, Greenwood-Van Meerveld B (2010) Elevated corticosterone in the amygdala leads to

persistent increases in anxiety-like behavior and pain sensitivity. Behavioural brain

research 214:465-469.

Mysorekar IU, Isaacson-Schmid M, Walker JN, Mills JC, Hultgren SJ (2009) Bone morphogenetic

protein 4 signaling regulates epithelial renewal in the urinary tract in response to

uropathogenic infection. Cell Host Microbe 5:463-475.

Neugebauer V, Carlton SM (2002) Peripheral metabotropic glutamate receptors as drug targets

for pain relief. Expert Opin Ther Targets 6:349-361.

Neugebauer V, Li W (2002) Processing of nociceptive mechanical and thermal information in

central amygdala neurons with knee-joint input. Journal of neurophysiology 87:103-112.

Qin C, Greenwood-Van Meerveld B, Foreman RD (2003) Spinal neuronal responses to urinary

bladder stimulation in rats with corticosterone or aldosterone onto the amygdala. Journal

of neurophysiology 90:2180-2189.

Rudick CN, Billips BK, Pavlov VI, Yaggie RE, Schaeffer AJ, Klumpp DJ (2010) Host-pathogen

interactions mediating pain of urinary tract infection. J Infect Dis 201:1240-1249.

Saban MR, Nguyen NB, Hammond TG, Saban R (2002) Gene expression profiling of mouse bladder inflammatory responses to LPS, substance P, and antigen-stimulation. Am J Pathol 160:2095-2110.

Schaeffer AJ, Schwan WR, Hultgren SJ, Duncan JL (1987) Relationship of type 1 pilus expression in Escherichia coli to ascending urinary tract infections in mice. Infect Immun 55:373-380.

Schwartz DJ, Chen SL, Hultgren SJ, Seed PC (2011) Population dynamics and niche distribution of uropathogenic Escherichia coli during acute and chronic urinary tract infection. Infect Immun 79:4250-4259.

Tran L, Greenwood-Van Meerveld B (2012) Lateralized amygdala activation: Importance in the regulation of anxiety and pain behavior. Physiology & behavior 105:371-375.

Varney MA, Gereau RW (2002) Metabotropic glutamate receptor involvement in models of acute and persistent pain: prospects for the development of novel analgesics. Curr Drug Targets CNS Neurol Disord 1:283-296.

Walker K, Reeve A, Bowes M, Winter J, Wotherspoon G, Davis A, Schmid P, Gasparini F, Kuhn R, Urban L (2001) mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurones mediate inflammatory hyperalgesia. Neuropharmacology 40:10-19.

Westropp JL, Buffington CA (2002) In vivo models of interstitial cystitis. The Journal of urology 167:694-702.

Wood MW, Breitschwerdt EB, Nordone SK, Linder KE, Gookin JL (2011) Uropathogenic E. coli Promote a Paracellular Urothelial Barrier Defect Characterized by Altered Tight Junction Integrity, Epithelial Cell Sloughing and Cytokine Release. J Comp Pathol.

Zhuo M, Gebhart GF (2002) Facilitation and attenuation of a visceral nociceptive reflex from the rostroventral medulla in the rat. Gastroenterology 122:1007-1019.

LARA WILEY CROCK

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EDUCATION

Medical Scientist Training Program Washington University, Saint Louis, MO	**2002-2005,** **2008-2013**
Barnard College, NY, NY **B.A. in Biochemistry** Honors Thesis: Role of CD36 in macrophage-mediated hydrogen peroxide production	**1997-2001**

AWARDS

National Institutes of Health NIDDK Ruth L. Kirschstein Predoctoral National Research Service Award #F30DK89969	**2010-2013**
Graduated with Honors, Magna Cum Laude	**2001**
The Marie Reimer Prize in Chemistry	**2000**
Dean's List	**1997-2001**

OTHER EXPERIENCES

Participated in Boston University Summer Institute in Geriatric Medicine	**2008**

RESEARCH EXPERIENCE/EMPLOYMENT HISTORY

Columbia University, NY, NY Role of CD36 in macrophage-mediated production of hydrogen peroxide in heart disease Laboratory of Dr. Samuel Silverstein M.D.	**1999-2001**
Massachusetts General Hospital, Boston, MA Cell cycle and ionizing radiation-induced DNA damage Laboratory of Dr. Simon Powell M.D.	**2001-2002**
Washington University, Saint Louis, MO Roles of Eubacterium Elegans and Eubacteium Rectale in the gut microbial environment Laboratory of Dr. Jefferey Gordon M.D.	**2003-2005**
Washington University, Saint Louis, MO Role of mGluR5 in Bladder Nociception Laboratory of Dr. Robert Gereau Ph.D.	**July 2008-** **Jan 2012**

