Journal of Molecular Neuroscience (2021) 71:1575–1588
https://doi.org/10.1007/s12031-020-01649-x

Intrabladder PAC1 Receptor Antagonist, PACAP(6-38), Reduces Urinary Bladder Frequency and Pelvic Sensitivity in Mice Exposed to Repeated Variate Stress (RVS)

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Received: 17 May 2020 / Accepted: 22 June 2020 / Published online: 1 July 2020

Abstract

Stress causes symptom exacerbation in functional disorders of the urinary bladder. However, the potential mediators and underlying mechanisms of stress effects on micturition reflex function are unknown. We have characterized PACAP (*Adcyap1*) and PAC1 receptor (*Adcyap1r1*) signaling in stress-induced urinary bladder dysfunction in mice. We determined PACAP and PAC1 transcripts and protein expressions in the urinary bladder and lumbosacral dorsal root ganglia (DRG) and spinal cord in repeated variate stress (RVS) or control mouse (handling only) groups. RVS in mice significantly ($p \leq 0.01$) increased serum corticosterone and urinary bladder NGF content and decreased weight gain. PACAP and PAC1 mRNA and protein were differentially regulated in lower urinary tract tissues with changes observed in lumbosacral DRG and spinal cord but not in urinary bladder. RVS exposure in mice significantly ($p \leq 0.01$) increased (2.5-fold) voiding frequency as determined using conscious cystometry. Intrabladder administration of the PAC1 receptor antagonist, PACAP(6-38) (300 nM), significantly ($p \leq 0.01$) increased infused volume (1.5–2.7-fold) to elicit a micturition event and increased the intercontraction interval (i.e., decreased voiding frequency) in mice exposed to RVS and in control mice, but changes were smaller in magnitude in control mice. We also evaluated the effect of PAC1 blockade at the level of the urinary bladder on pelvic sensitivity in RVS or control mouse groups using von Frey filament testing. Intrabladder administration of PACAP(6-38) (300 nM) significantly ($p \leq 0.01$) reduced pelvic sensitivity following RVS. PACAP/receptor signaling in the CNS and PNS contributes to increased voiding frequency and pelvic sensitivity following RVS and may represent a potential target for therapeutic intervention.

Keywords Repeated variate stress · Pain · Micturition · Urothelium · Dorsal root ganglia · Spinal cord

Introduction

Normal micturition involves the filling and storage of urine in the bladder and the periodic voiding of urine at socially appropriate times. Storage and elimination functions involve the reciprocal functions of the bladder, urethra, and external urethral sphincter, which are controlled by the coordination of the structural features of the bladder and complex neural pathways organized in the central nervous system (CNS) and peripheral nervous system (PNS) (Andersson 2004; Elbadawi 1996). Bladder pain syndrome (BPS)/interstitial cystitis (IC) is an urologic, chronic pelvic pain syndrome characterized by pelvic pain, pressure, or discomfort with urinary symptoms (Hanno and Sant 2001). Stress-induced symptom exacerbation (flares) among patients with BPS/IC is well recognized and attributed to multiple triggers (Nickel et al. 2016; Sutcliffe et al. 2014, 2018). Flares are bothersome, disruptive, and associated with increased pelvic pain and urologic symptoms (Nickel et al. 2016; Sutcliffe et al. 2014, 2018). Effective treatments and greater understanding of the stress contribution to BPS/IC are needed.

PACAP peptides are expressed in and exhibit diverse functions in multiple organ systems (e.g., endocrine, nervous, urinary, gastrointestinal, cardiovascular systems) and in the CNS and PNS, including sensory and autonomic ganglia (Arimura 1998; Arimura et al. 1991; Beaudet et al. 1998, 2000; Braas and May 1996, 1999; Braas et al. 1998; Brandenburg et al. 1997; May et al. 1998; May and Braas 1995; Cheppudira et al. 2009; Ghatei et al. 1993; Holgert et al. 1996; Klimaschewski et al. 1996; Koves et al. 1990, 1991; Masuo et al. 1993; Moller

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et al. 1997a, b; Nogi et al. 1997; Portbury et al. 1995; Shiotani et al. 1995; Sundler et al. 1996; Tatsuno et al. 1994). Neuropeptides are expressed in neural and non-neural components of the lower urinary tract (LUT) including afferent neurons and pathways, plasma, areas of tissue inflammation or injury, bladder fibroblasts, detrusor, and the urothelium. The balance of neuropeptides in LUT pathways can be affected by disease, neural injury, and target organ inflammation and contributes to a hyper- or a hypo-active reflex state of the LUT (Arms and Vizzard 2011). PACAP-IR is expressed in C-fiber bladder afferents in the dorsal root ganglia (DRG) and in nerve fibers within the urinary bladder smooth muscle, suburothelial nerve plexus, and surrounding blood vessels (Fahrenkrug and Hannibal 1998). The urothelium expresses the PACAP receptor, PAC1, and upon PACAP stimulation, the urothelial cells release ATP to stimulate receptors on underlying sensory nerve fibers in the suburothelial plexus (Girard et al. 2008). Mice with a genetic disruption or deletion of PACAP or vasoactive intestinal polypeptide (VIP) exhibit altered bladder and somatic function (May and Vizzard 2010; Studeny et al. 2008; Jensen et al. 2008). PACAP and PAC1 receptor expression exhibits neuroplastic changes in both the neural and non-neural components of the LUT following nerve injury or inflammation (Larsen et al. 1997; Moller et al. 1997a; Vizzard 2000c; Zhang et al. 1995, 1996).

Symptom exacerbation due to stress is prevalent in many disease states, including functional disorders of the urinary bladder (e.g., overactive bladder (OAB), BPS/IC) (Nickel et al. 2016; Sutcliffe et al. 2014; Sutcliffe et al. 2018); however, the mechanisms underlying the effects of stress on the micturition reflex function are unclear. The repeated variate stress (RVS) model has been used previously to examine stress-induced changes in bladder function and pelvic sensation in rats (Girard et al. 2017; Gonzalez et al. 2016; Merrill et al. 2013; Merrill and Vizzard 2014). In this study, we extend the use of the RVS paradigm to mice and examine the role of PACAP/PAC1 signaling to RVS-induced changes in voiding frequency and pelvic sensation. We have characterized PACAP/PAC1 expression and modulation with RVS exposure as well as the effect of PAC1 receptor blockade at the level of the urinary bladder, in RVS-induced urinary bladder dysfunction using biochemical, molecular, and functional approaches. Portions of this study have been presented in abstract (May et al. 2017b, 2019).

Methods

Animals

For these studies, we used a PACAP promoter-dependent EGFP BAC transgenic mice strain (PACAP-EGFP) in which PACAP-expressing cells and neurons are tagged with green fluorescent protein (GFP) for easy visualization and tracking (Condro et al. 2016; May et al. 2015, 2017a). PACAP-EGFP transgenic mice, Tg(Adcyap1-EGFP)FB22Gsat/Mmucd (RRID:IMSR_MMRRC:012011), were generated using the BAC (RP24-358O1) by the Gene Expression Nervous System Atlas (GENSAT) project and obtained from the Mutant Mouse Resource and Research Centers (Condro et al. 2016). The PACAP-EGFP construct is regulated by the endogenous promoter. Therefore, it is possible to examine whether altered physiology, including stress, can differentially regulate specific neuronal PACAP populations in the CNS and PNS. We have examined the effects of CYP-induced cystitis in PACAP promoter-dependent EGFP BAC transgenic mice (May et al. 2015, 2017a). Mice in the current study are also being used to define LUT neural pathways and neural populations in CNS and PNS following RVS with or without characterization of bladder function (May et al. 2017b). In pilot studies, we verified that control female PACAP-EGFP (handled but no stress exposure) mice had bladder capacity and somatic sensitivity similar to that of C57Bl/6 wild-type mice (Vizzard et al., unpublished observations) (Girard et al. 2016). Mice were bred locally at the Larner College of Medicine at the University of Vermont (UVM) animal facilities, and animal genotype was confirmed by PCR analyses. The UVM Institutional Animal Care and Use Committee approved all experimental protocols involving animal (IACUC #08-085, #13-030, #X9-020). The UVM Office of Animal Care Management oversaw all animal use in accordance with AAALAC and NIH guidelines. All efforts were made to minimize the potential for animal pain. Separate cohorts of female PACAP-EGFP mice were used in the following experiments. We used female mice because of the female predominance of IC/BPS (24, 67, 74). Estrous cycle status was not determined for mice in these studies.

