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## Accelerated onset of the vesicovesical reflex in postnatal NGF-OE mice and the role of neuropeptides

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### Abstract

The mechanisms underlying the postnatal maturation of micturition from a somatovesical to a vesicovesical reflex are not known but may involve neuropeptides in the lower urinary tract. A transgenic mouse model with chronic urothelial overexpression (OE) of NGF exhibited increased voiding frequency, increased number of non-voiding contractions, altered morphology and hyperinnervation of the urinary bladder by peptidergic (e.g., Sub P and CGRP) nerve fibers in the adult. In early postnatal and adult NGF-OE mice we have now examined: (1) micturition onset using filter paper void assays and open-outlet, continuous fill, conscious cystometry; (2) innervation and neurochemical coding of the suburothelial plexus of the urinary bladder using immunohistochemistry and semi-quantitative image analyses; (3) neuropeptide protein and transcript expression in urinary bladder of postnatal and adult NGF-OE mice using Q-PCR and ELISAs and (4) the effects of intravesical instillation of a neurokinin (NK)-1 receptor antagonist on bladder function in postnatal and adult NGF-OE mice using conscious cystometry. Postnatal NGF-OE mice exhibit age-dependent ( $R^2=0.996-0.998$ ;  $p < 0.01$ ) increases in Sub and CGRP expression in the urothelium and significantly ( $p < 0.01$ ) increased peptidergic hyperinnervation of the suburothelial nerve plexus. By as early as P7, NGF-OE mice exhibit a vesicovesical reflex in response to intravesical instillation of saline whereas littermate WT mice require perigenital stimulation to elicit a micturition reflex until P13 when vesicovesical reflexes are first observed. Intravesical instillation of a NK-1 receptor antagonist, netupitant (0.1  $\mu\text{g/ml}$ ), significantly ( $p < 0.01$ ) increased void volume and the interval between micturition events with no effects on bladder pressure (baseline, threshold, peak) in postnatal NGF-OE mice; effects on WT mice were few. NGF-induced pleiotropic effects on neuropeptide (e.g., Sub P) expression in the urinary bladder contribute to the maturation of the micturition reflex and are excitatory to the micturition reflex in postnatal NGF-OE mice. These studies provide insight into the mechanisms that contribute to the postnatal development of the micturition reflex.

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#### Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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## Keywords

postnatal development; Substance P; neurokinin-1 receptor antagonist; suburothelial nerve plexus

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## Introduction

In neonates of many species, the neural control of micturition undergoes marked changes during early postnatal development (de Groat, 1975, 1990, 1993; de Groat and Araki, 1999; de Groat and Ryall, 1969; Maggi et al., 1986). 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) (de Groat and Araki, 1999; Maggi et al., 1986). This reflex pathway consists of a somatic afferent limb in the pudendal nerve and a parasympathetic efferent limb in the pelvic nerve. Similar reflexes have been identified in human infants (Boehm and Haynes, 1966). As the CNS matures, a spinobulbospinal reflex pathway that is responsible for micturition in adult animals gradually replaces the spinal micturition reflex (de Groat, 1975, 1990, 1993; de Groat and Araki, 1999; de Groat and Ryall, 1969; Maggi et al., 1986). The spinobulbospinal micturition reflex is triggered by mechanoreceptors in the bladder and begins to elicit voiding in the rat between postnatal days (P) 16–18 (Capek and Jelinek, 1956; Maggi et al., 1986; Ng et al., 2007; Zvarova and Zvara, 2012) and continues to mature during postnatal weeks 3–6 (Capek and Jelinek, 1956; Ng et al., 2007). Studies demonstrate (Sugaya et al., 1997) that supraspinal pathways involved in adult micturition reflex patterns exist in neonatal animals but may not be functional or may function in an inhibitory manner prior to the emergence of a functional, adult micturition pattern (de Groat et al., 1998). Studies in human infants also support an anatomical connection to the cerebral cortex in advance of conscious or voluntary voiding (Sillen, 2001).

The mechanisms underlying the postnatal maturation of micturition are not known but it has been suggested that maturation of voiding function involves prominent reorganization of synaptic connections in bladder reflex pathways. This reorganization leads to down regulation of primitive spinal mechanisms and upregulation of mature supraspinal pathways (de Groat et al., 1998; de Groat and Araki, 1999). Previous studies have suggested the importance of neuroactive compounds in the process of maturation of the micturition reflexes during prenatal and early postnatal development (Ekstrom et al., 1994; Iuchi et al., 1994; Maggi et al., 1986; Sann et al., 1997). Neurochemical plasticity in central and peripheral micturition reflex pathways has been demonstrated with neural injury, disease, inflammation and during postnatal development (Arms and Vizzard, 2011; Corrow et al., 2010; de Groat et al., 1998; de Groat and Yoshimura, 2012; Merrill et al., 2013; Merrill et al., 2012; Merrill and Vizzard, 2014). For example, we have demonstrated neurochemical plasticity in neuropeptides (e.g., Sub P, CGRP, PACAP) after bladder inflammation in bladder afferent cells in DRG (Braas et al., 2006; Vizzard, 2000d, 2001) and in spinal segments involved in micturition reflexes (Vizzard, 2000a, c, d). Bladder inflammation and postnatal maturation also altered expression of neurotrophic factors (NTF), including NGF, in the urinary bladder (Vizzard, 2000b). NGF expression in the urinary bladder may directly affect urinary bladder maturation, urinary bladder function and micturition reflex pathways;

however, pleiotropic changes resulting from increased NGF expression may also play a role (Girard et al., 2010; Girard et al., 2011; Zvara and Vizzard, 2007). For example, numerous studies have demonstrated the NGF-dependence of neuropeptide expression (e.g., Sub P and CGRP) in sensory neurons (Dmitrieva and McMahon, 1996; McMahon, 1996; Ribeiro-da-Silva et al., 2000; Zvara and Vizzard, 2007).

We recently characterized a transgenic mouse model of chronic, urothelium-specific NGF-overexpression that represents a novel approach to exploring the role of NGF in urinary bladder inflammation and sensory function (Schnegelsberg et al., 2010). Functionally, NGF-OE mice exhibit frequent voiding, the presence of non-voiding contractions (NVCs) during the bladder filling phase and referred somatic pelvic hypersensitivity (Schnegelsberg et al., 2010). In addition, NGF-OE mice exhibit changes in the morphology and neurochemistry of the urinary bladder and increases in inflammatory cell infiltrates to the urinary bladder (Schnegelsberg et al., 2010). Neurochemical changes include increased expression of Sub P and CGRP in the suburothelial nerve plexus of the urinary bladder (Schnegelsberg et al., 2010). Our findings support and extend many previous studies in rodents demonstrating involvement of NGF in altered bladder sensory function and the development of referred hyperalgesia in response to bladder inflammation (Dmitrieva and McMahon, 1996; Guerios et al., 2006; 2008; Hu et al., 2005; Jaggar et al., 1999; Lamb et al., 2004; Zvara and Vizzard, 2007). We now expand upon these findings and begin to identify mechanisms that drive development of bladder innervation and function. We investigated the contributions of NGF and the NGF-dependence of neuropeptide expression in the developing bladder to the maturation of voiding reflexes.

## Materials and Methods

### Animals

**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 (Schnegelsberg et al., 2010) (Girard et al., 2011) (Girard et al., 2010). Animal genotype was confirmed by Southern and/or PCR analyses; all mice have the inbred genetic C57BL/6J background and were derived from F10 to F12 generations maintained through a hemizygous backcross strategy with C57BL/6J wildtype (WT) 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. Female and male mice of varying postnatal (P) age (P0-Adult (A)) from multiple dams were used to examine postnatal maturation of voiding reflexes. In these studies, P0 is defined as day of birth. As previously demonstrated (Girard et al., 2010; 2011; Schnegelsberg et al., 2010) and confirmed in this study, urinary bladder weight was significantly ( $p < 0.01$ ) increased in NGF-OE mice compared to littermate WT mice (Table 1). Wistar rats (Charles River Canada, St. Constant, Quebec) of both sexes and various postnatal ages (P0-A) were used to determine urination onset and to serve as a comparison for WT and NGF-OE mice. 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. Separate groups of littermate WT and NGF-OE mice were used in the following experiments. Both male and female NGF-OE and WT mice and Wistar rats were used in these studies.

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

Determination of NGF, Sub P, and CGRP transcript expression in the urinary bladder (urothelium, detrusor) of NGF-OE transgenic mice (n=6–8 for each age) and littermate WT mice (n=6–8 for each age) was determined using Q-PCR as previously described (Girard et al., 2010; 2011; Schnegelsberg et al., 2010). 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., 2010; 2011; Schnegelsberg et al., 2010). One µg 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, 10-fold serial dilutions of stock plasmids were prepared as quantitative standards. The range of standard concentrations was determined empirically. Complementary DNA templates, diluted 10-fold to minimize the inhibitory effects of the reverse transcription reaction components, were assayed using HotStart-IT SYBR Green qPCR Master Mix (USB, Cleveland, OH, USA) and 300 nM of each primer in a final 25 µl reaction volume.

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) 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 60–64°C depending on primers set for 30 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 to 95 °C. A single DNA melting profile was observed under these dissociation assay conditions demonstrating the amplification of a single unique product free of primer dimers or other anomalous products. Oligonucleotide primer sequences were: mouse NGF: forward primer (5'-AGTGAGGTGCATAGCGTAAT-3'); reverse primer (5'-CACATTGGTGGGAACAAA-3'); mouse CGRP: forward primer (5'-ATCCTGCAACACTGCCA -3'); reverse primer (5'-CACATTGGTGGGAACAAA-3'); Substance P (Sub P): forward primer (5'-CGGTGCCAACGATGATCTAAA -3'); reverse primer (5'-ACGCCTTCTTTCGTACTIONTCTG -3'). L32 primer sequences have been previously reported (Klinger et al., 2008).

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 ( $R_n$ ) was plotted as a function of cycle number and the threshold cycle was determined by the software as the amplification cycle at which the  $R_n$  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 L32.

### **Split bladder preparation and assessment of potential contamination of bladder layers**

The urothelium + suburothelium was dissected from the detrusor smooth muscle using fine forceps under a dissecting microscope as previously described (Corrow et al., 2010; Schnegelsberg et al., 2010). To confirm the specificity of our split bladder preparations, urothelium + suburothelium and detrusor samples were examined for the presence of  $\alpha$ -smooth muscle actin (1:1000; Abcam, Cambridge, MA) and uroplakin II (1:25; American Research Products, Belmont, MA) by western blotting or reverse transcription PCR (Corrow et al., 2010; Girard et al., 2011; Girard et al., 2013). In urothelium + suburothelium layers, only uroplakin II was present (data not shown). Conversely, in detrusor samples, only  $\alpha$ -smooth muscle actin was present (data not shown). In these studies, the use of the term urothelium refers to the urothelium and suburothelial layers.

### **Measurement of urinary bladder NGF, Sub P and CGRP by ELISAs**

Determination of NGF, Sub P, and CGRP protein content in the urinary bladder of NGF-OE transgenic mice and WT littermate controls was determined using enzyme-linked immunoassays (ELISAs) as previously described (Gonzalez et al., 2015; Schnegelsberg et al., 2010; Vizzard, 2000b). Individual mouse bladders were dissected, weighed, and placed in Tissue Protein Extraction Reagent (1 g tissue/20 ml; Pierce Biotechnology, Woburn, MA) with Complete protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany), and stored at  $-80^{\circ}\text{C}$ . On the day of assay, individual bladders were disrupted with a Polytron homogenizer until homogeneous and centrifuged (10,000 rpm for 10 min) (Gonzalez et al., 2015; Schnegelsberg et al., 2010; Vizzard, 2000b), and the supernatant was used for total protein estimation and CGRP and Sub P quantification. Total protein was determined by the Coomassie Plus Protein Assay Reagent Kit (Pierce) (Gonzalez et al., 2015), and NGF, CGRP and SP were quantified using standard 96-well ELISA plates (Phoenix Pharmaceuticals, Burlingame, CA) according to the manufacturer's recommendations (Gonzalez et al., 2015). Microtiter plates (R & D Systems, Minneapolis, MN) were coated with an anti-rat NGF antibody, anti-rat CGRP or anti-rat Sub P antibody. 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 ( $R^2 = 0.998$ ,  $p < 0.0001$ ) for tissue samples. The CGRP standard provided with this protocol generated a linear standard curve from 0 to 100 ng/ml ( $R^2 = 0.996$ ,  $p < 0.0001$ ) for bladder samples (Gonzalez et al., 2015). The Sub P standard provided with this protocol generated a linear standard curve from 0 to 25 ng/ml ( $R^2 = 0.997$ ,  $p < 0.0001$ ) for bladder samples (Gonzalez et al., 2015). 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, Sub P and CGRP content of samples was performed using a least squares fit as previously described (Gonzalez et al., 2015; Schnegelsberg et al., 2010; Vizzard, 2000b).

