Social stress in mice induces urinary bladder overactivity and increases (TRPV1) channel dependent afferent nerve activity

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Running head: TRPV1 mediates afferent activity

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Abstract

Social stress has been implicated as a cause of urinary bladder hypertrophy and dysfunction in humans. Using a murine model of social stress, we and others have shown that social stress leads to bladder overactivity. Here, we show that social stress leads to bladder overactivity, increased bladder compliance and increased afferent nerve activity. In the social stress paradigm, 6-week-old male C57BL/6 mice were exposed for a total of 2 weeks, via barrier cage, to a C57BL/6 retired breeder aggressor mouse. We performed conscious cystometry with and without intravesical infusion of the TRPV1 inhibitor capsazepine, and measured pressure-volume relationships and afferent nerve activity during bladder filling using an ex vivo bladder model. Stress lead to a decrease in intermicturition interval and void volume in vivo, which was restored by capsazepine. Ex vivo studies demonstrated that at low pressures, bladder compliance and afferent activity were elevated in stressed bladders as compared with unstressed bladders. Capsazepine did not significantly change afferent activity in unstressed mice, but significantly decreased afferent activity at all pressures in stressed bladders. Immunohistochemistry revealed that TRPV1 colocalizes with CGRP staining nerve fibers in unstressed bladders. Colocalization significantly increased along the same nerve fibers in the stressed bladders. Our results support the concept that social stress induces TRPV1-dependent afferent nerve activity, ultimately leading to the development of overactive bladder symptoms.

Key words: TRPV1, social stress, voiding frequency, cystometry
Introduction:

Stress exacerbates symptoms of urinary bladder dysfunction in overactive bladder, interstitial cystitis/bladder pain syndrome, bladder outlet obstruction and spinal cord injury (28). Notably, stress has been implicated in dysfunctional voiding in children (32). In this population, treatment options are limited and are often ineffective, due in part to a lack of understanding of the mechanisms underlying voiding dysfunction. Chang et al. showed that social stress leads to remodeling and hypertrophy of the bladder with abnormal voiding patterns (6). Recently, data by Long et al. have corroborated the above (19). In addition, using a stress model of maternal separation, van den Wijngaard and colleagues demonstrated that nerve growth factor (NGF) and transient receptor potential vanilloid type 1 (TRPV1) channels play a role in modulating the effects of stress on the gut (30).

Members of the TRP family of non-selective cation channels have variable permeability to calcium (8,9). Multiple members of the TRP channel vanilloid family (TRPV1, TRPV2, TRPV4) are expressed in the urinary bladder and may act as sensors of stretch and/or chemical irritation in the lower urinary tract (1, 3). TRPV1 channels have been detected in human and rodent lower urinary tract tissue, including the peripheral sensory nerve fibers of the bladder (5). It has been suggested that TRP channels, in particular TRPV1, have a functional role in overactive bladder (26).

In the current study, we sought to determine if social stress affects bladder afferent activity and whether TRPV1 plays a role in modulating that activity. These studies expand upon our existing work with a social stress model of bladder dysfunction where we have shown that stress leads to bladder overactivity (24). The current study addresses the mechanism underlying stress-induced bladder overactivity. During conscious cystometry in stressed mice, we observed a decreased intermicturition interval and voided volume, which was restored to normal by inhibition of TRPV1 channels. Utilizing a novel ex vivo bladder preparation, we demonstrated that social stress increases afferent bladder nerve activity and that this increased activity can be significantly attenuated with TRPV1 channel inhibition. These results suggest that the increase in bladder afferent activity induced by social stress is due, at least in part, to TRPV1 channel activity.
**Materials and Methods**

*Animal care and use*

Male C57BL/6 mice (Charles River Canada, St. Constant, Canada) (20-30 g) were used in these studies. Mice were housed in AAALAC accredited animal facilities and provided free access to food and water. All procedures involving animals were performed in accordance with the Institutional Animal Care and Use Committee at the University of Vermont, and were consistent with the Guide for the Care and Use of Laboratory Animals (8th edition).

