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Impacts of Chronic Stress on Urinary Bladder Function & Recovery

Through Modification of PACAP Signaling

Morgan Mathews

The University of Vermont

Abstract

Stress can cause or contribute to bladder dysfunction though specific effects remain unclear. Individuals with interstitial cystitis (IC)/bladder pain syndrome (BPS) experience increased symptom severity with stress, including pain and increased urgency and frequency of voiding. Further research can identify stress-related changes to urinary mechanisms, such as changes in sensory neurotransmitters and subsequent receptor expression. These factors could then become targets for future therapies providing bladder dysfunction relief. Alterations in PACAP and TRPV channel expression have been shown in sensory pathways in response to disease. My research studied the effects of chronic stress on bladder function and the potential for PACAP(6-38), a PACAP antagonist, to recover normal function. Mice went through a repeated variate stress regime followed by bladder tube implant and cystometrogram recording. Bladder, dorsal root ganglia (DRG), and spinal cord tissues were collected and immunostained for the presence of TRPV1, TRPV4, and PACAP expression. Chronic stress decreased inter-contraction interval (ICI) and bladder capacity while increasing bladder pressures. Both control and stressed male and female mice showed improvement following a 30-minute intravesical infusion of PACAP(6-38), with ICI and bladder capacity measures having the most consistent improvement across groups. PACAP and TRPV1 expression was upregulated in the bladder afferent pathway. These findings suggest that chronic stress can contribute to bladder dysfunction, and it seems to be mediated in part by upregulation of PACAP and TRPV channel activity. With further research, these factors may be valid molecular targets for therapy. As chronic stress

may present alone or in combination with other conditions (IC/BPS, injury, etc.), it is critical to understand stress-related changes in order to reduce or eliminate their impact.

Introduction

Normal bladder function is usually unappreciated until something goes wrong. An estimated 3.3 to 7.8 million adult females in the U.S. alone suffer symptoms of IC/BPS (Berry et al., 2011). IC/BPS is a debilitating inflammatory disease that impedes daily activities and makes participating in the community difficult through persistent discomfort and bladder dysfunction (Beckett et al., 2014). Chronic stress, related or unrelated to the disease, may exacerbate symptoms and thus reduce quality of life further (Robbins et al., 2007; Rothrock et al., 2001). Additionally, chronic stress, as an inflammatory condition, may be able to independently cause bladder dysfunction with a presentation similar to IC/BPS (Smith et al., 2011). Currently, the mechanisms by which stress contributes to bladder dysfunction are unknown, and there is no cure besides removing the stressor(s), which is not always possible. Also, there is no cure for IC/BPS. This research is intended to make progress towards these goals.

The Basics of Bladder Innervation

The bladder is composed of four layers – an inner endothelial layer of epithelium, the lamina propria (connective tissue and vasculature), the detrusor smooth muscle, and the perivesical soft tissue (fat and vasculature). The inferior aspect of the bladder is the bladder neck, containing the more superior internal sphincter (smooth muscle) and the external urethral sphincter (striated muscle).

These components all work together to achieve two modes of operation: storage and elimination.

Normal urinary bladder function is a consistent, repetitive cycle of storage and voiding (micturition) controlled by conscious and autonomic inputs (de Groat et al., 1993). In a mature urinary system, bladder filling occurs with somatic and sympathetic contraction of the sphincter muscles and sympathetic inhibition of detrusor smooth muscle activity (de Groat & Booth, 1980). Sensory nerves relay information about fullness from the bladder to the spinal cord, where there are localized reflex pathways that also project to the pontine micturition center and cerebral cortex (Mahony et al., 1977; Zhang et al., 2005). Parasympathetic innervation contracts the detrusor muscle whereas sympathetic and somatic innervation relaxes the sphincter muscles, which together allows for voiding (Edvardsen, 1972; Mahony et al., 1977).

Changes in these pathways produce voiding dysfunctions because neural control as well as detrusor muscle activity is often altered. This presents as changes (often increases) in voiding frequency, nocturia, urgency, incomplete emptying, urinary hesitancy, and suprapubic pain (Irwin et al., 2006; Koziol et al., 1993). These changes are studied functionally by measuring bladder capacity, ICI (the time between voids), and pressure values throughout a micturition cycle (Maggi et al., 1986; Schafer et al., 2002). Pressures are lowest right after voiding when the bladder is empty. Pressure remains steady throughout the filling phase until it dramatically increases just prior to voiding (Maggi et al., 1986). Unstable or

consistently elevated pressures throughout a micturition cycle suggest dysfunction (Malmgren, 1987; Neal et al., 1987).

States of urinary bladder dysfunction are usually caused by changes to autonomic control of the system (as opposed to conscious inputs). This can happen through changes in perceived bladder sensations, which occurs when expression of sensory molecules and their channels are altered in the bladder afferent relay. For example, two channels in the transient receptor potential (TRP) superfamily, transient receptor potential vanilloid family member 1 (TRPV1) and member 4 (TRPV4), are important in maintaining proper bladder sensation and activity, and have increased expression levels in IC/BPS (Everaerts et al., 2010; Wang et al., 2008). As such, studying these channels in their relation to bladder dysfunction has led to a better understanding of IC/BPS physiology and potential treatments. Relative location and density of these channels indicate specific functional contributions in supporting normal voiding behavior (Yamada et al., 2009).

TRPV1

TRPV1 channels are temperature-sensitive and respond to a variety of sensory molecules that will transmit nociception, including nerve growth factor (NGF), bradykinin, and capsaicin (Chuang et al., 2001). These channels are expressed in afferent nerves that relay information on bladder distension and chemical irritation from the urinary bladder (Yamada et al., 2009). Activation of these channels causes perceived pain, bladder hypersensitivity, and compromised bladder function. Birder and colleagues (2001) found TRPV1 channels in the urothelium cells as well. TRPV1(-/-) mice have more non-voiding bladder

contractions than control mice and a loss of unconscious reflexive voiding (Birder et al., 2002). This suggests a role for TRPV1 in normal bladder function, as there is dysfunction present without it. Desensitizing TRPV1 channels using capsaicin did not impact ICI in these mice, though it did have a negative effect on ICI in controls (Birder et al., 2002). These results suggest that the TRPV1 is important in detecting irritants like capsaicin and amplifying the signal as it travels towards spinal and brain centers to evoke a response. This indicates that TRPV1 acts in a physiologically relevant manner in bladder afferents.