### **Urinary bladder preparation, immunohistochemistry and image analysis**

The urinary bladder from littermate WT (n = 6–8) and NGF-OE mice at specified postnatal ages (P7-A) (n = 6–8 each) was dissected and placed in Krebs solution. The bladder was cut open along the midline and pinned to a Sylgard-coated dish. Notches were made on one side of the bladder neck for orientation purposes. The bladder was incubated for 1.5 h at room temperature in cold fixative (2% paraformaldehyde + 0.2% picric acid). Fine-tipped forceps and iris scissors were used to dissect the urothelium + suburothelium from the underlying detrusor smooth muscle with the aid of a dissecting microscope (Schnegelsberg et al., 2010). The tissue was blocked with PBS (pH 7.4) containing 20% normal goat serum, 0.2% Triton X-100 for 2 h at room temperature and the primary antibody was applied in PBS containing 4% normal goat serum, 0.2% Triton X-100 overnight at 4° C (Arms et al., 2013; Arms et al., 2010; Schnegelsberg et al., 2010). The following primary antibodies were used: a rabbit protein gene product (PGP) 9.5 antibody (1:6,000) (Ultraclone, UK), a rabbit calcitonin gene-related peptide (CGRP) antibody (1:500) (Sigma-Aldrich, St. Louis, MO), a rabbit substance P (Sub P) antibody (1:500)(Phoenix Pharmaceuticals, Inc., Burlingame, CA), a rabbit tyrosine hydroxylase (TH) antibody (1:300) (Chemicon, Temecula, CA), and a rabbit neurofilament 200 (NF200) antibody (1:150) (Chemicon). The tissue was washed in PBS (pH 7.4) containing 0.1% BSA, 0.1% Triton-X-100, 4 X for 15 min each at room temperature. The secondary antibody used for all whole-mount staining was a Cyanine Cy3 conjugated goat anti-rabbit antibody (1:500) (Jackson ImmunoResearch, West Grove, PA) incubated for 2 h at room temperature in the same solution as the primary antibody. The tissue was washed 3 X for 10 min each in PBS and mounted with mounting medium (Polysciences, Warrington, PA).

### **Assessment of immunohistochemical staining in suburothelial nerve plexus**

Immunohistochemistry and subsequent semi-quantification of neurochemical-IR in whole-mount preparations were performed on littermate WT and NGF-OE tissues simultaneously to reduce the incidence of staining variation that can occur between tissues processed on different days. Staining in experimental tissue was compared with that in experiment-matched negative controls. Whole mount preparations exhibiting immunoreactivity that was greater than the background level in experiment-matched negative controls were considered positively stained.

### **Visualization and semi-quantitative analyses of neurochemistry of suburothelial nerve plexus**

Whole mounts from littermate WT (n = 6–8) and NGF-OE of various selected postnatal ages (P7-A)(n = 6–8 each) groups were examined under an Olympus fluorescence photomicroscope as described above. Cy3 was visualized with a filter with an excitation range of 560–596 nm and an emission range of 610–655 nm. Semi-quantification of



neurochemical expression in the suburothelial nerve plexus was performed as previously described (Arms et al., 2013; Arms et al., 2010). Grayscale images acquired in tiff format were imported into MetaMorph image analysis software (version 4.5r4; Universal Imaging, Downingtown, PA) as previously described (Corrow et al., 2010; Corrow and Vizzard, 2007; Gonzalez et al., 2013) and images were thresholded. Images were acquired from the neck region of WT and NGF-OE mice for suburothelial nerve plexus analyses. 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 the suburothelial nerve plexus. The average optical density of neurochemical immunoreactivity in the suburothelial nerve plexus was then calculated. Immunoreactivity for all neurochemicals examined (PGP9.5, CGRP, Sub P, NF-200 and TH) in the suburothelial plexus was greatest in the bladder neck region. Semi-quantification of neurochemical immunoreactivity in the suburothelial plexus was determined in the bladder neck region for all WT and NGF-OE mice.

### Figure preparation

Digital images were obtained using a charge-coupled device camera (MagnaFire SP, Optronics, Optical Analysis, Nashua, NH) and LG-3 frame grabber attached to an Olympus microscope (Optical Analysis). Exposure times were held constant when images were acquired from WT and NGF-OE mice processed and analyzed on the same day. Images were imported into Photoshop 7.0 (Adobe Systems, San Jose, CA), where groups of images were assembled and labeled.

### Conscious, open outlet, continuous fill cystometry

Rodents (mice and rats) were anesthetized with isoflurane (3–4%), a lower midline abdominal incision was made, and polyethylene tubing (PE-10, Clay Adams, Parsippany, New Jersey) was inserted into the bladder dome and secured with a nylon purse-string sutures (6-zero) (Gonzalez et al., 2013; Schnegelsberg et al., 2010). 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 (Gonzalez et al., 2013; Schnegelsberg et al., 2010). The distal end of the tubing was sealed, tunneled subcutaneously and externalized at the back of the neck out of reach (Gonzalez et al., 2013; Schnegelsberg et al., 2010). Abdominal and neck incisions were closed with nylon sutures (6-zero). Rodents (>P21) that had been weaned received postoperative analgesics (buprenorphine, 0.5 mg/kg, s.c.) and were maintained for 72 h after survival surgery to ensure recovery. Rodents (<P21) that had not been weaned received a single preoperative dose of analgesic (buprenorphine, 0.5 mg/kg, s.c.) and were used in conscious cystometry studies on the same day after a 2 h recovery period. Attempts to return rodents (<P21) postoperatively to the home cage with the dam and litter were not successful in pilot studies. Some rodents (>P21) were also evaluated with conscious cystometry on the same day as bladder catheter implantation and no significant differences in the cystometric parameters measured (see below) were observed; data were pooled in analyses.

For cystometry in conscious rodents (mice and rats), 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, St. Albans, VT) was used for urodynamic measurements (Gonzalez et al., 2013; Schnegelsberg et al., 2010). Saline solution was infused at room temperature into the bladder at a rate of 25  $\mu$ l/min (mice) or 10 ml/hr (rats) to elicit repetitive bladder contractions. At least six reproducible micturition cycles were recorded after the initial stabilization period of 25–30 min (Gonzalez et al., 2013; Schnegelsberg et al., 2010). In rodents that did not exhibit a vesicovesical reflex, voiding was elicited by perigenital stimulation to elicit a somatovesical reflex. Manual bladder emptying was accomplished by rapidly stroking the perigenital area for 60 s with a cotton swab. The following cystometric parameters were recorded in each animal: baseline pressure (BP; pressure at the beginning of the bladder filling), threshold pressure (TP; bladder pressure immediately prior to micturition), peak micturition pressure (MP), intercontraction interval (ICI; time between micturition events), bladder capacity (BC), void volume (VV), presence and amplitude of non-voiding bladder contractions (NVCs) (Gonzalez et al., 2013; Schnegelsberg et al., 2010). NVCs were defined as rhythmic intravesical pressure increases 5 cm H<sub>2</sub>O above baseline, during the filling phase, without the release of fluid from the urethra. The presence of NVCs was determined in a micturition cycle, which was defined as the duration of time that includes bladder filling, at a specified rate, resulting in a voiding reflex. Bladder capacity (BC) was measured as the volume of saline infused into the bladder at the time when micturition commenced (Gonzalez et al., 2013; Schnegelsberg et al., 2010). Mice and rats in these studies had residual volume of less than 5  $\mu$ l; therefore, VV and BC were similar. At the conclusion of the experiment, the rodent was euthanized (5% isoflurane plus thoracotomy).

### **Conscious cystometry and effects of netupitant, a neurokinin (NK)-1 receptor antagonist**

The effects of netupitant, a highly potent and selective neurokinin (NK)-1 receptor antagonist (Greenwood-Van Meerveld et al., 2014; Haab et al., 2014; Zhang et al., 2012), on urinary bladder function in littermate WT and NGF-OE mice of specified postnatal ages (P7-A; n = 6–8) were assessed using conscious, open outlet, cystometry with continuous instillation of intravesical saline (Gonzalez et al., 2013; Schnegelsberg et al., 2010). For intravesical administration of netupitant, mice were anesthetized with 2% isoflurane and netupitant (<1.0 ml) was injected through the bladder catheter; the animals were maintained under anesthesia to prevent expulsion of netupitant via a voiding reflex. In this procedure, netupitant remained in the bladder for 30 min at which time, the drug was drained, the bladder washed with saline and animals recovered from anesthesia for 20 min before experimentation. The effectiveness of intravesical netupitant (Med Chem Express (MCE), Monmouth Junction, NJ; 0.1  $\mu$ g/ml) administration was evaluated in littermate WT and NGF-OE mice from various postnatal ages (P7-A). These experiments were performed in the same mice before and after treatment with netupitant. The concentration (0.1  $\mu$ g/ml) of netupitant used in these studies was based upon previous studies (Greenwood-Van Meerveld et al., 2014) and pilot studies. WT and NGF-OE mice receiving intravesical administration of vehicle (0.9% saline) (n = 6–8) were also evaluated. To summarize, the experimental design involves administration of a one time, intravesical infusion of netupitant (0.1  $\mu$ g/ml) with cystometric data collection occurring ~75 min after infusion. At the conclusion of the



experiment, the animal was euthanized (4% isoflurane plus thoracotomy). Experiments were conducted at similar times of the day to avoid the possibility that circadian variations were responsible for changes in bladder capacity measurements. An individual blinded to genotype analyzed the cystometric data; groups were decoded after data analysis (Gonzalez et al., 2013; Schnegelsberg et al., 2010).

### 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 pre- and/or postoperative analgesics (Gonzalez et al., 2013; Schnegelsberg et al., 2010). In the present study, no mice or rats were excluded from the study. In addition, behavioral movements such as grooming, standing, walking, and defecation rendered bladder pressure recordings during these events unusable.

### Urination Patterns

As previously described, the spinobulbospinal micturition reflex is triggered by mechanoreceptors in the bladder and begins to elicit voiding in the rat between P16–P18 (Capek and Jelinek, 1956). To determine onset of the micturition reflex in NGF-OE mice and littermate WT mice, male and female mice of both genotypes and of varying postnatal ages (P3-A) were separated from the dam (if litters were pre-weaning) and placed individually in cages lined with Whatman Grade 3 filter paper for a period of 2 hours (h) under continuous monitoring (Studený et al., 2008). Mice were habituated to cages for 1 h in the morning (9–11 am) on each of two consecutive days prior to data accumulation (Studený et al., 2008). As an additional comparison, the onset of the micturition reflex in individual postnatal (P3-A) Wistar rats of both sexes was also determined in the same manner. After 2 h, mice (NGF-OE and WT) or rats were returned to the litter (if pre-weaning) and the filter paper was examined under UV light to determine presence and size of urine spots. Urine spots were photographed under UV light (Studený et al., 2008), area (cm<sup>2</sup>) of spots determined with large (0.2 – 10 cm<sup>2</sup>) (Birder et al., 2002) and small (< 0.2 cm<sup>2</sup>) (Birder et al., 2002) spots being counted. An individual blinded to genotype analyzed these data were analyzed; groups were decoded after data analysis.