*Social stress protocol*

On consecutive days for a total of 2 weeks, submissive 6-week-old C57BL/6 mice were placed in direct contact with C57BL/6 retired breeder mice (Charles River Canada) until the C57BL/6 mice initiated aggressive behavior (e.g. biting sufficient to break the skin) usually within 5 minutes. The mice were then separated in the same cage by a clear plastic barrier with small holes that allowed for olfactory stimulation for a period of 1 hr. The submissive mouse was then returned to its own cage for 23 hours, and the process was repeated the next day with a different C57BL/6 breeder mouse. As a control, age-matched mice were placed in barrier cages without exposure to the C57BL/6 aggressor mouse for a total of 1 hour before being returned to their own cage.

*Open voiding cystometry in conscious, unrestrained mice*

The urinary bladder was exposed through a lower midline abdominal incision under general anesthesia (2-3% isoflurane). A saline-filled PE-10 cannula with the end flared by heat was inserted into the dome of the bladder and secured with a 6-0-nylon purse string suture. The distal end of the cannula was sealed. Muscle and skin layers were closed separately using absorbable and non-absorbable sutures, respectively. The distal part of the cannula was placed in the subcutaneous space, and the mice were returned to their home cages for 72 hours to ensure complete recovery. Postoperative analgesics were given for a period of 48 hours. Prior to cystometry, the mice were anesthetized and the cannula was exteriorized. After regaining consciousness, mice were placed unrestrained in recording cages with a balance and pan placed below the cage for urine collection and measurement. Intravesical pressure changes were recorded using a Small Animal Cystometry System (Med Associates, Inc., St Albans, VT, USA). The exteriorized cannula was connected to one port of a pressure transducer; the other
port was connected to a syringe pump. Either saline solution alone or saline with the TRPV1 antagonist capsazepine (10 µM) was infused at a rate of 10 µl/min. At least four reproducible micturition cycles were recorded after an initial stabilization period of 25 to 30 minutes. Voided saline was collected to determine void volume. Intermicturition interval, bladder capacity, baseline pressure and non-voiding contractions (NVC) were also measured. For these studies, NVCs were defined as rhythmic intravesical pressure rises (greater than 5cm from baseline pressure). Mice were excluded from studies when any of the following adverse events occurred: ≥ 20% reduction in body weight post-surgery, lethargy, pain or distress not relieved by our IACUC-approved regimen of post-operative analgesics, or another significant postoperative adverse event. In the present studies, three mice were excluded due to iatrogenic bladder outlet obstruction. Behavioral movements, such as grooming, standing, ambulation and defecation, also rendered bladder pressure recordings during these events unusable; these events were excluded from the analysis. In the majority of animals, experiments were conducted at similar times of day to avoid the possibility that circadian variations were responsible for changes in bladder capacity measurements. Mice were euthanized at the conclusion of the study by isoflurane (5%) anesthesia followed by thoracotomy.

Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using the STAT-60 total RNA/mRNA isolation reagent (Tel-Test Inc., Friendswood, TX, USA) as previously described (12, 17). cDNA was synthesized from 1 mg RNA per sample with M-MLV reverse transcriptase (Promega Corp., Madison, WI USA) using a mix of random hexamer and oligo dT primers in a final reaction volume of 25 µl. cDNA templates, diluted 10-fold to minimize the inhibitory effects of the reverse transcription reaction components, were assayed using HotStart-IT SYBR Green qPCR Master Mix (USB, Cleveland, OH, USA) and each primer (300 nM) in a final reaction volume of 25 µl. The following primer pairs were used: TrpV1, 5’-TAC TTT TCT TTG TAC AGT CAC T-3’ (forward) and 5’-TCA ATC ATG ACA GCA TAG AT-3’ (reverse); 18S rRNA, 5’-AGT CGC CGT GCC TAC CAT-3’ (forward) and 5’-GCC TGC TGC CTT CCT TG-3’ (reverse). Real-time quantitative PCR was performed on an Applied Biosystems 7500 Fast real-time PCR system (Foster City, CA, USA) using the following standard conditions: initial denaturation at 94°C for 2 minutes, followed by 45 cycles at 94°C for 15 seconds and 60-65°C (depending on the primer set) for 30 seconds. The specificity of the reaction was confirmed by subjecting amplified products to a SYBR Green I melting analysis in which dissociation was monitored as the temperature was gradually raised from
60°C to 95°C. A single DNA melting profile was observed under these conditions, demonstrating amplification of a single, unique product free of primer dimers or other reaction artifacts.