TRPV4

Previous studies have indicated that TRPV4 channels may function as a sensor of mechanical pressure in the bladder. Extensive TRPV4 expression has been found in the urothelium (Yamada et al., 2009). Alessandri-Haber and colleagues (2003) demonstrated that TRPV4 acts on primary afferent nerve fibers in response to changes in osmolarity and/or mechanical stretch. It seems that these fibers are mechanosensitive but capsaicin-insensitive nociceptive (pain) fibers (Aizawa et al., 2011). Activation of TRPV4 caused pain-related behavior in rats, supporting the idea that the channel is involved in sensory transmission (Alessandri-Haber et al., 2003). These behaviors were not seen in TRPV4 knockout mouse models (Alessandri-Haber et al., 2003). This suggests that TRPV4 signaling is required for hypotonic-related nociception. Guler and colleagues (2002) also found that TRPV4 channels are activated in response to warm temperatures, with increased sensitivity to heat responses in hypo-osmotic solutions. This suggests that TRPV4 could be a

multimodal receptor, responding to and integrating at least two types of sensory information.

The functional significance of TRPV4 has not been fully elucidated, but clear behavior changes have been found. Mice without TRPV4 channels in the urinary bladder have fewer voiding contractions (Gevaert et al., 2007). Similarly, research by Everaerts and colleagues (2010) has also shown difficulty in producing high cystitis-like voiding frequencies in mice TRPV4 (-/-) mice. Merrill and colleagues (2012) found increased expression levels of TRPV4 in chronic cystitis. Similar bladder function changes have been seen with stress models. Merrill & Vizzard (2014) found increased TRPV4 expression in the urinary bladder following stress. Similar results occurred in control mice with TRPV4 agonist administration, suggesting that increased TRPV4 expression may contribute to bladder dysfunction (Merrill & Vizzard, 2014). These decreases in bladder capacity with increases in TRPV4 expression suggest that this channel has an important mechanosensor role in normal bladder function.

In control and cystitis-model mice, TRPV4 antagonist action increases bladder capacity and reduces ICI (Thorneloe et al., 2008). There was also an improvement in bladder capacity and ICI following TRPV4 antagonist administration in stressed animal models (Merrill & Vizzard, 2014). By detecting changes in bladder wall stretch, these receptors signal apparent bladder fullness. These results suggest the involvement of TRPV4 in normal bladder function, as well as a possible role in the response to stress and cystitis.

Pituitary adenylate cyclase-activating polypeptide (PACAP)

PACAP-38, first isolated by Miyata and colleagues (1989), is a well-conserved sensory neurotransmitter in mammals with a widespread presence throughout the central and peripheral nervous systems. Dun and colleagues (1996) found PACAP-immunoreactivity (PACAP-IR) in the dorsal horn of the spinal cord, with light expression in ventral horn. A distribution primarily in the dorsal horn suggests involvement in sensory and autonomic activity. With administration of capsaicin, the number and intensity of PACAP-IR nerve fibers were reduced in the lower urinary tract (LUT; comprised of the bladder, urethra, and prostate) (Fahrenkrug & Hannibal, 1998). This led them to propose that PACAP expression depends on sensory input. Helyes and colleagues (2007) went on to show that PACAP is released by capsaicin-sensitive neurons into systemic circulation. This would support the idea that PACAP does respond to sensory information, as we know that capsaicin-responsive neurons are located in the afferent limb of bladder innervation. This also suggests co-localization of PACAP with TRPV1 channels in urinary bladder afferents.

Variations in PACAP levels have been shown to cause a variety of functional changes in the micturition process. PACAP-38 was found to produce relaxation of the pig urinary bladder neck through PACAP receptor activation and subsequent inhibitory signaling (Hernandez et al., 2006). The bladder neck is the sphincter smooth muscle that, when relaxed, allows for voiding. High levels of PACAP may then prompt repeated relaxations of this muscle that may contribute to high levels of non-voiding contractions of the detrusor. This effect was diminished with administration of the antagonist PACAP(6-38) (Hernandez et al., 2006). PACAP (-/-)

mice show increased bladder capacity, ICI, and void volume (May & Vizzard, 2010). The detrusor was contracted for significantly longer than controls and there was a large residual volume (May & Vizzard, 2010). PACAP has been observed in spinal cord (L1, L2, and L4-S1) and DRG (L1, L2, L6, and S1), with an increase in expression after chronic cystitis (Vizzard, 2000). This shows further presence in afferent signaling.

Girard and colleagues (2013) demonstrated an increase in TRPV4 expression in the lumbosacral (L1, L2, L6-S1) DRGs in transgenic mice with a chronic overexpression of nerve growth factor (NGF), which regulates PACAP and its receptors. Mice with chronic overexpression of NGF at the level of the urinary bladder exhibit significantly increased voiding frequency. This suggests that TRPV4 channel changes may show similarities to changes in PACAP, which aids in detrusor muscle contraction for voiding. Combined with the understanding that TRPV1 and PACAP are expressed in capsaicin-sensitive neurons, these factors seem to be co-expressed.

An increase in PACAP expression with cystitis could explain bladder overactivity. The blockade of PACAP-selective channels using the antagonist PACAP(6-38) reduces urinary frequency in rats with bladder inflammation induced by cyclophosphamide (CYP) (Braas et al., 2005). This is supported by research demonstrating a decrease in bladder overactivity with PACAP(6-38) administration in rodent models of chronic spinal cord injuries, another inflammatory condition (Yoshiyama & de Groat, 2008). Because stress can lead to inflammation and previous cystitis models shown upregulation in PACAP and TRPV channels (Braas et

al., 2005; Girard et al., 2013; Vizzard, 2000), the expression of both PACAP and TRPV channels after prolonged stress is expected to be similar to that seen with cystitis. Previous research has also suggested that PACAP and TRPV channels are both expressed in small diameter DRG neurons (Helliwell et al., 1998; Moller et al., 1993). Because of similar function and co-expression, alleviation of stress-related urinary symptoms may be possible through alterations of these mechanisms. This may provide insight into possible targets for drugs to help provide relief for voiding changes seen in inflammatory conditions.

Bladder Function & Stress

Bladder dysfunction reduces quality of life through psychosocial factors, including financial, social, physical, and emotional stress. Severe symptoms can limit patients' confidence to travel far from their homes. IC/BPS has been shown to lead to increased likelihood of missing or quitting work through collective symptom impact, depressive symptoms, and co-morbidities (Beckett et al., 2014). These comorbidities may often be mental health concerns. For example, there is an increased prevalence of IC/BPS in those diagnosed with anxiety disorders (Clemens, Brown, & Calhoun, 2008). Life stressors can exacerbate bladder dysfunction. Rothrock and colleagues (2001) have found that higher levels of stress are related to increased IC/BPS symptoms.

Although many functional changes in LUT physiology have been implicated in IC/BPS, stress-related changes to urinary sensory mechanisms are largely unknown. These physiological changes, however, are very important to understand and create therapies for stress-related bladder dysfunction. This research will

include studying changes in the relay of the message via modulation of ion channel and neuropeptide expression at key locations (e.g., urinary bladder, spinal cord, and DRG) in the voiding reflex, and advancing this knowledge may eventually contribute toward providing relief to millions with bladder dysfunction. Following chronic stress, I hypothesize that there is increased expression and co-expression of TRPV1 and TRPV4 channels with PACAP in the lumbosacral (L1, L2, L6, and S1) DRG. I also hypothesize that chronic stress will increase voiding frequency and threshold pressure, which will be alleviated with PACAP(6-38) administration into the urinary bladder.