### Statistical Analyses

Statistical analyses did not demonstrate differences between male and female mice of either genotype. Thus, mouse data were pooled for statistical analyses of immunoassays, Q-PCR, immunohistochemical analyses and cystometry. All values are means  $\pm$  S.E.M. Linear regression analyses were performed to determine the relationship between postnatal age and NGF, Sub P or CGRP mRNA or protein expression in urinary bladder. Comparisons among experimental groups were made using ANOVA. Percentage data from image analysis were arcsin transformed to meet the requirements of the statistical test. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. When *F* ratios exceeded the critical value ( $p < 0.05$ ), the Newman-Keul's post hoc test was used to compare experimental means among groups.

## Results

### Bladder weight increases in NGF-OE postnatal mice

NGF-OE transgenic mice developed normally with no adverse clinical signs or altered behaviors. NGF-OE mice of both sexes had similar body weight to age-matched (P0-Adult, A), littermate WT control mice. Male and female NGF-OE mice (P0-A) exhibited a significant ( $p < 0.01$ ) increase in urinary bladder weight and a 2.9- to 3.2-fold increase in bladder-to-body weight ratios compared with age-matched, sex-matched, littermate WT mice (P0-A) (Table 1).

### NGF transcript and protein expression is increased in urothelium of postnatal WT and NGF-OE mice with no changes in detrusor

Consistent with our previous studies (Cheppudira et al., 2008; Schnegelsberg et al., 2010), NGF transcript and protein expression were significantly ( $p < 0.01$ ) increased in urothelium of NGF-OE mice at all postnatal (P) ages examined (P0, P7, P14, P21, P28, P36 and Adult (A))(Fig. 1A, Fig. 2A) compared to age-matched littermate WT mice. NGF transcript and protein expression in urothelium of NGF-OE mice was correlated with increasing postnatal age ( $R^2=0.978-0.989$ ;  $p < 0.001$ )(Fig. 1A, Fig. 2A). In contrast, no changes were observed in NGF transcript and protein expression in detrusor smooth muscle between postnatal NGF-OE and littermate WT mice (Fig. 1B, Fig. 2B). No correlation with postnatal age was demonstrated for NGF transcript and protein expression in detrusor smooth muscle of postnatal NGF-OE or WT mice (Fig. 1B, Fig. 2B).

### Substance P (Sub P) transcript and protein expression in urothelium and detrusor of postnatal WT and NGF-OE mice

Sub P transcript and protein expression were significantly ( $p < 0.01$ ) increased in urothelium of NGF-OE mice compared to age-matched WT mice at all postnatal ages examined (P7, P14, P21, P28, P36, A)(Fig. 3A, Fig. 5A). Sub P transcript expression was significantly ( $p < 0.01$ ) greater at P7, P28, P36 and A compared to P14 in urothelium of NGF-OE mice (Fig. 3A). Sub P transcript expression was significantly ( $p < 0.01$ ) greater in detrusor of NGF-OE mice compared to age-matched WT mice at each postnatal age examined (Fig. 3B). Sub P protein expression in urothelium of NGF-OE mice was correlated with increasing postnatal age ( $R^2=0.977$ ;  $p < 0.001$ )(Fig. 5A). Sub P protein expression in the urothelium of NGF-OE mice was significantly ( $p < 0.01$ ) greater at P36 and A compared to other postnatal ages (P7, P14, P21, P28)(Fig. 5A). Sub P protein expression in urothelium of WT mice was also correlated with increasing postnatal age ( $R^2=0.982$ ;  $p < 0.001$ ) (Fig. 5A). Although Sub P transcript was significantly increased in the detrusor of NGF-OE mice compared to WT mice at all postnatal ages examined (P7, P14, P28, P36, A)(Fig. 3B), no changes were observed in Sub P protein expression in detrusor smooth muscle (Fig. 5B) between NGF-OE mice and WT mice. Sub P protein expression in the detrusor of NGF-OE and littermate WT mice was correlated ( $R^2=0.979$ ;  $p < 0.001$ ) with increasing postnatal age (Fig. 5B). Sub P protein expression in the detrusor of NGF-OE and WT mice was greatest at P28, P36 and A (Fig. 5B).

### Calcitonin gene-related peptide (CGRP) transcript and protein expression in urothelium and detrusor of postnatal WT and NGF-OE mice

CGRP transcript expression was significantly ( $p < 0.01$ ) decreased in urothelium of WT mice compared to age-matched NGF-OE mice at all postnatal ages examined (P7, P14, P28, P36, A)(Fig. 4). CGRP transcript expression was significantly ( $p < 0.01$ ) greater in urothelium of P7–P28 WT mice compared to A WT mice (Fig. 4). CGRP transcript expression in urothelium of WT mice was negatively correlated with increasing postnatal age ( $R^2=0.987$ ;  $p < 0.001$ )(Fig. 4). No changes in CGRP transcript expression in detrusor of NGF-OE mice were observed during postnatal development at the ages examined (P7, P14, P28, P36, A) (data not shown). CGRP protein expression in the urothelium of NGF-OE mice was significantly ( $p < 0.01$ ) greater at P36 and A compared to NGF-OE mice of other postnatal ages (P7, P14, P21, P28)(Fig. 6A). CGRP protein expression in urothelium of NGF-OE mice was significantly ( $p < 0.01$ ) greater than that observed in age-matched WT mice at every age examined (Fig. 6A). CGRP protein expression in urothelium of WT mice and NGF-OE mice was correlated with increasing postnatal age ( $R^2=0.976-0.988$ ;  $p < 0.001$ )(Fig. 6A). CGRP protein content was significantly ( $p < 0.01$ ) greater in urothelium of NGF-OE mice at each age examined (P7, P14, P21, P28, P36, A)(Fig. 6A) compared to age-matched WT mice. No differences in CGRP protein content were demonstrated in detrusor smooth muscle between age-matched NGF-OE and WT mice at any postnatal age examined (Fig. 6B). CGRP protein content in detrusor of NGF-OE and WT mice was correlated with increasing postnatal age ( $R^2=0.985-0.992$ ;  $p < 0.001$ )(Fig. 6B).

### Sensory and sympathetic hyperinnervation in the urinary bladders of NGF-OE transgenic mice

We characterized the subpopulations of neurons contributing to the generalized hyperinnervation observed in urinary bladder of postnatal NGF-OE transgenic mice at postnatal ages (P7, P14, P21, P28)(Figs. 7–9) using whole-mount immunostaining of bladder urothelium using a panel of neuronal markers. Using the pan-neuronal marker, protein gene product, PGP9.5, a significant ( $p < 0.01$ ) increase in total nerve fiber density was seen in the urinary bladders of NGF-OE compared to littermate WT mice (Figs. 7, 8, 9A). This dense network was composed of CGRP- (Figs. 7, 8, 9D) and Sub P-positive (Figs. 7, 8, 9B) unmyelinated C-fiber sensory afferents, NF200-positive myelinated sensory afferents (Figs. 7, 8, 9C) and TH-positive, postganglionic sympathetic nerve fibers (Figs. 7, 8, 9E). In contrast to CGRP-, Sub P- and NF-200-positive nerve fibers that exhibited increased density in NGF-OE vs. WT urinary bladders at all postnatal ages examined (P7, P14, P21, P28), no changes were observed in TH-positive nerve fibers density in NGF-OE vs. WT urinary bladders from any postnatal age examined. The increased nerve fiber density observed in the NGF-OE bladders was evident in both the neck (Figs. 7, 8) and the dome (data not shown) regions of the urinary bladder. However, consistent with published literature, we observed a higher innervation density within the urinary bladder neck region compared with the dome (Gabella and Davis, 1998; Schnegelsberg et al., 2010) in both WT and NGF-OE mice for all postnatal ages examined (data not shown).

### **Onset of micturition reflex function in postnatal NGF-OE and littermate WT mice**

The spinobulbospinal micturition reflex is triggered by mechanoreceptors in the urinary bladder and begins to elicit voiding in the rat between P16–P18 (Capek and Jelinek, 1956). To determine the onset of the micturition reflex in postnatal NGF-OE and littermate WT mice, individual male and female mice of varying postnatal ages (P0–A) were separated from the dam (if litters were pre-weaning) and placed on filter paper for a period of 2 h under continuous monitoring. As an additional comparison, the onset of the micturition reflex in individual postnatal (P0–A) Wistar rats of both sexes was also determined in the same manner. For NGF-OE mice, the onset of the micturition reflex occurred as early as P6 with all NGF-OE mice examined exhibiting voiding behaviors by P7 (Fig. 10A). In contrast, littermate WT mice first exhibited micturition as early as P13 with all WT mice examined exhibiting voiding behaviors by P14 (Fig. 10A). Consistent with previous studies, postnatal Wistar rats first exhibited micturition as early as P15 with all Wistar rats studied exhibiting micturition behaviors by P16 (Fig. 10A). No differences in the onset of the micturition reflex were observed between sexes for postnatal NGF-OE, littermate WT or Wistar rats.

### **Number and area of urine spots during postnatal development of NGF-OE and WT mice**

The number of urine spots and the size of the urine spots varied during postnatal development between NGF-OE and littermate WT mice (Fig. 10B). For NGF-OE mice, the number of urine spots increased ( $p < 0.01$ ;  $R^2=0.998$ ) with postnatal age from P5 to P12 and then the number of urine spots remained consistent from P14–A (Fig. 10B). Up to P12, the sizes of the urine spots voided by NGF-OE mice were small in area ( $<0.2 \text{ cm}^2$ ) (Fig. 10B). After P12, NGF-OE mice voided both small and large urine spots; however, the number of small urine spots was significantly ( $p < 0.01$ ) greater at each postnatal age examined (Fig. 10B). The urine spots voided by littermate WT mice were not correlated with increasing postnatal age and the number of urine spots remained consistent from P12–A. Littermate WT mice expressed both small and large size urine spots with the number of large urine spots being significantly ( $p < 0.01$ ) greater from P12–P16 and from P28–A (Fig. 10B). No differences in the number or area of urine spots were observed between sexes for postnatal NGF-OE or littermate WT mice.

### **Conscious, open-outlet cystometry in postnatal NGF-OE and WT mice**

Conscious, open outlet cystometry with continuous intravesical infusion of saline was performed in separate groups ( $n = 6$  each) of female WT and NGF-OE during postnatal development (P7, P14, P28 and A) to determine urinary bladder function (Fig. 11). In contrast to P7 NGF-OE mice that exhibited micturition reflexes with continuous intravesical infusion of saline, age-matched, littermate WT mice exhibited no micturition reflexes in the absence of perigenital stimulation (Fig. 11A, B). This finding with cystometry is consistent with the absence of urine spots from the voiding on filter paper assay. NGF-OE mice exhibited micturition reflexes with cystometry at the earliest postnatal age examined (P7) and void volumes were significantly ( $p < 0.01$ ) smaller than those of age-matched WT mice at all ages examined (P7, P14, P28 and A) where micturition reflexes were present in the absence of perigenital stimulation (Figs. 11, 12A). The intercontraction interval (ICI) was significantly ( $p < 0.01$ ) reduced in NGF-OE mice compared to littermate WT mice at

postnatal ages examined (P7, P14, P28, A) (Figs. 11, 12B). NGF-OE (P7, P14, P28 and A) mice exhibited a significantly ( $p < 0.01$ ) greater number of non-voiding contractions (NVCs) in comparison to age-matched WT mice (Figs. 11, 13A). Baseline pressure (BP), threshold pressure (TP) and peak micturition pressure (MP) were comparable between age-matched WT and NGF-OE mice (P7, P14, P28 and A) (Fig. 13B). No differences in the cystometric parameters analyzed were observed between sexes for postnatal NGF-OE or littermate WT mice.