Data were analyzed at the termination of each assay using sequence-detection software (Sequence Detection Software, version 1.3.1; Applied Biosystems, Norwalk, CT, USA). In standard assays, default baseline settings were selected. The increase in SYBR Green I fluorescence intensity (ΔRn) was plotted as a function of cycle number, and the threshold cycle was determined by the software as the amplification cycle at which the ΔRn first intersects the established baseline. Relative expression levels were determined by expressing the threshold cycle number for the gene of interest normalized to that of the housekeeping gene 18S. These data were converted to copy number by reference to a standard curve constructed by amplification of 10-fold serially diluted plasmids containing the target sequence. These quantitative PCR standards for all transcripts were prepared using the amplified cDNA products ligated directly into the pCR2.1 TOPO vector using the TOPO TA cloning kit (Life Technologies Carlsbad, CA, USA). The nucleotide sequences of the inserts were verified by automated fluorescent dideoxy dye terminator sequencing (Vermont Cancer Center DNA Analysis Facility). The concentration range of PCR standards in standard curves was determined empirically.

**Western blot analysis**

Whole bladders were placed in Tissue Protein Extraction Reagent (TPER; Thermo Scientific, Waltham, Massachusetts, USA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Total bladder proteins were then extracted using MP Biomedicals Lysing Matrix D (MP Biomedical, Santa Ana, CA, USA), and the protein concentration was measured using a Coomassie Plus (Bradford) Assay Kit (Thermo Scientific). Reagents and instruments used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein transfer were all purchased from Invitrogen. Samples (35 μg of protein) were resolved by electrophoresis on 4-12% Tris-glycine gels (Life Technologies, Waltham, MA). Separated proteins were transferred to (polyvinylidene difluoride (PVDF) membranes using an iBLOT Dry Blotting System (Life Technologies, Waltham, MA). Membranes were blocked by incubating for 2 hours at room temperature in 3% bovine serum albumin in Tris-buffered saline containing 0.05% Tween. Membranes were then incubated overnight at 4°C with rabbit anti-TRPV1 (1:400) and anti-actin (1:10,000) primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Washed membranes were incubated for 2 hours at room temperature with species-appropriate horseradish peroxidase-conjugated secondary antibodies, followed by chemiluminescence detection with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA). Images were
captured using a Versa Doc 4000 MP Imaging System (BioRad) at the VT Cancer Center DNA Analysis Facility. The adjusted f each band was analyzed, and background intensities were subtracted using Quantity One software (BioRad).

**Immunofluorescence for TRPV1 and calcitonin gene related peptide (CGRP)**

Whole bladders were harvested before or after 14 days of stress, and immediately were placed in 4% paraformaldehyde for 24 hours at 4°C. Tissues were then transferred to 30% sucrose in 1xPBS, pH 7.4 overnight at 4°C. Tissues were embedded in optimal cutting temperature compound (Tissue-Tek, Batavia, IL) and kept at -20°C for 24 hours. Tissues were sectioned (20-μm) and mounted on 0.5% gelatin-coated slides. Tissues were blocked with 10% donkey serum diluted in PBS with 0.5% Triton-X (Sigma-Aldrich, St. Louis, MO) for 1 hour and then incubated overnight at room temperature with the following primary antibodies: goat anti-TRPV1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-CGRP (1:1000; Phoenix Pharmaceuticals Burlingame, CA). The following day, tissues were washed (PBS, 3 × 15 min), and then incubated for 2 hours at room temperature with the following secondary antibodies: donkey anti-goat Alexa Fluor 555 (1:500; Invitrogen Life Technologies Waltham, MA) and donkey anti-rabbit Cy2 (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). Following washing (PBS, 3 × 15 min), nuclear staining was performed using DAPI 1:500 (Invitrogen Life Technologies, Waltham, MA). Coverslips were then mounted on the slides with an anti-fading media (Citifluor, Fischer Scientific, Pittsburgh, PA).

**Confocal Scanning Laser Microscopy**

All tissue sections (4 randomly selected sections containing urothelium and detrusor from each animal) were imaged with a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) operating in multitrack mode. Each track was imaged sequentially. The DAPI (nuclear) signal was excited with a 405 nm blue diode laser and collected with a 420-480 nm band pass filter; the Cy2 with an argon ion laser and collected with a 505-530 nm band pass filter; and the Alexa 555 with helium neon ion laser and collected with a 560-615 nm band pass. Images were acquired with a PlanApochromat 63X (1.4 NA) objective. The confocal pinholes were initially set to 1.0 Airy Unit diameters for the 543 nm excitation, and an optical section thickness of 0.9 μm was matched for the 405 nm, 488nm and 633 nm channels when imaging with the 63X objective. Images were acquired at 12-bit data depth, and all settings (including laser power, amplifier gain, and amplifier offset) were
established using a look-up table to provide optimal gray-scale intensities. Primary antibody-exposed samples and secondary-only controls were imaged under identical instrument settings.