Methods

The work was completed in the Vizzard Laboratory in the Given Building and in the Given Animal Care Facility at the UVM College of Medicine. Dr. Vizzard and other laboratory members including a senior research technician and postdoctoral associate were available to provide day-to-day guidance and support. All experimental protocols involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Repeated Variate Stress

PACAP-GFP transgenic mice donated by Dr. James A. Waschek (UCLA) were bred in-house onto a C57BL/6 background. Adult PACAP-GFP mice (female n=12, male n=15) were randomly assigned by cage to control or stressed groups. All animals remained housed as they were before the experiment (single or colony), were maintained on a 12-hour light/dark cycle, and had access to food and water *ad libitum*. Enrichment was prohibited, including huts. The stressed mice underwent

seven days of repeated variate stress, with one stressor per day as seen in Table 1. This paradigm works to best resemble life stress, as there are multiple different sources of stress that change frequently.

Table 1. The schedule and length of stressor events in the chronic variate stress protocol.

Day	Stressor	Duration
1	Pedestal	30 minutes
2	Swim	5 minutes
3	Foot Shock	two at 5 seconds each, separated by one minute of rest
4	Restraint	1 hour
5	Oscillation	30 minutes
6	Swim	5 minutes
7	Foot Shock	two at 5 seconds each, separated by one minute of rest

Pedestal: Mice were placed on a pedestal platform (7"x7") three feet from the ground for thirty minutes. There was one mouse per platform and the two pedestals were placed in the center of black netting to catch any mice that jump or fall off of the platform. The platforms were sanitized with an alcohol solution in between mice to remove urine and fecal pellets.

Swim: Mice were forced to swim individually for five minutes in room temperature water deep enough that their tails could not touch the bottom of the container.

Afterward, they were placed in a holding cage with super absorbent bedding for five minutes before being returned to their home cages.

Foot Shock: Mice were put in a 30 × 25 × 35 cm (L × W × H) Plexiglas conditioning chamber (Med Associates, St. Albans, VT) to acclimate for five minutes. Then, two five-second 0.29 mA shocks were delivered one minute apart through the chamber floor. The drop tray was sanitized in between animals to remove urine and fecal pellets.

Restraint: Mice were placed headfirst into 50mL tubes that were modified such that the bottom portion was cut off to provide ventilation and a slit towards the top of the tube allowed the tail to rest more naturally outside of the tube. A rubber plug with additional ventilation holes was placed into the tubes behind the mouse to restrict backward motion. Blocks were placed on either end of the tubes so that they could not roll. Mice remained in the tubes for one hour.

Oscillation: Mice were placed individually into clean and empty standard mouse housing cages that were placed on a multi-purpose rotator (Fisher Scientific, Morris Plains, NJ) to oscillate for 30 minutes at slow to medium speed.

Bladder Tube Implant

After seven days of stress, the mice underwent an intravesical catheter implant. Using 2% isoflurane (3% for stressed animals) as general anesthesia, a lower midline abdominal incision was made to expose the bladder. Polyethylene tubing was inserted into the dome of the bladder and secured using a 6-0 nylon purse-string suture. The sealed distal end was tunneled subcutaneously to the back of the neck to remain in the subcutaneous space during recovery. The abdominal

incision was closed with a chromic gut suture through the abdominal muscle and then a 4-0 nylon running stitch externally. The neck incision was closed with 4-0 nylon stitch and both incision sites were covered with GLUture. Buprenorphine (0.03 μ l dose of .05 mg/ml) and carprofen (0.01 ml/g dose of 0.05 mg/ml) administered subcutaneously were given as analgesics prior to surgery and for 72 hours post-operatively.

Tube Externalization

After the recovery period, the mice were anesthetized with isoflurane (2%) to exteriorize the distal end of the tubing for cystometry. The stitches in the back of the neck were removed, the extra length of tubing was removed from the subcutaneous space, and the neck was resealed with GLUture. Tape was used to cover the externalized tubing to prevent any chewing damage.

Bladder function evaluation with cystometry

The mice were placed unrestrained in a cystometry recording cage with a wire floor. The tubing was connected to a pressure transducer port, which was connected to a syringe pump that infused saline at a constant rate (25 μ l/min) to produce repetitive bladder contractions. Measuring began after a 15-minute anesthesia recovery period and acclimation time. A pan on a balance placed below the cage was used to catch and measure urine output. The minimum pressure, fill pressure, voiding threshold pressure, maximal voiding pressure, ICI, bladder capacity, and void volume was recorded by a small Animal Cystometry System (Med Associates, St. Albans, VT). Bladder capacity was measured as the infused volume between micturitions. To measure the effects of a PACAP blockade, the mice were

given a continuous 300 nM infusion of PACAP(6-38), a PAC1 receptor antagonist, for thirty minutes. This was done by first disconnecting the mouse's tubing, removing remaining saline from the tubing, filling with PACAP antagonist solution, and then reconnecting the tubing and restarting the infusion pump. Following this period, the line was again flushed to remove remaining antagonist before a second round of recording with the saline infusion.

Tissue Collection & Preparation

The mice were euthanized via isoflurane (5%) and thoracotomy. The brain, spinal cord, DRG, and bladder were removed. Detrusor and urothelium layers were separated from each other. Tissues were placed in a 4% paraformaldehyde solution in a phosphate buffered saline (PBS) solution overnight, which was replaced by a 30% sucrose solution in PBS for the following week. Detrusor and urothelium were cross-sectioned through the longer plane using surgical scissors. The other tissues were embedded in an OCT compound within a cryomold cassette for sectioning. The tissue was sectioned using a cryostat and mounted onto slides (DRG, 10 μ m) or free-floated in wells (brain and spinal cord sections, 40 μ m).

Immunohistochemistry

Immunohistochemistry using anti-TRPV4-ATTO-550 (#ACC-034-AO, Alomone Labs, Jerusalem, Israel) and VR1 primary antibody (R-130, Santa Cruz, Santa Cruz, CA) was used to determine the expression of TRPV1 and TRPV4 channels. Species-specific secondary antibodies tagged with fluorophores distinct from GFP were used to label both TRPV1 and TRPV4. Antibody specificity was previously demonstrated using DRG and spinal cord tissue harvested from TRPV1

and TRPV4 null mice. In tissues from TRPV1 and TRPV4 null mice, no TRPV1- or TRPV4-immunoreactivity, respectively, was demonstrated. Additionally, the transgenic mice (PACAP-GFP) are designed to exhibit green fluorescent protein (GFP) expression at sites with PACAP expression. Then the slides were viewed with a fluorescence microscope to determine the location and expression density of the TRPV channels of interest. Optical density and co-expression software with confocal microscopy were used to quantify expression, changes in expression and co-expression of TRPV channels and PACAP.