### **Effects of intravesical netupitant, neurokinin (NK)-1 receptor antagonist, on urinary bladder function in NGF-OE and WT mice**

Intravesical netupitant (0.1  $\mu\text{g/ml}$ ) significantly ( $p < 0.01$ ) increased void volume in NGF-OE mice at each postnatal age examined (P7, P14, P28 and A) (Fig. 12A). In contrast, intravesical netupitant did not affect void volume in WT mice (P28, A) but did increase ( $p < 0.05$ ) void volume in P14 WT mice (Fig. 12A). Intravesical netupitant significantly ( $p < 0.01$ ) increased ICI in NGF-OE mice at P7, P14, P28 and A (Fig. 12B). In contrast, intravesical netupitant did not affect the ICI in age-matched WT mice (Fig. 12B). Intravesical netupitant (0.1  $\mu\text{g/ml}$ ) was without effect on the number of NVCs observed in postnatal (P7, P14, A) NGF-OE or WT mice except for a significant ( $p < 0.05$ ) reduction in NVCs in NGF-OE mice at P28 (Fig. 13A). Intravesical netupitant had no effect on BP, TP or peak MP in either NGF-OE mice or age-matched WT mice (P7, P14, P28, A) (Fig. 13B). Residual volume in NGF-OE and age-matched littermate WT mice was small ( $< 5 \mu\text{l}$ ) and was unaffected by intravesical netupitant administration. No differences in the effects of intravesical netupitant on the cystometric parameters analyzed were observed between sexes for postnatal NGF-OE or littermate WT mice.

## **Discussion**

These studies, involving early postnatal mice with chronic urothelial overexpression of NGF, have produced novel insights into the mechanisms that drive the development of normal bladder innervation and function. As demonstrated with filter paper void assays and open-outlet, continuous fill cystometry in conscious mice, early postnatal NGF-OE mice exhibit the onset of the micturition reflex in the absence of perigenital stimulation (i.e., vesicovesical reflex) by P6. In contrast, littermate WT mice first exhibit micturition in the absence of perigenital stimulation by P13 similar to the onset of micturition in rats by P15. In association with this early onset of micturition in postnatal NGF-OE mice, these mice also exhibit hyperinnervation of the urinary bladder and increased bladder mass at all postnatal ages examined. The neurochemical coding of bladder hyperinnervation reflected immunoreactivity (IR) for neuropeptides (i.e., Sub P and CGRP) and NF200 with no increase in TH-IR. Sub P and CGRP protein exhibited an age-dependent increase in expression in the urothelium of NGF-OE mice during postnatal development. Intravesical instillation of the neurokinin-1 (NK-1) receptor antagonist, netupitant (0.1  $\mu\text{g/ml}$ ) increased the void volume and the interval between micturition events in NGF-OE mice during postnatal development (P7, P21, A) with no effects of micturition pressure (baseline, threshold or peak). These studies highlight the contribution of urothelial NGF and NGF-

mediated pleiotropic effects on resulting neuropeptides expression on postnatal maturation of micturition reflexes.

The maturation of peptidergic afferent pathways of the LUT of the neonatal rat may be involved in development of adult patterns of micturition (Maggi et al., 1986; 1987; 1988). In contrast, it has been demonstrated that regional noradrenergic and cholinergic innervation of the rat urinary bladder is relatively stable as the rat matures (Johnson et al., 1988). Bladder afferents contain a variety of neuropeptides, including: CGRP, Sub P, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating polypeptide, cholecystokinin and enkephalins (Arms and Vizzard, 2011; de Groat et al., 1983; Keast and De Groat, 1992; Vizzard, 2000d, 2001). The innervation of the urinary bladder arises primarily from neuronal cell bodies located at a distance from the urinary bladder; however, some neuronal cell bodies appear transiently in the bladder wall during development and early postnatal life, with few remaining by adulthood (Keast et al., 2015; Zvarova and Vizzard, 2005). The functions of these intramural ganglion neurons are not known but their chemical phenotype more closely resembles autonomic rather than sensory neurons (Forrest et al., 2014; Keast et al., 2015; Zvarova and Vizzard, 2005).

We demonstrated age-dependent increases in Sub P and CGRP protein content in the urothelium of postnatal NGF-OE mice and expression was significantly greater in the urothelium of NGF-OE mice compared to littermate WT mice at all postnatal ages examined. Sub P could be expressed by urothelial cells as well as by nerve fibers in the suburothelial nerve plexus both of which have been previously shown to express Sub P-IR (Andersson, 2002; Arms and Vizzard, 2011; Birder and Andersson, 2013; Birder et al., 2010; Heng et al., 2011; Schnegelsberg et al., 2010). Sub P and CGRP protein expression in detrusor smooth muscle also exhibited age-dependent increases in expression but no differences in protein expression were demonstrated in detrusor between NGF-OE and WT mice at any postnatal age examined. In contrast, Sub P and CGRP mRNA did not exhibit age-dependent differences in expression in urothelium or detrusor in NGF-OE mice. Sub P mRNA was consistently greater in urothelium and detrusor of NGF-OE mice at every postnatal age examined. CGRP mRNA was significantly reduced in urothelium of NGF-OE mice compared to WT and CGRP mRNA expression exhibited an age-dependent decrease in detrusor expression in WT mice. Increased Sub P and CGRP protein content in the urothelium of NGF-OE mice correlated well with increased Sub P- and CGRP-immunoreactivity (IR) in the suburothelial nerve plexus of the urinary bladder, which primarily consists of afferent nerve fibers (Birder and Andersson, 2013). Future studies should consider: (1) co-labeling of Sub P and CGRP in the suburothelial nerve plexus and urothelium and (2) differences in the degree of co-labeling in nerve fibers and urothelial cells among the various postnatal ages, between mouse strains and as a function of onset of the micturition reflex. Hyperinnervation of the urinary bladder of NGF-OE mice during early postnatal development is consistent with our previous studies in adult NGF-OE mice (Schnegelsberg et al., 2010). In addition to increased peptidergic innervation, the suburothelial nerve plexus in NGF-OE mice also exhibited increased NF-200-IR but did not exhibit increased adrenergic-IR.



To determine if increased peptidergic innervation of the urinary bladder was associated with Sub P/NK-1 receptor signaling that affected urinary bladder function, we intravesically infused a NK-1 receptor antagonist, netupitant (0.1 µg/ml) (Greenwood-Van Meerveld et al., 2014; Haab et al., 2014; Zhang et al., 2012), and determined the effects on micturition function in postnatal NGF-OE and WT mice using conscious cystometry. Intravesical infusion of a NK-1 receptor antagonist had little effect on any micturition parameter measured in conscious WT mice (P7, P28, A), which is similar to a previous report in rats (Ishizuka et al., 1994). However, there was an effect on void volume in P14 WT mice where intravesical netupitant (0.1 µg/ml) significantly increased void volume but this effect was not observed at any other age examined (P7, P28, A). In contrast, intravesical instillation of netupitant (0.1 µg/ml) significantly increased void volume and the interval between micturition events (ICI) in NGF-OE mice at P7, P14, P28 and adult. Intravesical netupitant (0.1 µg/ml) did not affect bladder pressure (baseline, threshold or peak micturition pressure) in NGF-OE, littermate WT at any postnatal age examined (P7, P14, P28, A). Intravesical instillation of netupitant significantly reduced the number of NVCs in NGF-OE mice at P7 and P28 with no effects on the number of NVCs in WT mice. The distribution of NK receptor subtypes in the urinary bladder of NGF-OE mice is not known but given the effects demonstrated via intravesical instillation of a NK-1 receptor antagonist, it is likely that NK-1 receptors are located on bladder nerve terminals and/or urothelial cells (Meini et al., 1994). However, evidence of urothelial expression of NK-1 receptors is not widely reported in rat (Heng et al., 2011; Ishizuka et al., 1995) and is unknown in mouse. Although NK receptor subtypes are likely present throughout the detrusor (Meini et al., 1994; Steinhoff et al., 2014), a lack of effect on detrusor NK receptor subtypes (as evident by the absence of effects on micturition pressures), may reflect an inability of the NK-1 receptor antagonist to reach the detrusor due to high transepithelial resistance of the urothelium or a higher concentration of the NK-1 receptor antagonist may be necessary to see an effect on micturition pressures. The results of future studies involving protamine sulfate (to disrupt the urothelial barrier) (Greenwood-Van Meerveld et al., 2015; Klinger and Vizzard, 2008) plus NK-1 receptor antagonist or intravesical instillation of a NK-2 receptor antagonist would be informative when compared to the NK-1 receptor antagonist data generated in this study.

In many species, the neuropeptide Sub P is present throughout the LUT and is released in the spinal cord or in the bladder by capsaicin-induced stimulation of nociceptive afferent nerves (Lecci and Maggi, 2001). Sub P modulates bladder activity by acting on three NK receptor subtypes (NK-1, NK-2, and NK-3) expressed by cells and tissues of the LUT including: bladder smooth muscle cells, urothelial cells, afferent nerves, or spinal cord neurons (Steinhoff et al., 2014) (Arms and Vizzard, 2011; de Groat and Yoshimura, 2009; Lecci and Maggi, 2001). However, considerable differences in the distribution of NK receptors in the LUT among species have been reported and tachykinin/receptor interactions in the LUT are complex (Regoli et al., 1994). The most consistent literature addresses the use of NK-1 receptor antagonists to reduce urinary frequency following neural injury, disease or inflammation. Clinical studies using NK-1 receptor antagonists have demonstrated a reduction in overactive bladder (OAB) symptoms (Green et al., 2006; Frenkl et al., 2010) and a reduction in hyperactive bladder reflexes induced by spinal cord injury, following cyclophosphamide-induced bladder inflammation or chemical irritation of the

urinary bladder (Abdel-Gawad et al., 2001; Doi et al., 1999; 2000). It has been demonstrated that these effects reflect NK-1 receptor antagonists acting on CNS and/or PNS components of micturition reflex pathways (Lecci and Maggi, 1995; 2001; Seki et al., 2005). At the level of the urinary bladder, afferent nerves release Sub P to affect afferent nerve activity either indirectly by inducing smooth muscle contractions (Lecci and Maggi, 2001) or directly via an autofeedback mechanism (Sculptoreanu et al., 2009; Sculptoreanu et al., 2008; Sculptoreanu and de Groat, 2007). The regulation of bladder activity by Sub P/NK receptor signaling depends on species, the tissue distribution and the NK receptor subtypes expressed (Lecci and Maggi, 2001). A few studies have examined the pharmacological profiles of NK receptor subtypes in control mouse LUT (Burcher et al., 1986; Nsa Allogho et al., 1997; Regoli et al., 1994); however, no studies have examined NK receptor subtype distribution in early postnatal or adult LUT in the NGF-OE mouse. We are using Q-PCR and immunohistochemical assays on LUT tissues from NGF-OE mice to address this void in our knowledge.

Micturition in normal adult animals and humans occurs through activation of a vesicovesical reflex organized at supraspinal levels and triggered through distension of the urinary bladder and activation of mechanoreceptors (de Groat and Araki, 1999; de Groat et al., 1998; Maggi et al., 1986). It is widely accepted that the vesicovesical reflex is absent in newborn animals and that this reflex is not fully developed or functional at birth (Maggi et al., 1986; Zvarova and Zvara, 2012). In early postnatal animals (e.g. rats, cats, guinea pigs) micturition is activated via a somatovesical reflex when the mother licks the perineal area (Capek and Jelinek, 1956; de Groat and Araki, 1999; de Groat et al., 1998; Ng et al., 2007; Maggi et al., 1986). Interestingly, transneuronal tracing studies from the urinary bladder in early postnatal rats (P2 and P12) demonstrated that the supraspinal micturition reflex pathways are intact but do not function until the third postnatal week (Sugaya et al., 1997). In this study, NGF-OE mice exhibited early onset of micturition that was demonstrated using voiding on filter paper assays and conscious cystometry. The early onset of micturition in NGF-OE mice by P5 occurred in the absence of perigenital stimulation (i.e., somatovesical reflex). In contrast, maternal separation has been shown to increase the duration of the somatovesical reflex but had no effect on the appearance of spontaneous voiding (i.e., vesicovesical reflex). In postnatal and adult NGF-OE mice, intravesical instillation of a NK-1 receptor antagonist, netupitant (0.1 µg/ml), increased void volume and ICI in NGF-OE mice; however, effects of NK-1 receptor blockade on micturition onset could not be determined. Given the chronic urothelial overexpression of NGF in the NGF-OE mice (Schnegelsberg et al., 2010), designing an experiment using pharmacological tools (NK-1 receptor antagonist) to block the long-term effects of NGF did not seem plausible. One way to address the contribution of peptidergic pathways to onset of micturition in NGF-OE mice could begin with developing new mouse lines that result from crossing TRPV1<sup>-/-</sup> mice (Birder et al., 2002) with NGF-OE mice or from crossing NK-1<sup>-/-</sup> mice (Saban et al., 2000) with NGF-OE mice.