**Colocalization analysis**

Images taken with the 63X objective were subject to colocalization analysis using Velocity version 6.3.0 software (Perkin Elmer, Waltham, MA, USA). Tissue areas of interest were manually selected. A scatter plot of the channels to be analyzed was used to manually threshold the image to exclude background. Once the appropriate threshold was determined, colocalization was calculated according to Manders et al. (22).

**Ex vivo bladder preparation**

The urinary bladder and urethra with attached post-ganglionic nerves, major pelvic ganglion and pelvic nerves were dissected and placed in a recording chamber recirculated with physiologic saline solution (PSS; containing (mM): 118.5 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgCl2, 2.0 CaCl2, 24 NaHCO3, 7 glucose, pH 7.4). PSS was bubbled with biological atmosphere gas (2% O2, 5% CO2, 83% N2) and maintained at 37°C. The bladder was cannulated via the urethra and infused with saline at a constant rate (1.8 ml/hr), during which intravesical pressure and afferent nerve activity were recorded. Bladder afferent nerve activity was recorded by a suction electrode attached to one of the pelvic nerves. The suction electrode was connected to a Neurolog head stage (NL100AKS Digitimer), where the extracellular action potentials were amplified with an AC amplifier (NL104, Digitimer), filtered (band pass 200-4000 Hz, Digitimer NL125/NL126), and digitized with a Power1401 analog-to digital interface (Cambridge Electronic Design, Cambridge, UK). Afferent nerve activity was acquired at a rate of 25,000 Hz and bladder pressure at 100 Hz. Data analysis was performed offline using Spike 2 software (Cambridge Electronic Design, Cambridge, UK). The threshold for action potential detection was set at twice the root mean square of the recorded signal in the absence of action potentials. The TRPV1 antagonist capsazepine (10 µM) was applied to the bladder by addition to the superfusate. Baseline afferent activity was measured by binning afferent activity in 1-second
bins and taking the average of the lowest bins of afferent activity within 10 seconds either side of the selected pressure. Amplitudes and integrals of spontaneous phasic contractions (SPCs) and corresponding afferent activity were measured using LabChart Pro software (AD Instruments, Colorado Springs, CO, USA). Bladder compliance was measured at 5mmHg intervals. Compliance was calculated as the change in infused volume per change in pressure.

**Statistical analysis**

For comparisons of two samples of equal variance, statistical significance between groups was established using two-tailed, unpaired Student’s t-tests (α=0.05). For samples of unequal variance, the Mann-Whitney U test was used (α=0.05). For multiple sample comparisons, two-way ANOVA was used followed by Bonferroni’s post hoc analysis to compare individual means. Calculations were performed using Microsoft Excel (Microsoft Corporation, USA) or GraphPad Prism (GraphPad Software Inc., USA). Values are expressed as means ± standard error of the mean (SEM). Differences with p-values < 0.05 were considered statistically significant.
Results

The TRPV1 antagonist capsazepine decreases in vivo urinary bladder overactivity that is induced by stress

Stress negatively affects the function of organs, including the urinary bladder, where it has been shown to exacerbate symptoms of bladder overactivity (20, 28). We performed conscious cystometry on both stressed and unstressed mice. The stressed mice showed a significant decrease in intermicturition interval, voided volume and bladder capacity (volume of urine in the bladder prior to micturition) as compared to non-stressed mice, indicative of bladder overactivity (Fig. 1A). Continuous infusion of the TRPV1 antagonist capsazepine (10 µM) did not significantly alter any measured cystometric parameters in non-stressed mice (Fig. 1B-D). However, in stressed mice, intermicturition interval, voided volume and bladder capacity were significantly increased as compared to vehicle controls (Fig. 1B-D). A significant reduction in bladder pressure in the stressed mice pre- and post-capsazepine was also present (Fig. 1E). Stressed mice did not exhibit a change in NVCs post-capsazepine. Taken together, these data suggest that stress induces sensory TRPV1 channel activity, which contributes to bladder overactivity.

