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Transcriptional and Translational Plasticity in Rodent Urinary Bladder TRP Channels with Urinary Bladder Inflammation, Bladder Dysfunction, or Postnatal Maturation

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Abstract These studies examined the transcriptional and translational plasticity of three transient receptor potential (TRP) channels (TRPA1, TRPV1, TRPV4) with established neuronal and non-neuronal expression and functional roles in the lower urinary tract. Mechanosensor and nociceptor roles in either physiological or pathological lower urinary tract states have been suggested for TRPA1, TRPV1, and TRPV4. We have previously demonstrated the neurochemical, organizational, and functional plasticity in micturition reflex pathways following induction of urinary bladder inflammation using the antineoplastic agent, cyclophosphamide. More recently, we have characterized similar plasticity in micturition reflex pathways in a transgenic mouse model with chronic urothelial overexpression (OE) of nerve growth factor (NGF) and in a transgenic mouse model with deletion of vasoactive intestinal polypeptide (VIP). In addition, the micturition reflex undergoes postnatal maturation that may also reflect plasticity in urinary bladder TRP channel expression. Thus, we examined plasticity in urinary bladder TRP channel expression in diverse contexts using a combination of quantitative, real-time PCR and western blotting approaches. We demonstrate transcriptional and translational plasticity of urinary bladder TRPA1, TRPV1, and TRVP4 expression. Although the functional significance of urinary bladder TRP channel plasticity awaits further investigation, these studies demonstrate context- (inflammation, postnatal development, NGF-OE, VIP deletion) and tissue-dependent (urothelium + suburothelium, detrusor) plasticity.

Keywords TRPA1 · TRPV1 · TRPV4 · Cyclophosphamide · NGF · VIP

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Introduction

The transient receptor potential (TRP) channels are a novel superfamily of nonspecific ion channels with modest permeability to Ca^{2+} (Chung et al. 2011; Mandadi et al. 2011; Moran et al. 2011). There are more than 50 TRP channels described in diverse species from yeast to human and 28 known mammalian TRP channels in seven subfamilies: TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), TRPML (mucolipin), and TRPN (no mechanopotential) (Everaerts et al. 2010; Moran et al. 2011). Multiple TRP channels, including TRPV1, TRPV2, TRPV4, TRPM8, and TRPA1, are expressed in the urinary bladder and may act as sensors of stretch and/or chemical irritation in the lower urinary tract (LUT) (Andersson et al. 2010; Araki 2011; Eid 2011). In neonates of many species, the neural control of micturition undergoes marked changes during early postnatal development (de Groat et al. 1998). In newborn rats and cats, micturition is dependent upon a spinal reflex pathway that is activated when the mother licks the perineal region of the young animal (perineal-to-bladder reflex). Similar reflexes have been identified in human infants (de Groat et al. 1998). As the CNS matures, the spinal micturition reflex is gradually replaced by a spinobulbospinal reflex pathway, triggered by mechanoreceptor afferents in the bladder, that is responsible for micturition in adult animals (de Groat et al. 1998). Involvement of TRP channels in normal micturition

reflex function as well as bladder dysfunction has been suggested (Andersson et al. 2010; Araki 2011; Eid 2011).

Studies of TRP channels in the LUT have primarily focused on TRPA1, TRPV1, or TRPV4, and functional roles (Andersson et al. 2010) in overactive bladder (OAB) and bladder pain syndrome (BPS) have been suggested (Nilius et al. 2007; Everaerts et al. 2008). TRPV1 has been detected in neuronal and non-neuronal human and rodent LUT tissues including the suburothelial nerve plexus, smooth muscle, interstitial cells, dorsal root ganglion (DRG), neurons and urothelium (Nilius et al. 2007) (Andersson et al. 2010); however, its expression pattern has recently been questioned in the urothelium (Yamada et al. 2009; Andersson et al. 2010; Yu et al. 2010). Intravesical administration of the TRPV1 activators, namely capsaicin or resiniferatoxin, which desensitize afferent neurons, has been introduced into clinical practice for OAB treatment with mixed success highlighting the need for the identification of additional targets to improve urinary bladder function (Lecci and Maggi 2005; Moran et al. 2011). TRPV4 expression has been demonstrated in basal and intermediate urothelial cells of mice and rats (Gevaert et al. 2007) with confirmation from independent laboratories (Birder et al. 2007; Kullmann et al. 2009; Mochizuki et al. 2009; Yamada et al. 2009). TRPV4 is also expressed in the detrusor muscle but urothelial TRPV4 expression is ~20–36-fold greater (Thorneloe et al. 2008; Xu et al. 2009) compared to detrusor. In addition, TRPV4 expression has been demonstrated in DRG neurons innervating viscera and has been implicated in tonicity-induced neurogenic inflammation (Vergnolle et al. 2010). Functional contributions of TRPV4 to normal bladder function as well as to bladder dysfunction following cyclophosphamide (CYP)-induced bladder inflammation have also been demonstrated (Gevaert et al. 2007).

The sole mammalian member of the TRPA subfamily, TRPA1, is also implicated in urinary bladder function based upon distribution in neuronal and non-neuronal tissues of the LUT as well as pharmacological activation or blockade of TRPA1 channels (Nilius et al. 2007; Andersson et al. 2010). TRPA1 immunoreactivity (IR) has been demonstrated in suburothelial nerve fibers, detrusor, urothelium, and most abundantly in lumbosacral (L6-S1) DRG neurons that co-express TRPV1 and calcitonin gene-related peptide IR (Andrade et al. 2006; Du et al. 2007; Streng et al. 2008; Andersson et al. 2010). Roles for TRPA1 in bladder sensation and inflammatory diseases of the urinary bladder have been suggested based upon induction of detrusor overactivity with intravesical administration of TRPA1 channel activators (Streng et al. 2008). A functional role for TRPA1 in OAB following spinal cord injury has also been suggested (Andrade et al. 2006).

The current studies expand upon existing work by beginning to address transcriptional and translational plasticity in TRP channel expression following urinary bladder inflammation, during postnatal development and in transgenic mouse models with documented urinary bladder dysfunction (Studeny et al. 2008; Schnegelsberg et al. 2010). Specifically, we examine TRP (TRPA1, TRPV1, TRPV4) channel expression using real-time quantitative polymerase chain reaction (Q-PCR) and western blotting approaches in the urinary bladder (urothelium + suburothelium and/or detrusor smooth muscle): (1) of rats after CYP-induced bladder inflammation of varying duration (Arms et al. 2010); (2) of transgenic mice with urothelial overexpression (OE) of nerve growth factor (NGF) under the control of the uroplakin II promoter and of vasoactive intestinal polypeptide null ($VIP^{-/-}$) mice (Studeny et al. 2008; Schnegelsberg et al. 2010); and (3) of C57Bl/6 mice during early postnatal maturation (postnatal day (P)6–P36).