RVS

Mice were randomly assigned to control or RVS groups and weighed daily. Mice assigned to the RVS group (hereafter RVS mice) were exposed to 7 days of stressors with a single stressor being presented on each day between 9 am and 12 pm as described previously (Hammack et al. 2009; Merrill et al. 2013; Merrill and Vizzard 2014) (Fig. 1A, B). Control mice were handled daily and remained in home cages in the animal facility following weighing. All mice within the RVS group were exposed to the same order of stressors for the same duration (Fig. 1B):

Oscillation stress: Mice were placed inside a plastic chamber 25 × 16 × 13 cm (L × W × H), which was secured to a clinical rotator (Fisher Scientific, Morris Plains, NJ) and oscillated at low to medium speed for 30 min (min). *Forced swim*: Mice were placed in a cylindrical container 29 × 37 cm (D × H) that was filled with room temperature water

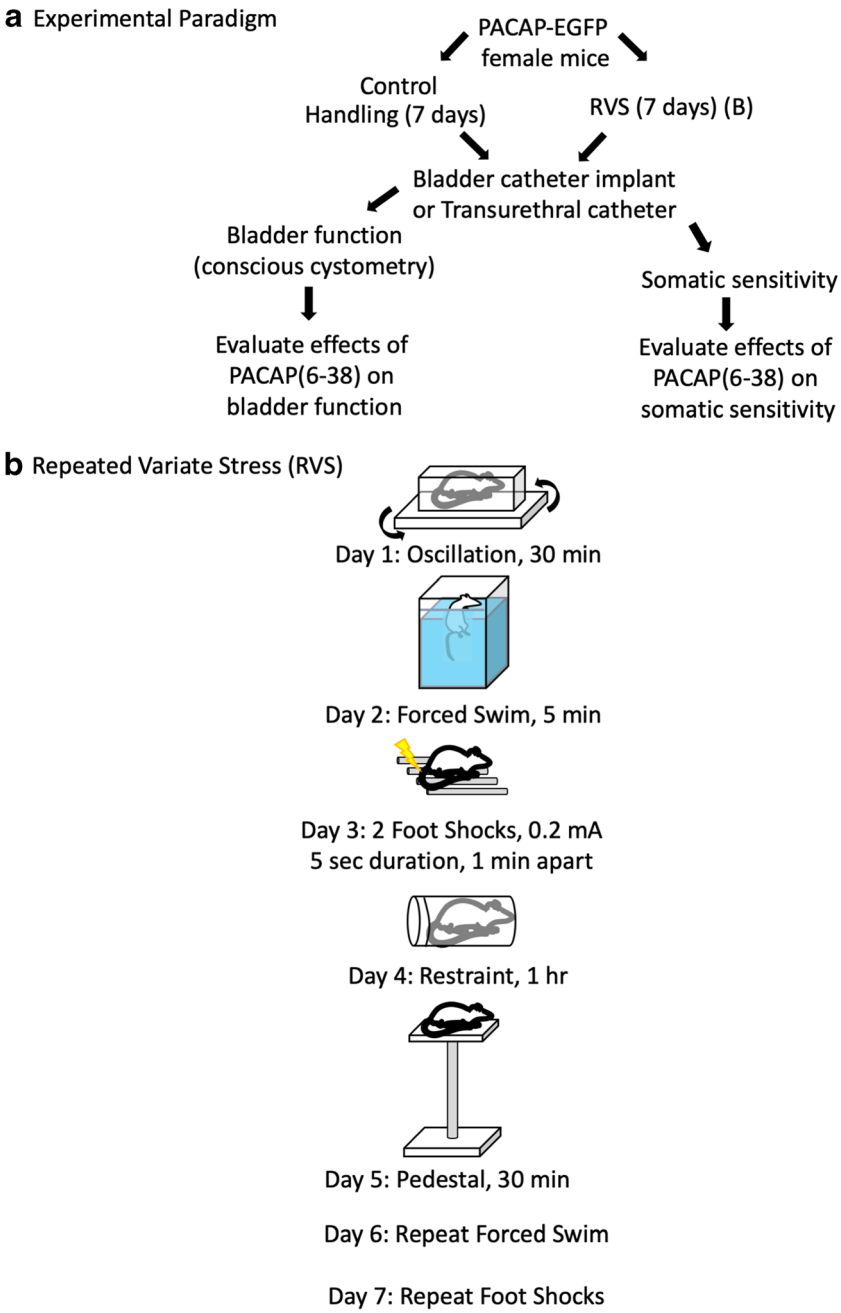

Fig. 1 **A** Experimental paradigm. Overview of studies using control mice (handled only) and mice exposed to repeated variate stress (RVS) and the two primary outcome measures measured in this study: urinary bladder function and referred somatic sensitivity of the pelvic region. **B** Repeated variate stress (RVS) protocol (7 days) in the order of stressor presentation. Five different stressors and the duration of each stressor for each day they are administered in the RVS protocol (Longden et al. 2014a, b; Merrill et al. 2013; Merrill and Vizzard 2014) are indicated. Swim and foot shock stressors are repeated on the last 2 days of RVS. hr hour, s seconds, min minutes

to a depth that prevented the tail from touching the bottom of the container. After 5 min of monitored swimming, mice were placed in a holding chamber for 30 min prior to being returned to their home cage. *Electrical foot shock*: Mice were placed inside a Plexiglass conditioning chamber (Med Associates, St. Albans, VT) 30 × 25 × 35 cm (L × W × H). After a 5-min acclimation period, two 0.2-mA, 5-s scrambled foot shocks were delivered through the grid floor with a 1 min intertrial interval. *Restraint*: Mice were placed in a cylindrical restraining device 30 × 115 mm (D × L) for 60 min. *Pedestal*: Mice were placed on an elevated (60 cm) platform 20 × 20 cm (L × W) for 30 min.

Euthanasia and Tissue Harvest

Female (control and RVS mice, $n = 8$ each) mice were deeply anesthetized with isoflurane (5%) and then euthanized via thoracotomy. The urinary bladder and lumbosacral (L6-S1) DRG and spinal cord were quickly dissected under RNase-free conditions as previously described (Girard et al. 2010; Girard et al. 2013). The bladder was cut open along the midline and pinned to a sylgard-coated dish, and the urothelium was removed with the aid of fine forceps and a dissecting microscope. All tissues were snap-frozen on dry ice prior to processing as previously described (Arms et al. 2010).

Real-Time Q-PCR

The urothelium + suburothelium was dissected (Schnegelsberg et al. 2010; Corrow et al. 2010) from the detrusor, and the specificity of the split bladder preparations was examined for the presence of α-smooth muscle actin (1:1000; Abcam, Cambridge, MA) and uroplakin II (1:25; American Research Products, Belmont, MA) by western blotting or Q-PCR (Corrow et al. 2010; Girard et al. 2011, 2013). We determined PACAP and PAC1 transcript expression in the urinary bladder (urothelium + suburothelium, detrusor), lumbosacral (L1, L2, L5-S1) spinal cord, and DRG of control and RVS mice ($n = 8$ each) using Q-PCR as described (Girard et al. 2010, 2011, 2013).

