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Activation of spinal extracellular signal-regulated kinases (ERK) 1/2 is associated with the development of visceral hyperalgesia of the bladder

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Abstract

Activation of extracellular signal-regulated kinases (ERK) 1/2 in dorsal horn neurons is important for the development of somatic hypersensitivity and spinal central sensitization after peripheral inflammation. However, data regarding the roles of spinal ERK1/2 in the development of visceral hyperalgesia are sparse. Here we studied the activation of ERK1/2 in the lumbosacral spinal cord following innocuous and noxious distention of the inflamed (cyclophosphamide-treated) and non-inflamed urinary bladder in mice. We also correlated the spinal ERK1/2 activation to distention-evoked bladder nociception as quantified by the abdominal visceromotor response (VMR). Cyclophosphamide treatment (bladder inflammation) evoked increased bladder hyperalgesia and allodynia to bladder distention, as evident from an upward and leftward shift of the VMR stimulus-response curve compared to that of non-inflamed mice. Development of bladder hyperalgesia was associated with robust enhancement of ERK1/2 activation in the dorsal horn and deeper laminae bilaterally in the L6-S1 spinal cord. Functional blockade of spinal ERK1/2 activity via intrathecal administration of the upstream MEK inhibitor U0126 attenuated distention-evoked bladder nociception, and caused a significant downward shift of the VMR stimulus-response curve. In summary, we have provided functional and immunohistochemical evidence that activation of lumbosacral spinal ERK1/2 is associated with the development of primary visceral (bladder) hyperalgesia. Our results suggest that aberrant processing of visceral nociceptive information at the level of the lumbosacral spinal cord via activation of ERK1/2 signaling may contribute to chronic bladder pain in the context of inflammation.

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Keywords

Urinary Bladder; Visceral Pain; Inflammatory Pain; Visceromotor Response; ERK Signaling; Central Sensitization

Introduction

Extracellular signal-regulated kinases (ERK) belong to a family of serine/threonine kinases (mitogen-activated protein kinases, MAPK) that regulate intracellular signal transduction to a variety of stimuli [13; 35]. Activation of ERK (by phosphorylation) via MEK (MAPK/ERK kinase) leads to the regulation of gene transcription [6; 12; 24] as well as acute post-translational changes such as phosphorylation of ion channels [1; 17; 18]. In the nervous system, these neuromodulatory effects may contribute to long-term changes in neural plasticity [22]. Recent evidence suggests that ERK signaling also modulates nociceptive processing in somatic pain models [25]. After noxious stimulation of the hind paw (e.g. with formalin-induced inflammation), ERK1 and ERK2 are rapidly activated in dorsal horn neurons [2; 15; 23; 27; 28]. ERK activation leads to phosphorylation and modulation of K_v4.2-containing ion channels, increased dorsal horn neuronal excitability, and spinal central sensitization. Blockade of ERK1/2 activity attenuates mechanical hyperalgesia and spinal neuronal hyperexcitability that is induced in the context of acute or persistent inflammation [2; 17–20; 26; 27].

Previous studies have shown that spinal ERK1/2 activation is associated with the development of somatic hyperalgesia and central sensitization after peripheral inflammation. However, data regarding the potential roles of spinal p-ERK1/2 in the development of visceral hyperalgesia are sparse. In a colon inflammation model, ERK1/2 activation is associated with the development of *secondary* mechanical hyperalgesia of the abdominal wall and the hind paw [14]. However, it is unclear whether ERK activation is associated with the development of *primary* hyperalgesia of the visceral organ itself (e.g. increased nociception to colon distention), or whether the association may apply to the urinary bladder. In the colon inflammation study, the secondary mechanical hyperalgesia was reversed by systemic blockade of ERK1/2 activity [14]. Since systemic MEK/ERK inhibition (intravenous administration of U0126) was performed, it was unclear whether central or peripheral p-ERK, or both, contributes to visceral hyperalgesia. More recently, immunohistochemical studies revealed that lumbosacral ERK1/2 is phosphorylated following distention and/or inflammation of the bladder, suggesting that spinal ERK is activated after increased bladder sensory input [10–12]. However, without any quantification of bladder nociception (e.g. using visceromotor response, VMR) or pharmacologic ERK blockade (e.g. using intrathecal U0126), the functional significance of spinal ERK activation in the context of bladder nociception/hyperalgesia cannot be ascertained. In this study, we investigate the functional association between spinal ERK1/2 activation and the development of *primary* visceral hyperalgesia of the bladder. Bladder inflammation and/or phasic distention were used. Spinal ERK activation and bladder nociception was quantified by immunostaining and the VMR, respectively, in the presence and absence of intrathecal MEK/ERK inhibitors.