Additional factors that may contribute to the initiation of the micturition reflex in adults include the myogenic contractile activity of the detrusor muscle and maturation of central micturition pathways (Maggi et al., 1986). The neonatal bladder exhibits high-amplitude, low-frequency, spontaneous contractions during postnatal weeks 1–3 prior to evidence of mature voiding (Kanai et al., 2007; Maggi et al., 1986; Ng et al., 2007). With the

development of mature supraspinal reflex pathways, spontaneous bladder activity decreases. This spontaneous activity in neonatal bladders is highly organized and originates in the urothelium/suburothelium near the bladder dome (Kanai et al., 2007). Although the bladder exhibits myogenic activity that is independently driven by the detrusor smooth muscle, parasympathetic and sympathetic pathways are the effectors for coordinated urinary bladder storage and release. Thus, target organ (i.e., urinary bladder) changes need to be integrated with the PNS and CNS to fully understand the mechanisms underlying bladder innervation and the maturation of voiding reflexes. Maturation of central pathways may also be a factor in the postnatal development of bladder reflexes. Various transmitters including serotonin (Bregman, 1987), CRF/urocortin/receptors (CRFR1, CRFR2) (LaBerge et al., 2006; Puder and Papka, 2001; Studeny and Vizzard, 2005), somatostatin (Marti et al., 1987), NADPH-diaphorase and nNOS (Vizzard et al., 1994) appear or increase in prominence in the lumbosacral spinal cord during the second to third postnatal week.

## Conclusions

These studies have begun to provide insights into some factors that contribute to the maturation of the micturition reflex from a somatovesical to a vesicovesical reflex. There is a large gap in our understanding of the mechanisms that underlie the postnatal maturation of voiding reflexes. Using a transgenic mouse model with chronic urothelial overexpression of NGF (Schnegelsberg et al., 2010) has given us the opportunity to examine the contribution of target-derived growth factors as well as secondary events resulting from NGF-OE including increased neuropeptides (e.g., Sub P and CGRP) expression in the urinary bladder (i.e., urothelium and suburothelial nerve plexus). We demonstrated an age-dependent increase in Sub P and CGRP expression in the urothelium and suburothelial nerve plexus in postnatal NGF-OE mice. In addition to an early onset of micturition in NGF-OE mice in the absence of a perineal-to-bladder reflex, postnatal NGF-OE mice exhibit increased voiding frequency, shorter intervals between void events and the presence of NVCs in comparison to littermate WT mice. Intravesical instillation of a NK-1 receptor antagonist reduced voiding frequency and increased void volume in NGF-OE mice with few effects on WT mice. In addition to providing key insights into developmental maturation of voiding reflexes, these studies may also provide important information relevant to adult OAB and neurogenic voiding disorders. For example, NK-1 receptor antagonists have been used to improve bladder dysfunction in adult overactive bladder and manage neurogenic disorders of micturition (Abdel-Gawad et al., 2001; Doi et al., 1999; 2000; Frenkl et al., 2010; Green et al., 2006). In addition, studies of the development of micturition reflexes may provide insights into the management of neurogenic disorders of micturition. For example, injuries or disorders of the adult spinal cord that lead to the reemergence of primitive functions (reflex voiding and incontinence), prominent during early development, may represent a return to early voiding patterns (de Groat et al., 1998; de Groat and Yoshimura, 2012; Keast et al., 2015). Thus, the study of the maturation of voiding reflexes not only has merit on its own but also has the potential to improve our understanding of OAB and neurogenic voiding disorders.

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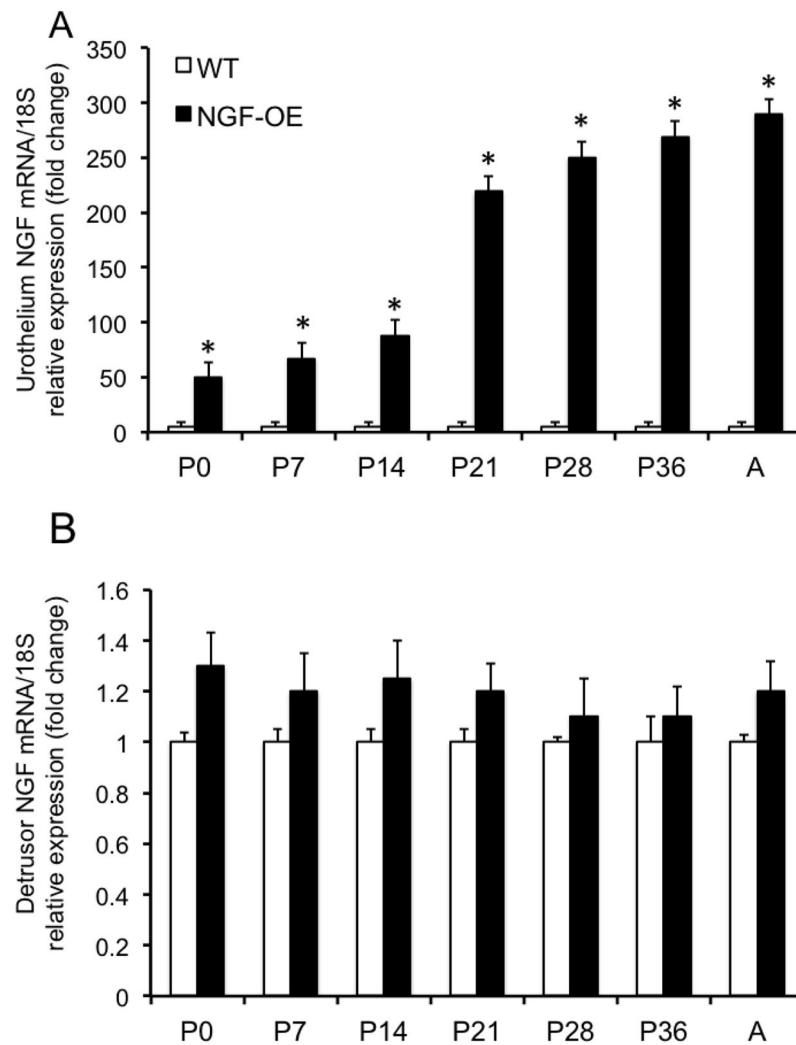


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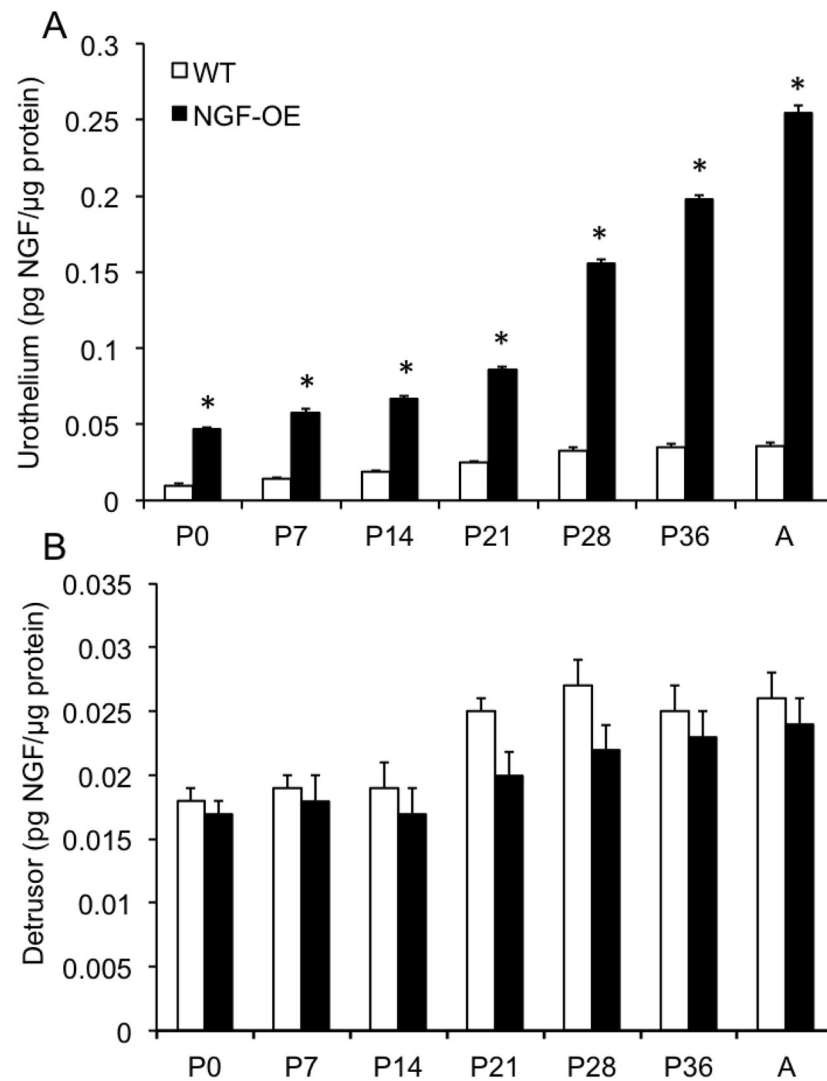


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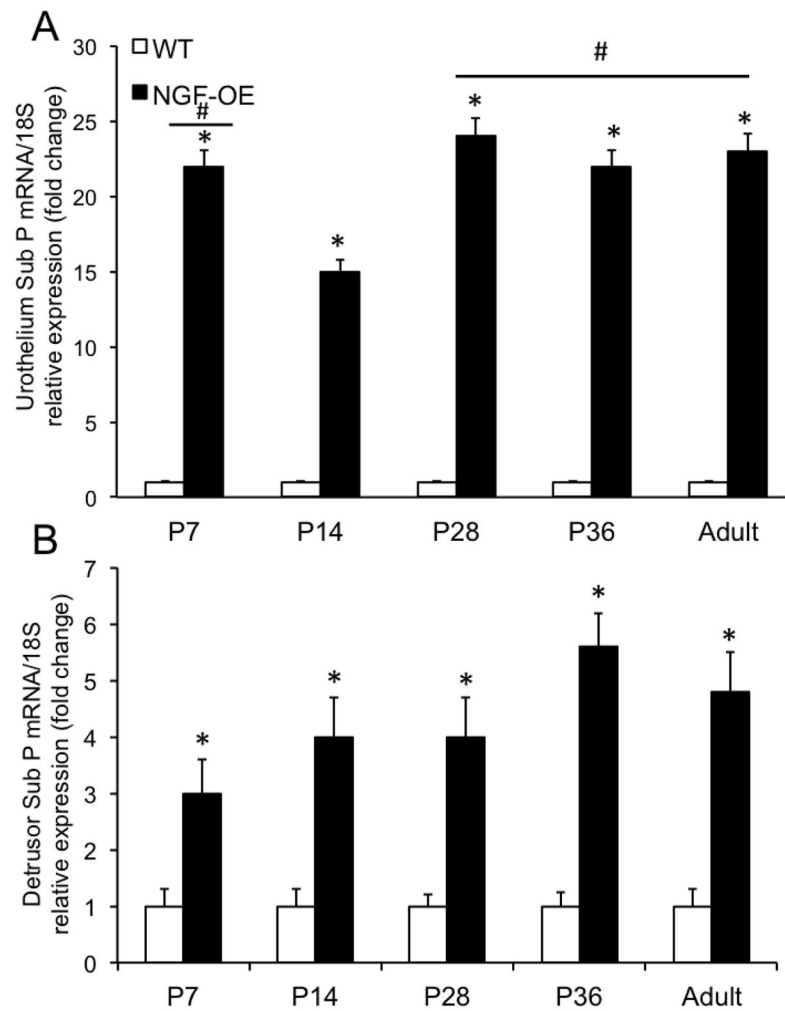
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**Figure 1.** Characterization of nerve growth factor (NGF) mRNA in urinary bladder from NGF-overexpressing (OE) mice at different postnatal (P) ages (P0, P7, P14, P28, P36, Adult (A)) by quantitative PCR. NGF transcript expression in urothelium/suburothelium (A) and detrusor layers (B) in NGF-OE and wildtype (WT) mice normalized to expression of the housekeeping gene 18S. Values are means  $\pm$  S.E.M. (n = 6–8 for each group). \*p < 0.01: NGF-OE urothelium/suburothelium vs. WT urothelium/suburothelium (A).