Measurement of Urinary Bladder, L6-S1 DRG, and Spinal Cord PACAP, PAC1, NGF, or Serum Corticosterone

We determined PACAP and PAC1 (PACAPR1) protein content in the urinary bladder of control ($n = 8$) or RVS mice ($n = 8$) using ELISA kits (MBS2503456, PACAP; MBS033808, PACAPR1; MyBioSource, Inc., San Diego, CA); tissue processing and ELISAs were performed as described previously (Vizzard 2000b; Guo et al. 2018). We determined serum corticosterone from control and RVS mice ($n = 8$ each) using ELISAs (ADI-900-097, Enzo Life Sciences, Farmingdale, NY). One hour after the last stressor of the RVS paradigm (day 7), blood from the abdominal aorta was collected in serum tubes between 9 am and 12 pm and allowed to coagulate at room temperature for 1 h. Samples were centrifuged in an Eppendorf 5415R centrifuge at 10,000 rpm for 10 min. Serum was aliquoted and frozen at – 20 °C until analysis. We determined NGF protein content in the urinary bladder of control ($n = 8$) and RVS mice ($n = 8$) using ELISAs (R&D Systems, Minneapolis, MN) as previously described (45, 75, 85). No samples were diluted, and all samples had absorbance values on the linear portion of the standard curve. Curve fitting of standards and evaluation of PACAP, PACAPR1, or corticosterone content of samples were performed using a least squares fit.

Conscious, Open Outlet, Continuous Fill Cystometry

Mice were anesthetized with isoflurane (3–4%), a lower midline abdominal incision was made, and polyethylene tubing (PE-10, Clay Adams, Parsippany, NJ) was inserted into the bladder dome and secured with a nylon purse-string suture (6-zero) (Schnegelsberg et al. 2010; Gonzalez et al. 2013; Girard et al. 2019). The end of the PE tubing was heat flared, but the catheter did not extend into the bladder body or neck, and it was not associated with inflammation or altered cystometric function (Schnegelsberg et al. 2010; Gonzalez et al. 2013; Girard et al. 2019). The distal end of the tubing was sealed, tunneled subcutaneously, and externalized at the back of the neck (Schnegelsberg et al. 2010; Gonzalez et al. 2013; Girard et al. 2019). Abdominal and neck incisions were closed with nylon sutures (6-0). Mice received postoperative analgesics (subcutaneous carprofen, 5.0 mg/kg, once a day for 2 days) and recovered from survival surgery for 72 h before performing cystometry.

For cystometry in conscious mice, an unrestrained animal was placed in a Plexiglas cage with a wire bottom. Before the start of the recording, the bladder was emptied and the catheter was connected via a T-tube to a pressure transducer (Grass Model PT300, West Warwick, RI) and microinjection pump (Harvard Apparatus 22, South Natick, MA). A Small Animal Cystometry Lab Station (MED Associates, Fairfax, VT) was used for urodynamic measurements (Schnegelsberg et al. 2010; Gonzalez et al. 2013; Girard et al. 2019). Saline solution was infused at room temperature into the bladder at a rate of 25 μl/min to elicit repetitive bladder contractions. At least six reproducible micturition cycles were recorded after the initial stabilization period of 25–30 min (Schnegelsberg et al. 2010; Gonzalez et al. 2013; Girard et al. 2019). The following cystometric parameters were recorded in each animal: baseline pressure (pressure at the beginning of the bladder filling), threshold pressure (bladder pressure immediately prior to micturition), peak micturition pressure, intercontraction interval (ICI; time between micturition events), infused volume (IV), and void volume (VV) (Schnegelsberg et al. 2010; Gonzalez et al. 2013; Girard et al. 2019). Mice in these studies had residual volume of less than 10 μl. At the conclusion of the experiment, the mouse was euthanized (5% isoflurane plus thoracotomy).

Conscious Cystometry and Effects of a PAC1 Selective Receptor Antagonist, PACAP(6-38), on Bladder Function in Control and RVS Mice

The effects of PACAP(6-38), a PAC1 selective receptor antagonist, on urinary bladder function in control mice and RVS mice were assessed using conscious, open outlet, cystometry with continuous instillation of intrabladder saline (Girard et al. 2019; Gonzalez et al. 2013; Schnegelsberg et al. 2010; Girard et al. 2016) (Fig. 1A, B). Two groups of mice were evaluated: control mice receiving intrabladder administration of vehicle (0.9% saline) and PACAP(6-38) ($n = 8$) and RVS mice receiving intrabladder administration of vehicle (0.9% saline) and PACAP(6-38) ($n = 8$). For intrabladder administration of PACAP(6-38) or vehicle, mice were anesthetized with 2% isoflurane and PACAP(6-38) (< 200 μl) was injected through the bladder catheter; the animals were maintained under anesthesia to prevent expulsion of PACAP(6-38) or vehicle via a voiding reflex. In this procedure, PACAP(6-38) or vehicle remained in the bladder for 30 min at which time, the bladder

was drained and washed with saline and animals recovered from anesthesia for 20 min before experimentation. These experiments were performed in the same mice before and after treatment with PACAP(6-38). The concentration (300 nM) of PACAP(6-38) (Bachem, Torrance, CA) used was based upon previous studies (Braas et al. 2006; Girard et al. 2016). To summarize, the experimental design involves administration of a one time, intrabladder infusion of PACAP(6-38) (300 nM) with cystometric data collection occurring ~ 75 min after infusion. At the conclusion of the experiment, the animal was euthanized (5% isoflurane plus thoracotomy). Experiments were conducted at similar times of the day (9 am–12 pm) to avoid the possibility that circadian variations were responsible for changes in bladder capacity measurements (Girard et al. 2019). An individual blinded to treatment or group analyzed the cystometric data; groups were decoded after data analysis.

Exclusion Criteria

Mice were removed from the study when adverse events occurred that included a significant postoperative event, lethargy, pain, or distress not relieved by our IACUC-approved regimen of postoperative analgesics (Cheppudira et al. 2008; Schnegelsberg et al. 2010). In the present study, no mice were excluded from the study. In addition, behavioral movements such as grooming, standing, walking, and defecation rendered bladder pressure recordings during these events unusable.

Mechanical Sensitivity Testing in Control and RVS Mice

Referred (secondary) hyperalgesia was measured by testing the frequency of withdrawal responses to the application of calibrated von Frey monofilaments to the abdominal (Cheppudira et al. 2008; Schnegelsberg et al. 2010; Girard et al. 2019; Girard et al. 2016) region overlying the urinary bladder with PACAP antagonist, PACAP(6-38) (300 nM), or vehicle (0.9% saline) delivered into the bladder (intrabladder) via a transurethral catheter in control and RVS mice ($n = 10$ each) (Fig. 1A, B) (Girard et al. 2019). Four separate groups ($n = 10$ each) of mice were evaluated: control mice with vehicle, RVS mice with vehicle, control mice with PACAP(6-38), and RVS mice with PACAP(6-38). For these studies, a transurethral bladder catheterization method was used to avoid the need for an abdominal incision (Girard et al. 2019). In this procedure, PACAP(6-38) (< 200 μl) or vehicle remained in the bladder for 30 min at which time, the bladder was drained and washed with saline, the catheter removed, and animals recovered from anesthesia for 20 min before testing. Mechanical sensitivity assessment was performed using von Frey monofilaments (Stoelting, Wood Dale, IL) with forces of 0.1–4 g applied to the pelvic region (Cheppudira et al. 2008; Schnegelsberg et al. 2010; Girard et al. 2019). All mice were first habituated in a clear acrylic testing chamber 20 min/day for 4 days with a fan to generate ambient noise. On the day of testing, the mice were placed in the acrylic testing chamber on top of a metal mesh floor (IITC Life Science Inc., Woodland Hills, CA) and habituated again for 10 min before the application of von Frey filaments in an up-down method for 1–3 s with a minimum interstimulus interval of 2 min (Cheppudira et al. 2008; Schnegelsberg et al. 2010). The following behaviors were considered positive responses to pelvic region stimulation: sharp retraction of the abdomen, jumping, or immediate licking or scratching of the pelvic area (Cheppudira et al. 2008; Schnegelsberg et al. 2010). Separate cohorts of mice were used for cystometry and somatic sensitivity testing. All mechanical sensitivity testing was performed in a blinded manner. The groups were decoded after data analysis.