Materials and Methods

All animal experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP published in PAIN® [41]. The experimental protocol was approved by the Washington University Institutional Animal Care and Use Committee. Female C57BL/6 mice aged 12–14 weeks were used for all experiments. They were bred in the Washington

University Animal Facility from mice originally obtained from Jackson Laboratory. Animals were maintained on a 12h light/dark cycle and allowed ad libitum access to food and water.

1. Cyclophosphamide-induced cystitis, phasic bladder distention, and VMR measurements

The metabolite of cyclophosphamide, acrolein, is a potent chemical irritant and causes intense bladder inflammation [3; 4]. Chronic cyclophosphamide (CYP) cystitis was induced by intraperitoneal injection of CYP (75 mg/kg) on days 0, 3, 6, and 9 into 12 to 14 week-old female C57BL/6 mice (19–23g). Control mice received intraperitoneal saline injections on the same schedule. On day 10, the mice were anesthetized with 2% isoflurane. Silver wire electrodes were placed on the superior oblique abdominal muscle and subcutaneously across the abdominal wall (as a ground) to allow differential amplification of the abdominal visceromotor response (VMR) signals. A lubricated angiocatheter was inserted into the bladder via the urethra for bladder distention. After completion of the surgical preparation, isoflurane anesthesia was reduced to ~0.875% until a flexion reflex response was present (evoked by pinching the paw) but spontaneous escape behavior and righting reflex were absent. The animals were not restrained in any fashion. Body temperature was maintained using an overhead radiant light and monitored throughout the experiment. The conditions were optimized to establish a stable depth of anesthesia and consistent baseline VMR activity. 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. The distention stimulus applied 20–80 mmHg pressure for 20 seconds every 2 min. The VMR signal was relayed in real-time using a Grass CP511 preamplifier to a PC via a WinDaq DI-720 module. The 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 s to quantify the area under the curve (see area A in Fig 1 inset). The VMR was presented in arbitrary units. The investigator who quantified the VMR was blinded to treatment.

Bladder nociception was quantified by the abdominal VMR, which is the electromyographic response of the abdominal muscle to noxious bladder stimulation. Behaviorally, the VMR corresponds to the abdominal withdrawal and guarding reflex when the animal experiences pain. While it is acknowledged that the VMR may not exclusively reflect a response to nociceptive processing, it is a validated technique to measure visceral pain and has been used in numerous studies as a pseudo-affective surrogate measure of bladder nociception [5; 21; 31; 32; 34; 40]. The VMR is potentiated by pro-nociceptive processes (e.g. inflammation), shows a dose-response relation with distention pressure, and is inhibited by analgesics (e.g. intravesical lidocaine or systemic morphine) [31; 32].

2. Spinal cord phospho-ERK1/2 immunoreactivity

Female C57BL/6 mice treated with either CYP or saline (on days 0, 3, 6, 9) underwent a series of phasic bladder distentions on day 10. Animals received noxious bladder distention (60 mmHg, 20 s each, 5 distentions) or innocuous bladder distention (20 mmHg, 20 s each, 5 distentions). Immediately after the 5th distention cycle, the mice were perfused transcardially with warm saline, followed by 50 ml of ice-cold 4% paraformaldehyde solution. The L6-S1, L1-L2, and cervical (control) spinal cord sections were removed and post-fixed overnight at 4°C for 4 hours in 4% paraformaldehyde, followed by cryoprotection at 4°C in 30% sucrose. Transverse sections (40 µm) were cut using a cryostat and sections were transferred to PBS (pH 7.4) for immunohistochemical staining. The primary antibodies only recognize the activated (phosphorylated) forms of ERK1/2 (p-ERK1/2) but not total ERK1/2. Spinal p-ERK1/2 immunostaining was performed according to published protocols [27]. After rinsing, sections were blocked for 1 hr in 5% normal donkey serum with PBST