Materials and Methods

Animals

$VIP^{-/-}$ Mice The $VIP^{-/-}$ mouse model was prepared using a VIP gene disruption strategy with confirmation of targeted mutation in mice and subsequent backcrossing to the C57BL/6 strain for at least ten generations (Dr. James Waschek, University of California, LA, USA) (Colwell et al. 2003). Mice used were bred locally at The University of Vermont, and the lack of the VIP gene was confirmed by PCR genotyping of tail snips. Mice were housed (12-h light/dark cycle) in groups (five) in the UVM animal vivarium with water and food provided ad libitum. $VIP^{-/-}$ and wild-type (WT) controls from the same litter were analyzed in most cases (Jensen et al. 2008). When a WT littermate was unavailable, an age-matched control mouse from another C57BL/6 litter was used. Previous studies (Girard et al. 2006) have demonstrated that the closely related neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), does not compensate for VIP in $VIP^{-/-}$ mice. Thus, the VIP and PACAP systems appear distinct.

NGF-OE Mice NGF-OE transgenic mice were generated at Roche Palo Alto (material transfer agreement with Roche Palo Alto and Dr. Debra Cockayne) in collaboration with Dr. Henry Sun at New York University Medical School as previously described (Cheppudira et al. 2008; Schnegelsberg et al. 2010). Animal genotype was confirmed by Southern blot and/or PCR analyses; all mice have the inbred genetic C57BL/6J background and were derived from F2 to F4 generations maintained through a hemizygous backcross strategy with C57BL/6J wild-type mice. Mice used in this study were bred locally at The University of Vermont College of Medicine. The litters were of normal size and weight and behaviors (feeding, drinking, activity patterns) appeared normal.

Wistar Rats Adult, female Wistar rats (205–225 g) were used in studies where bladder inflammation was induced by CYP treatment.

C57Bl/6 Mice Female, C57Bl/6 mice of varying postnatal age (P6–P36) from multiple dams were used to examine postnatal maturation of TRP channel expression in urinary bladder. Mouse pups were born to timed pregnant mice and several postnatal ages were studied from each litter born. Developmental ages prior to (P0–P17) or following (P21) the development of the spinobulbospinal micturition reflex were selected and analyzed (Capek and Jelinek 1956).

All experimental protocols involving animal use were approved by the University of Vermont (UVM) Institutional Animal Care and Use Committee. Animal care was under the supervision of the UVM Office of Animal Care Management in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress, or distress.

Measurement of Urinary Bladder NGF by Enzyme-Linked Immunoassays (ELISAs)

Determination of NGF content in the urinary bladder of NGF-OE transgenic mice and WT littermate controls was done using ELISAs as previously described (Vizzard 2000a; Cheppudira et al. 2008; Schnegelsberg et al. 2010). Microtiter plates (R & D Systems, Minneapolis, MN, USA) were coated with a mouse anti-rat NGF antibody (R & D Systems). Sample and standard solutions were run in duplicate. A horseradish peroxidase–streptavidin conjugate was used to detect the antibody complex. Tetramethyl benzidine was the substrate and the enzyme activity was measured by the change in optical density. The NGF standard provided with this protocol generated a linear standard curve from 15 to 1,000 pg/ml (R^2=0.997, $p \leq 0.0001$) for tissue samples. The absorbance values of standards and samples were corrected by subtraction of the background absorbance due to nonspecific binding. No samples fell below the minimum detection limits of the assay and no samples were diluted prior to use. Curve fitting of standards and evaluation of NGF content of samples was performed using a least squares fit as previously described (Vizzard 2000a; Schnegelsberg et al. 2010).

Induction of CYP-Induced Cystitis

Rats were anesthetized with isoflurane (2 %) and received intraperitoneal (i.p.) injection(s) of CYP (Sigma-Aldrich, St. Louis, MO, USA) to produce urinary bladder inflammation. To induce chronic bladder inflammation, CYP was injected (75 mg/kg; i.p.) every third day for 10 days with euthanasia occurring on the tenth day (Arms et al. 2010). To induce acute bladder inflammation, CYP was injected (150 mg/kg; i.p.) with euthanasia occurring 4 or 48 h after injection (Vizzard 2000b; Hu et al. 2003; Arms et al. 2010). Control rats received no treatment. The CYP model of bladder inflammation produces an increase in voiding frequency with small micturition volumes and is associated with inflammatory cell infiltrates in the urinary bladder including mast cells, macrophages, and neutrophils and somatic sensitivity (Vizzard 2000b; Hu et al. 2003; LaBerge et al. 2006).

Euthanasia and Tissue Harvest

WT and NGE-OE littermate (n=10–14 for each; 15–16 weeks of age) mice of both genders, WT and $VIP^{-/-}$ littermate (n=10–14 for each; 15–16 weeks of age) mice of both genders, female Wistar rats (n=10–14 for each; 16 weeks of age), and female C57Bl/6 mice (P6–P36; n=10–12 for each) were deeply anesthetized with isoflurane (3–4 %) and then euthanized via thoracotomy. The urinary bladder was quickly dissected under RNase-free conditions. In some instances, 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, and all tissues were snap-frozen on dry ice prior to processing as previously described (Arms et al. 2010). The urothelium has suburothelial structures, including the lamina propria, associated with it; the term urothelium in this paper refers to both urothelial and suburothelial structures. To confirm the specificity of our split bladder preparations, urothelium + suburothelium and detrusor samples were examined for the presence of α-smooth muscle actin (1:1,000; Abcam, Cambridge, MA, USA) and uroplakin II (1:25; American Research Products, Belmont, MA, USA) by western blotting or RT-PCR (Corrow and Vizzard 2007; Cheppudira et al. 2008). In urothelium+ suburothelium layers, only uroplakin II was present (data not shown). Conversely, in detrusor samples, only α-smooth muscle actin was present (data not shown). When we have insufficient numbers of animals to do analyses of bladder substructures, the whole urinary bladder is harvested for total RNA extraction.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (Q-PCR)

Total RNA was extracted using the STAT-60 total RNA/mRNA isolation reagent (Tel-Test "B", Friendswood, TX, USA) as previously described (Girard et al. 2002; Klinger et al. 2008). One microgram of RNA per sample was used to synthesize complementary DNA using a mix of random

hexamer and oligo dT primers with M-MLV reverse transcriptase (Promega Corp.) in a 25-μl final reaction volume. The quantitative PCR standards for all transcripts were prepared with the amplified cDNA products ligated directly into pCR2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen). The nucleotide sequences of the inserts were verified by automated fluorescent dideoxy dye terminator sequencing (Vermont Cancer Center DNA Analysis Facility). To estimate the relative expression of the receptor transcripts, tenfold serial dilutions of stock plasmids were prepared as quantitative standards. The range of standard concentrations was determined empirically.

