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PACAP/VIP AND RECEPTOR CHARACTERIZATION IN MICTURITION PATHWAYS IN MICE WITH OVEREXPRESSION OF NGF IN UROTHELIUM

Beatrice M. Girard¹, Susan E. Malley², Karen M. Braas¹, Victor May¹, and Margaret A. Vizzard^{1,2}

¹ University of Vermont, Department of Anatomy and Neurobiology, Burlington, VT, 05405 (U.S.A.)

² University of Vermont, Department of Neurology, Burlington, VT, 05405 (U.S.A.)

Abstract

Urothelium-specific overexpression of NGF in the urinary bladder of transgenic mice stimulates neuronal sprouting or proliferation in the urinary bladder, produces urinary bladder hyperreflexia and results in increased referred somatic hypersensitivity. Additional NGF-mediated changes might contribute to the urinary bladder hyperreflexia and pelvic hypersensitivity observed in these transgenic mice such as upregulation of neuropeptide/receptor systems. Chronic overexpression of NGF in the urothelium was achieved through the use of a highly urothelium-specific, uroplakin II promoter. In the present study, we examined PACAP, VIP and associated receptors (PAC1, VPAC1, VPAC2) transcripts or protein expression in urothelium and detrusor smooth muscle and lumbosacral DRG in NGF-overexpressing and littermate wildtype mice using real-time quantitative reverse transcription-polymerase chain reaction and immunohistochemical approaches. Results demonstrate upregulation of PAC1 receptor transcript and PAC1-immunoreactivity in urothelium of NGF-OE mice whereas PACAP transcript and PACAP-immunoreactivity were decreased in urothelium of NGF-OE mice. In contrast, VPAC1 receptor transcript was decreased in both urothelium and detrusor smooth muscle of NGF-OE mice. VIP transcript expression and immunostaining was not altered in urinary bladder of NGF-OE mice. Changes in PACAP, VIP and associated receptors transcripts and protein expression in micturition pathways resemble some, but not all, changes observed after induction of urinary bladder inflammation known to involve NGF production.

Index entries

NGF; urinary bladder; neuropeptides; urothelium

Introduction

Nerve growth factor (NGF) has been suggested to play a role in urinary bladder dysfunction by mediating inflammation as well as morphological and functional changes in sensory and sympathetic neurons innervating the urinary bladder. Many previous studies in rodents have demonstrated the importance of NGF in bladder sensory function and the development of referred hyperalgesia in response to bladder inflammation (Jaggar et al., 1999; Zvara and Vizzard, 2007; Guerios et al., 2008; Arms et al., 2009). We recently examined the role of NGF in urinary bladder dysfunction by generating a mouse model of urinary bladder hypersensitivity

based on the hypothesis that chronic urothelial NGF overexpression would induce sensory neuronal hypersensitivity and increased urinary bladder reflex function (Schnegelsberg et al., 2009). Chronic overexpressing of NGF in the urothelium was achieved through the use of a highly urothelium-specific, uroplakin II promoter (Lin et al., 1995; Liang et al., 2005). Our studies (Schnegelsberg et al., 2009) 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 with interstitial cystitis (IC)/bladder pain syndrome (BPS) (77) and in the urothelium of individuals with IC/BPS or other painful bladder conditions (63). More recently, it was demonstrated that urinary NGF levels are increased in patients with overactive bladder (OAB) symptoms associated with detrusor overactivity (DO), stress urinary incontinence, or bladder outlet obstruction (BOO) (47; 48; 57–62; 77; 103).

Additional NGF-mediated changes might contribute to the urinary bladder hyperreflexia and pelvic hypersensitivity observed in these mice (Schnegelsberg et al., 2009), such as stimulation/recruitment of bladder mast cells, modulation of local neuroinflammatory responses, upregulation of neuropeptide/receptor systems and ion channels as well as changes in the expression of other neurotrophins and associated receptors. NGF-mediated changes in urinary bladder function and altered referred somatic sensitivity (Nicol and Vasko, 2007) may involve changes in expression of nociception-related molecules, including brain-derived neurotrophic factor, tropomyosin-related receptor tyrosine kinases, TrkA and TrkB, as well as p75^{NTR} (Vizzard, 2000d; Vizzard, 2001; Braas et al., 2006; de Groat and Yoshimura, 2009).

In the present study, we examined PACAP, VIP and associated receptors transcripts or protein expression in urothelium and detrusor smooth muscle in NGF-overexpressing (OE) and littermate wildtype mice using real-time quantitative reverse transcription-polymerase chain reaction (Q-PCR) and immunohistochemical approaches.

Materials and Methods

Animals

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., 2009). Animal genotype was confirmed by Southern 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 wildtype mice. Female 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. All experimental protocols involving animal use were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC # 08-085). Animal care was under the supervision of the University of Vermont's Office of Animal Care Management in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) 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 ELISA

Determination of NGF content in the urinary bladder and dorsal root ganglia (DRG; L1-S1) of NGF-OE transgenic mice and WT littermate controls was determined using enzyme-linked immunoassays (ELISAs) as previously described (Vizzard, 2000c; Cheppudira et al., 2008;

Schnegelsberg et al., 2009). Microtiter plates (R & D Systems, Minneapolis, MN) were coated with a mouse anti-rat NGF antibody (R & D Systems, Minneapolis, MN). 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 1000 pg/ml ($R^2 = 0.998$, $P \leq 0.0001$) 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, 2000c; Schnegelsberg et al., 2009).