TRPV1 mRNA and protein expression in whole bladders of stressed and unstressed is unchanged

To determine whether exposure to stress alters TRPV1 expression, we examined TRPV1 mRNA and protein levels in whole urinary bladders of stressed mice by Q-PCR and Western blotting, respectively. There was no significant change in TRPV1 protein expression (Fig. 2A, B) in urinary bladder, but there was a significant increase in mRNA in urinary bladder in mice exposed to stress (Fig. 2C). This suggests that stress leads to an up-regulation of TRPV1 mRNA with no change in protein expression.

TRPV1 and CGRP immunofluorescence and colocalization in bladders of stressed and unstressed mice

To determine the cellular sites of expression for TRPV1, and to determine if there was a change in this expression when exposed to stress, we performed immunofluorescence staining for TRPV1 and CGRP (a marker for sensory nerves) in bladder muscle and urothelium of unstressed (Fig. 3A, B) and stressed (Fig. 3E, F) mice. Nuclei were stained with DAPI (Fig. 3C). TRPV1-immunoreactivity colocalized with CGRP-positive nerve cells in bladder muscle
and urothelium of both unstressed and stressed mice (Fig. 3D, H). Nerve fibers staining positively for both TRPV1 and CGRP is significantly (p ≤ 0.05) increased in the bladders from stressed mice compared to controls in both muscle (Fig. 4E) and urothelium (Fig. 4F) (p≤0.05). The increased correlation coefficient of these two markers suggests that stress increased TRPV1-immunoreactivity in afferent nerve fibers.

Social stress increases ex vivo bladder volume and afferent activity

To test the hypothesis that social stress increases bladder afferent nerve activity, we studied the effects of social stress on bladder pressure-volume relationships and afferent nerve activity using an ex vivo bladder preparation. Constant infusion of PSS into the excised bladder steadily increased bladder intravesical pressure and afferent activity as recorded from the pelvic nerve (Fig. 5A). A comparison of the pressure-volume curves of urinary bladders from unstressed and stressed mice revealed that bladder volume was significantly increased in mice in the stressed group compared with mice in the unstressed group (Fig. 5B, C). We also examined bladder compliance and afferent nerve activity during bladder filling. For this analysis the pressure-volume curve was divided into 5 mmHg intervals to elucidate the range of bladder pressures most affected by stress. Bladder compliance was significantly increased in stressed compared with unstressed animals at low pressure (0-5 mmHg) (Fig. 5D). Baseline afferent activity was also increased in stressed compared to unstressed mice at low pressures (0-10 mmHg) (Fig. 5E). These findings suggest that stress caused an increase in bladder volume and also augmented afferent activity at physiologic filling pressures.

TRPV1 inhibition decreases baseline afferent nerve activity and afferent nerve activity evoked spontaneous phasic contractions in stressed mice during bladder filling

To examine the effect of TRPV1 inhibition on baseline afferent nerve activity, we measured baseline afferent activity during bladder filling in ex vivo bladders from unstressed and stressed mice in the presence or absence of the TRPV1 inhibitor capsazepine (10 µM). Capsazepine had no effect on bladders from unstressed mice (Fig. 6A), but significantly reduced afferent activity in stressed bladders (Fig. 6B).

Spontaneous phasic contractions (SPCs) are transient increases in pressure that have been previously reported in isolated bladder strips and cannulated bladder preparations (ex vivo and in vivo) (11, 13). The function of SPCs is presently unknown, but they may increase afferent activity and convey information related to bladder fullness (1, 11, 14, 25, 27). To study the role of TRPV1 channels in the response of afferent nerves to SPCs, ex vivo bladders were treated
with the TRPV1 inhibitor, capsazepine (10 µM). SPCs, measured as the pressure integral during an event was unaffected by stress (Fig. 7B), suggesting that stress does not affect spontaneous detrusor contractility. However, afferent activity during SPCs was significantly increased with stress (Fig. 7C). Capsazepine (10 µM) significantly reduced afferent activity in stressed but not unstressed bladders. Furthermore, the afferent activity per mmHg of SPC was significantly increased with stress (Fig. 7D), and also significantly reduced by capsazepine (10 µM).