Statistical Analyses

All values represent means \pm SE. Cystometry data were compared using paired *t*-tests and ANOVA, with each animal serving as its own control. Immunohistochemical data were compared with independent *t*-tests. Statistics were run using SPSS software obtained through The University of Vermont.

Results

Bladder Function

Generally, a healthy bladder results in relatively high bladder capacity with low pressure and therefore relatively long inter-micturition intervals. All bladder pressure values should be relatively low, with the peak micturition pressure having the highest value.

Females. There was no significant difference between control and stressed functional values before PACAP(6-38) infusion. Both control and stressed female groups saw improvement on all functional measures after infusion. Control mice saw significant improvement post-infusion on ICI measures ($t(5) = 3.461$, $p \leq 0.05$)

and bladder capacity ($t(5) = 3.466, p \leq 0.05$) (Fig. 1 & 2). Threshold, minimum, fill, and maximum pressures all decreased post-infusion but not significantly (Fig. 3). The stressed cohort saw significant improvement post-infusion on ICI measures ($p \leq 0.05$) and bladder capacity ($p \leq 0.05$) (Fig. 4 & 5). While all pressure measures did decrease post-infusion, no changes were significant (Fig. 6).

Males. There were no significant differences between control and stressed functional values before PACAP(6-38) infusion. Both control and stressed male groups saw improvement on all functional measures after infusion. Control mice had non-significant increases in ICI and bladder capacity (Fig. 7 & 8) and decreases in all pressure values (Fig. 9). Stressed mice had significant increases in ICI ($t(6) = 4.312, p \leq 0.01$) and bladder capacity ($t(6) = 4.310, p \leq 0.01$) (Fig. 10 & 11). All four pressure values were decreased, with minimum pressure ($t(6) = 3.065, p \leq 0.05$) and fill pressure ($t(5) = 2.452, p \leq 0.05$) being significantly so (Fig. 12).

Histology

In the DRG, there was increased expression of TRPV1-IR and PACAP-IR cells in the stressed mice (Fig. 12 & 13). These increases were significant at the L1 ($p \leq 0.001$), L2 ($p \leq 0.01$), L6 ($p \leq 0.01$), and S1 ($p \leq 0.001$) levels. PACAP-IR was also increased under stress conditions at all levels of the DRG, with significant increases at L1 ($p \leq 0.001$), L5 ($p \leq 0.05$), and S1 ($p \leq 0.05$). The number of cells expressing both TRPV1-IR and PACAP-IR was similar between stressed and control samples at all levels of the DRG.

Discussion

Bladder Function

The repeated variate stress regime decreased ICI and bladder capacity while increasing bladder pressures in both male and female mice. This presentation is remarkably similar to that seen in animal models of IC/BPS. These results suggest that chronic levels of stress are capable of producing functional changes in the urinary system. Stress was the only experimental condition in this research, which suggests that these urinary changes may occur in the absence of other contributing factors, such as disease. When combined with another condition like IC/BPS or spinal cord injury, stress could then exacerbate current symptoms or add new complications.

Intravesical PACAP(6-38) Infusion

PACAP(6-38) was shown to aid urinary bladder function across all experimental groups. Improvement was seen most consistently in ICI and bladder capacity values, with female control, female stressed, and male stressed cohorts showing significant improvement. This provides supporting evidence to target PACAP for symptom relief.

Female stressed and control mice showed better functional values after PACAP(6-38) infusion. Neither group showed significant changes from their pre-infusion values. Because both stressed and controls saw similar levels of functional improvement, the effect of PACAP(6-38) infusion on bladder function may be universally beneficial. This would also suggest, then, that the magnitude of effect of PACAP(6-38) on the urinary bladder is not improved in the presence of stress.

PACAP(6-38) could be working in a non-specific manner to generally improve the function of the bladder without addressing the specific mechanism(s) altered by stress. Brass and colleagues (2005) have found evidence for PACAP(6-38) in cystitis symptom alleviation. This provides support for a positive effect on inflammatory states, despite the method of impact.

This equal improvement in both experimental groups was not the case in the male mice. While all functional values of stressed and control male mice improved with infusion, stressed mice showed more improvement than controls. This was seen in the significant decreases in minimum and fill pressures for stressed mice post-infusion. This would suggest that stress might increase the magnitude of effect for this therapy, whether it is caused by an increase in general health of the system or by improvement of a stress-specific mechanism.

Additionally, both theories may be right and there may be sex differences in urinary innervation, control, and response to stress. Overall, these sex differences in response to therapy may also suggest that the PACAP(6-38) infusion is having sex-dependent effects on bladder function. This could be the result of differing mechanisms of bladder innervation or varying levels of contribution the PACAP mechanisms may have on overall function.

Mouse estrous cycles were not controlled for in this experiment, which could be a confounding variable in the female response to stress. Other experiments on bladder function have made efforts to control for estrous cycles (Wang et al., 2008). Shea and colleagues (2000) have suggested that sensory innervation to the bladder could be sensitive to hormonal status, especially mechanoreceptive input. Johnson

and Berkley (2002) found that threshold pressure of inflamed bladders, but not normal bladders, was influenced by estrous state. This could be a direct result of hormones or, more likely, the interaction between hormones and sensory neurotransmitters. This could contribute to the increased variability seen in female mice groups, and should be taken into consideration with housing style and timing of future experiments.

Neither of the stressed groups was significantly worse in baseline functional values when compared to their control counterparts. This may raise questions to the validity of the repeated variate stress regime and if the stressed cohorts were actually stressed to a substantial degree to be considered a good model for chronic stress. However, functional values for stressed groups were on average worse than controls before administration of PACAP(6-38). All values trended in the expected direction under stress despite significance. These animals were also visibly stressed, as seen by anxiety-like behaviors including increased startle, frequent urination/defecation, and hair loss. These behaviors were not measured.

Further measures like blood cortisol levels could be taken to determine stress level. Preliminary cortisol measurements done in new groups of male control (n=4) and stressed (n=5) and female control (n=5) and stressed mice (n=5) showed that stressed groups had higher average cortisol levels than control groups. Neither female ($p = .102$) nor male ($p = .068$) was significantly above the control condition. Being that this is preliminary testing, a larger sample size could lead to significance and a stronger argument for substantial stress under the repeated variate stress regime.