Complementary DNA templates, diluted tenfold 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 300 nM of each primer in a final 25 μl reaction volume. Rat and mouse primers (Table 1) were designed with the upper primer bridging an intron/exon boundary to exclude DNA amplification. Primer sequences for the ribosomal protein L32, used as a reference gene in these studies, have been previously reported (Klinger et al. 2008).

Q-PCR was performed (Applied Biosystems 7500 Fast real-time PCR System, Foster City, CA, USA) using the following standard conditions: (1) serial heating at 94 °C for 2 min and (2) amplification over 45 cycles at 94 °C for 15 s and 55–65 °C for 30 s. The amplified product from these parameters was subjected to SYBR Green I melting analysis by ramping the temperature of the reaction samples from 60 to 95 °C. A single DNA melting profile was observed under these dissociation assay conditions demonstrating amplification of a single unique product free of primer dimers or other anomalous products.

For data analyses, a standard curve was constructed by amplification of serially diluted plasmids containing the target sequence. 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. All data are expressed as the relative quantity of the gene of interest normalized to the relative quantity of the reference gene L32.

Western Blotting for TRPV1 and TRPV4

Whole urinary bladders (control, 4 h, 48 h, and chronic) were homogenized (1 g, 20 ml) separately in a tissue protein extraction agent (T-PER; Roche, Indianapolis, IN, USA), a mild zwitterionic dialyzable detergent in 25 mM bicine, 150 mM sodium chloride (pH 7.6) containing a protease inhibitor mix (Sigma-Aldrich; 16 μg/ml benzamidine, 2 μg/ml leupeptin, 50 μg/ml lima bean trypsin inhibitor, and 2 μg/ml pepstatin A), and aliquots were removed for protein assay. Samples (375μg) were suspended in sample buffer for fractionation on gels and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and efficiency of transfer was evaluated with Ponceau S stain (Sigma-Aldrich). Membranes were blocked overnight in a solution of 5 % milk and 3 % bovine serum albumin in Tris-buffered saline with 0.1 % Tween. For immunodetection, the following antibodies were used overnight at 4 °C: rabbit anti-TRPV1 [1:400; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA (catalog #sc-28759)] and goat anti-TRPV4 [1:400; Santa Cruz Biotechnology, Inc. (catalog #sc-47527)]. Washed membranes were incubated in species-specific secondary antibodies for 2 h at room temperature for enhanced chemiluminescence detection (Pierce, Rockford, IL, USA). Blots were exposed to Biomax film (Kodak, Rochester, NY, USA) and developed. Blots were analyzed using the Versa Doc 4000 MP Imaging System (BioRad, Hercules, CA, USA). The adjusted volume of each band was analyzed and background intensities subtracted using Quantity One software (BioRad) (VT Cancer Center DNA Analysis Facility). Images were scanned with a flatbed

Table 1 Primer sequences for TRPA1, TRPV1, and TRPV4 designed for use with the species (mouse, rat) indicated

Species	Primers	Sequences
Mouse	TRPA1 upper	5′-TCGTGTGAAGTGCTGAATAT-3′
	TRPA1 lower	5′-AACTTTACACTTTCAACTTGATTTT-3′
Rat	TRPA1 upper	5′-CTGAATTTCCAAGATGCCTT-3′
	TRPA1 lower	5′-TATGCCAGTCATTCCTGAA-3′
Mouse/rat	TRPV1 upper	5′-TACTTTTCTTTGTACAGTCACT-3′
	TRPV1 lower	5′-TCAATCATGACAGCATAGAT-3′
Mouse	TRPV4 upper	5′-TTCGTAGGGATCGTTGGTCCT-3′
	TRPV4 lower	5′-TACAGTGGGGCATCGTCCGT-3′
Rat	TRPV4 upper	5′-ACTGGCAAGATCGGGGTCTT-3′
	TRPV4 lower	5′-GAGGAGAGGTCGTAGAGAGAAGAAT-3′

scanner, the contrast was corrected, and the images imported and figures assembled with Adobe Photoshop (San Jose, CA, USA). In this and previous studies (Corrow et al. 2010), we have used actin as a loading control because expression of actin (1:1,000; Santa Cruz Biotechnologies, Inc.; catalog #sc-1616) protein is not changed in urinary bladder with CYP treatments. Nonspecific reactions were assessed by preabsorption treatment with 10^{-6} M of the antigen peptide when available [blocking peptide for TRPV4, Santa Cruz Biotechnology, Inc. (catalog #sc-47527)]. Use of the TRPV4 blocking peptide eliminated immunostaining at ~105 kDa (data not shown). Specificity of TRPV1 expression was confirmed in TRPV1 null mice (Jackson Laboratories, ME, USA) and TRPV1 null mice lack immunostaining at ~100 kDa (data not shown). Both molecular weights (TRPV1, TRPV4) are consistent with the manufacturer's information. Although immunoblotting for TRPA1 was attempted with multiple antibodies from diverse sources, the presence of numerous bands coupled with the inability to demonstrate specificity has prevented our use of these data.

Statistical Analyses

Values are expressed as means ± SEM from *n* mice or rats. Statistical comparisons between groups were made using one-way analysis of variance. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. When *F* ratios exceeded the critical value ($p \leq 0.05$), Dunnett's post hoc test was used to compare the control to experimental (CYP) treatments, WT to transgenic groups, or the youngest postnatal age examined (P6) to older postnatal ages (P14–P36). To test for the effect of age on TRP expression, linear regression analysis was performed. No differences were observed when mice of different genders were used; therefore, data from both genders were pooled for analyses. Differences were considered statistically significant if $p \leq 0.05$.

Results

TRPA1, TRPV1, and TRPV4 Transcript Expression in Urothelium + Suburothelium (uro) or Detrusor (det) After Cyclophosphamide (CYP)-Induced Cystitis in Rats

Cystitis (48 h or chronic) significantly ($p \leq 0.01$) increased TRPA1 transcript expression in the uro of female Wistar rats (Fig. 1a). No changes in TRPA1 transcript expression were observed in the uro with acute (4 h) CYP treatment (Fig. 1a). In addition, no changes in TRPA1 transcript expression were observed in det with any duration of CYP treatment (Fig. 1a). Cystitis (4 or 48 h) significantly ($p \leq 0.01$) decreased TRPV1 transcript expression in the uro of female Wistar rats (Fig. 1b). No changes in TRPV1 transcript expression were observed in the uro with chronic CYP treatment (Fig. 1b). Chronic CYP