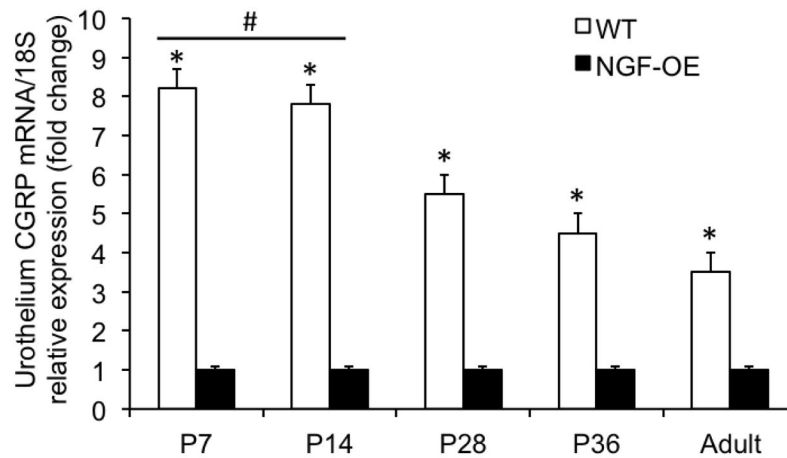


**Figure 2.** NGF content in the urinary bladders of WT and NGF-OE transgenic mice of different postnatal (P) ages. NGF content in urothelium/suburothelium (A) and detrusor (B) mice was determined in WT and NGF-OE mice of different postnatal (P) ages, including P0, P7, P14, P28, P36, and Adult (A). NGF content was significantly ( $*p < 0.01$ ) greater in NGF-OE vs. WT urothelium at each postnatal age examined (A). No differences in NGF content were observed in detrusor of NGF-OE and WT mice at any postnatal age examined (B). Values are means  $\pm$  S.E.M. ( $n = 6-8$  for each group).



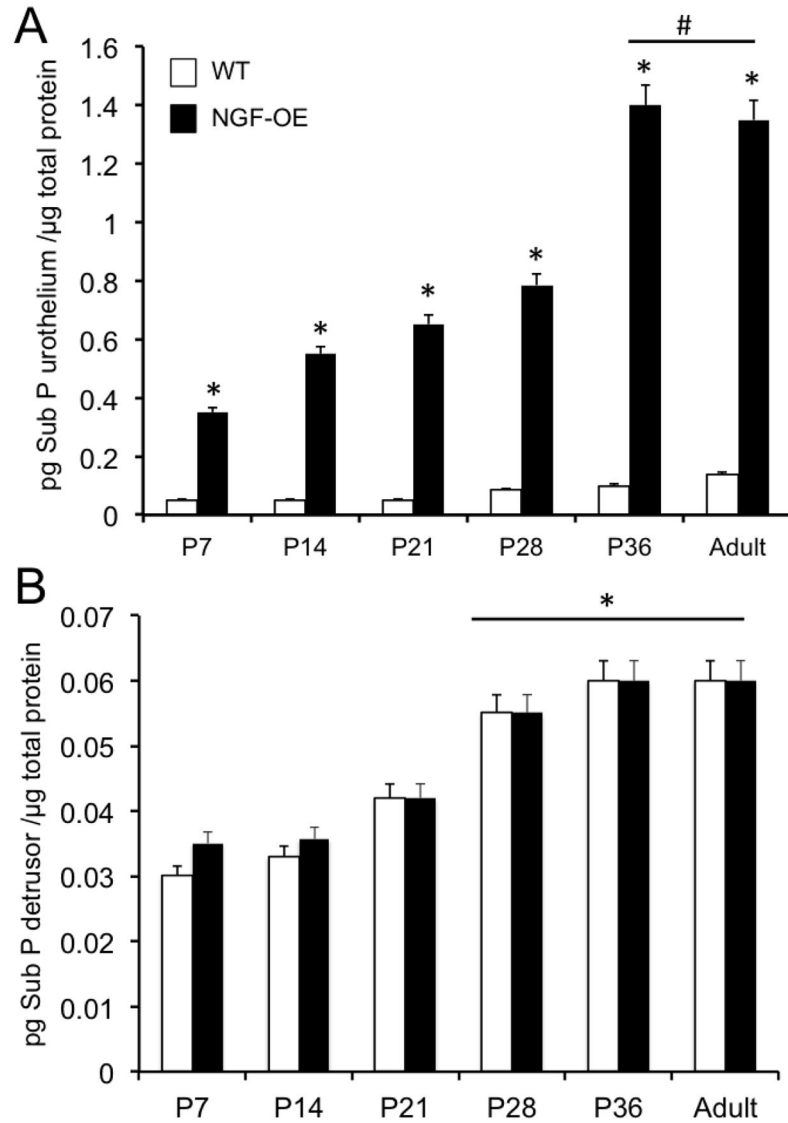
**Figure 3.**

Characterization of substance P mRNA in urinary bladder from NGF-overexpressing (OE) mice at different postnatal (P) ages (P7, P14, P28, P36, Adult (A)) by quantitative PCR. Substance P transcript expression in urothelium/suburothelium (A) and detrusor layers (B) in NGF-OE and wildtype (WT) mice normalized to expression of the housekeeping gene 18S. Values are means  $\pm$  S.E.M. (n = 6–8 for each group). \*p < 0.01: NGF-OE urothelium/suburothelium vs. WT urothelium/suburothelium (A). #, p < 0.01: NGF-OE urothelium/suburothelium at P7, P28, P36 and A vs. NGF-OE urothelium/suburothelium at P14 (A). \*p < 0.01: NGF-OE detrusor vs. WT detrusor (B).

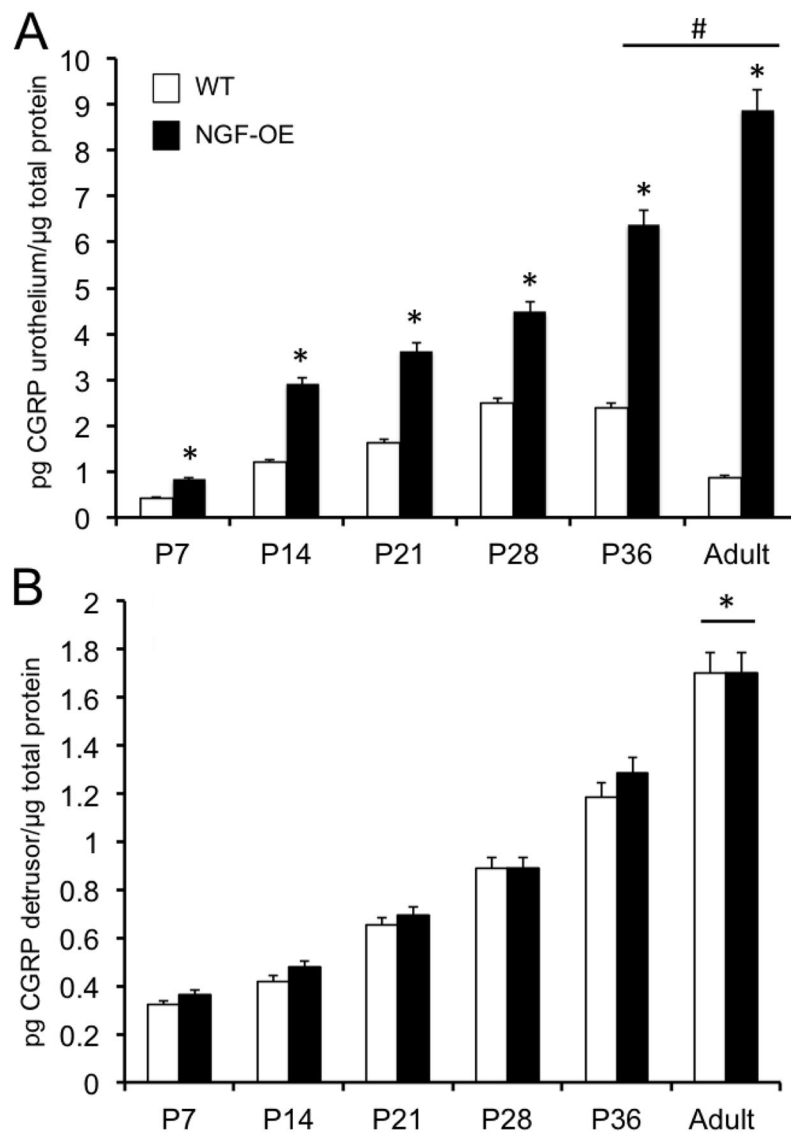


**Figure 4.** Characterization of CGRP mRNA in urinary bladder from NGF-overexpressing (OE) mice at different postnatal (P) ages (P7, P14, P28, P36, Adult (A)) by quantitative PCR. CGRP transcript expression in urothelium/suburothelium in NGF-OE and wildtype (WT) mice normalized to expression of the housekeeping gene 18S. Values are means  $\pm$  S.E.M. (n = 6–8 for each group). \*p < 0.01: WT urothelium/suburothelium vs. NGF-OE urothelium/suburothelium. #, p < 0.01: WT urothelium/suburothelium at P7, P14 vs. WT urothelium/suburothelium at P28, P36 and A.