Histology

The stressed male mice saw significantly increased rates of both PACAP- and TRPV1-IR cells in the DRG. This would support the theory that PACAP is a sensory neurotransmitter that participates in the relay of sensory information, especially nociception. The increase in expression suggests that more noxious input is sent to CNS control centers, contributing to higher levels of reflexive urge to urinate/bladder contractions and perceived pain or discomfort. This, then, could account for accommodating changes in bladder function in an attempt to react to this abnormal sensory information. It could also explain the increased awareness of the urinary bladder state (notably pain), resulting in psychological symptom development. Therapies targeting PACAP, then, are targeting the afferent branch of this loop, reducing information transmission before it reaches the central relay centers.

PACAP-IR cells in the bladder provide support for the presence of this powerful substrate in peripheral tissue, with physiological functions in the immediate area. PACAP may also have an immediate effect on release, as it has been shown before to act in a localized manner on smooth muscle inhibition and indirect excitation (Fox-Threlkeld et al., 1999). The location and function of the detrusor smooth muscle in bladder reflexology lends itself as a possible target for PACAP upregulation.

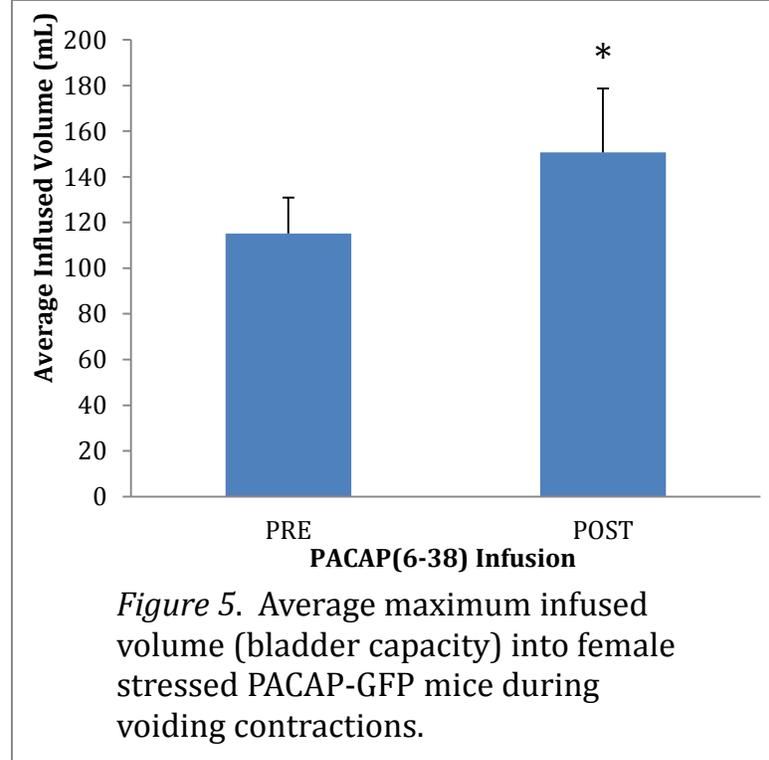
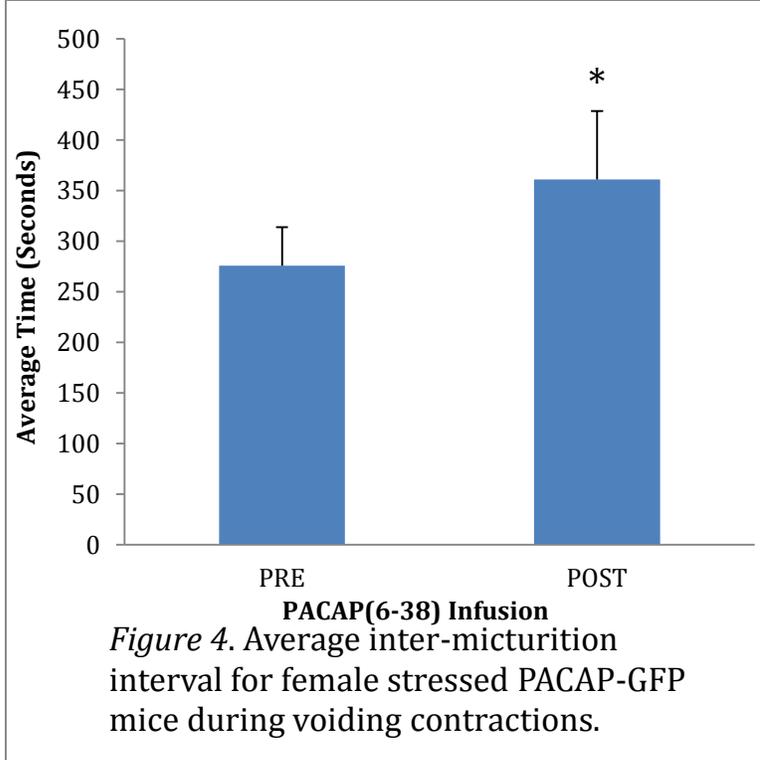
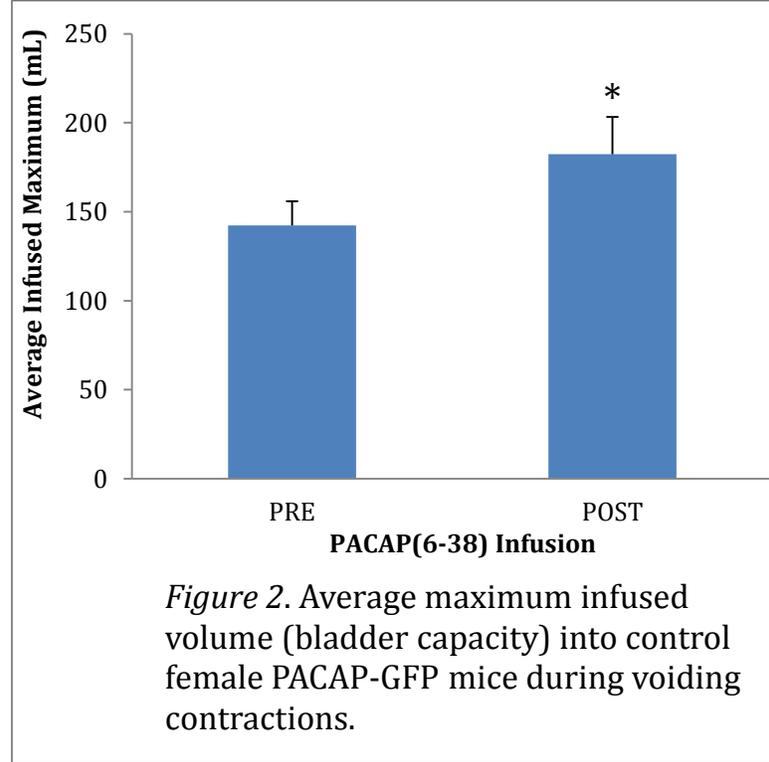
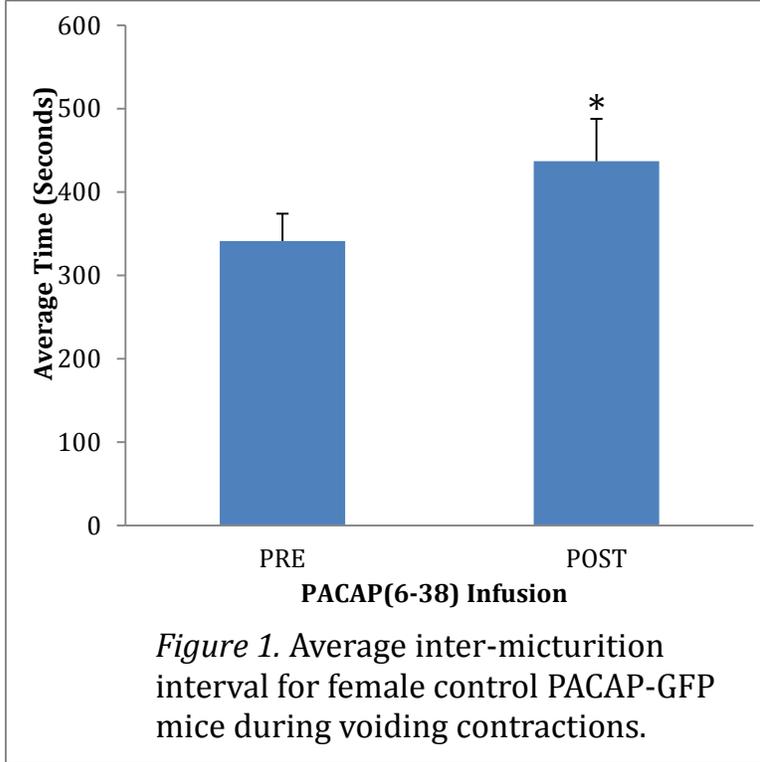
Future Directions