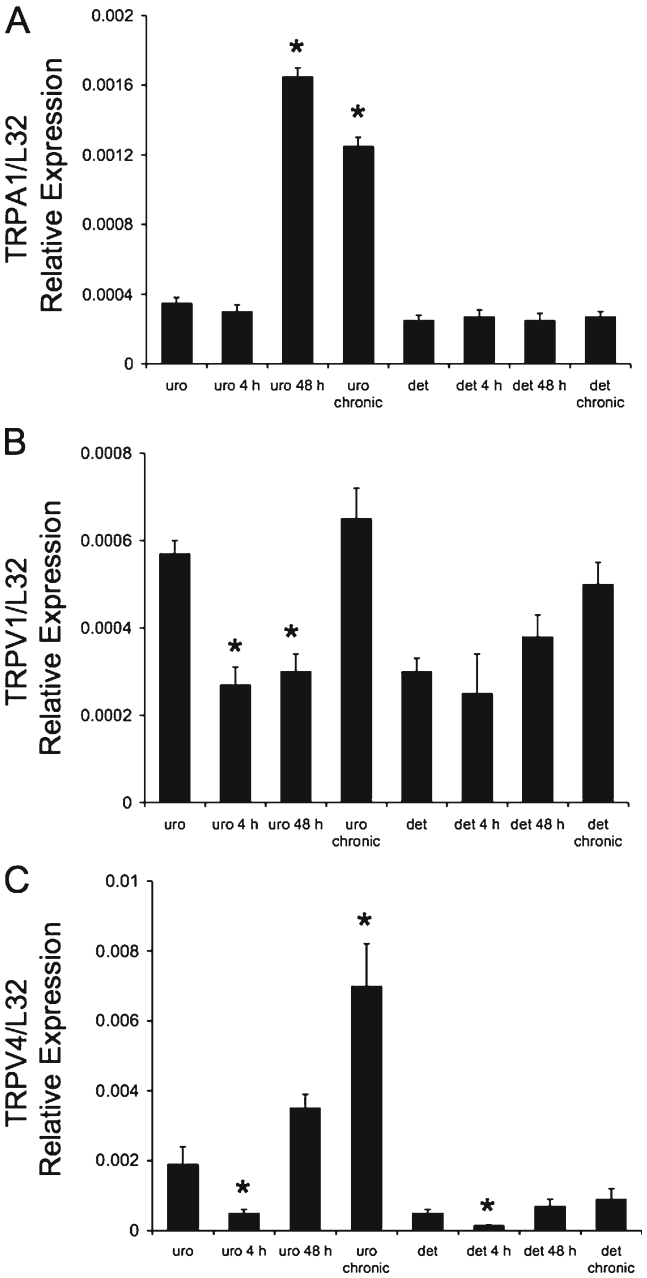

Fig. 1 TRPA1 (**a**), TRPV1 (**b**), and TRPV4 (**c**) transcript levels in rat urothelium + suburothelium (uro) and detrusor (det) smooth muscle with or without cyclophosphamide (CYP) treatment of varying duration (4 h, 48 h, chronic). Relative expression of TRPA1 (**a**) transcript expression in uro or det in control and CYP-treated rats normalized to the relative expression of the reference gene, L32. Relative expression of TRPV1 (**b**) transcript expression in uro or det in control and CYP-treated rats normalized to the relative expression of the reference gene, L32. Relative expression of TRPV4 (**c**) transcript expression in uro or det in control and CYP-treated rats normalized to the relative expression of the reference gene, L32. Samples size are *n* of 10–14; **p*=0.01 versus uro or det without CYP

treatment was the only duration of CYP treatment that significantly ($p \leq 0.01$) increased TRPV1 transcript expression in det (Fig. 1b). Cystitis (4 h) significantly ($p \leq 0.01$) decreased TRPV4 transcript expression in uro and det, but chronic CYP treatment significantly ($p \leq 0.01$) increased TRPV4 expression in uro (Fig. 1c).

TRPV1 and TRPV4 Protein Expression in Whole Urinary Bladder After Cyclophosphamide (CYP)-Induced Cystitis in Rats

Cystitis (4 or 48 h) significantly ($p \leq 0.01$) increased TRPV1 transcript expression in the whole urinary bladder of female Wistar rats (Fig. 2a). No changes in TRPV1 protein expression were observed in the bladder with chronic CYP treatment (Fig. 2a). Each duration of CYP-induced cystitis evaluated (4 h, 48 h, or chronic) significantly ($p \leq 0.01$) increased TRPV4 transcript expression in the whole urinary bladder of female Wistar rats (Fig. 2b).

NGF Expression in Urothelium and Detrusor of WT and NGF-OE Mice

Consistent with our previous studies (Cheppudira et al. 2008; Schnegelsberg et al. 2010), NGF transcript and protein expression was significantly ($p \leq 0.001$) increased in the urothelium of NGF-OE mice; no changes were observed in detrusor smooth muscle between NGF-OE and littermate WT mice (data not shown).

TRPA1, TRPV1, and TRPV4 Transcript Expression in Urothelium + Suburothelium (uro) or Detrusor (det) of Littermate WT and NGF-OE Mice

TRPA1 (Fig. 3a) and TRPV4 (Fig. 3e) transcript expression was significantly ($p \leq 0.01$) increased in uro of NGF-OE mice compared to littermate WT mice with no changes being observed in the det (Fig. 3b, f). In contrast, TRPV1 transcript expression was similar in uro of littermate WT and NGF-OE mice, whereas TRPV1 transcript expression was significantly ($p \leq 0.01$) decreased in the det of NGF-OE mice compared to littermate WT mice (Fig. 3c, d).

TRPA1, TRPV1, and TRPV4 Transcript Expression in Whole Urinary Bladder of $VIP^{-/-}$ Mice and Littermate WT Mice

Littermate WT and $VIP^{-/-}$ mice exhibited similar TRPA1 (Fig. 4a) or TRPV4 (Fig. 4c) transcript expression in whole urinary bladder. Whole urinary bladder of $VIP^{-/-}$ mice exhibited significantly decreased TRPV1 transcript expression (Fig. 4b).