Statistical Analyses

All values represent mean ± SEM. Comparisons among experimental groups were made using analysis of variance (ANOVA), repeated measures ANOVA, and paired or unpaired t test where appropriate. When F test statistic exceeded the critical value at $\alpha = 0.05$, the Sidak's multiple comparisons test was used to compare group means.

Results

Serum Corticosterone Is Increased, and Body Weight Is Decreased in Mice Exposed to RVS

RVS (7 days) significantly ($p \leq 0.01$) increased (4.4-fold) serum corticosterone measured 1 h after the final stressor presented on day 7 compared with control mice (handling only) (Fig. 2A). RVS significantly ($p \leq 0.01$) decreased body weight in mice exposed to RVS by day 4 of the RVS paradigm. Significant ($p \leq 0.01$) weight reductions were maintained through day 7 of the RVS paradigm (Fig. 2B). RVS significantly ($p \leq 0.01$) increased (2.1-fold) whole urinary bladder NGF in RVS mice measured on day 7 of the RVS paradigm (Fig. 2C).

PACAP and PAC1 Transcript and Protein Expression Is Increased in LUT Tissues in Mice Exposed to RVS

No changes in PACAP transcript expression were observed in urothelium or detrusor from RVS mice (Fig. 3A); however, mice exposed to RVS exhibited significantly ($p \leq 0.01$) increased PACAP transcript expression in L1, L2, L6, and S1 dorsal root ganglia (DRG) (Fig. 3B) and S1 spinal cord (SC) (Fig. 3C). No changes in PAC1 transcript expression were observed in urothelium or detrusor smooth muscle or in any DRG

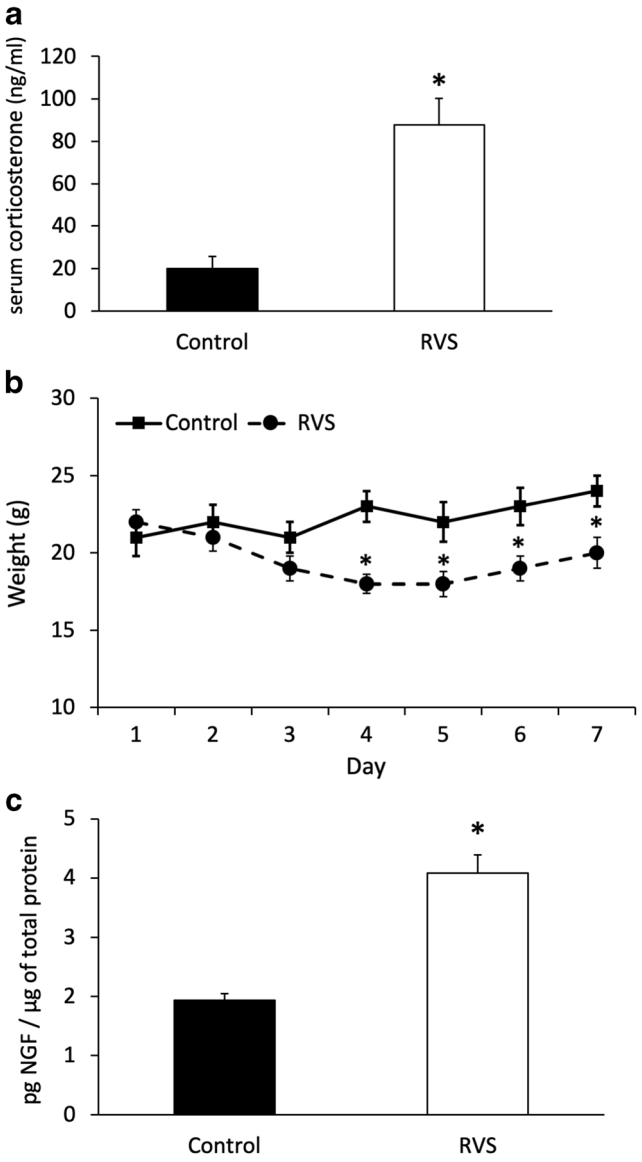

Fig. 2 RVS effects on serum corticosterone, body weight, and whole urinary bladder NGF content in mice. **A** Serum corticosterone significantly increased with RVS exposure measured 1 h after the final day RVS (7 days). **B** Changes in body weight of mice during 7-day RVS. Beginning on day 4 and lasting for the full duration of 7-day RVS, mice exhibited significant weight gain attenuation compared with control mice. Body weights were significantly ($p \leq 0.01$) decreased in the RVS group on days 4–7 of RVS. **C** Whole urinary bladder nerve growth factor (NGF) content was significantly ($p \leq 0.01$) increased following 7-day RVS in mice. Samples size are $n = 8$. Values are means ± SEM. *$p \leq 0.01$

level examined (Fig. 3D, E). PAC1 transcript expression was significantly increased in L5 and S1 SC segments with RVS exposure (Fig. 3F). RVS exposure did not affect PACAP or PAC1 protein expression in the whole urinary bladder (Fig. 4A); however, PACAP expression was significantly ($p \leq 0.01$) increased in L6 and S1 DRG and spinal cord segments in RVS mice (Fig. 4A). RVS mice also exhibited significantly ($p \leq 0.01$) increased PAC1 expression in L6 and S1 DRG (Fig. 4B); lumbosacral spinal cord segments were not examined.

PAC1 Receptor Blockade with Intrabladder Infusion of PACAP(6-38) Reduces Voiding Frequency and Increases Functional Bladder Capacity in Mice Exposed to RVS

Conscious cystometry with continuous intrabladder infusion of vehicle (0.9% saline) was performed in control (handling only) and RVS-exposed female mice before intrabladder infusion of PACAP(6-38) to establish baseline voiding frequency, intercontraction interval, and infused volume (functional bladder capacity). Baseline voiding characteristics of control female PACAP-EGFP mice were similar to wild-type mice that we have previously characterized (Girard et al. 2019) (Figs. 5A, B and 6A–C). RVS-exposed mice exhibited significant ($p \leq 0.01$) reductions (2.5-fold) in the infused volume (IV) of saline required to induce a micturition event (functional bladder capacity) (Figs. 5E, F and 6A) and significant ($p \leq 0.01$) reductions in intercontraction interval (ICI;3.1-fold) that resulted in increased voiding frequency (Figs. 5E, F and 6B). This increase in voiding frequency with RVS exposure is consistent with previous studies in rats (Merrill et al. 2013; Merrill and Vizzard 2014). Following intrabladder infusion of the PAC1 receptor antagonist, PACAP(6-38) (300 nM), the same control PACAP-EGFP mice exhibited significantly ($p \leq 0.01$) increased IV (1.5-fold) (Figs. 5C, D and 6A) and ICI (1.5-fold) (Figs. 5C, D and 6B). No changes in bladder pressure were observed in control PACAP-EGFP mice treated with PACAP(6-38) (300 nM) (Fig. 6C). The same PACAP-EGFP mice exposed to RVS also exhibited significantly ($p \leq 0.01$) increased IV (2.7-fold) (Figs. 5G, H and 6A) and ICI (2.9-fold) (Figs. 5G, H and 6B) and significant ($p \leq 0.01$) reduction in baseline (filling) bladder pressure (Fig. 6C) following intrabladder infusion of the PAC1 receptor antagonist, PACAP(6-38) (300 nM). All changes in urinary bladder function with intrabladder instillation of PACAP(6-38) persisted for the duration of the data collection period (~ 75 min).