Euthanasia and Tissue Harvest

Wildtype (WT) and NGE-OE littermate ($n = 5 - 7$ for each; 5–6 weeks of age) mice were deeply anesthetized with isoflurane (3–4%) and then euthanized via thoracotomy. The urinary bladder and lumbosacral DRG were quickly dissected under RNase-free conditions. 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 (Zvarova and Vizzard, 2005; Arms et al., 2009). The urothelium has suburothelial structures associated with it; the term urothelium in this paper refers to both urothelial and suburothelial structures. DRG were identified and isolated as previously described (Vizzard, 1997; Vizzard, 2000a; Vizzard, 2000d). Lumbosacral DRG (L6, S1) were specifically chosen for real-time quantitative reverse transcription-polymerase chain reaction (Q-PCR) analysis based upon the previously determined segmental representation of urinary bladder circuitry (Donovan et al., 1983; Keast and de Groat, 1992; Nadelhaft and Vera, 1995). Bladder afferents are not distributed within the L4–L5 DRG (Donovan et al., 1983; Keast and de Groat, 1992) that contain only somatic afferents nor are neurons that are involved in urinary bladder function observed in the L4–L5 spinal segments (Nadelhaft and Vera, 1995). Harvest of DRG was restricted to L6-S1 because previous studies have demonstrated the presence of PAC1 and VPAC2 receptors in these tissues using non-quantitative reverse-transcription polymerase chain reaction (Braas et al., 2006).

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 to 2 mg of RNA per sample (urinary bladder or DRG) was used to synthesize complementary DNA using SuperScript II reverse transcriptase and a mix of random hexamer and oligo dT primers with the SuperScript II Pre-amplification System (Invitrogen, Carlsbad, CA, USA) in a 20 ml final reaction volume.

The quantitative PCR standards for all transcripts were prepared with the amplified PAC1, VPAC1, VPAC2, PACAP, VIP, NGF and 18S 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, 10-fold serial dilutions of stock plasmids were prepared as quantitative standards. The range of standard concentrations was determined empirically.

Real-time quantitative PCR was performed using SYBR Green I detection (Girard et al., 2002; Klinger et al., 2008; Arms et al., 2009). Complementary DNA templates, diluted 5-fold to minimize the inhibitory effects of the reverse transcription reaction components, were

assayed using SYBR Green I JumpStart™. Taq ReadyMix™ (Sigma, St. Louis, MO, USA) containing 5 mM MgCl₂, 200 mM dATP, dGTP, dCTP and dTTP, 0.64 U Taq DNA polymerase and 300 nM of each primer in a final 25-ml reaction volume. The real-time quantitative PCR was performed on an Applied Biosystems 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) (Girard et al., 2002; Klinger et al., 2008; Arms et al., 2009) using the following standard conditions: (i) serial heating at 50 °C for 2 min and 94 °C for 2 min; (ii) amplification over 40 cycles at 94 °C for 15 s and 60 to 64 °C depending on primers set for 40 s.

The amplified product from these amplification parameters was subjected to SYBR Green I melting analysis by ramping the temperature of the reaction samples from 60°C 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. Oligonucleotide primer sequences for PACAP (Girard et al., 2002), VIP (Girard et al., 2002), PAC1 (Braas and May, 1999), VPAC1 (Girard et al., 2006), VPAC2 (Girard et al., 2006), NGF (Schneegelsberg et al., 2009) and 18S (Girard et al., 2002; Klinger et al., 2008) used in these studies have been previously described.

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 the Sequence Detection Software version 1.3.1 (Applied Biosystems, Norwalk, CT). In standard assays, default baseline settings were selected. The increase in SYBR Green I fluorescence intensity (DR_n) was plotted as a function of cycle number and the threshold cycle was determined by the software as the amplification cycle at which the DR_n 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 housekeeping gene 18S. WT samples are set equal to 100%.

Immunohistochemistry—Bladder cryosections (10 μm) from wildtype (n = 5) and NGF-OE (n = 5) mice were prepared for immunohistochemistry using an on-slide processing technique (Klinger et al., 2008; Klinger and Vizzard, 2008; Arms et al., 2009). For immunohistochemical processing, the cryosections were incubated overnight at room temperature with rabbit anti-PAC₁ (1:2000) (Braas and May, 1999), rabbit anti-PACAP (1:500; Bachem Americas, Inc., Torrance, CA) or rabbit anti-VIP (1:1000; Immunostar, Inc., Hudson, WI) diluted in 0.1M potassium phosphate buffered saline (KPBS) containing 1% goat serum. After washing, the preparations were incubated with a Cy3-conjugated species-specific secondary antibody (1:500) for 2 h at room temperature, rinsed and mounted with antifade medium (Citifluor Ltd., London) for fluorescent microscopy. Methodological and procedural controls were performed including preabsorption of PAC₁, PACAP or VIP antisera with appropriate immunogen (1–3 μg/ml) that reduced staining to background levels.