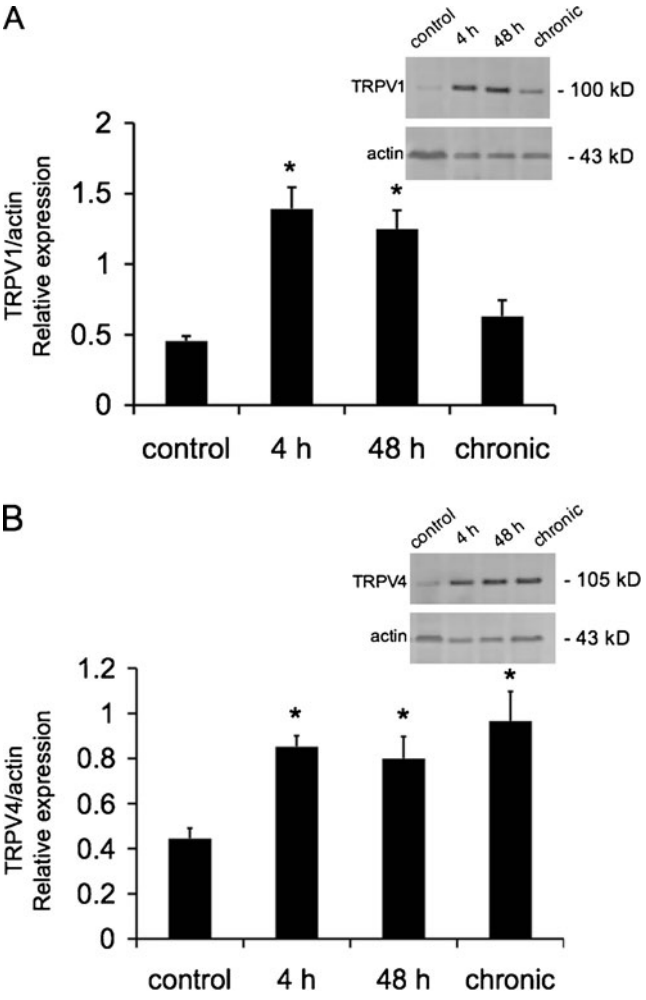

Fig. 2 Western blot analyses of TRPV1 (**a**) and TRPV4 (**b**) in whole urinary bladder from rats treated with cyclophosphamide (CYP) of varying durations (4 h, 48 h, chronic). Representative western blot of TRPV1 protein expression in whole urinary bladder from rats treated with CYP (**a**). Summary histogram of the relative expression of TRPV1 protein normalized to actin expression in urinary bladder from control and CYP-treated rats (**a**). Representative western blot of TRPV4 protein expression in whole urinary bladder from rats treated with CYP (**b**). Summary histogram of the relative expression of TRPV1 protein normalized to actin expression in urinary bladder from control and CYP-treated rats (**b**). Samples size are *n* of 10–12; **p*= 0.01 versus control

TRPV1 and TRPV4 Protein Expression in Whole Urinary Bladder of Littermate WT and NGF-OE Mice and Littermate WT and $VIP^{-/-}$ Mice

TRPV1 protein expression was similar in whole urinary bladder of littermate WT and NGF-OE mice (Fig. 5a). In contrast, TRPV1 protein expression was significantly ($p \leq 0.01$) increased in whole urinary bladder of $VIP^{-/-}$ mice compared to littermate WT mice (Fig. 5a). TRPV4 protein expression was similar in whole urinary bladder of littermate WT and NGF-OE mice (Fig. 5b). In contrast, TRPV4 protein expression was significantly ($p \leq 0.01$) increased in

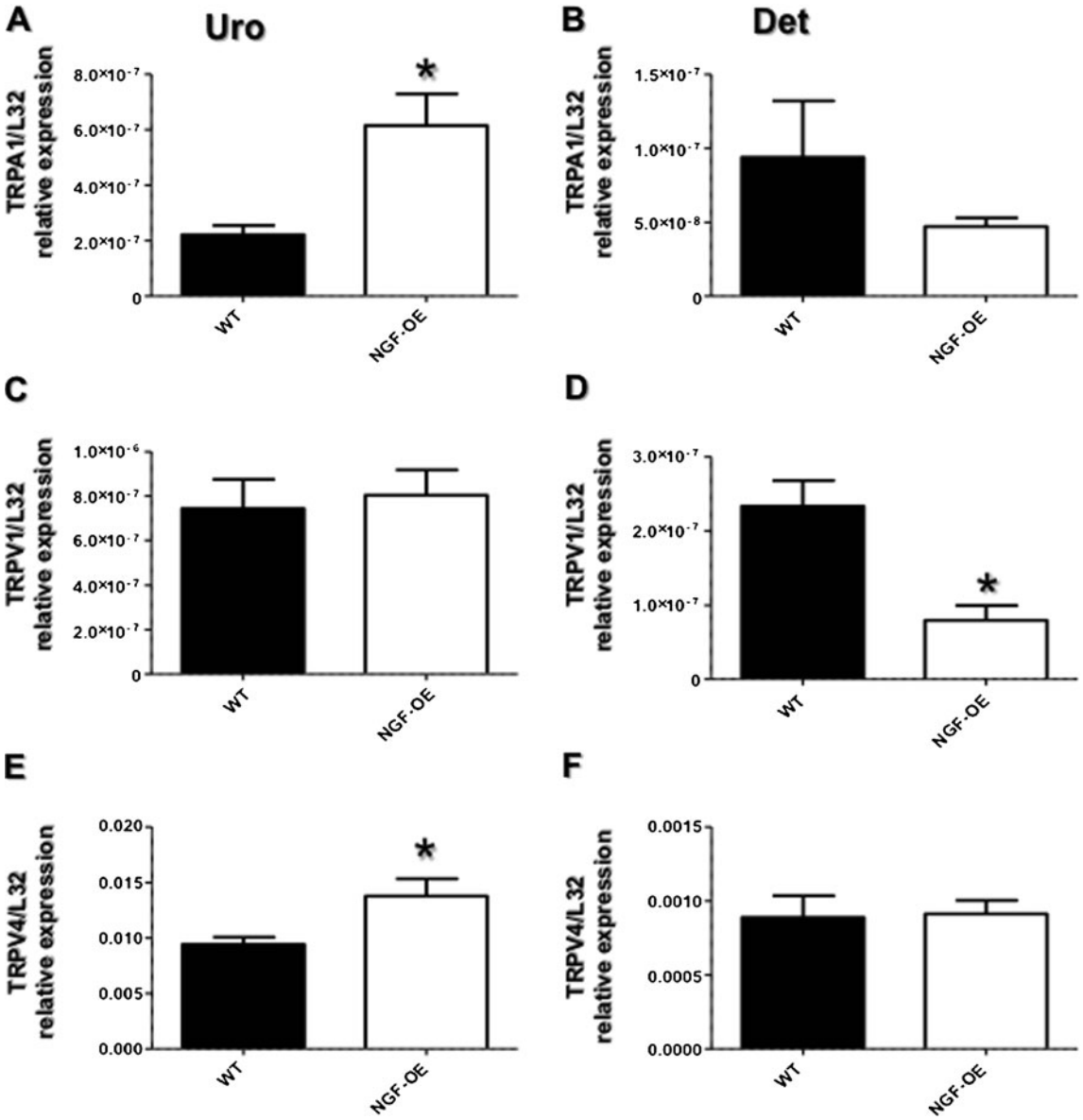

Fig. 3 TRPA1 (**a**, **b**), TRPV1 (**c**, **d**), and TRPV4 (**e**, **f**) transcript levels in urothelium + suburothelium (uro: **a**, **c**, **e**) or detrusor smooth muscle (det: **b**, **d**, **f**) from wild-type (WT) and nerve growth factor overexpressing (NGF-OE) mice. Relative expression of TRPA1 (**a**, **b**), TRPV1 (**c**, **d**), and TRPV4 (**e**, **f**) transcript expression is normalized to the relative expression of the reference gene, L32. Samples size are *n* of 10–14; **p*=0.01

whole urinary bladder of VIP$^{-/-}$ mice compared to littermate WT mice (Fig. 5b).