PAC1 Receptor Blockade with Intrabladder Infusion of PACAP(6-38) Reduces Pelvic Somatic Sensitivity in Mice Exposed to RVS

Pelvic somatic sensitivity was similar in control female PACAP-EGFP mice treated with intrabladder saline (vehicle) to wild-type mice previously studied (Girard et al. 2019; Schnegelsberg et al. 2010). Female PACAP-EGFP mice exposed to RVS and given intrabladder saline exhibited significantly ($p \leq 0.01$) decreased somatic sensitivity in the pelvic region with all monofilament forces evaluated (0.1 to 4 g) (Fig. 7). Intrabladder infusion of PACAP(6-38) (300 nM) significantly ($p \leq 0.01$) decreased the somatic sensitivity in the pelvic region in female PACAP-EGFP mice exposed to RVS (Fig. 7) with all monofilament forces evaluated (0.1 to 4 g) but

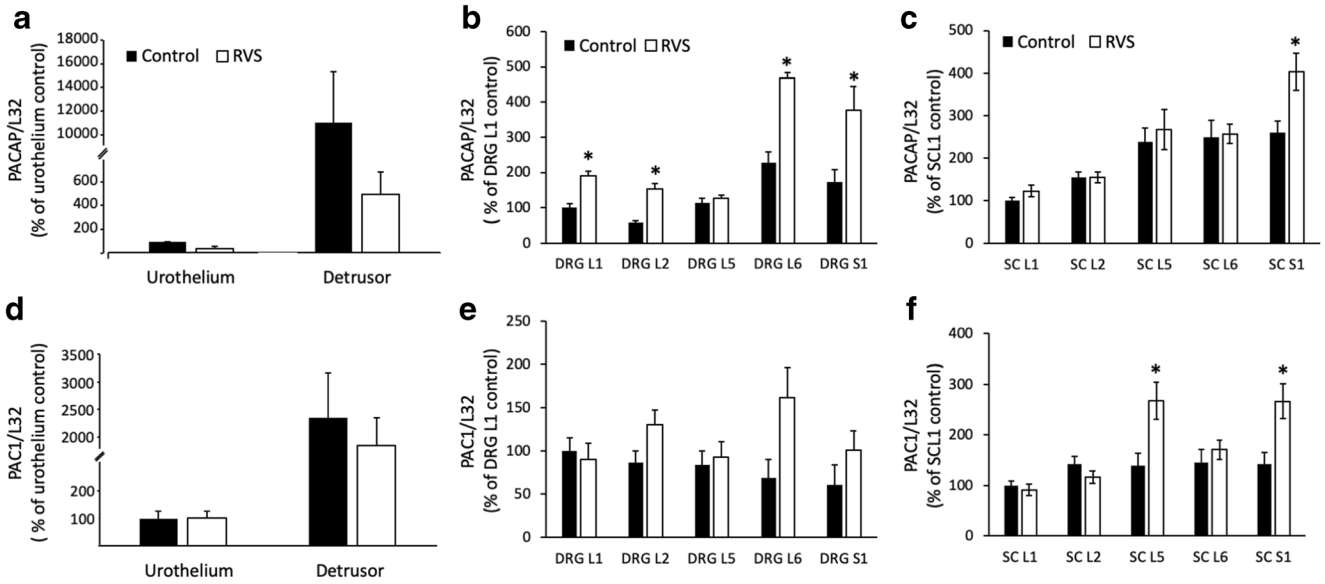

Fig. 3 Regulation of PACAP and PAC1 receptor transcript expression in lower urinary tract tissues from control and RVS mice. PACAP (**A**–**C**) and PAC1 (**D**–**F**) receptor expressions in control (handled only) and RVS-exposed mice in urothelium and detrusor smooth muscle (**A**, **D**), lumbosacral (L1, L2, L5-S1) dorsal root ganglia (DRG; **B**, **E**), and lumbosacral (L1, L2, L5-S1) spinal cord (SC; **C**, **F**). Relative expression of transcripts is expressed as a percentage of control urothelium (A, D), control L1 DRG (B, E), or control L1 SC (C, F) and normalized to the relative expression of the housekeeping gene, L32. Values are means ± SEM. Samples size are $n = 8$; $^*p \leq 0.01$ versus control

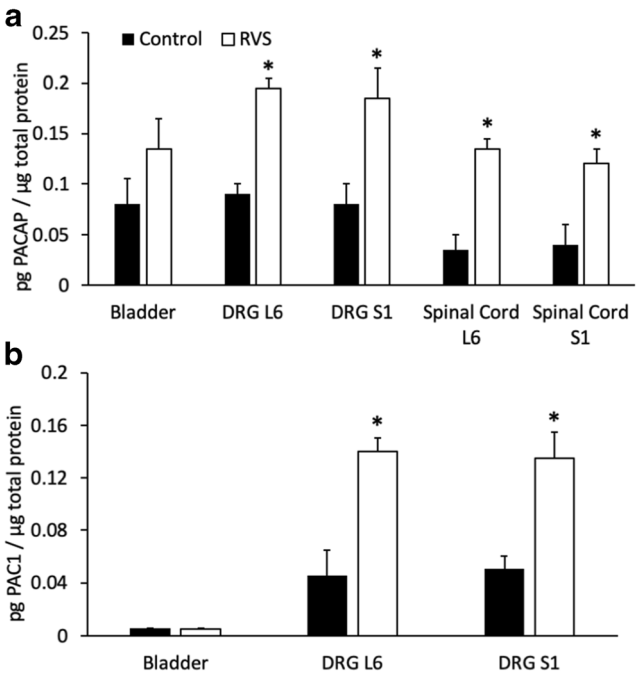

Fig. 4 Regulation of PACAP and PAC1 protein expression in LUT tissues from control and RVS mice. PACAP (**A**) and PAC1 (**B**) receptor protein expressions in control (handled only) and RVS-exposed mice in whole urinary bladder, lumbosacral (L6, S1) dorsal root ganglia (DRG), and lumbosacral (L6, S1) spinal cord. No changes in PACAP or PAC1 receptor protein expression were observed in urinary bladder following 7-day RVS; however, PACAP (**A**) and PAC1 (**B**) receptor expressions were increased with RVS exposure in lumbosacral DRG and spinal cord. Values are means ± SEM. Samples size are $n = 8$; $^*p \leq 0.01$ versus control

was without effect in control mice (handling only) treated with intrabladder infusion of PACAP(6-38) (300 nM) (Fig. 7).