containing 0.3% Triton X-100, incubated at 4°C overnight in rabbit anti-human phospho-ERK1/2 (p44/p42sMAPK) antibodies (1:4000 dilution, Cell Signaling Technology). Sections were then rinsed in PBST and incubated with Cy3-conjugated fluorescent anti-rabbit IgG secondary antibodies at room temperature for 1.5 hours (1:600 dilution, Jackson ImmunoResearch). Sections were mounted onto glass slides, coverslipped and observed for evidence of p-ERK1/2 immunostaining. The number of p-ERK1/2 positive cells was quantified in the lumbosacral L6-S1 spinal cord (n=7 saline 20 mmHg, n=7 saline 60 mmHg, n=6 CYP 20 mmHg, n=8 CYP 60 mmHg).

We have included animals from different treatment groups and harvested their spinal cords on the same day. To ensure uniform staining intensity across sections from different treatment groups, we processed all the cryoprotected cords and applied primary and secondary antibodies to the sections together on the same day. The researcher who quantified the number of p-ERK1/2 positive cells was blinded to the treatment groups to reduce any bias in counting.

3. Intrathecal pharmacologic experiments

Female C57BL/6 mice were treated with intraperitoneal CYP (75 mg/kg) or saline (control) on days 0, 3, 6, and 9 as described above. On day 10, in addition to silver electrode placement for VMR measurement and urethral catheter placement for bladder distention, a catheter was also placed acutely into the intrathecal space. A longitudinal incision was made over the spine to expose the L5–L6 intervertebral space. A tapered P10 catheter was then threaded into the subarachnoid space, sutured onto paraspinal muscle, and externalized. Phasic bladder distention (20–80 mmHg, 20 s distention, every 2 min) was performed and the baseline VMR was recorded. The MEK inhibitor U0126 (2 µg in 5 µL 0.8% DMSO; Tocris) or U0124 (negative control analog, 2 µg in 5 µL 0.8% DMSO; Tocris) was then delivered to the intrathecal space. A second set of phasic bladder distentions were then performed 30 minutes later, and the post-treatment VMR was compared to the baseline VMR. The intrathecal U0126 dose was chosen based on our previous publications on nocifensive behavioral experiments after peripheral inflammation [2]. We believe this dose will give maximal response without the risks of affecting gross motor function (e.g. rotarod performance) and muscle EMG activity (VMR) independent of the nociceptive response. Position of the intrathecal catheter was confirmed by injection of a dye after the animal was sacrificed. For experiments involving intrathecal drug administration, the investigator was blinded to the intrathecal drug treatment (U0126 vs. U0124). To confirm that intrathecal U0126 inhibits spinal p-ERK, CYP animals were pre-treated with either intrathecal U0126 or U0124. 30 minutes later, the bladder was distended with five cycles of 20s 60 mmHg distention. L6-S1 spinal cord was then fixed for p-ERK1/2 immunostaining.

Data Analysis

The areas under the curve of the rectified VMR signal were averaged for each group at each distention pressure level. Groups were compared using a two-way ANOVA with repeated measures and a Bonferroni post-test. A p-value <0.05 was considered significant. All statistical analyses were conducted using Graphpad Prism 5.01 software (La Jolla, CA).