TRPA1, TRPV1, and TRPV4 Transcript Expression in Whole Urinary Bladder of C57Bl/6 Mice During Early Postnatal Development (P6–P36)

Significant ($p \leq 0.01$) increases in TRPA1 transcript expression in whole urinary bladder were observed at two points during the postnatal ages examined, P17 and P36 (Fig. 6a). TRPV1 (Fig. 6b) and TRPV4 (Fig. 6c) transcript expression in urinary bladder exhibited an age-dependent increase (R^2=0.967–0.987) in expression from P6–P21 or from P6–P28, respectively. TRPV1 transcript expression was significantly ($p \leq 0.01$) increased in urinary bladder of postnatal rats at P17 and P21 compared to the youngest postnatal age examined, P6 (Fig. 6b). TRPV4 transcript expression was significantly increased in urinary bladder of postnatal rats at P21 and P28 compared to the youngest postnatal age examined, P6 (Fig. 6c).

Discussion

Multiple TRP channels are expressed in the LUT, and mechanosensory and nociceptor roles in micturition reflex function under physiological and pathophysiological states

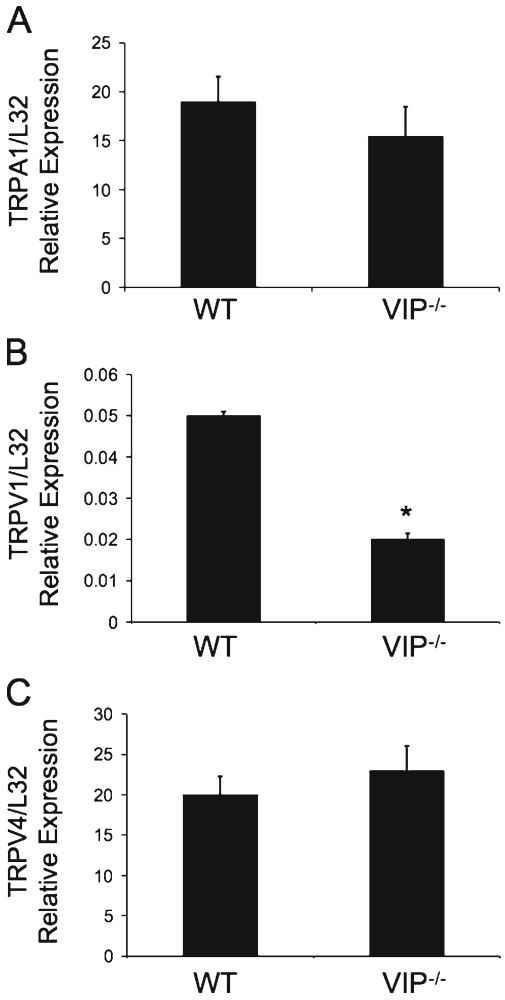

Fig. 4 TRPA1 (**a**), TRPV1 (**b**), and TRPV4 (**c**) transcript levels in whole urinary bladder from wild-type (WT) and vasoactive intestinal polypeptide null ($VIP^{-/-}$) mice. Relative expression of TRPA1 (**a**), TRPV1 (**b**), and TRPV4 (**c**) transcript expression is normalized to the relative expression of the reference gene, L32. Samples size are *n* of 10–14; **p*=0.01

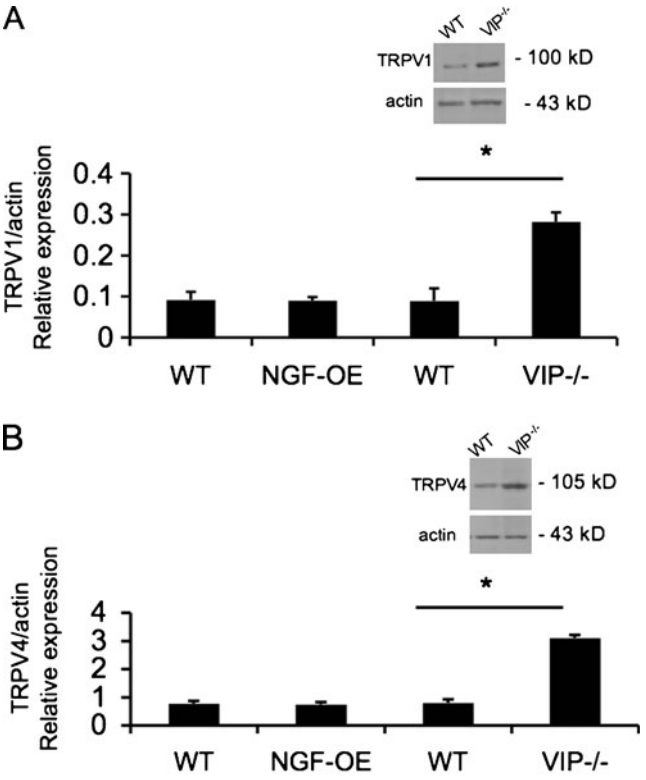

Fig. 5 Western blot analyses of TRPV1 (**a**) and TRPV4 (**b**) in whole urinary bladder from littermate wild-type (WT) and nerve growth factor overexpressing (NGF-OE) mice and littermate WT and vasoactive intestinal polypeptide null ($VIP^{-/-}$) mice. Representative western blot of TRPV1 protein expression in whole urinary bladder from littermate WT and $VIP^{-/-}$ mice (**a**). Summary histogram of the relative expression of TRPV1 protein normalized to actin expression in urinary bladder from littermate WT and NGF-OE mice and littermate WT and $VIP^{-/-}$ mice (**a**). Representative western blot of TRPV4 protein expression in whole urinary bladder from littermate WT and $VIP^{-/-}$ mice (**b**). Summary histogram of the relative expression of TRPV4 protein normalized to actin expression in urinary bladder from littermate WT and NGF-OE mice and littermate WT and $VIP^{-/-}$ mice (**b**). Samples size are *n* of 10–14; **p*=0.01 versus WT

have been suggested. We have focused these initial studies on TRPA1, TRPV1, and TRPV4 channels that exhibit LUT expression and function. These studies demonstrate considerable transcriptional plasticity of urinary bladder TRPA1, TRPV1, and TRPV4 channels with bladder inflammation, postnatal development or in transgenic NGF-OE or $VIP^{-/-}$ mice. A lack of correlation between transcript and protein expression was also observed that may reflect changes in posttranscriptional and posttranslational mechanisms including increased protein stability. Of note in the present study is the demonstration that NGF-OE in the urinary bladder results in significant increases in TRPV4 as well as TRPA1 transcript expression in the urothelium and suburothelium. Urinary bladder of $VIP^{-/-}$ mice exhibited a significant decrease or no change in TRPV1 or TRPV4 transcript expression but demonstrated significant increases in TRPV1 and TRPV4 protein expression. CYP-induced cystitis increased urinary bladder TRPA1 protein expression. Postnatal maturation was associated with age-dependent increases in urinary bladder TRPA1, TRPV1, and TRPV4 transcript expression but patterns of expression differed. The functional significance of TRP channel plasticity in the urinary bladder in these diverse contexts remains to be determined.