Visualization and semi-quantitative analysis of PAC₁, PACAP, or VIP in urothelium:

PAC₁, PACAP or VIP staining in bladder sections was visualized and images were captured using an Olympus fluorescence photomicroscope. The filter was set with an excitation range of 560–569 nm and emission range of 610–655 nm for visualization of Cy3. Images were captured, acquired in tiff format and imported into Meta Morph image analysis software (version 4.5r4; University Imaging, Downingtown, PA) (Klinger et al., 2008; Arms et al., 2009). The free hand drawing tool was used to select the urothelium and the urothelium was measured in total pixels area (Klinger et al., 2008; Arms et al., 2009). A threshold encompassing an intensity range of 100–250 grayscale values was applied to the region of interest in the least brightly stained condition first. The threshold was adjusted for each experimental series using concomitantly processed negative controls as a guide for setting background fluorescence. The same threshold was subsequently used for all images. Immunoreactivity was considered to be

positive only when the staining for the marker of interest (PAC1, PACAP, VIP) exceeded the established threshold. Percent marker expression above threshold in the total area selected was calculated.

Visualization and semi-quantitative analysis of PAC1, PACAP or VIP in detrusor smooth muscle:

Visualization of PAC1, PACAP and VIP-IR in detrusor smooth muscle of cryostat sections was identical to that described for the urothelium (above). Semi-quantification of PAC1, PACAP or VIP-IR in detrusor smooth muscle was performed as previously described (Klinger et al., 2007; Cheppudira et al., 2008) and modified from Brady et al. (Brady et al., 2004). Grayscale images acquired in tiff format were imported into Image J (Abramoff et al., 2004) and images were thresholded. Images of detrusor smooth muscle were acquired from the dome, body and neck region of the urinary bladder in WT and NGF-OE mice. A rectangle of fixed dimension (500 × 500 pixels) was placed on the section according to a random selection of x and y coordinates. This process was repeated seven times for each image of detrusor. The average optical density of PAC1, PACAP or VIP-IR in detrusor smooth muscle was then calculated. PAC1, PACAP and VIP-IR in the detrusor exhibited equivocal expression throughout detrusor of the dome, body and neck of the urinary bladder; thus, data from each region are pooled and presented as PAC1, PACAP or VIP-IR in detrusor smooth muscle.

Digital images were obtained using a CCD camera (MagnaFire SP; Optronics; Optical Analysis Corp., Nashua, NH) and LG-3 frame grabber (Scion Corp; Frederick, MD). Exposure times, brightness and contrast were held constant when acquiring images from experimental or control animals processed and analyzed on the same day. Images were imported into Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA) assemblage and labeling.

Statistical Analyses

One-way analysis of variance was used to evaluate differences among groups for Q-PCR. Percentage data from image analysis were arcsin transformed to meet the requirements of this statistical test. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. Differences were considered statistically significant if $p \leq 0.05$. When F ratios exceeded the critical value ($p \leq 0.05$), the Newman-Keul's post-hoc test was used to compare the experimental means.

Results

NGF expression in urothelium, detrusor and lumbosacral DRG of WT and NGF-OE mice

Consistent with our previous studies (Cheppudira et al., 2008; Schnegelsberg et al., 2009), NGF transcript and protein expression was significantly ($p \leq 0.01$) increased in urothelium of NGF OE mice; no changes were observed in detrusor smooth muscle between NGF OE and littermate WT mice (data not shown). Similarly, no changes in NGF transcript were detected in any DRG level examined (L1-S1) between NGF OE and littermate WT mice (data not shown).

PAC1, VPAC1, and VPAC2 receptor transcript expression in urothelium and detrusor of WT and NGF-OE mice

Consistent with previous studies in rats (Braas et al., 2006; Girard et al., 2008), PAC1, VPAC1 and VPAC2 receptor transcripts were expressed in the urothelium and detrusor smooth muscle of mouse urinary bladder (Fig. 1A–C). In urothelium of NGF-OE mice, PAC1 receptor transcript exhibited a significant ($p \leq 0.05$) increase in expression whereas no changes were exhibited in detrusor smooth muscle between wildtype (WT) and NGF-OE mice (Fig. 1A). PAC1 receptor transcript expression was also significantly greater in detrusor smooth muscle compared to urothelium in both WT and NGF-OE mice (Fig. 1A). In urothelium and detrusor

smooth muscle of NGF-OE mice, VPAC1 receptor transcript expression was significantly ($p \leq 0.01$) decreased compared to WT with no changes in receptor transcript expression observed between tissues (urothelium vs. detrusor) (Fig. 1B). In detrusor smooth muscle of NGF OE mice, VPAC2 receptor transcript expression was significantly ($p \leq 0.01$) increased whereas no changes were observed in the urothelium or between detrusor and urothelium in WT and NGF-OE mice (Fig. 1C).

PAC1-Immunoreactivity (IR) in urothelium and detrusor of WT and NGE-OE mice

Changes in PAC1-IR in tissue sections paralleled changes in PAC1 receptor transcript expression. PAC1-IR was significantly increased in urothelium of NGF-OE compared to WT (Fig. 2A–C, F). PAC1-IR was observed in WT urothelium, primarily in apical cells, consistent with previous demonstrations of PAC1-IR in rat urothelium (Braas et al., 2006). In urothelium of NGF-OE mice, PAC1-IR was increased in apical urothelial cells (Fig. 2B–C) and PAC1-IR was also observed in other urothelial layers (intermediate and basal). No changes in PAC1-IR were observed in detrusor smooth muscle between WT and NGF-OE mice (Fig. 2D–F).