Results

1. Cyclophosphamide induces bladder hyperalgesia and allodynia to bladder distention

Cyclophosphamide (CYP) was used to induce chronic inflammation of the bladder, while phasic bladder distention was used to evoke bladder/visceral nociception. Distention-evoked bladder nociception was quantified by the abdominal VMR, a pseudo-affective surrogate

measure of visceral pain. In saline treated mice (n=10), the VMR (area under the curve) was small when the distention pressure was within physiologic, innocuous range (≤ 20 –30 mmHg, Figure 1). When the bladder was distended with higher pressure, a significant increase of the VMR was observed, consistent with increasing nociception ($p < 0.0001$). Bladder inflammation (CYP treatment, n=8) augmented the VMR at each pressure, and shifted the VMR stimulus-response curve upwards and leftwards compared to saline treatment ($p < 0.0001$). This corresponded to the development of bladder hyperalgesia at noxious pressures (e.g. 60 mmHg) and bladder allodynia at innocuous pressures (e.g. 20 mmHg) after CYP treatment. These results demonstrate that CYP treatment induces bladder hyperalgesia and allodynia that is quantifiable by the VMR.

2. Development of bladder hyperalgesia is associated with L6-S1 spinal ERK1/2 activation

Next, we studied the activation of ERK1/2 in lumbosacral spinal cord in response to CYP-induced bladder inflammation and following noxious and innocuous distention of the bladder. As depicted in Figure 2, distending the bladder with 60 mmHg noxious pressure evoked robust ERK1/2 activation in the L6-S1 lumbosacral spinal cord in CYP-treated mice (CYP-60 mmHg). p-ERK1/2 immunoreactivity was not observed in cervical (C6) and thoracolumbar (L1–L2) spinal sections in CYP-treated animals when their bladder was distended with 60 mmHg. The staining intensity was consistent across all animals in each group.

We have quantified the number of p-ERK1/2-positive cells in the L6-S1 lumbosacral spinal cord (figure 3B). Significant increase in the total number of p-ERK1/2 positive cells was noted when the bladder was stimulated with both CYP inflammation and 60 mmHg noxious distention (comparing CYP-60 mmHg to saline-20 mmHg, $p < 0.01$). CYP treatment alone did not appear to be sufficient to increase the number of p-ERK1/2 positive cells (comparing saline-20 mmHg to CYP-20 mmHg, or saline-60 mmHg to CYP-60 mmHg, $p > 0.05$). When the distention pressure was increased from innocuous (20 mmHg) to noxious (60 mmHg), an increase in p-ERK1/2-positive cells was noted only in CYP animals (comparing CYP-20 mmHg to CYP-60 mmHg, $p < 0.05$), but not in saline-treated mice (comparing saline-20 mmHg to saline-60 mmHg, $p > 0.05$), suggesting ERK1/2 activation is evoked by noxious distention in “sensitized” bladders.

The specific areas of ERK1/2 activation were the deep dorsal commissure (DCM) around lamina X, spinal parasympathetic nuclei (SPN) around lamina V, lateral dorsal horn (LDH), and medial dorsal horn (MDH) bilaterally in the L6-S1 lumbosacral cord. These results demonstrate that spinal ERK1/2 is activated in multiple regions of the lumbosacral spinal cord during noxious bladder distention in CYP-treated animals. Significant changes were noted in the deeper DCM and SPN regions, but not in the superficial lateral and medial dorsal horns (figures 3C-F).

3. Functional blockade of spinal ERK1/2 activation attenuates distention-evoked bladder nociception

To further evaluate whether there is a functional association between spinal ERK1/2 activation and the development of bladder hyperalgesia, we used an upstream inhibitor of ERK signaling (MEK inhibitor) to block spinal ERK activation. MEK (MAPK/ERK kinase) is the upstream kinase that phosphorylates and activates ERK1/2. U0126 is a MEK inhibitor and thus inhibits ERK signaling. U0124 is an inactive structural analog of U0126 that does not inhibit ERK1/2 activation and serves as a negative control. Blockade of spinal ERK1/2 activity with intrathecal administration of the MEK inhibitor, U0126, significantly reduced noxious distention-evoked bladder nociception (VMR) in CYP-treated mice (Figure 4A, n=12). No significant reduction of the noxious distention evoked VMR was observed when

intrathecal U0124 (negative control analog) was administered to CYP-treated mice (Figure 4B, n=7). Intrathecal administration of U0126 had no effect on the performance of mice on an accelerating rotarod (not shown) suggesting that the change in VMR was not due to an effect on gross motor function. To confirm that intrathecal U0126 inhibits spinal p-ERK, CYP animals were pre-treated with either intrathecal U0126 or U0124. 30 minutes later, the bladder was distended with 60 mmHg. The number of L6-S1 spinal p-ERK1/2 positive cells was significantly lower after U0126 than after U0124 pre-treatment (Figure 5, p=0.0065). Our results provide functional evidence that spinal ERK1/2 activation is associated with the development of bladder hyperalgesia in a mouse model of bladder inflammation.