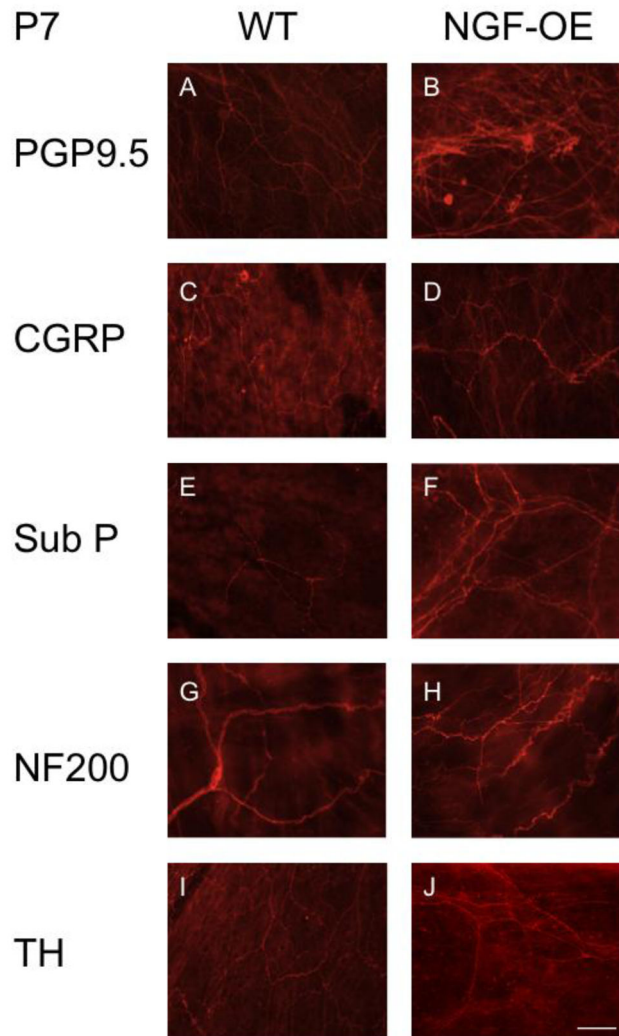


**Figure 5.**

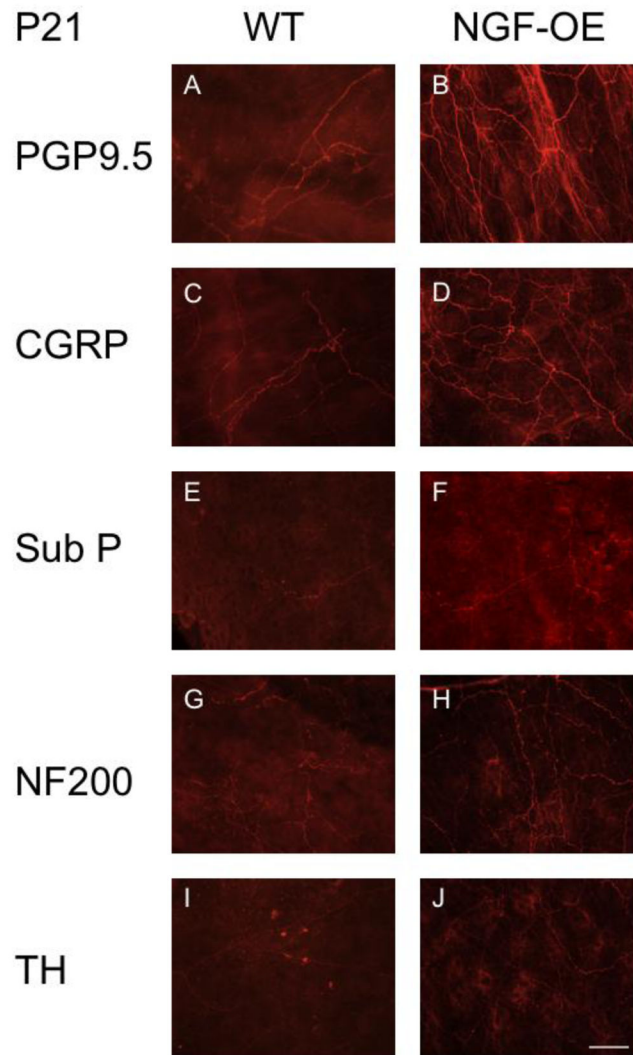
Substance P (Sub P) content in the urinary bladders of WT and NGF-OE transgenic mice of different postnatal (P) ages. Sub P content in urothelium/suburothelium (A) and detrusor (B) mice was determined in WT and NGF-OE mice of different postnatal (P) ages, including P7, P14, P28, P36, and Adult (A). Sub P content was significantly (\* $p < 0.01$ ) greater in NGF-OE vs. WT urothelium at each postnatal age examined (A). # $p < 0.01$ : NGF-OE urothelium/suburothelium at P36 and A vs. NGF-OE urothelium/suburothelium at P7, P14, P21, P28. No differences in Sub P content were observed in detrusor of NGF-OE vs. WT mice at any postnatal age examined (B). \* $p < 0.01$ : Sub P content in detrusor at P28, P36, A vs. P7, P14, P21 for both WT and NGF-OE. Values are means  $\pm$  S.E.M. ( $n = 6-8$  for each group).



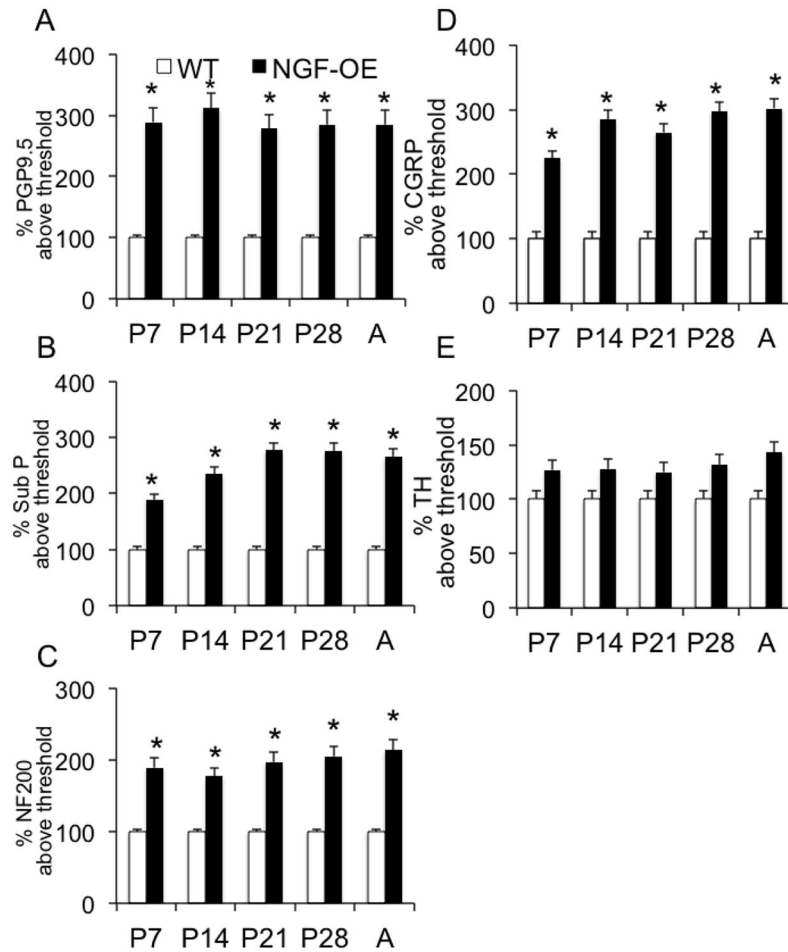
**Figure 6.** CGRP content in the urinary bladders of WT and NGF-OE transgenic mice of different postnatal (P) ages. CGRP content in urothelium/suburothelium (A) and detrusor (B) mice was determined in WT and NGF-OE mice of different postnatal (P) ages, including P7, P14, P28, P36, and Adult (A). CGRP content was significantly (\* $p < 0.01$ ) greater in NGF-OE vs. WT urothelium at each postnatal age examined (A). # $p < 0.01$ : NGF-OE urothelium/suburothelium at P36 and A vs. NGF-OE urothelium/suburothelium at P7, P14, P21, P28. No differences in CGRP content were observed in detrusor of NGF-OE vs. WT mice at any postnatal age examined (B). \* $p < 0.01$ : CGRP content in detrusor at A vs. P7, P14, P21, P28, P36 for both WT and NGF-OE. Values are means  $\pm$  S.E.M. ( $n = 6-8$  for each group).



**Figure 7.** Neuronal hyperinnervation in the urinary bladders of NGF-OE mice at postnatal (P) day 7. Representative fluorescence images of PGP 9.5 (A, B), CGRP (C, D), Sub P (E, F), NF200 (G, H), and TH (I, J) immunoreactivity in the bladder neck region in urothelial whole-mount preparations of urinary bladders from P7 WT (A, C, E, G, I) and NGF-OE (B, D, F, H, J) mice (n = 6–8). Scale bar: 50  $\mu$ m. Protein Gene Product (PGP 9.5), calcitonin gene-related protein (CGRP), substance P (Sub P), neurofilament (NF 200), tyrosine hydroxylase (TH).

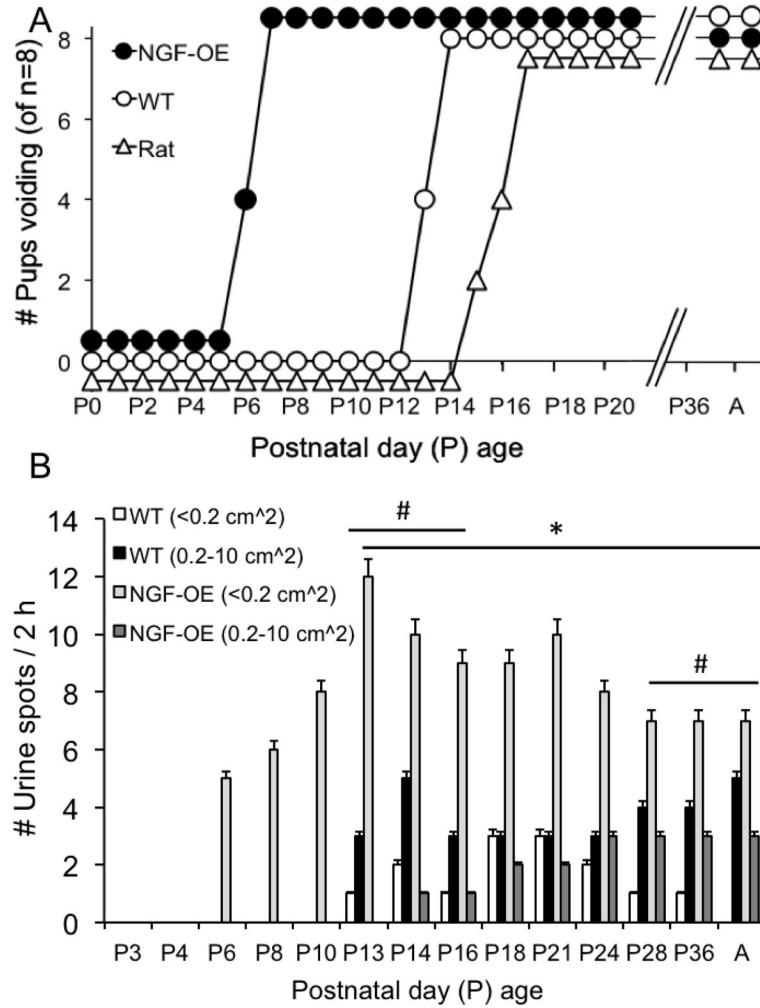


**Figure 8.** Neuronal hyperinnervation in the urinary bladders of NGF-OE mice at postnatal (P) day 21. Representative fluorescence images of PGP 9.5 (A, B), CGRP (C, D), Sub P (E, F), NF200 (G, H), and TH (I, J) immunoreactivity in the bladder neck region in urothelial whole-mount preparations of urinary bladders from P7 WT (A, C, E, G, I) and NGF-OE (B, D, F, H, J) mice (n = 6–8). Scale bar: 50  $\mu$ m. Protein Gene Product (PGP 9.5), calcitonin gene-related protein (CGRP), substance P (Sub P), neurofilament (NF200), tyrosine hydroxylase (TH).



**Figure 9.**

Semi-quantitative analyses of neurochemistry in urothelium of NGF-OE mice at various postnatal ages (P7, P14, P21, P28, A). Histograms of neurochemical expression in the urothelium during postnatal development expressed as a percentage of expression above threshold and averaged for all bladders from specific postnatal ages for NGF-OE and WT mice. Values are means  $\pm$  S.E.M. ( $n = 6-8$  for each group). At each postnatal age examined (P7, P14, P21, P28, Adult (A)), the percent expression of Protein Gene Produce (PGP)9.5 (A), Substance P (Sub P) (B), neurofilament (NF200) (C) and calcitonin gene-related peptide (CGRP) (D) was significantly ( $*p < 0.01$ ) above threshold in NGF-OE vs. WT mice. No changes were observed for tyrosine hydroxylase expression in NGF-OE vs. WT mice at any age examined (E).