Taken together, these data suggest that TRPV1 channel activation does not contribute to normal urinary bladder sensory outflow, but it is induced by stress.
Discussion

In this study, we expand upon our current knowledge of the effects of social stress on bladder function. Consistent with our previous work, stress in mice leads to bladder overactivity, defined as a decrease in intermicturition interval, voided volume and bladder capacity during conscious cystometry (24). Our *ex vivo* studies show that social stress leads to a significant increase in afferent nerve activity in stressed bladders at sub-threshold pressures between 0-10 mmHg. Since cystometry recordings revealed a reduced capacity in stressed bladders, we expected a similar finding of reduced volume in stressed *ex vivo* bladder preparations. However, the bladder volume in stressed *ex vivo* bladders was significantly increased. These seemingly contradictory findings can be explained by the increased afferent activity recorded from the stressed bladders. The increased sensory activity from the stressed bladders may play a key role in signaling to the central nervous system that a partially full bladder has reached capacity, leading to earlier micturition. Support for this concept comes from cystometric recordings with the infusion of capsazepine. Inhibition of TRPV1 channels in stressed mice abrogates the stress-induced elevation of afferent activity and increases the intermicturition interval and bladder volume. Furthermore, inhibition of TRPV1 activity with capsazepine significantly decreased the afferent activity underlying both SPCs and baseline afferent activity in the stressed bladder, with no effect on control bladders. These findings suggest a role for TRPV1 channel activity in the elevation of sensory outflow following stress on *in vivo* and *ex vivo* bladder activity.

TRPV1 is a non-selective cation channel that is activated by both endogenous and exogenous physical and chemical stimuli (8). It has been demonstrated that TRPV1 expression is increased with detrusor overactivity caused by spinal cord injury at the level of the suburothelial nerves and is reduced after intravesical instillation of capsaicin, resiniferatoxin or onabotulinumtoxin A (2, 10). Stress has previously been implicated in modulating the activity of TRPV1 (30, 31). In a rat model of neonatal maternal separation with acute water-avoidance stress, capsazepine was shown to inhibit post stress hypersensitivity, as measured by the visceromotor response (30). Our work is the first to show a role for TRPV1 in modifying afferent activity in a stressed bladder. However, cystometric studies designed to indirectly address the role of afferent output in the micturition reflex have shown that TRPV1-knockout mice have an increased bladder capacity and attenuated voiding contractions (9). These changes were hypothesized to result from reduced afferent output based on the observed reduction in the
number of cFos-immunopositive neurons in the sacral spinal cord in the TRPV1-knockout mouse (4). Although these researchers used a TRPV1-knockout model, we saw a similar increase in bladder capacity and decrease in intermicturition level with the application of capsazepine, albeit only in the stressed mice. These findings are supported by our ex vivo studies, which showed increased capsazepine-sensitive afferent activity.

More recently, Daly et al. (9) investigated the role of TRPV1 receptors in modulating bladder afferent activity using both pharmacological blockade and genetic deletion of TRPV1. They reported that, in wild-type preparations, distention of the bladder to a maximal pressure of 40 mmHg produced a graded increase in afferent activity. The application of capsazepine (10 µM) to the wild-type mice significantly attenuated afferent activity. Our findings agree with those of Daly et al. at pressures up to 25 mmHg. We chose this maximal pressure because it is closer to physiologic pressure; had we extended our recordings to higher pressures, we might have observed the same phenomenon. Our findings suggest that TRPV1 only plays a role in increasing afferent sensory activity under pathologic conditions, as we found that capsazepine had no effect on afferent activity in the non-stressed mouse bladder. Daly et al. also reported that the altered sensitivity they observed in their experiments was not due to a change in the pressure–volume relationship during filling, whereas we observed significant differences in sensitivity at low filling pressure (0-10 mmHg). The latter may be due to stress affecting increased sensation early during bladder filling.

The results of our ex vivo bladder studies show that TRPV1 modifies both baseline and SPC-evoked afferent nerve activity. It is not known if this modification occurs via similar or different nerve populations. Two types of fibers have been identified in whole pelvic nerve bundles: low-threshold fibers with distention thresholds <20 mmHg, and high-threshold fibers with distention thresholds >20 mmHg (16). It is unlikely that this activity occurs through two populations of nerves, as social stress caused increased afferent activity at pressures less than 20 mmHg. Our results are consistent with normal sensory outflow occurring through Aδ fibers, which generally lack TRPV1 channels and that stress induces activity of small-diameter, unmyelinated C-fibers, which are known to express TRPV1 channels (23, 29).