Discussion

Previous studies have shown that spinal ERK1/2 activation is associated with the development of somatic hyperalgesia and central sensitization after peripheral inflammation [2; 17–20; 26; 27]. However, data regarding the roles of spinal p-ERK1/2 in the development of visceral hyperalgesia are sparse. Recent studies revealed that lumbosacral ERK1/2 is phosphorylated following distention and/or inflammation of the bladder [10–12]. However, without any quantification of bladder nociception or the use of intrathecal MEK/ERK inhibitors, the functional significance of spinal ERK activation cannot be ascertained in those immunohistochemical studies. In the present study, we have provided unequivocal functional (VMR) and pharmacologic (intrathecal U0126) evidence that spinal ERK1/2 activation is associated with the development of *primary* visceral hyperalgesia of the bladder. After visceral (colon) inflammation, ERK1/2 activation is associated with the development of *secondary* mechanical hyperalgesia of the abdominal wall and the hind paw [14]. The present study provides functional evidence that spinal ERK1/2 activation is also associated with the development of *primary* hyperalgesia of the bladder (e.g. increased nociception to bladder inflammation and distention). Functional blockade of spinal ERK1/2 signaling with intrathecal inhibitors significantly attenuates the noxious distention-evoked VMR. Chronic visceral pain is one of most common reasons patients visit their physicians. The observation that MEK/ERK inhibitors are effective in alleviating visceral pain is an exciting one, since it may lead to new treatments of functional visceral pain syndromes such as interstitial cystitis (IC) or irritable bowel syndrome.

Previously, it has been shown that ERK1/2 is rapidly activated in the dorsal horn following noxious hind paw and colonic stimulation, as well as following chronic, constrictive nerve injury. Inhibition of spinal ERK signaling reduces (1) nocifensive behavior after subcutaneous injection of formalin, capsaicin, carrageenan or CFA [2; 15; 23; 27; 28], (2) secondary mechanical hyperalgesia evoked by intracolonic capsaicin stimulation [14], and (3) neuropathic pain induced by chronic constriction injury of the sciatic nerve [36]. In this study, we demonstrate that spinal ERK1/2 is also activated following noxious distention of the bladder, and that inhibition of spinal ERK1/2 signaling reduces distention-evoked bladder nociception. Because similar results have been observed across multiple experimental pain models (bladder, colon, hind paw inflammation, nerve injury) with different forms of noxious stimulation, spinal ERK signaling may be a common signaling pathway that modulates many different forms of pain hypersensitivity.

Unlike the case of somatic pain where ERK1/2 activation is restricted to the ipsilateral spinal laminae I-II in the superficial dorsal horn, in the case of bladder pain, ERK1/2 activation is also observed in the deep spinal cord, particularly around laminae X (dorsal commissure, DCM) and V (spinal parasympathetic nuclei, SPN) bilaterally (Figure 3C-D). Previous NeuN co-staining studies have localized this p-ERK activity to neurons [10]. The bilaterality and the more diffuse pattern of ERK1/2 activation are consistent with the spinal areas known to contain neurons responsive to bladder stimulation [16; 29; 30]. Results from

our mouse study are similar to those obtained from a rat study that showed that L6 p-ERK1/2 activation occurred after noxious bladder distention and CYP bladder inflammation (CYP + 60 mmHg noxious distention > CYP + 15 mmHg innocuous distention > saline + distention in the rat study) [10]. Even though thoracolumbar (L1–L2) spine also receives afferent input from the bladder via the hypogastric nerve (the bladder has dual autonomic innervation), p-ERK immunoactivity was most intense in the lumbosacral (L6–S1) spine which receives input via the pelvic plexus.