Discussion

Stress-induced symptom exacerbation or "flare" is common in functional bladder disorders (e.g., OAB, BPS/IC) (Sutcliffe et al. 2014, 2018) and may be partly due to hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Nazif et al. 2007; Westropp and Buffington 2002). The prevalence of micturition disorders is high among individuals with anxiety disorders; however, the mechanisms underlying stress effects on micturition reflex function are unclear. Growing evidence indicates that PACAP (*Adcyap1*) and the PAC1 receptor (*Adcyap1r1*) are novel stress mediators (Dore et al. 2013) with PACAP/PAC1 receptor transcripts increased in the limbic system following RVS exposure, CNS PACAP signaling is anxiogenic and anorexic, and PAC1 receptor antagonists block chronic stress-induced anxiety-related behaviors (Hammack et al. 2009; Lezak et al. 2014a, b; Missig et al. 2014; Roman et al. 2014). Furthermore, PACAP/receptor expression and signaling contributes to LUT function and dysfunction (Zvara and Vizzard 2007; Vizzard 2000c; Braas et al. 2006; Girard et al. 2008, 2010, 2012; Herrera et al. 2006): (1) PAC1 receptor is expressed in bladder nerve fibers (Braas et al. 2006) and tissues (Braas et al. 2006; Girard et al. 2010), (2) PACAP facilitates detrusor contractility (Braas et al. 2006) and ATP release from the urothelium (Girard et al. 2008), (3) PACAP and PAC1 mRNA and protein

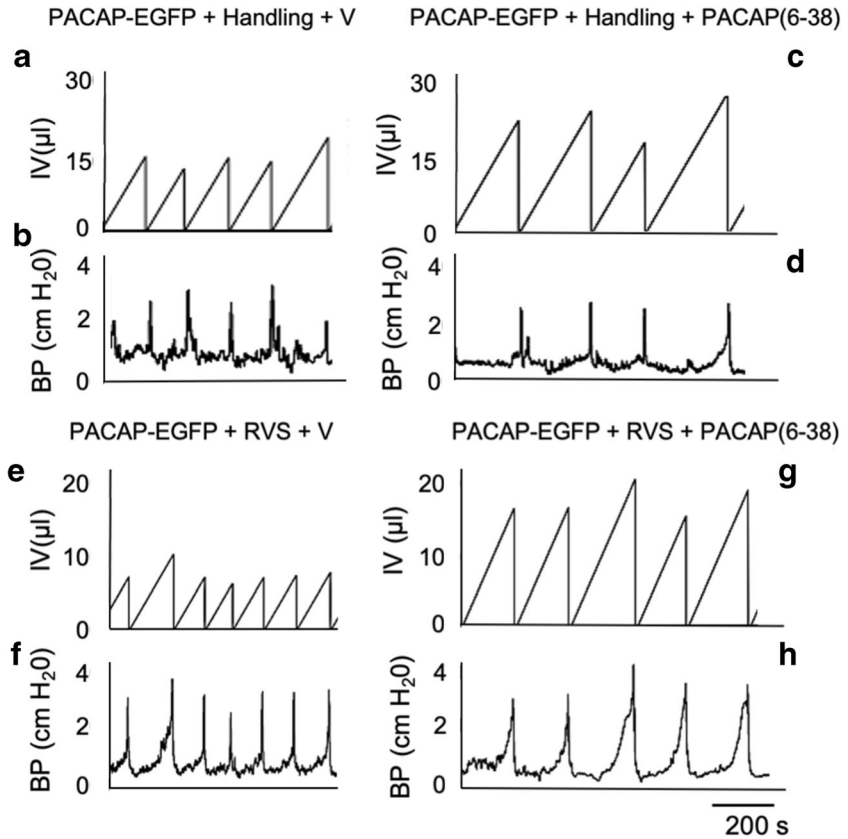

Fig. 5 Representative bladder function recordings from the same control (**A**–**D**) PACAP-EGFP or PACAP-EGFP RVS (**E**–**H**) mouse before (**A**, **B**; **E**, **F**) and after intrabladder instillation of PACAP(6-38) (300 nM) (**C**, **D**; **G**, **H**). **A**, **B** Control PACAP-EGFP mice (handling only) infused with vehicle (V) exhibit an infused volume (IV) necessary to elicit a micturition event (i.e., functional bladder capacity) that is similar to wild-type mice previously characterized. **C**, **D** After PACAP(6-38) instillation, control mice exhibited a reduction in voiding frequency (i.e., increased IV). **E**, **F** Prior to PACAP(6-38) instillation, RVS mice infused with V exhibit reduced infused volume (IV; **E**, **F**) required to elicit a micturition that results in increased voiding frequency compared with control mice. **G**, **H** After PACAP(6-38) instillation, RVS mice exhibit a reduction in voiding frequency (i.e., increased IV). BP bladder pressure

expression are regulated in LUT tissues in mice with chronic urothelial overexpression of NGF (NGF-OE) (Girard et al. 2010) or with CYP-induced cystitis (Girard et al. 2012; Gonzalez et al. 2016), and (4) the PAC1 receptor antagonist, PACAP(6-38), decreases voiding frequency and pelvic sensitivity in NGF-OE mice (Schnegelsberg et al. 2010) and in rats with CYP-induced cystitis (Braas et al. 2006). Thus, we examined whether PACAP/PAC1 signaling at the level of the urinary bladder could affect bladder function and pelvic sensitivity in mice exposed to 7-day repeated variate stress (RVS) that results in increased voiding frequency and somatic sensitivity. Intrabladder administration of PACAP(6-38) (300 nM) significantly ($p \leq 0.01$) increased bladder capacity and the intercontraction interval and decreased filling pressure in RVS mice with similar effects (i.e., bladder capacity and ICI) on control mice being significant ($p \leq 0.01$) but lesser in magnitude. Intrabladder administration of PACAP(6-38) (300 nM) also significantly ($p \leq 0.01$) reduced pelvic sensitivity in RVS mice but was without effect in control mice. These studies add to growing evidence that blockade of urinary bladder PACAP/PAC1 signaling can reduce voiding frequency and pelvic sensitivity regardless of cause: CYP-induced cystitis (May et al. 2017a, b), urothelial overexpression of NGF (NGF-OE) (May et al. 2017a, b), or RVS.

A RVS paradigm, previously used to examine PACAP mRNA expression in the bed nucleus of the stria terminalis (BNST) (Hammack et al. 2009; King et al. 2017), was found to be anxiogenic, most likely mediated by BNST PACAP (Hammack et al. 2009; Lezak et al. 2014a, b; Missig et al. 2014; Roman et al. 2014). The RVS paradigm (Hammack et al. 2009; King et al. 2017) has multiple advantages including the following: (1) lack of habituation with novel stressor exposure, (2) reproducible and robust changes in urinary bladder function (Merrill et al. 2013; Merrill and Vizzard 2014), and (3) reproducible decrease (~ 10–25%) in weight gain during RVS as demonstrated in rats (Merrill et al. 2013; Merrill and Vizzard 2014) and confirmed in mice in the current study. In the current study, we demonstrated that serum corticosterone is increased in RVS mice, compared with control mice (handling only), when measured 1 h after the final stressor. Future studies should determine serum corticosterone levels in the RVS group compared with a control group that receives