PACAP, VIP transcript expression in urothelium and detrusor of WT and NGE-OE mice

PACAP transcript expression significantly ($p \leq 0.05$) decreased in the urothelium of NGF-OE mice compared to WT (Fig. 3A). PACAP transcript expression was significantly ($p \leq 0.05$) greater in detrusor smooth muscle compared to urothelium in both WT and NGF-OE mice (Fig. 3A). No changes in VIP transcript expression were detected in urothelium or detrusor of WT or NGF-OE mice (Fig. 3B). However, VIP transcript expression was significantly ($p \leq 0.01$) greater in detrusor smooth muscle compared to urothelium in both WT and NGF-OE mice (Fig. 3B).

PACAP, VIP-immunoreactivity (IR) in urothelium and detrusor of WT and NGE-OE mice

Changes in PACAP-IR in tissue sections paralleled changes in PACAP receptor transcript expression. PACAP-IR was significantly ($p \leq 0.01$) decreased in urothelium of NGF-OE compared to WT (Fig. 4A–C). PACAP-IR was observed in WT urothelium in all cell layers (apical, intermediate and basal) (Fig. 4A, arrows). Some evidence of PACAP-IR in putative suburothelial nerves was also observed in WT mice (Fig. 4A). In urothelium of NGF-OE mice, PACAP-IR was decreased throughout the urothelium (Fig. 4B). No changes in PACAP-IR were observed in detrusor smooth muscle between WT and NGF-OE mice (Fig. 4C). No changes in VIP-IR were observed in urothelium or detrusor smooth muscle in WT and NGF-OE mice (Fig. 5A–D). In urothelium from WT and NGF-OE mice, VIP-IR was expressed throughout all urothelial cell layers (Fig. 5A, B). VIP-IR in putative suburothelial nerves was observed in urinary bladder sections from both WT and NGF-OE mice (Fig. 5A–C, arrows) consistent with previous studies (Fahrenkrug et al., 1998; Lasanen et al., 1992).

PAC1, VPAC1, and VPAC2 receptor transcript expression in L6-S1 dorsal root ganglia (DRG) of WT and NGE-OE mice

Consistent with previous rat studies (Braas et al., 2006; Girard et al., 2008), PAC1, VPAC1 and VPAC2 receptor transcripts are expressed in mouse lumbosacral (L6-S1) DRG (data not shown). No changes in PAC1, VPAC1 or VPAC2 receptor transcript expression was observed between WT and NGF-OE mice or between L6 and S1 DRG (data not shown)

PACAP and VIP in L6-S1 dorsal root ganglia (DRG) of WT and NGE-OE mice

No changes in PACAP or VIP transcript expression were observed between WT and NGF-OE mice in L6 or S1 DRG (Fig. 6A–B). In S1 DRG, PACAP transcript expression was significantly greater in both WT and NGF-OE mice compared to L6 levels (Fig. 6A). In S1 DRG, VIP transcript expression was significantly greater in WT mice compared to L6 levels (Fig. 6B).

Discussion

We examined PACAP, VIP and associated receptors (PAC1, VPAC1, VPAC2) transcripts or protein expression in urothelium and detrusor smooth muscle and lumbosacral DRG in NGF-overexpressing (OE) and littermate wildtype mice using real-time quantitative reverse transcription-polymerase chain reaction and immunohistochemical approaches. PAC1 receptor transcript and PAC1-immunoreactivity was significantly increased in urothelium of NGF-OE mice whereas PACAP transcript and PACAP-immunoreactivity were decreased in urothelium of NGF-OE mice. In contrast, VPAC1 receptor transcript was decreased in both urothelium and detrusor smooth muscle of NGF-OE mice. VPAC2 receptor transcript was significantly increased in the detrusor smooth muscle in NGF-OE mice. VIP transcript expression and immunostaining was not altered in urinary bladder of NGF-OE mice. Changes in PACAP, VIP and associated receptors transcripts and protein expression in micturition pathways resemble some, but not all, changes observed after induction of urinary bladder inflammation (Girard et al., 2008) known to involve NGF production (Oddiah et al., 1998; Vizzard, 2000c; Bjorling et al., 2001; Dupont et al., 2001; Murray et al., 2004; Guerios et al., 2008; Klinger and Vizzard, 2008).

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 (Okragly et al., 1999; Kim et al., 2005; Kim et al., 2006; Liu and Kuo, 2007; Liu and Kuo, 2008a; Liu and Kuo, 2008b). 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; Schnegelsberg et al., 2009). IC/BPS is a chronic inflammatory bladder disease of unknown etiology characterized by urinary frequency, urgency, and suprapubic/pelvic pain (Driscoll and Teichman, 2001; Sant and Hanno, 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 hyper-responsive to normally innocuous stimuli such as bladder filling (Driscoll and Teichman, 2001; Sant and Hanno, 2001). A few studies have demonstrated increased innervation of the bladder suburothelial and detrusor layers by PGP9.5-positive, substance P-positive or sympathetic nerve fibers in IC/BPS patients (Christmas et al., 1990; Lundeberg et al., 1993; Hoyle et al., 1998; Peeker et al., 2000). In NGF-OE mice, there was a marked increase in the density of CGRP- and substance P-positive C-fiber sensory afferents, neurofilament 200 (NF200) myelinated sensory afferents and tyrosine hydroxylase-positive sympathetic nerve fibers within the suburothelial nerve plexus of the urinary bladder (Schnegelsberg et al., 2009). In this study, we have begun to determine whether chronic NGF-OE in the urothelium causes additional neurochemical changes in expression of PACAP, VIP and associated receptors (PAC1, VPAC1, VPAC2) in the urinary bladder and lumbosacral DRG that might contribute to the bladder hyperreflexia and pelvic hypersensitivity in NGF-OE mice.