Precisely how p-ERK1/2 modulates visceral nociceptive transmission in the spinal cord is not clear at this time. p-ERK has been shown to regulate the expression of the immediate early gene, *c-fos*, in the superficial and deep spinal cord after noxious bladder stimulation [12]. In superficial dorsal horn neurons, ERK1/2 has also been shown to phosphorylate and modulate $K_v4.2$ -containing ion channels, leading to increased neuronal excitability and spinal central sensitization [2; 17–20; 26; 27]. We hypothesize that ERK-mediated alteration in neuronal excitability (spinal central sensitization) may contribute to the development of bladder hyperalgesia, but clearly the molecular signaling mechanisms underlying ERK-mediated modulation of spinal processing of bladder nociception deserve further investigation.

The intrathecal U0126 experiments showed VMR inhibition that was modest, in the range of about 20%. This reflects the fact that in the CYP model there is intense bladder inflammation and peripheral sensitization of bladder nociceptors in addition to central sensitization at the level of spinal cord. The VMR reflects nociception modulated by peripheral and central processes. In light of the continuous barrage of afferent signals arising from the bladder due to CYP cystitis, the ability of U0126 to have any statistically significant reduction on the VMR at all attests even stronger to the importance of spinal MEK/ERK signaling. The apparent mismatch in the magnitude of the reduction in number of p-ERK positive cells and the modest change in the VMR evoked by this dose of U0126 may be explained by the lack of perfect correlation between the number of immuno-positive cells, p-ERK activity (which may be better quantified with western blotting), and functional outcomes (e.g. VMR).

The CYP method utilized here to model bladder pain was originally developed to study the classic inflammatory form of interstitial cystitis (IC) – a chronic, debilitating bladder/pelvic pain syndrome of unknown etiology affecting millions of patients [7]. CYP-treated mice show micturition and spontaneous nocifensive behaviors as well as histological indicators that mimic IC [3; 4; 37; 39]. Here we have provided additional functional evidence that the CYP model reproduces the two clinical features that are most consistently reported among IC patients: i.e. visceral hyperalgesia and allodynia to bladder distention [38]. A fully distended bladder may result in mild pain in normal individuals, but would be very painful in IC patients (bladder hyperalgesia). A moderately distended bladder may cause urinary urgency (but no pain) in normal individuals, but would induce pain in IC patients (bladder allodynia). There are heated debates among researchers as to what constitutes a valid animal model to study IC, primarily because the pathophysiology of the human disease is unknown. We agree that there are dissimilarities between the model used in the present study and the human disease. However, by combining the methodologies of inflammation, distention, and VMR, we have a robust tool to study the signaling mechanisms that underlie bladder hyperalgesia in inflammatory bladder pain conditions.

In a sensitized inflamed bladder, bladder hypersensitivity is characterized not only by increased nociception and visceral hyperalgesia as measured by VMR, but also by bladder overactivity and uninhibited bladder contractions as measured by cystometry [8; 10]. Here we have shown that intrathecal U0126 is effective in attenuating bladder nociception. Two

previous studies have also shown that intrathecal administration of PD98059 (another MEK inhibitor) and intravesical administration of U0126 are both effective in reducing bladder overactivity and uninhibited bladder contractions in the CYP model [8; 10]. Taken together, these results demonstrate that in rodents, spinal ERK1/2 play important roles in modulating nociceptive processing and bladder contraction of a sensitized bladder. Although it remains to be determined whether spinal ERK is activated in any human disease, results from animal studies nevertheless raise the possibility of investigating the use of specific ERK/MAPK inhibitors to treat conditions associated with sensitized bladders (e.g. interstitial cystitis, bladder overactivity).