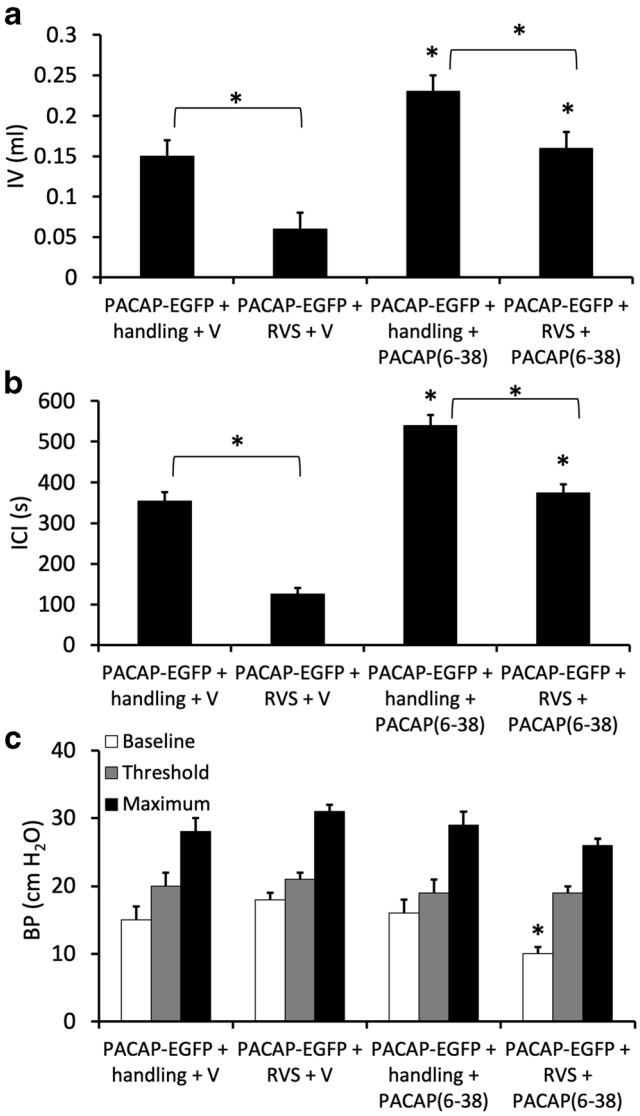

Fig. 6 Summary histograms of infused volume (IV), intercontraction interval (ICI), and bladder pressure (BP) measured from bladder function testing in control ($n = 8$) and RVS mice ($n = 8$) before and after intrabladder instillation of PACAP(6-38) (300 nM). RVS mice exhibited significantly ($*p \leq 0.01$) reduced IV (**A**) and ICI (**B**) compared with control (handled) mice. Intrabladder PACAP(6-38) significantly ($*p \leq 0.01$) increased IV (**A**) and ICI (**B**) in RVS and control mice. Brackets indicate comparisons made between groups (e.g., control vs. RVS). Asterisks indicate comparisons of treatment with a group (e.g., control vs. control + PACAP(6-38)). **C** In RVS mice, intrabladder instillation of PACAP(6-38) significantly ($*p \leq 0.01$) reduced baseline (i.e., filling) bladder pressure with no effects on threshold pressure or maximum pressure. PACAP(6-38) was without effect on bladder pressure in WT mice. Values are means ± SEM. Samples size are $n = 8$; $*p \leq 0.01$ versus control

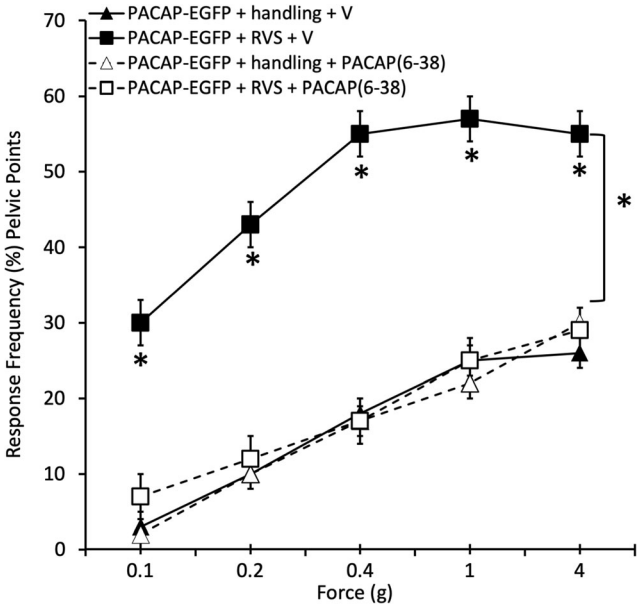

Fig. 7 Effects of RVS on pelvic region sensitivity. Pelvic region testing with calibrated von Frey filaments was determined in control and RVS mice treated with intrabladder vehicle (V) or PAC1 receptor antagonist, PACAP(6-38). Stimulation was confined to the lower abdominal area overlying the urinary bladder. RVS mice ($n = 10$) had a significantly ($*p \leq 0.01$) increased pelvic response frequency with all von Frey hairs (0.1–4 g) tested compared with control mice ($n = 10$). In RVS mice, intrabladder instillation of PACAP(6-38) significantly ($*p \leq 0.01$) reduced pelvic response frequency with all von Frey filaments evaluated. No changes in pelvic sensitivity were observed in control mice (handling) following intrabladder PACAP(6-38) instillation. All somatic testing was performed in a blinded manner. Values are means ± SEM. Samples size are $n = 10$; $*p \leq 0.01$ PACAP-EGFP + RVS + V compared with all other groups. Bracket and asterisk indicate comparison between groups PACAP-EGFP + RVS + V and PACAP-EGFP + RVS + PACAP(6-38)

only the single, last stressor. This approach will determine if increased serum corticosterone reflects 7-day RVS or the single, last stressor. We have previously used the RVS protocol to characterize effects on bladder function and somatic sensitivity (Merrill et al. 2013; Merrill and Vizzard 2014) in rats. RVS in rats (Merrill et al. 2013; Merrill and Vizzard 2014) produced similar changes in bladder function and somatic sensation observed in the present study, including increased urinary frequency and somatic sensitivity of the hindpaw and pelvic region as well as increased NGF content in the urinary bladder. In addition, RVS in rats also caused additional changes in the inflammatory milieu of the urinary bladder including, changes in histamine, myeloperoxidase, and the chemokine, CXCL12 (Merrill et al. 2013; Merrill and Vizzard 2014). Current studies are examining more prolonged periods of RVS (2 or 4 weeks) and potential effects on bladder function and expression of various neuromodulators in LUT pathways. In addition, ongoing studies are using natural voiding assays (Urovoid assays) (Girard et al. 2019), without the need for survival surgery or bladder catheter implant, following daily stressor exposure in the RVS paradigm to assess bladder function.

We have previously examined the expression of PACAP, VIP, and associated receptors in the urinary bladder and lumbosacral DRG in NGF-OE mice (Girard et al. 2010) and in rodents with CYP-induced cystitis (May et al. 2015, 2017a; both animal models exhibit increased voiding frequency.