Pituitary adenylate cyclase activating polypeptide (PACAP) peptides have diverse functions in the endocrine, nervous, gastrointestinal and cardiovascular systems (Braas and May, 1996; Arimura, 1998) and differential effects on nociception (Sandor et al., 2009) through PAC1, VPAC1 and VPAC2 G protein-coupled receptors. High levels of PACAP and VIP expression have been identified in many central nervous system neurons and in sensory and autonomic ganglia (Arimura et al., 1991; Sundler et al., 1996; Moller et al., 1997a; Moller et al., 1997b; Arimura, 1998; Braas et al., 1998). Both PACAP- and VIP-immunoreactivity have been identified in urinary bladder (Fahrenkrug and Hannibal, 1998; Mohammed et al., 2002). PACAP and VIP peptides regulate smooth muscle function, either directly or by facilitating cholinergic and nitric oxide mechanisms, in a tissue- and species-specific manner (Mizumoto et al., 1992; Onaga et al., 1998; Fox-Threlkeld et al., 1999; Seebeck et al., 2002; Zizzo et al.,

2004). A number of studies have implicated PACAP in lower urinary tract function (Vizzard, 2000d; Zvarova et al., 2005; Braas et al., 2006; Herrera et al., 2006).

In the present study using mice with chronic overexpression of NGF in the urothelium, we have demonstrated increased PAC1 receptor transcript expression and immunostaining in the urothelium. These data are consistent with previous studies demonstrating that NGF increases PAC1 mRNA levels in rat PC12 cells (Jamen et al., 2000; Jamen et al., 2002) and that reduced PAC1 receptor mRNA expression is present in NGF null embryos (Andres et al., 2008). In this study, VPAC1 receptor transcript expression was reduced in both the urothelium and detrusor smooth muscle in NGF-OE mice and no changes in VPAC2 receptor transcript expression were observed in either urothelium or detrusor in NGF-OE mice. PACAP transcript expression and immunostaining was decreased in urothelium of NGF-OE mice whereas VIP transcript expression and immunostaining was not altered in urinary bladder of NGF-OE mice. In contrast, enhanced target-derived NGF availability has been shown to increase PACAP expression in small nociceptive neurons in DRG (Vizzard, 2000d; Jongsma Wallin et al., 2001; Jongsma Wallin et al., 2003). In experimental inflammation paradigms, the expression of several sensory peptides including PACAP is increased while VIP levels, by contrast, are unchanged (Donaldson et al., 1992; Lu et al., 2005). NGF does not increase VIP expression in DRG that may be consistent with the target tissue neurotrophic model to selectively augment PACAP expression in DRG (Mulder and Lindsay, 1990) (V. May and K. Braas, unpublished observations). Although many studies have demonstrated similarities in receptors and channels expressed and neurochemical properties between urothelial cells and DRG cells (Birder, 2005; Birder, 2006; Birder, 2009), the present study demonstrates differences in NGF regulation of PACAP expression between these cell types.

VPAC1 and VPAC2 receptor transcript expression was differentially affected in detrusor smooth muscle of NGF-OE mice. VPAC1 receptor transcript expression was reduced whereas VPAC2 receptor expression was increased in detrusor smooth muscle in NGF-OE mice. Changes in transcript expression in detrusor in mice with chronic overexpression of NGF in the urothelium suggest urothelial-mesenchymal interactions whereby urothelial NGF expression may influence detrusor smooth muscle properties as previously demonstrated for development and differentiation of detrusor smooth muscle (Liu et al., 2000; Baskin et al., 2001). In contrast to observed changes in VIP/PACAP and receptor systems in the urinary bladder of NGF-OE mice, no changes were observed in lumbosacral (L6-S1) DRG of NGF-OE mice. This was not surprising given the absence of increased NGF content in these DRG as demonstrated with ELISAs and suggests absence of retrograde NGF transport from urinary bladder to DRG in these mice.

It is clear from the present studies that chronic overexpression of NGF in the urothelium results in some, but not all of the same changes in VIP, PACAP and receptors transcript or protein expression in micturition pathways demonstrated in rodent models of CYP-induced bladder inflammation (Vizzard, 2000d; Girard et al., 2008). We have previously demonstrated involvement of many chemical mediators (e.g., neurotrophins, cytokines, chemokines, neuropeptides) produced in micturition reflex pathways with cystitis that may underlie changes in neurochemical (Vizzard, 2001; Zvarova et al., 2004; Braas et al., 2006; Klinger and Vizzard, 2008), organizational (Vizzard, 1999; Vizzard, 2000b) and electrophysiological (Yoshimura and de Groat, 1999) properties of micturition pathways and referred somatic sensitivity (Guerios et al., 2006; Guerios et al., 2008). Although changes in PAC1 receptor transcript and protein in NGF-OE mice paralleled changes observed in the rodent model of CYP-induced cystitis, VPAC1, VPAC2 and PACAP transcript and protein expression did not change or changed in an opposite manner compared to the CYP-induced bladder inflammation model. These results suggest that chemical mediators upregulated with CYP-induced bladder inflammation other than or in addition to NGF (e.g., neurotrophins (Vizzard, 2000c), cytokines

(Malley and Vizzard, 2002), chemokines (Yuridullah et al., 2006; Arms et al., 2009), neuropeptides (Vizzard, 2001; Braas et al., 2006) contribute to altered VPAC1, VPAC2 and PACAP transcript and protein expression in micturition pathways following CYP-induced cystitis. Given the increased PAC1 receptor transcript and protein expression in urothelium of NGF-OE mice, current studies are addressing the contribution of PACAP/PAC1 signaling to demonstrated bladder hyperreflexia and referred pelvic hypersensitivity in NGF-OE mice (Schnegelsberg et al., 2009).