The following caveats should be considered when interpreting the present results. First, the precise site of action of intrathecal U0126 is not well defined. Even though we have shown that intrathecal U0126 reduces spinal p-ERK immunoreactivity (Figure 5), intrathecal U0126 may theoretically also act on the central terminals of afferent nerves or in dorsal root ganglia. CYP inflammation has previously been shown to activate ERK in the bladder tissue [8; 33] and in the lumbosacral dorsal root ganglia [9]. The VMR reflects the sum of peripheral sensitization of bladder nociceptors and central sensitization of second order neurons. Even though we have examined the association between VMR and spinal ERK activation, our results do not strictly demonstrate that the spinal cord is the exclusive site of pain modulation by ERK signaling in the context of bladder inflammation. Peripheral ERK may also play important roles in the modulation of inflammatory bladder pain.

In conclusion, our results show that spinal ERK1/2 activation contributes to the development of inflammation-induced hypersensitivity of the bladder to distension. Using our animal model, we hope to gain insights into the molecular signaling mechanisms underlying the development of visceral hypersensitivity in inflammatory bladder pain conditions such as interstitial cystitis.

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Figure 1.

Cyclophosphamide induces bladder hyperalgesia and allodynia to bladder distention. The 2 curves are statistically different ($p < 0.0001$). Difference is also noted at noxious distention pressures (50–80 mmHg). * $p < 0.05$, ** $p < 0.01$, 2-way ANOVA with Bonferroni posttests, $n = 8$ in CYP group, $n = 10$ in saline group. A sample of the distention-evoked VMR signal is shown in the inset. “A” designates the time interval (20s) used to rectify and integrate the signal to calculate the area under the curve.

Figure 2.

Lumbosacral L6-S1 spinal p-ERK1/2 immunoreactivity in CYP-treated and saline-treated mice after 20 mmHg innocuous bladder distention (CYP-20 mmHg, Saline-20 mmHg), or 60 mmHg noxious bladder distention (CYP-60 mmHg, Saline-60 mmHg) were presented in the top 2 panels. Only the activated forms of ERK1/2 (p-ERK1/2) were stained by the antibodies. The right column is a magnification of the left column. Cervical (C6) and lumbar (L1–L2) spinal sections after 60 mmHg noxious bladder distention in CYP animals are presented in the bottom panel for comparison.

Figure 3.

The number of p-ERK1/2-positive cells in the (B) lumbosacral L6-S1 spinal cord, and in the L6-S1 (C) the deep dorsal commissure (DCM), (D) spinal parasympathetic nuclei (SPN), (E) lateral dorsal horn (LDH), and (F) medial dorsal horn (MDH) after various bladder inflammation (CYP vs. saline) and distention (60 vs. 20 mmHg) paradigms. n=7 saline 20 mmHg, n=7 saline 60 mmHg, n=6 CYP 20 mmHg, n=8 CYP 60 mmHg. * p<0.05, **p<0.01.

Figure 4.

Female mice were treated with CYP on days 0, 3, 6, 9. On day 10, immediately after a phasic distention cycle (baseline), the mice were treated with either intrathecal i.t. (A) U0126 (MEK inhibitor, 2 μ g in 5 μ L, n=12) or (B) U0124 (negative control analog of U0126, n=7). The phasic distention cycle was then repeated 30 mins later. (C) The U0126 experiments were performed on naive female mice (n=10). In CYP treated mice, functional blockade of spinal ERK1/2 activation significantly reduced noxious distention evoked VMR (50–80 mmHg). In naive mice, reduction of noxious distention evoked VMR was noted only at 60 and 80 mmHg, but not consistently at other noxious pressures (no difference at 50 or

70 mmHg) after i.t. U0126. * $p < 0.05$, ** $p < 0.01$, 2-way repeated measure ANOVA with Bonferroni posttests.

Figure 5.

CYP animals were pre-treated with either intrathecal U0126 or U0124 (each 2 μ g in 5 μ L). 30 minutes later, the bladder was distended with 5 cycles of 60 mmHg. The L6-S1 spinal cord was then fixed for p-ERK1/2 immunostaining. The number of L6-S1 spinal p-ERK1/2 positive cells was significantly lower after U0126 than after U0124 pre-treatment. * $p < 0.05$, 2-tailed t-test.