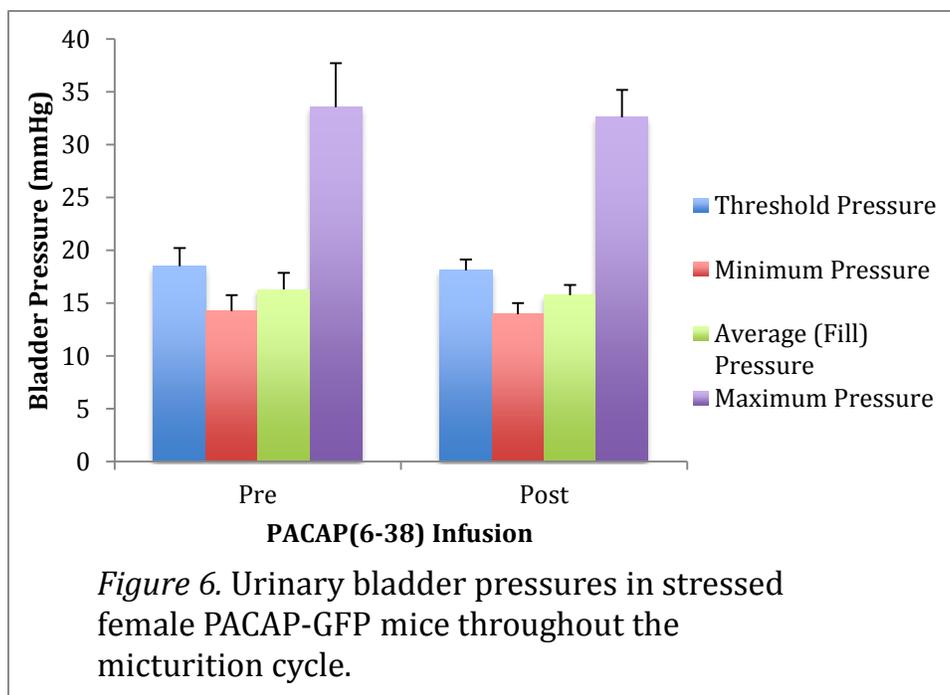
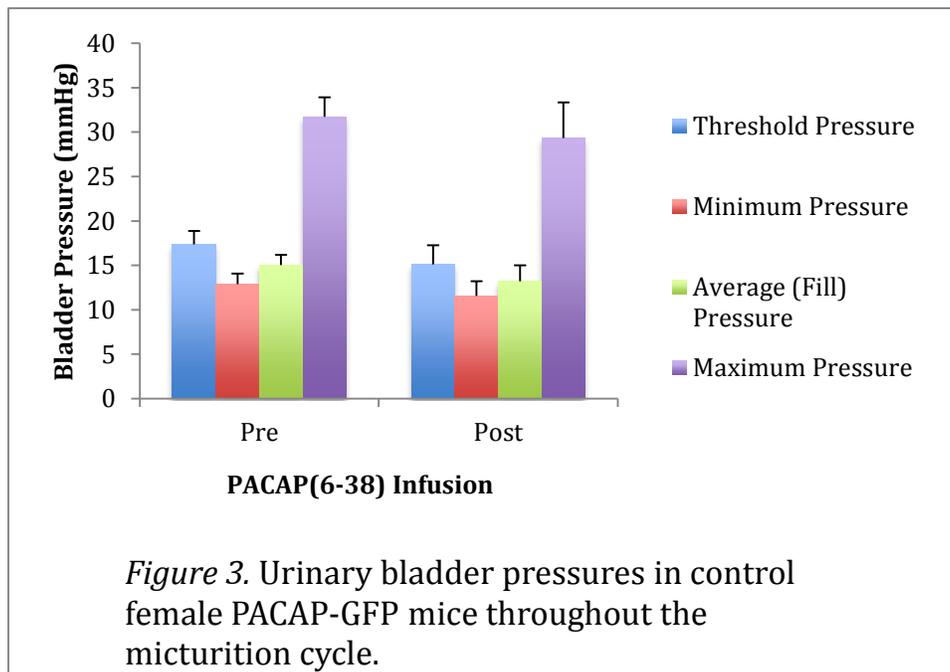
This work should be continued in an effort to fully describe the effect of chronic stress on the body. Further research on both sexes with control for estrous

cycle will help to determine the real differences in control and organization of the urinary bladder innervation that may lead to varying presentations of stress and responses to therapeutics. It is unclear what factor of urinary control and output may be causing these apparent sex differences in this experiment.

PACAP(6-38) infusion improved bladder function overall and therefore PACAP receptors may be a valid target for therapy for those with bladder dysfunction caused or exacerbated by stress. Because chronic stress presents with symptoms similar to other conditions, notably cystitis, PACAP(6-38) administration should be evaluated in other bladder dysfunction states.