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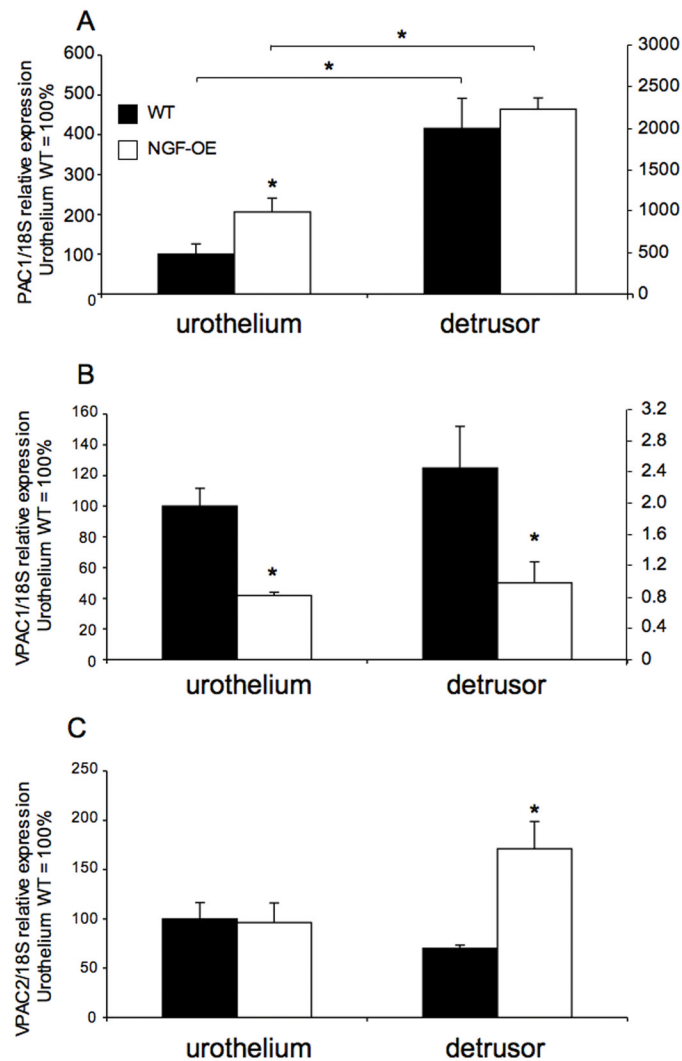


Figure 1. Regulation of PAC1, VPAC1 and VPAC2 receptor transcript levels in littermate wildtype (WT) and NGF overexpressing (NGF-OE) mice in urothelium and detrusor smooth muscle. Relative expression of the urothelium and detrusor receptor transcripts are expressed as a percentage of WT urothelium and normalized to the relative expression of the housekeeping gene, 18S. **A:** PAC1 mRNA expression. **B:** VPAC1 mRNA expression. **C:** VPAC2 mRNA expression. Samples size are n of 5 – 7; *, $p \leq 0.01$ versus control.