Pharmacological approaches as well as the use of TRPV1 knockout mice have begun to elucidate the contributions of TRPV1 to micturition reflex function (Nilius et al. 2007). TRPV1 knockout mice show a higher frequency of non-voiding bladder contractions compared to wild-type animals, as well as a reduction in reflex voiding during bladder filling (Birder et al. 2002). Additionally, the TRPV1 knockout mouse does not develop bladder overactivity

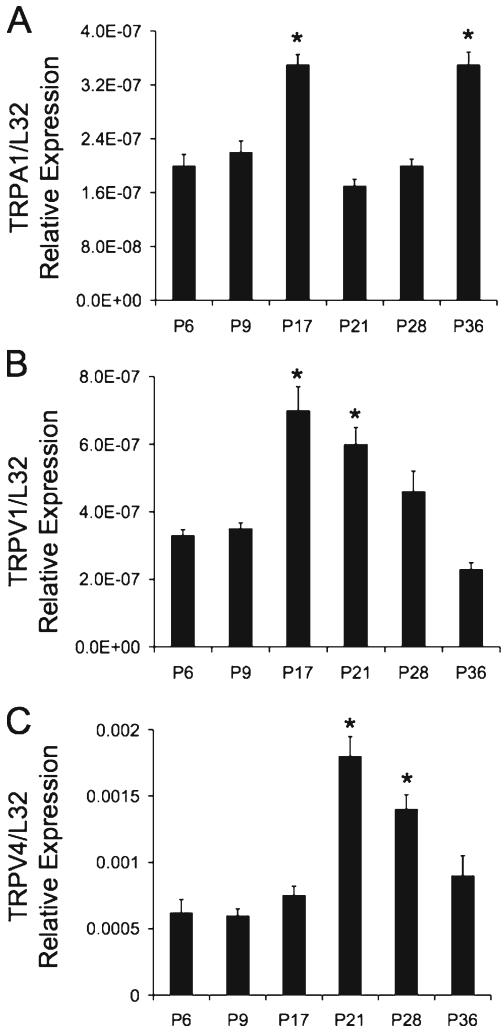

Fig. 6 TRPA1 (**a**), TRPV1 (**b**), and TRPV4 (**c**) transcript levels in whole urinary bladder from C57Bl/6 mice during postnatal development (postnatal age (P) P6–P36). Relative expression of TRPA1 (**a**), TRPV1 (**b**), and TRPV4 (**c**) transcript expression is normalized to the relative expression of the reference gene, L32. Samples size are *n* of 10–14; **p*=0.01 compared to P6

during acute bladder inflammation, suggesting that TRPV1 is involved in bladder hyperreflexia induced by inflammation (Nilius et al. 2007). The function of the TRPV4 channel in bladder function is also beginning to be established. An abnormal urine voiding pattern has been reported in the TRPV4 knockout mice (Gevaert et al. 2007; Angelico and Testa 2010), characterized by a decrease in voiding frequency and an increase in bladder capacity and void volume (Gevaert et al. 2007; Thorneloe et al. 2008; Everaerts et al. 2010). TRPV4 channel agonists increase bladder hyperactivity, whereas TRPV4 antagonists result in a decrease in bladder activity, making it a promising target for OAB and other bladder disorders (Janssen et al. 2011). Studies suggest that that TRPV4 channels are strong candidates as mechanosensors in the urinary bladder in a number of species including humans and suggest that blockade of TRPV4 channels could represent a novel therapy for overactive bladder and storage dysfunction (Gevaert et al. 2007; Araki 2011; Olsen et al. 2011).

CYP-induced cystitis significantly increased TRPV1 (4 and 48 h) and TRPV4 (4 h, 48 h, chronic) protein expression in the urinary bladder; however, TRPV1 transcript expression was significantly reduced in urothelium and suburothelial tissues with 4 and 48 h cystitis but increased in detrusor with chronic CYP-induced cystitis. TRPV4 transcript expression was significantly reduced in urothelium and suburothelial tissues and detrusor with 4 h CYP-induced cystitis but TRPV4 transcript expression significantly increased in urothelium and suburothelial tissues with chronic CYP-induced cystitis. Thus, the lack of correlation between TRPV1 and TRPV4 transcript and protein expression may reflect changes in posttranscriptional and posttranslational mechanisms including increased protein stability. Increased TRPV1 protein expression demonstrated in the current study is consistent with the suggestion that TRPV1 is involved in bladder hyperreflexia in inflammation (Nilius et al. 2007). Immunoblotting demonstrated significant increases in TRPV4 protein expression in urinary bladder with each duration of CYP-induced cystitis evaluated. This finding is consistent with the demonstration that a selective TRPV4 antagonist increases functional bladder capacity and reduces micturition frequency in mice and rats with acute cystitis (24 h) (Everaerts et al. 2010). These studies also suggest that chronic urinary bladder inflammation may involve TRPV4 and that chronic cystitis may also improve with TRPV4 channel antagonism (Everaerts et al. 2010).

TRPA1 receptor expression has been described in rodent and human bladders, in both C fibers and urothelial cells, where it is suggested to act as a mechanosensor and nociceptor in either physiological or pathological states (Du et al. 2007; Streng et al. 2008). Addition of TRPA1 agonists, allyl isothiocyanate (AI) and cinnamaledhyde (CA), to bladder strips induced concentration-dependent contraction (Andrade et al. 2006). Furthermore, intravesical administration of AI increased maximum bladder pressures and decreased voided volume, infused volume, and intercontraction interval (Streng et al. 2008), and CA administration decreased threshold pressure and intercontraction intervals during cystometry (Du et al. 2007). Moreover, it has been recently demonstrated that TRPA1 expression is upregulated in the bladder mucosa of patients with OAB caused by bladder outlet obstruction (Du et al. 2007) and in the bladder and in DRG with OAB induced by spinal cord injury (Andrade et al. 2006). These findings suggest that TRPA1 may not only be involved in normal urinary bladder function, but that it also may be considered a new potential target for the study of bladder disorders. We demonstrated significant increases in TRPA1 transcript expression with 48 h or

chronic CYP-induced cystitis, but due to difficulty confirming the specificity of TRPA1 immunoblotting results, no conclusions can be made concerning bladder TRPA1 protein expression with CYP-induced cystitis.