The mechanism(s) responsible for a modification in TRPV1 activity is not certain, since TRPV1 transcript expression is increased in stress whereas protein product remains unchanged. It is possible that determining a change TRPV1 protein expression is difficult, especially if changes in expression are in nerves that represent a small fraction of the total protein content of the urinary bladder. Immunofluorescence revealed that TRPV1 and CGRP colocalize, indicating that TRPV1 is expressed in close approximation to sensory elements in
bladder muscle and urothelium (but not in urothelial cells). More importantly, there is a significant increase in colocalization seen in both the urothelium and muscle of the stressed bladders. This further supports our findings that changes in TRPV1 activity occur only under pathologic conditions. However, since CGRP can be expressed in different types of sensory nerve fibers, there are several possible explanations for this phenomenon. One explanation includes the recruitment of otherwise quiescent nerve fibers (C-fibers). Alternatively, the increase in TRPV1 activity may be due to the recruitment of different sensory nerve fibers. In addition to C-fibers, TRPV1 has been localized to some medium diameter, thinly myelinated Aδ fibers (23). TRPV1 modification may also be due to the recruitment of different populations of C-fibers. While C-fibers can be subdivided into peptidergic and non-peptidergic classes, it is unclear to what extent TRPV1 expression follows a population-specific regulation. In the mouse, TRPV1 appears to predominate in the peptidergic population (33). Further investigation suggests that TRPV1 may also be present in a substantial portion of non-peptidergic neurons (15). Thus, in pathologic states such as stress, it is conceivable that both populations of C-fibers could be recruited and lead to a form of synaptic remodeling.

Increased TRPV1 activation may also be explained by stress causing the concomitant release of several endogenous activators. Previously, we showed that social stress leads to an increase in bladder urothelial expression of histamine and NGF (24). Literature reports have implicated peripheral mast cells as the cellular link between the brain and end organs during the stress response (7). Mast cell degranulation releases mediators, including NGF, which is known to be a strong inducer of TRPV1 sensitization (21). There are several other mechanisms by which NGF-mediated modulation of TRPV1 could be induced following stress. In addition to increasing the numbers of TRPV1 expressing neurons, TRPV1 activity could be increased during stress via post-translational modifications including phosphorylation, or by enhanced TRPV1 trafficking to the cell membrane (18). All of these possible explanations warrant further investigation.

The current findings increase our understanding of the important role that TRPV1 channels play in bladder afferent nerve function in the setting of social stress. In particular, we demonstrate that social stress leads to an increase in baseline afferent activity and increased neural activity in SPCs that are attenuated by TRPV1 inhibition. Importantly this TRPV1 activity is not observed in control mice. These findings increase our understanding of the possible mechanisms underlying the role of stress in bladder dysfunction and represent a novel foundation for future studies that will identify potential lower urinary tract targets for treatments for children with stress related bladder disorders. In particular, silencing TRPV1 channels or the
fibers which contain them, may be a therapeutic approach.
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Disclosures

No conflicts of interest are declared by the authors.
Figure legends

Figure 1: A. Representative tracing of cystometrograms from no stress and stressed mice, before and after infusion of 10 µM capsazepine. B-E. Summary bars graphs of intermicturition interval (B), void volume (C), bladder capacity (D) and baseline pressure (E).*: p≤0.05 vs. no stress. †: p≤0.05 vs. drug control. N=4.

Figure 2: A. Representative TRPV1 western blot showing no difference in TRPV1 protein expression between whole bladders of stressed and control mice. B, C. Summary histograms showing no change in TRPV1 protein expression and an increase in mRNA expression following stress. *p≤0.05 vs. control.

Figure 3: A-H. Representative immunofluorescent staining for CGRP (green) and TRPV1 (red) in no stress (A-D) and stressed (E-H) bladder sections. D, H: Overlay image showing TRPV1 (red), CGRP (green), and DAPI (nuclear stain; blue) fluorescence. Images are representative of n ≥ 5 animals in each group (no stress and stressed). Calibration bar = 50 µm.

Figure 4: A-D. Representative images used for colocalization analysis. White represents overlapping pixels of CGRP and TRPV1. E, F. Correlation coefficient reflecting the percentage of the TRPV1 positive staining that colocalizes with CGRP positive staining in muscle (A, C) and urothelium (B, D) from control (no stress, A, B) and stressed (C, D) mice. *p≤0.05 vs. control. Calibration bar = 50 µm.