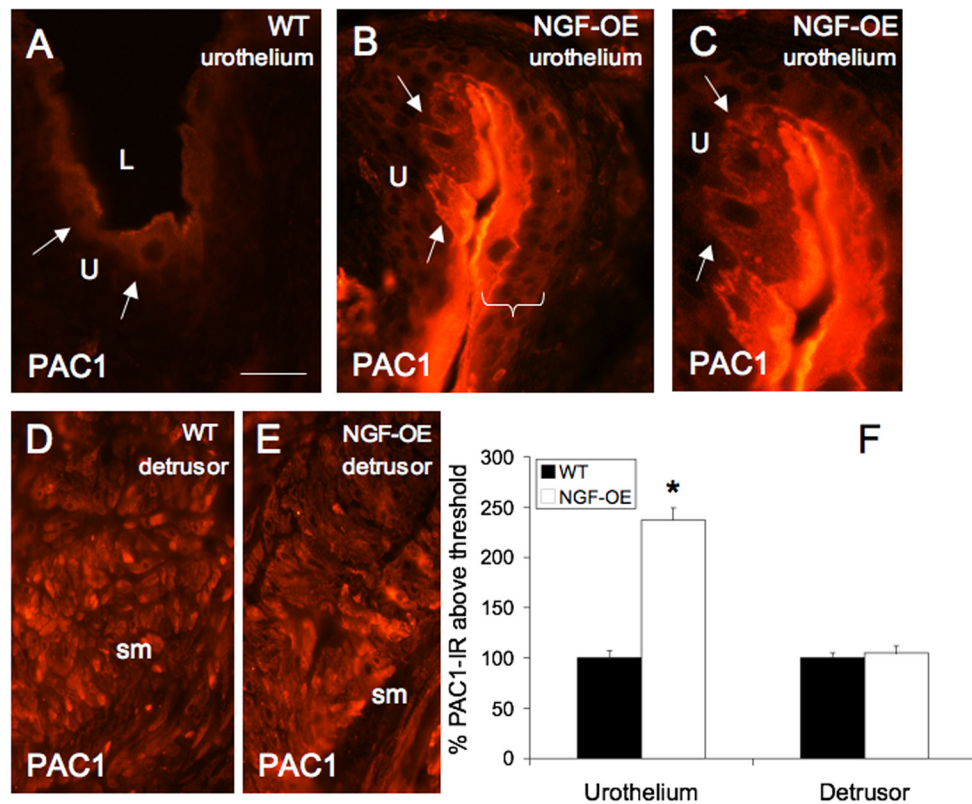


Figure 2.

PAC1-immunoreactivity in urothelium (U) (A–C) and detrusor smooth muscle (sm) (D–E) of littermate wildtype (WT) and NGF overexpressing (NGF-OE) mice. Faint PAC1-IR was present in apical urothelial cells of WT urothelium (A, arrows). In NGF-OE mice, PAC1-IR was increased in apical urothelial cells (B–C, arrows) but was also expressed in all urothelial cell layers (B, bracket). PAC1-IR was expressed in detrusor smooth muscle of WT and NGF-OE mice (D–E). F. Summary histogram of PAC1 expression in the U and detrusor sm in WT and NGF-OE mice. Values are mean \pm SEM; (n = 5). *, $p \leq 0.01$. L, lumen. Calibration bar equals 50 μ m.

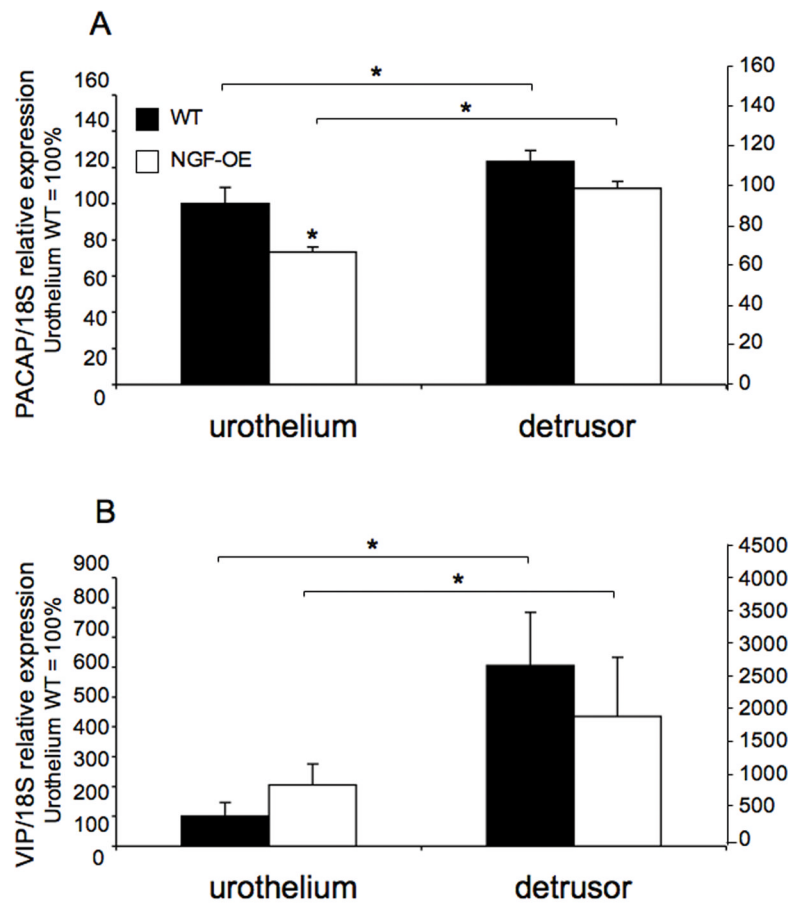


Figure 3. Regulation of PACAP and VIP transcript levels in littermate wildtype (WT) and NGF overexpressing (NGF-OE) mice in urothelium and detrusor smooth muscle. Relative expression of the urothelium and detrusor receptor transcripts are expressed as a percentage of WT urothelium and normalized to the relative expression of the housekeeping gene, 18S. **A:** PACAP mRNA expression. **B:** VIP mRNA expression. Samples size are n of 5 – 7; *, $p \leq 0.01$ versus control.

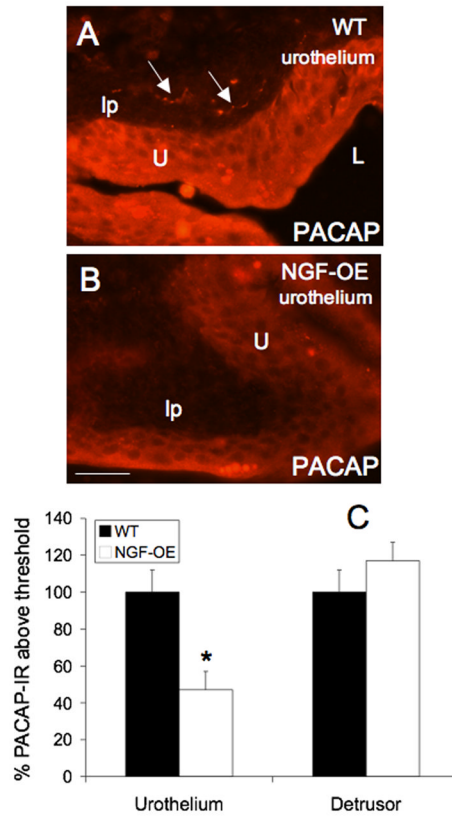


Figure 4.