PACAP mRNA expression in the urothelium may reflect expression in urothelial cells, axon terminals, as well as cells in the lamina propria (Ojala et al. 2018; Girard et al. 2017). PACAP mRNA expression in the detrusor may reflect expression in detrusor smooth muscle cells, axon terminals, as well as intramural ganglia (Ojala et al. 2018; Girard et al. 2017). Changes in PACAP, VIP, and associated receptor transcripts and protein expression in micturition pathways of NGF-OE mice resemble some, but not all, changes observed after CYP-induced cystitis (Girard et al. 2008) known to involve NGF production (Vizzard 2000c; Guerios et al. 2008; Klinger and Vizzard 2008; Bjorling et al. 2001). We demonstrated upregulation of PAC1 receptor transcript and PAC1-immunoreactivity (IR) in urothelium of NGF-OE mice, whereas PACAP transcript and PACAP-IR were decreased in urothelium (Girard et al. 2010); no changes in PACAP or associated receptors transcript expression were observed in lumbosacral DRG (Girard et al. 2010). In contrast, enhanced target-derived NGF availability has been shown to increase PACAP expression in small nociceptive neurons in DRG (Jongsma Wallin et al. 2001; Jongsma Wallin et al. 2003). With CYP-induced cystitis (48 h and chronic), PACAP and PAC1 transcript expressions were significantly increased in urinary bladder and L6 DRG (Girard et al. 2008). Consistent with CYP-induced cystitis (Vizzard 2000b), RVS exposure significantly increased urinary bladder NGF content measured at the conclusion of the RVS paradigm. In the present study, a major difference in the regulation of PACAP/PAC1 transcript or protein expression in LUT tissues with RVS exposure is the absence of change in the urinary bladder (urothelium or detrusor), whereas regulation was still observed in lumbosacral DRG and spinal cord. The absence of changes in PACAP/PAC1 regulation in the urinary bladder despite changes in urinary bladder function following RVS exposure may reflect the absence of a direct insult to the urinary bladder as achieved with CYP-induced cystitis (Vizzard 2000a, b, c) and the absence of urothelial transgene expression achieved in NGF-OE mice (Cheppudira et al. 2008; Schnegelsberg et al. 2010). Thus, changes in urinary bladder function as well as increased bladder NGF content may reflect secondary effects mediated by peripheral (lumbosacral DRG) and/or central (lumbosacral spinal cord) inputs. In addition, changes in PAC1 receptor transcript expression in L5 DRG in RVS mice were surprising given the lack of bladder innervation originating from L5. However, upregulation in L5 DRG may reflect the more systemic nature of the RVS. It would be of interest in future studies to determine hindpaw sensitivity in RVS mice. It would also be of interest in future studies to determine combined effects of CYP-induced cystitis or NGF-OE combined with RVS to address stress exacerbation on existing urinary bladder inflammation (Nazif et al. 2007; Westropp and Buffington 2002; Rothrock et al. 2001a, b; Lutgendorf et al. 2000).

In our previous studies using a CYP-induced cystitis model (Braas et al. 2006), the intrathecal or intrabladder administration of PAC1 receptor antagonist, PACAP(6-38), reduced cystitis-induced urinary frequency in rats and the intrabladder administration of PACAP(6-38) reduced voiding frequency in mice (May et al. 2015, 2017a). Intrabladder instillation of PACAP(6-38) similarly reduced voiding frequency in transgenic mice with chronic urothelial overexpression of NGF (NGF-OE). Our current results demonstrating intrabladder PACAP(6-38) reductions in urinary frequency in RVS mice are consistent with these previous studies. Although the site(s) of action of PACAP(6-38) in the current study is not known, PACAP(6-38) may affect PACAP/PAC1 signaling at the urothelium, in peripheral nerve terminals of DRG cells located in close proximity to the urothelium with demonstrated PAC1 expression (Braas et al. 2006). However, reductions in baseline (i.e., filling) pressure with intrabladder instillation of PACAP(6-38) suggest that detrusor smooth muscle cells may also be potential targets. The urothelium exhibits high transepithelial resistance (TER), and the effects of RVS on TER have not been studied; thus, the potential of PACAP(6-38) to reach the detrusor is unknown making the urothelium and adjacent nerve terminals more likely sites of action. Cold immobilization stress in rats (Ercan et al. 2001) produces morphological changes in the urinary bladder including urothelial barrier disruption; thus, future studies should consider changes in urothelial structure and function as an underlying mechanism contributing to increased voiding frequency with RVS.

Patients with disorders of the LUT and associated disease states report a worsening of symptoms during stress (Nazif et al. 2007; Westropp and Buffington 2002). A majority of these patients report exacerbation of symptoms by clinical stress (Rothrock et al. 2001a, b), and experimental stress increases bladder pain and urgency in these individuals (Lutgendorf et al. 2000). In addition, animal models of stress demonstrate symptoms of bladder dysfunction (e.g., increased micturition frequency, urgency, pain) as well as anxiety-like behavior (Birder and Andersson 2013) that may be due, in part, to disruption of the HPA axis (Pierce and Christianson 2015). Cortisol, by feeding back on the HPA axis, normally acts to attenuate inflammation; however, abnormalities in the feedback loop may cause dysregulation of the inflammatory response. Children exposed to chronic early life stress (ELS) (Taylor 2010) are at significant risk of developing HPA abnormalities (Pierce and Christianson 2015) (Fuentes and Christianson 2018), and individuals with functional bladder disorders have a higher incidence of ELS or trauma (Pierce and Christianson 2015; Fuentes and Christianson 2018). Therefore, patients with bladder dysfunction disorders may have abnormalities in the HPA axis, and stress could be attributed to the worsening of bladder symptoms (Nazif et al. 2007; Westropp and Buffington 2002).

Neural mechanisms and pathways linking psychosocial stress to altered behaviors and physiological disorders are still unclear. We are using PACAP-EGFP transgenic mice (Condro et al. 2016) in this study to map specific neuronal PACAP populations in the CNS and PNS and to determine if this transgenic mouse model can be used to examine and compare changes in PACAP populations with RVS and/or RVS and bladder function testing as suggested for other manipulations (e.g., facial nerve axotomy) (Condro et al. 2016). With 7-day RVS followed by bladder function testing, numbers of PACAP-EGFP+ cells increased dramatically, compared with RVS alone, in lumbosacral spinal cord segments and DRG involved in micturition reflexes, urothelial cells, and supraspinal locations including the following: locus coeruleus, Barrington's nucleus, rostral ventrolateral medulla, PAG, raphe, and amygdala (May et al. 2019). These supraspinal regions, in turn, can project to and receive input from the HPA axis which may be a component of the anatomical substrates connecting stress, bladder function, and HPA regulation/dysregulation (Fuentes and Christianson 2018; Grundy et al. 2018; Pierce and Christianson 2015). Additional studies are needed to understand how stress or the inability to attenuate stress signaling can result in maladaptations and cumulative long-term damages (i.e., increased HPA and sympathetic reactivity) that can manifest a variety of disorders including those involving the urinary bladder (Grundy et al. 2018; Pierce and Christianson 2015; FitzGerald et al. 2009; Lovallo 2013; Mahon et al. 2013; Marshall and Garakani 2002; Mihaljevic et al. 2016; Turner-Cobb 2005).

Acknowledgments The authors thank Dr. James A. Waschek for his generous gift of transgenic PACAP-EGFP mice breeding pairs. The authors also gratefully acknowledge the research efforts of summer undergraduate research students, some of whom were supported by a NINDS Summer Research Experience in Neuroscience for Undergraduates (R25 NS090623) through the Summer Undergraduate Neuroscience Fellowship Program at UVM. Student researchers included William (Trey) Walker, Michelle Hernandez, Hina Rattu, Neysharie Sánchez-Torres, Jenna McQuesten, Morgan Mathews, Diana Grinberg, Phat (Tony) Chan, Eric Hauke, and Gabrielle Krumgalz.

Funding Information This work was funded by National Institutes of Health (NIH) grants DK051369 (MAV), DK060481 (MAV), DK065989 (MAV), and DK120108 (MAV). This publication was also supported by grants from the National Center for Research Resources (5 P30 RR 032135) and the National Institute of General Medical Sciences (8 P30 GM 103498) from the NIH.

Compliance with Ethical Standards

The UVM Institutional Animal Care and Use Committee approved all experimental protocols involving animal (IACUC #08-085, #13-030, #X9-020). The UVM Office of Animal Care Management oversaw all animal use in accordance with AAALAC and NIH guidelines.

Conflict of Interest The authors declare that they have no conflict of interest.

Disclaimer The funding entity, NIH, had no role in the studies described including the following: design, data collection and analysis of studies performed in the Vizzard laboratory, decision to publish, or the preparation of the manuscript. The contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH.

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