Figure 5: Pressure-volume curves and summary data of afferent nerve activity recorded during bladder distension. A. Representative pressure volume curve (upper trace) and afferent activity during a single filling cycle to 25 mmHg (filling rate = 1.8 ml/hr.) in an unstressed bladder. Small amplitude SPCs are evident on the pressure volume curve. Afferent nerve activity increased as the bladder filled. Raw afferent nerve activity (bottom trace) recorded during bladder filling. Mean frequency of afferent nerve activity (middle trace). B, C. Representative pressure-volume curves and baseline summary graph from no-stress and stress bladders. D, E. Bladder compliance (D), baseline afferent activity (E), expressed in 5-mmHg changes in pressure. *p≤0.05 vs. control at the same pressure.
Figure 6: The TRPV1 inhibitor capsazepine (10 µM) did not significantly inhibit baseline afferent activity in no-stress mice (A), but significantly reduced baseline afferent activity in stressed mice (B). *p≤0.05 vs. control. in non-normalized data.

Figure 7: Pressure-volume curve and summary data of afferent nerve activity recorded during bladder distension. A. Representative pressure volume curve (upper trace) and afferent activity during a single filling cycle to 10 mmHg (filling rate = 1.8 ml/hr.). Small amplitude SPCs are noted on the pressure volume curve. Afferent nerve activity increased as the bladder filled. Raw afferent nerve activity (bottom trace) recorded during bladder filling. Mean frequency of afferent nerve activity (middle trace). B, C. Analysis of SPC amplitude and area up to 10 mmHg. D. Afferent frequency measured per mmHg increase in SPC amplitude. *: p≤0.05 vs. no stress. †: p≤0.05 vs. drug control
References


5. **Cavanaugh DJ, Chesler AT, Braz JM, Shah NM, Julius D, and Basbaum AI.** Restriction of transient receptor potential vanilloid-1 to the peptidergic subset of primary afferent neurons follows its developmental downregulation in nonpeptidergic neurons. *J Neurosci* 31: 10119-10127, 2011.


Figure 1.

A. 

<table>
<thead>
<tr>
<th>Infused Volume (ml)</th>
<th>Pressure (mmHg)</th>
<th>Void Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stress</td>
<td>Stressed</td>
<td>Vehicle</td>
</tr>
<tr>
<td>Capsazepine</td>
<td></td>
<td>Vehicle</td>
</tr>
</tbody>
</table>

| Time (seconds) |
|---------------|----------------|
| 500s          |                |

<table>
<thead>
<tr>
<th>0.3</th>
<th>0.1</th>
<th>0.0</th>
<th>0.2</th>
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</thead>
<tbody>
<tr>
<td>45</td>
<td>30</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>0.3</td>
<td>0.1</td>
<td>0.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

A figure showing the effects of vehicle capsazepine on infused volume, pressure, and void volume under no stress and stressed conditions over time.
Figure 1.
Figure 2.

A. 

No Stress | Stressed
---|---
TRPV1 | 100 kD
Actin | 43 kD

B.

<table>
<thead>
<tr>
<th>Relative Expression</th>
<th>No Stress</th>
<th>Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=6</td>
<td>N=6</td>
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</tbody>
</table>

C.

<table>
<thead>
<tr>
<th>TRPV1/18S mRNA (x10^6)</th>
<th>No Stress</th>
<th>Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=5</td>
<td>N=5</td>
</tr>
</tbody>
</table>
Figure 3.

A. No Stress
   CGRP
   50 µm

B. TRPV1

C. DAPI

D. Overlay

E. Stressed
   CGRP
   50 µm

F. TRPV1

G. DAPI

H. Overlay
Figure 4.

A. No Stress
Muscle

B. No Stress
Urothelium

C. Stressed
Muscle

D. Stressed
Urothelium

E. Muscle

![Graph showing M2 Correlation coefficient for Muscle with bars for No Stress and Stressed conditions. The Stressed condition shows a higher correlation coefficient with an asterisk indicating significance.]

F. Urothelium

![Graph showing M2 Correlation coefficient for Urothelium with bars for No Stress and Stressed conditions. The Stressed condition shows a higher correlation coefficient with an asterisk indicating significance.]

50 µm
Figure 5.
Figure 6.

A. No Stress

B. Stress

Afferent activity normalized to control at each pressure

- Control
- 10 μM capsazepine

n = 4

Pressure (mm Hg):
0 5 10 15 20 25
Figure 7.