PACAP-immunoreactivity in urothelium (U) (A–C) and detrusor smooth muscle (sm) (C) of littermate wildtype (WT) and NGF overexpressing (NGF-OE) mice. Robust PACAP-IR was present throughout the WT urothelium (A). Some evidence of PACAP-IR in presumptive suburothelial nerve fibers was observed (A, arrows). In NGF-OE mice, PACAP-IR was decreased in all urothelial cell layers (B). C. Summary histogram of PACAP expression in the U and detrusor sm in WT and NGF-OE mice. No changes in PACAP-IR were observed in WT or NGF-OE sm. Values are mean \pm SEM; (n = 5). *, $p \leq 0.01$. L, lumen; lp, lamina propria. Calibration bar equals 50 μ m.

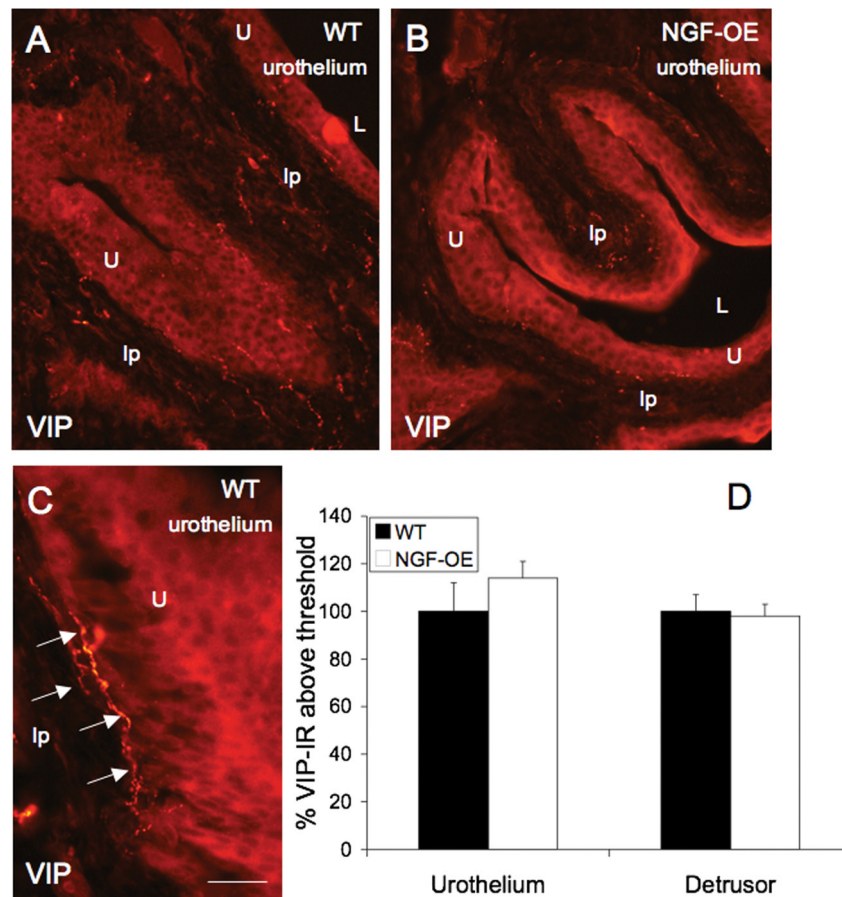


Figure 5. VIP-immunoreactivity in urothelium (U) (A–C) and detrusor smooth muscle (sm) (C) of littermate wildtype (WT) and NGF overexpressing (NGF-OE) mice. VIP-IR was present throughout the WT urothelium (A, C) and NGF-OE (B) urothelium. VIP-IR in presumptive suburothelial nerve fibers was observed in WT and NGF-OE urinary bladder (C, arrows). C. Summary histogram of VIP expression in the U and detrusor sm in WT and NGF-OE mice. No changes in VIP-IR were observed in WT or NGF-OE U or sm. Values are mean \pm SEM; (n = 5). *, $p \leq 0.01$. L, lumen; Ip, lamina propria. Calibration bar equals 100 μ m in A, B and 30 μ m in C.

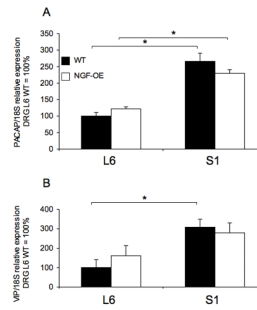


Figure 6. Regulation of PACAP and VIP mRNA expression in lumbar (L6-S1) dorsal root ganglia (DRG) in littermate wildtype (WT) and NGF overexpressing (NGF-OE) mice. Relative expression of the L6 and S1 receptor transcripts are expressed as a percentage of WT L6 DRG and normalized to the relative expression of the housekeeping gene, 18S. **A:** PACAP mRNA expression. **B:** VIP mRNA expression. Samples size are no 5 to 7; *, $p \leq 0.01$ versus control.