A number of diverse and conflicting roles for VIP have been demonstrated in the urinary bladder from numerous species (Erol et al. 1992; Igawa et al. 1993; Uckert et al. 2002; Hernandez et al. 2006). Previous studies from our laboratory have demonstrated that the $VIP^{-/-}$ mouse exhibits morphological changes of the lower urinary tract and functional changes involving the micturition reflex (Studeny et al. 2008). We demonstrated increased bladder mass and fewer but larger urine spots on filter paper in $VIP^{-/-}$ mice (Studeny et al. 2008). $VIP^{-/-}$ mice exhibit decreased void volumes and intercontraction intervals with continuous intravesical infusion of saline in conscious, unrestrained mice (Studeny et al. 2008). The changes in neural control of bladder function that manifest as bladder hyperreflexia and increased somatic sensitivity in $VIP^{-/-}$ mice may reflect increased basal expression of NGF (Jensen et al. 2008) or proinflammatory cytokine production in urinary bladder (Girard et al. 2008) as previously demonstrated.

In addition to roles as neurotransmitter or neuromodulator in autonomic nervous system pathways, VIP exhibits considerable anti-inflammatory properties (Said 1991; Voice et al. 2002; Szema et al. 2006), mediated through $VPAC_1$ receptors on inflammatory cells (Delgado et al. 2000). It has been shown that VIP inhibits the production of proinflammatory compounds including, TNFα, iNOS, IL-1, and IL-12 (Chorny et al. 2006) and upregulates the production of the anti-inflammatory cytokine, IL-10 (Delgado et al. 1999). In diverse experimental models of inflammation, VIP has shown to improve symptoms and survival (Delgado et al. 1999; Abad et al. 2003; Bik et al. 2004; Juarranz et al. 2005; Martinez et al. 2005; Newman et al. 2005; Gonzalez-Rey and Delgado 2006). In the present study, $VIP^{-/-}$ mice exhibited a significant reduction or no change in urinary bladder TRPV1 or TRPV4 transcript expression, respectively, but significant upregulation of TRPV1 and TRPV4 protein expression in urinary bladder. The lack of correlation between transcript and protein expression may also reflect posttranslational and posttranslational processes. Changes in TRPV1 and TRPV4 protein expression may reflect a change in the balance of growth factor as well as cytokine expression in the urinary bladder. Pharmacological manipulation of TRPV1 and TRPV4 channels in $VIP^{-/-}$ mice can begin to address the contribution of these TRPV channels to altered bladder dysfunction in $VIP^{-/-}$ mice.

Increased urinary bladder NGF content may underlie many of the sensory changes that occur in patients with OAB symptoms or IC/BPS, including irritative voiding symptoms and pain in the case of IC/BPS (Kim et al. 2005; Kim et al. 2006). Altered NGF content is associated with urinary bladder inflammation and dysfunction in rodents (Vizzard 2001; Zvarova et al. 2004; Braas et al. 2006; Klinger and Vizzard 2008). IC/BPS is a chronic inflammatory bladder disease of unknown etiology characterized by urinary frequency, urgency, and suprapubic/pelvic pain (Driscoll and Teichman 2001). Pain and altered bladder/visceral hypersensitivity in IC/BPS patients may involve organizational or functional changes in peripheral bladder afferents and central pathways such that bladder afferent neurons become sensitized and hyperresponsive to normally innocuous stimuli such as bladder filling (Driscoll and Teichman 2001). Our studies (Schnegelsberg et al. 2010) revealed that urothelium-specific overexpression of NGF in the urinary bladder of transgenic mice (1) stimulates neuronal sprouting or proliferation in the urinary bladder, (2) produces local inflammatory changes in the urinary bladder, (3) produces urinary bladder hyperreflexia, and (4) results in increased referred somatic hypersensitivity. Elevated levels of neurotrophins have also been detected in the urine of women (Okragly et al. 1999) and in the urothelium of individuals with BPS/IC or other painful bladder conditions (Lowe et al. 1997). More recently, it was demonstrated that urinary NGF levels are increased in patients with OAB symptoms associated with detrusor overactivity, stress urinary incontinence, or bladder outlet obstruction (Ochodnicky et al. 2011; Liu and Kuo 2008a, b). In addition to NGF, additional NGF-mediated changes might contribute to the urinary bladder hyperreflexia and pelvic hypersensitivity observed in these mice (Girard et al. 2010, 2011; Schnegelsberg et al. 2010), such as stimulation/recruitment of bladder mast cells, modulation of local neuroinflammatory responses, and upregulation of neuropeptide/receptor systems and neurotrophin/receptor systems and ion channels in the urinary bladder, including TRP channels. The current studies are the first to demonstrate increased TRPA1 and TRPV4 transcript expression in the urothelium of NGF-OE mice. However, the current studies are consistent with previous studies demonstrating that TRPA1 as well as TRPV1 function and modulation in sensory neurons is dependent upon target tissue expression of NGF and glial-derived neurotrophic factor (Malin et al. 2011). Furthermore, previous studies demonstrate that early neonatal colon injury results in a long-lasting visceral hypersensitivity, possibly driven by an early increase in growth factor expression and long-term changes in TRPA1 function (Christianson et al. 2010). Ongoing studies are determining the effects of TRPA1 or TRPV4 antagonism on altered micturition reflex function in the NGF-OE mouse model.

During postnatal maturation, primitive reflex pathways organized at the spinal level are replaced by a spinobulbospinal reflex leading to the emergence of voluntary voiding (de Groat and Araki 1999). Postnatal maturation of voiding function may involve prominent reorganization of synaptic

connections in bladder reflex pathways in the brain, spinal cord, and periphery. This reorganization leads to downregulation of primitive spinal mechanisms and upregulation of mature supraspinal pathways (de Groat and Araki 1999). Previous studies have suggested the importance of neuroactive compounds (Ekstrom et al. 1994; Iuchi et al. 1994; Sann et al. 1997) in the process of maturation of micturition reflexes. To our knowledge, the current studies are the first to examine TRP channel plasticity during early postnatal development with the long-term goal of determining if altered bladder TRP channel expression contributes to the maturation of micturition reflexes. Current studies are examining micturition reflex function in TRPA1, TRPV1, and TRPV4 knockout mice to see if these transgenic mice exhibit an altered maturation of micturition reflex function reflecting mechanosensory roles for these TRP channels in bladder function.

Conclusions and Future Directions

These studies reveal transcriptional and translational plasticity in urinary bladder TRPA1, TRPV1, and TRPV4 expression in the context of urinary bladder inflammation, postnatal maturation of bladder reflexes and in transgenic mice with NGF-OE in the urothelium (Schnegelsberg et al. 2010), or VIP deletion (Studeny et al. 2008). Additional studies are necessary to determine not only the functional role(s) of these TRP channels in these diverse contexts but it will also be of importance to demonstrate the tissue distribution of these TRP channels in the urinary bladder in these different contexts. Ongoing studies will provide important information on the regulation of TRP channel expression in the urinary bladder and add to the growing discussion of the utility in targeting urinary bladder TRP channels to improve urinary bladder dysfunction (Gevaert et al. 2007; Andersson et al. 2010; Araki 2011; Eid 2011).

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