Appendix





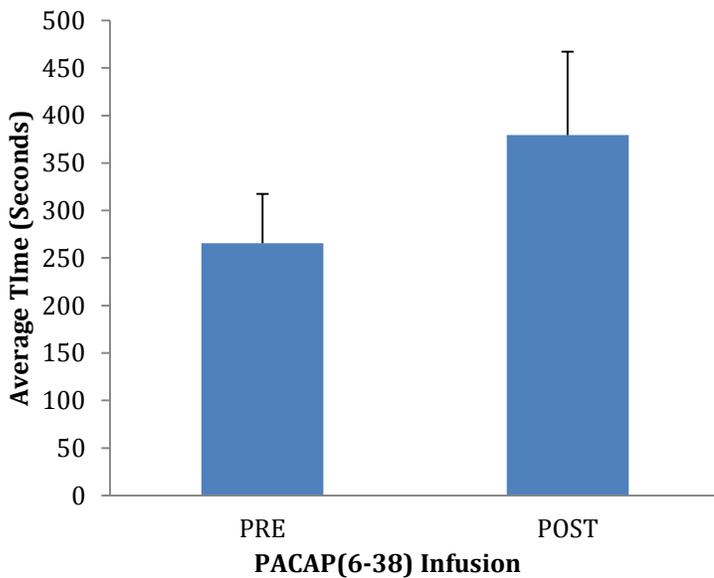
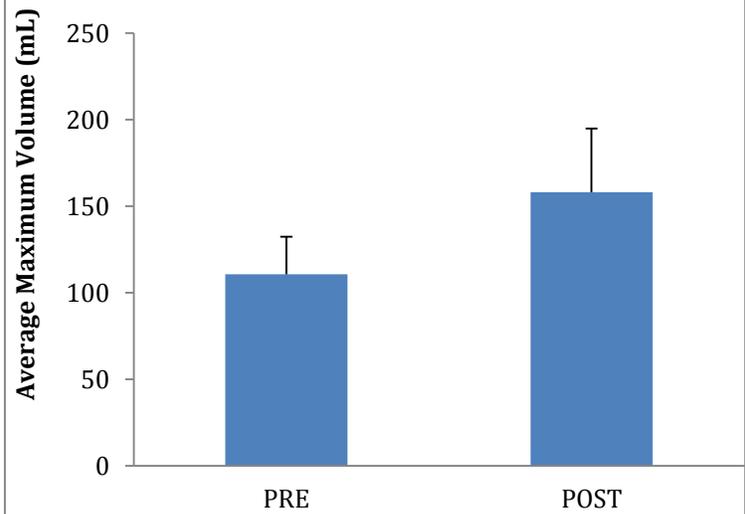


Figure 7. Average inter-micturition interval for control male PACAP-GFP mice during voiding contractions.



PACAP(6-38) Infusion
Figure 8. Average maximum infused volume (bladder capacity) for control male PACAP-GFP mice during voiding contractions.

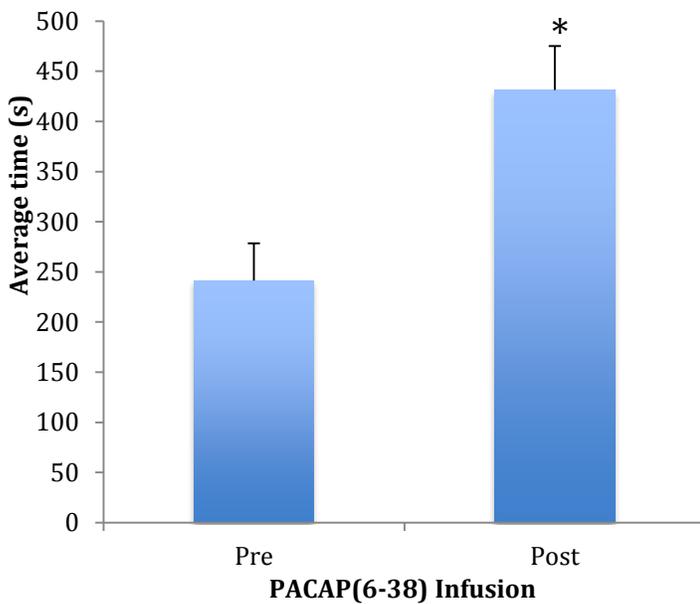
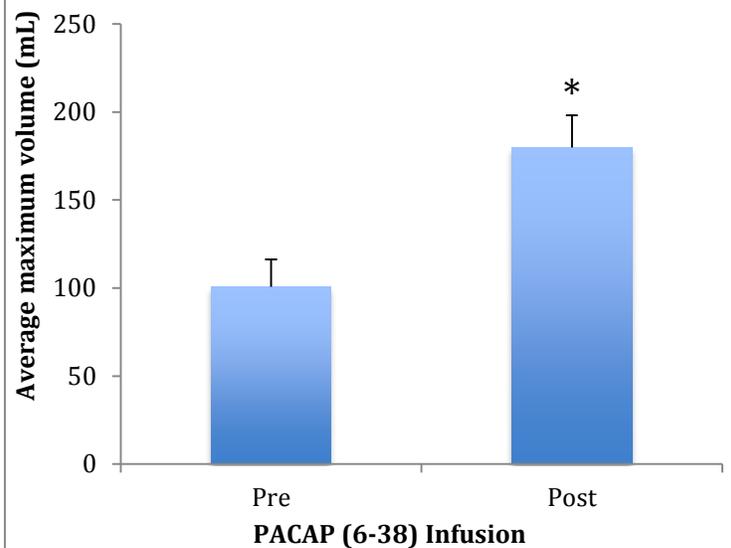
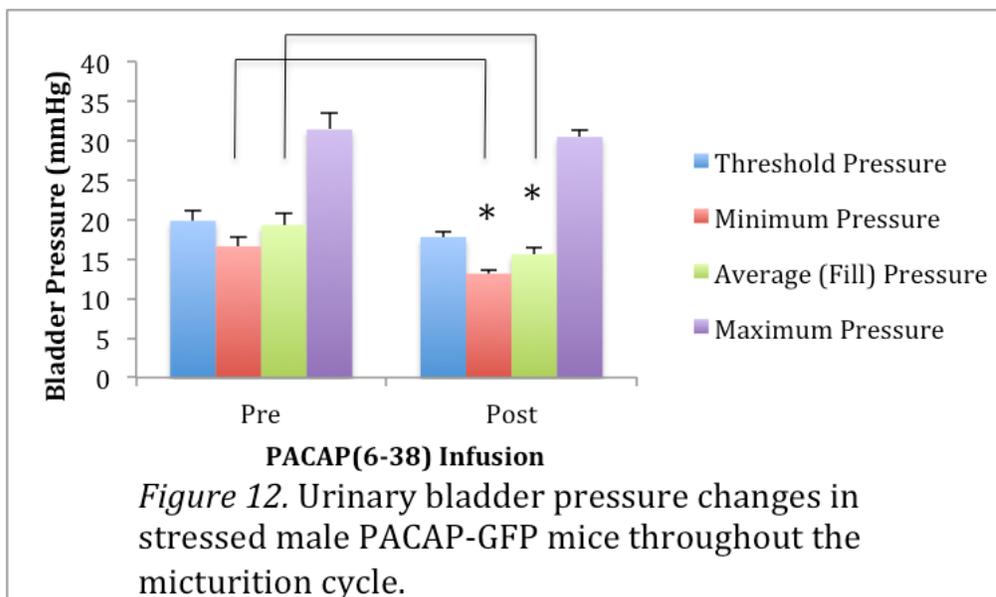
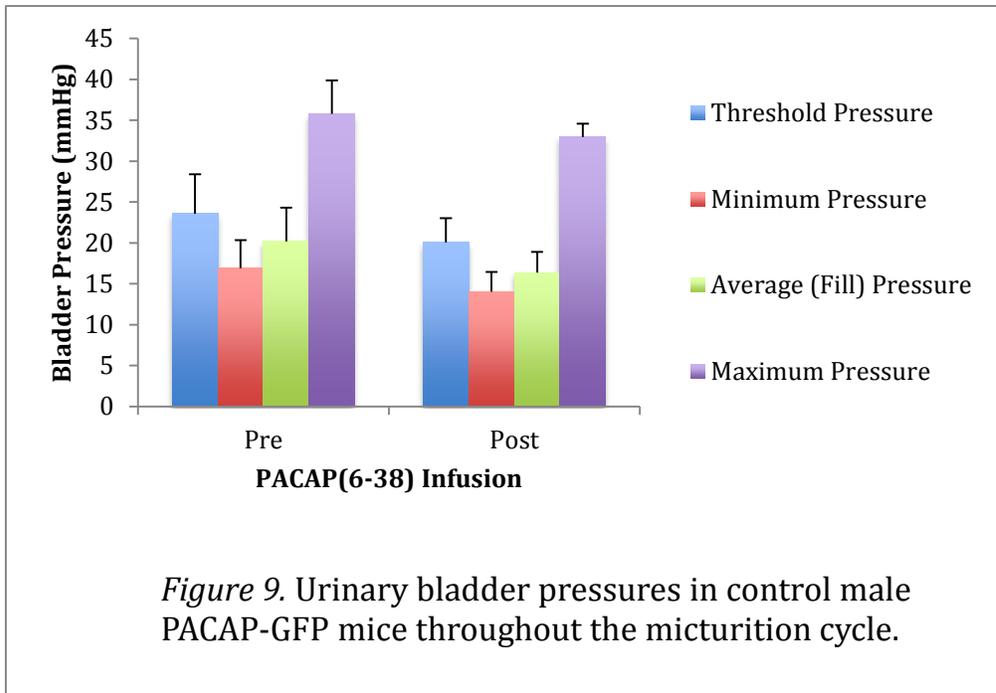