TEACHING EXPERIENCE

Teaching Assistant- Histology Laboratory Washington University in St. Louis School of Medicine	**2004**

ABSTRACTS/POSTERS

Crock LW; Silverstein SC; Sethy-Coraci I. Sustained production of hydrogen peroxide by human mononuclear phagocytes cultured on collagen IV matrices containing oxidized low-density lipoproteins. Annual American Society of Cell Biology Meeting. December 8-12 2001, Abstract #195A.

Crock LW, Qui CS, Lai HH, and Gereau RW. The Role of Metabotropic Glutamate Receptor 5 (mGluR5) in Bladder Pain. MSTP 40th Anniversary Poster Session, April 9th 2010.

Crock LW, Stemler KM, Qiu CS, Song DG, Lai HH, Mysorekar IU, Gereau RW. mGluR5 is necessary for the full expression of both inflammatory and non-inflammatory bladder pain. Society for Neuroscience Annual Meeting in Washington D.C., November 11-15th 2011, Poster #180.08/OO21

Lai HH, Qiu CS, **Crock LW**, Morales MEP, Ness TJ, Gereau RW. Activation of spinal extracellular signal-regulated kinases (ERK) 1/2 is associated with the development of visceral hyperalgesia of the bladder. Society for Neuroscience Annual Meeting in Washington D.C., November 11-15th 2011, Poster #584.08/JJ1

Crock LW, Song DG, Abbosh P, Qiu CS, Gereau RW, Lai HH. Metabotropic glutamate receptor subtype 5 (mGluR5) in micturition and bladder pain. Society for Urodynamics and Female Urology Annual Meeting in New Orleans LA, Feburary 28th-March 3rd 2012

Crock LW, Kolber BJ, Morgan CD, Vogt SK, Lai HH, Bruchas MR, Gereau RW. Role of mGluR5 in bladder pain. America Pain Society Annual Meeting in Honolulu HI, May 16th-19th 2012, Poster #326

Crock LW, Kolber BJ, Morgan CD, Vogt SK, Bruchas MR, Gereau RW. Central amygdala mGluR5 in the modulation of visceral pain. Society for Neuroscience Annual Meeting in New Orleans LA, October 13th-17th 2012, Poster #473.19/HH9

PUBLICATIONS

Sethy-Coraci I, **Crock LW**, Silverstein SC (2005) PAF-receptor antagonists, lovastatin, and the PTK inhibitor genistein inhibit H2O2 secretion by macrophages cultured on oxidized-LDL matrices. J Leukoc Biol 78(5):1166-74.

Mahowald MA, Rey FE, Seedorf H, Turnbaugh PJ, Fulton RS, Wollam A, Shah N, Wang C, Magrini V, Wilson RK, Cantarel BL, Coutinho PM, Henrissat B, **Crock LW**, Russell A, Verberkmoes NC, Hettich RL, Gordon JI (2009) Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla. Proc Natl Acad Sci USA. 106(14):5859-64.

Lai HH, Qiu CS, **Crock LW**, Morales MEP, Ness TJ, Gereau RW (2011) Activation of spinal extracellular signal-regulated kinases (ERK) 1/2 is associated with the development of visceral hyperalgesia of the bladder. Pain 152(9):2117-2.

Crock LW, Stemler KM, Song DG, Abbosh P, Vogt SK, Qiu CS, Mysorekar IU, Lai HH, Gereau RW (2012). Metabotropic glutamate receptor 5 (mGluR5) regulates bladder nociception. Mol Pain: March 26: 8:20.

Crock LW, Kolber BK, Morgan CD, Sadler KE, Vogt SK, Bruchas MR, Gereau RW (2012). Central amygdala metabotropic glutamate receptor 5 in the modulation of visceral pain. J Neurosci 32(41):14217-26.

Crock LW, Stemler KM, Lai HH, Mills JC, Gereau RW, Mysorekar IU (2013) Protamine sulfate induced bladder injury protects from distention induced bladder pain. J Urology 189(1):343-51.

TALKS

Woman's Reproductive Health Seminar, Invited Speaker   **October 26th 2011**

VOLUNTEER WORK

Saturday Neighborhood Health Clinic- Free student-run health clinic   **2002-2003, 2008**

Neuroscience Outreach   **2008, 2009**