Figure 10. Average inter-micturition interval for stressed male PACAP-GFP mice during voiding contractions.



PACAP (6-38) Infusion
Figure 11. Average maximum infused volume (bladder capacity) for stressed male PACAP-GFP mice during voiding contractions.



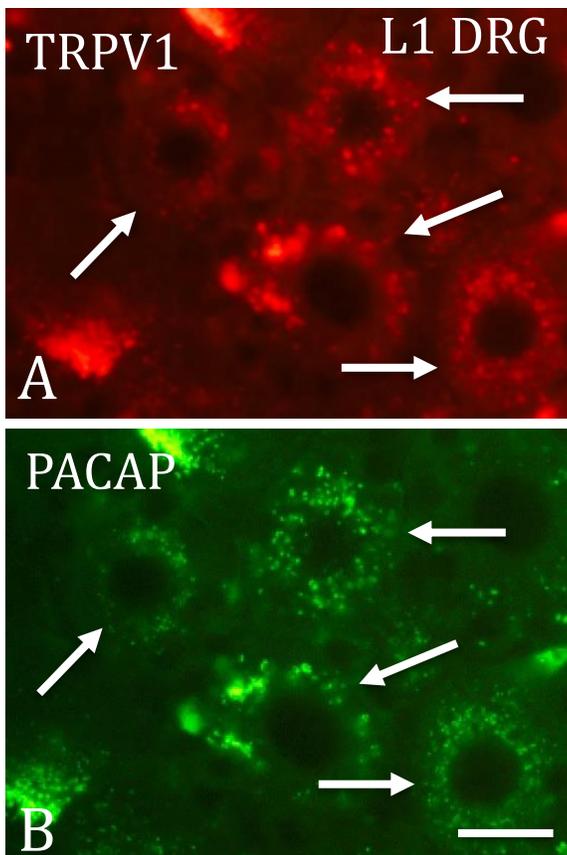


Figure 12. TRPV- and PACAP-IR in DRG sections at the L1 level of a stressed mouse.

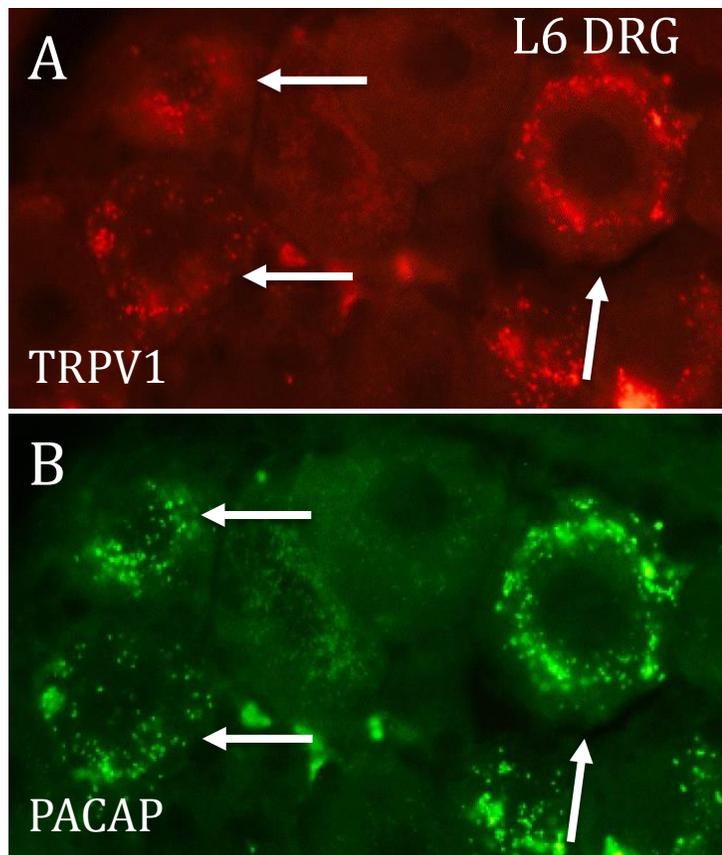


Figure 13. TRPV- and PACAP-IR in DRG sections at the L6 level of stressed mouse.

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