**Figure 10.**

Early onset of the vesicovesical reflex in postnatal NGF-OE mice. (A). Summary representation of the onset of spontaneous voiding as determined from filter paper void assays in NGF-OE mice, littermate WT mice, and Wistar rats of both sexes from various postnatal (P) ages (P0-Adult, A). NGF-OE mice exhibited spontaneous voiding by P5 with all NGF-OE mice exhibiting spontaneous voiding by P7. Littermate WT mice first exhibited spontaneous voiding on P13 with all WT mice exhibiting spontaneous voiding by P14. Wistar rats first exhibited spontaneous voiding by P15 with Wistar rats exhibiting spontaneous voiding by P17.  $n = 8$  for NGF-OE mice, WT mice and Wistar rats. (B) Summary histogram of the number (#) and area of urine spots on filter paper over a 2 h period of time from NGF-OE mice and littermate WT mice of both sexes from various postnatal (P) ages (P3-Adult, A). NGF-OE mice exhibited an early onset of spontaneous voiding with a large number of voids of small area ( $< 0.2 \text{ cm}^2$ ). For NGF-OE mice, the area of urine spots increased during postnatal development but the number of voids of small area were significantly ( $*p < 0.01$ ) greater than the number of voids of larger area ( $0.2 - 10 \text{ cm}^2$ ) at all postnatal ages examined. WT mice exhibited spontaneous voiding by P13. In WT mice, the number voids of larger area ( $0.2 - 10 \text{ cm}^2$ ) were significantly ( $\#p < 0.01$ ) greater at



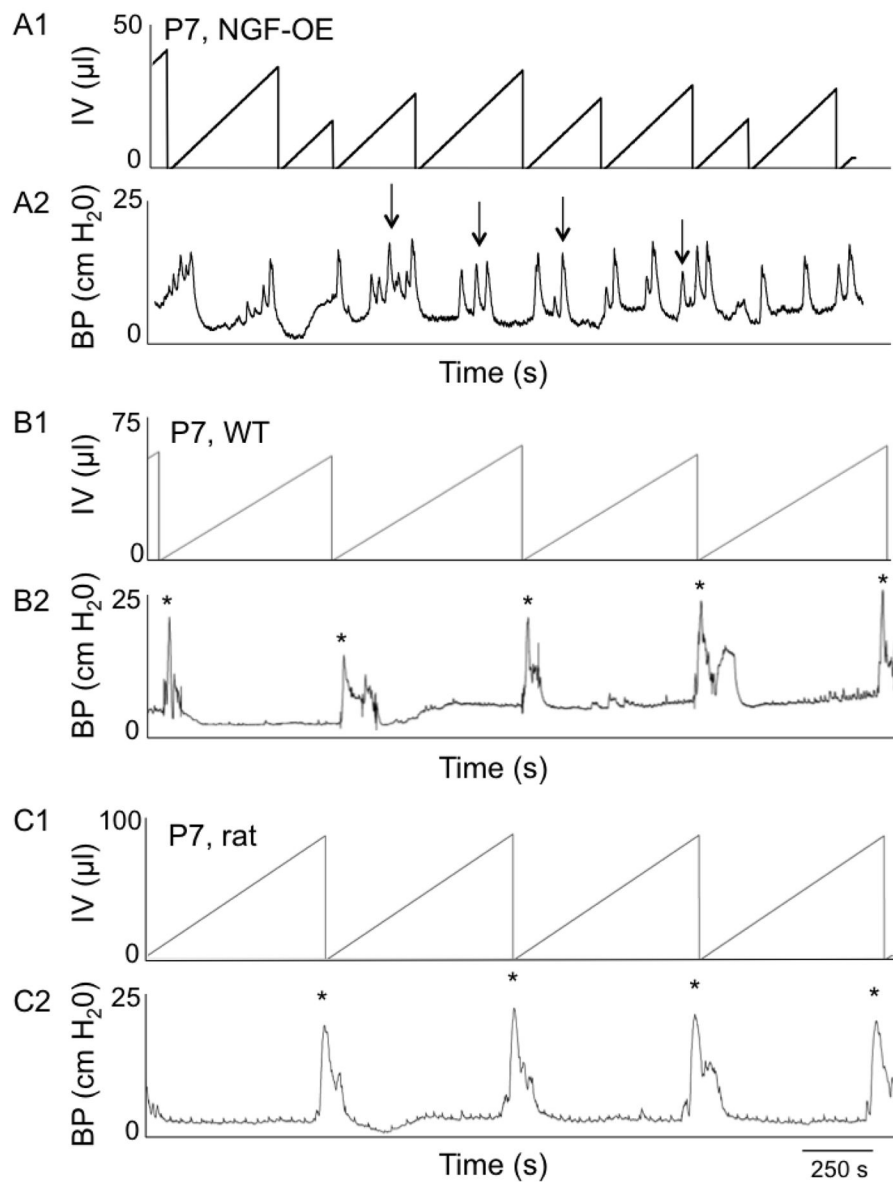
P13–P16 and P24-A with other ages (P18, P21) having equal numbers of small and larger area urine spots. Values are means  $\pm$  S.E.M. (n = 6–8 for each group).

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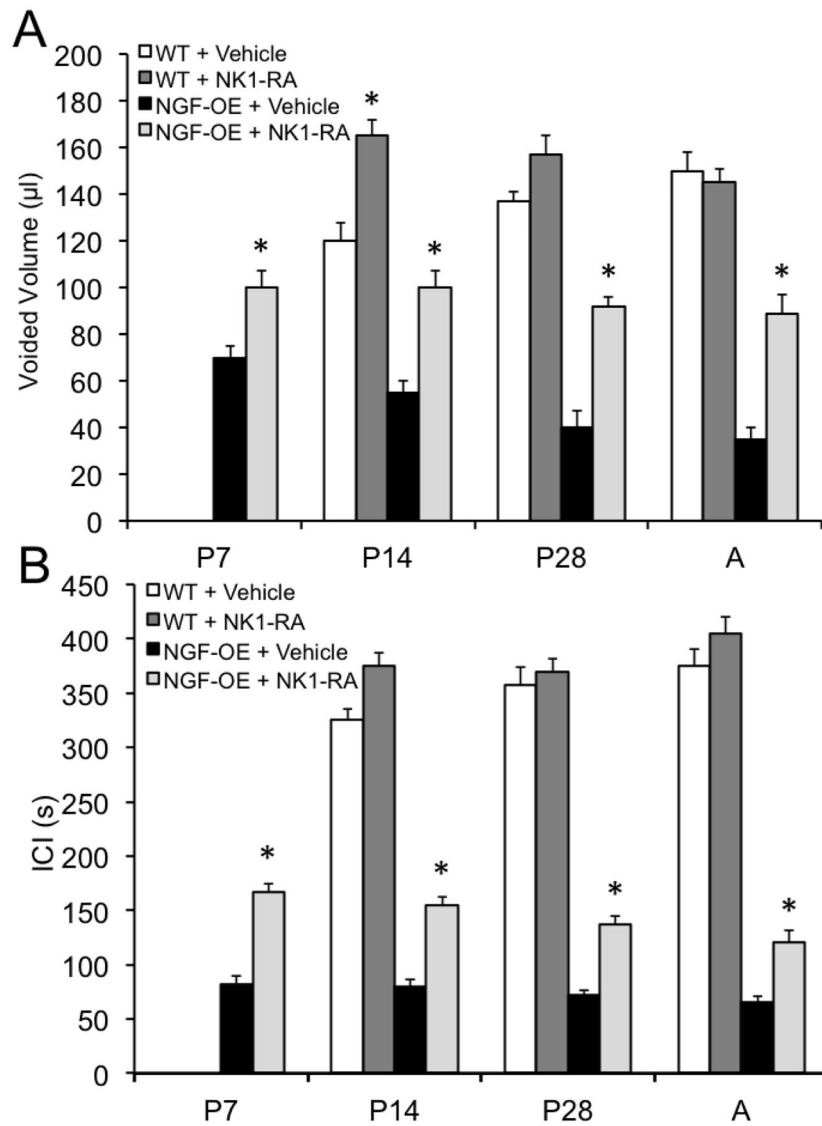
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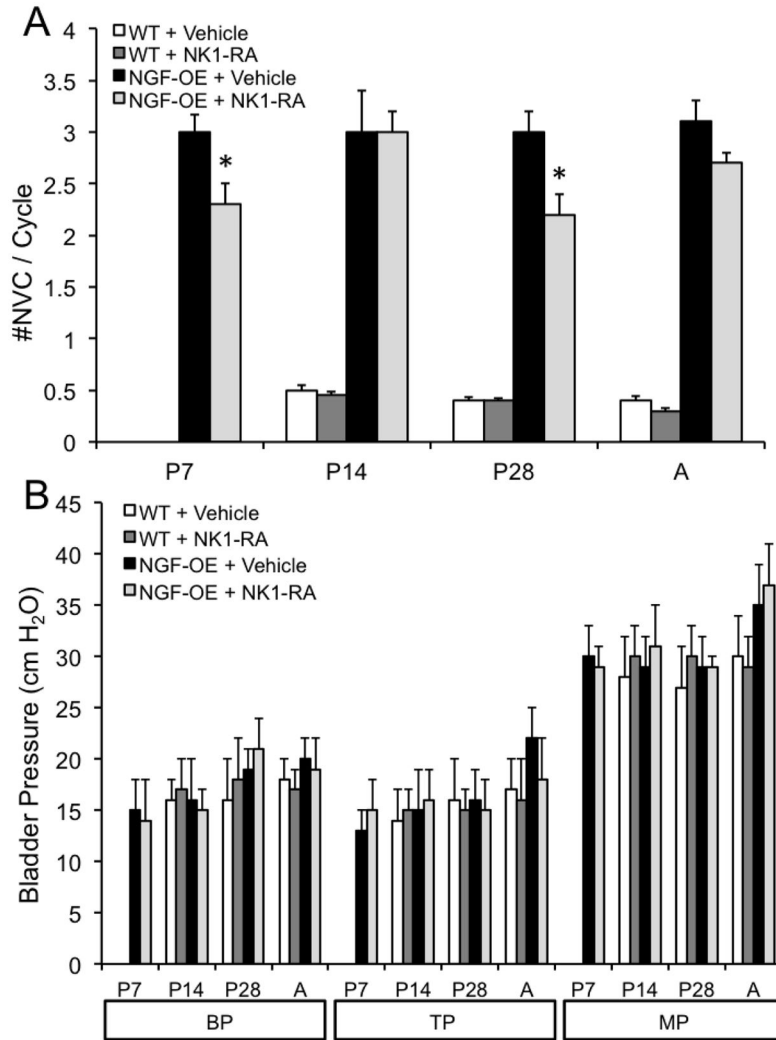
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**Figure 11.**

Open outlet, continuous fill cystometry in conscious NGF-OE mice, littermate WT mice and Wistar rats at postnatal (P) day 7. Representative cystometrogram trace from a P7 conscious, unrestrained NGF-OE (A) mouse, a littermate WT (B) mouse and a Wistar rat (C) during a continuous intravesical infusion (25  $\mu$ l/min for mice; 10 ml/hr for rats) of room temperature saline. A. At P7, a NGF-OE mouse exhibits micturition reflexes in the absence of perigenital stimulation. B. At P7, a WT mice (B) and a Wistar rat (C) only exhibit micturition reflexes with perigenital stimulation (\*). Infused volume (IV,  $\mu$ l)(A1, B1, C1) and bladder pressure (BP, cm H<sub>2</sub>O) (A2, B2, C2) are shown. Arrows indicate examples of nonvoiding bladder contractions (A). seconds, s.





**Figure 13.**

Effects of a neurokinin (NK)-1 receptor antagonist (RA), netupitant, on micturition reflex function from open outlet, continuous fill cystometry in NGF-OE mice and littermate WT mice at postnatal (P) day 7, P14, P28 and Adult (A). Intravesical netupitant (0.1  $\mu$ g/ml) significantly ( $p < 0.01$ ) decreased the number of nonvoiding contractions (NVCs) in NGF-OE mice at P7 and P28 but was without effect on NVCs in WT mice that exhibited few NVCs (A). Intravesical netupitant (0.1  $\mu$ g/ml) was without effect on bladder pressures (baseline (BP), threshold pressure (TP), peak micturition pressure (MP)) in NGF-OE and WT mice at any postnatal age examined (B). \*,  $p < 0.01$  compared to vehicle. Values are means  $\pm$  S.E.M. ( $n = 6-8$  for each group).

**Table 1**

Urinary bladder and body weights of WT and NGF-OE transgenic mice during postnatal development. Values are mean  $\pm$  S.E.M. for WT and NGF-OE male (M) and female (F) mice of both sexes from postnatal (P) day 0-Adult (A)

Urinary bladder, body weight (Wt) and bladder Wt/Body Wt of littermate WT and NGF-OE transgenic male (M) and female (F) mice during postnatal (P) (P0, P7, P14, P21, P28, P36, Adult (A) development. Values are means  $\pm$  S.E.M.

N	Sex	Age	Genotype	Bladder Wt, g	Body Wt, g	Bladder Wt/Body Wt
5	F	P0	WT	0.009 $\pm$ 0.001	1.80 $\pm$ 0.24	5.00 $\times$ 10 <sup>-3</sup> $\pm$ 0.42 $\times$ 10 <sup>-2</sup>
5	F	P0	NGF-OE	0.028 $\pm$ 0.002*	1.92 $\pm$ 0.23	1.45 $\times$ 10 <sup>-2</sup> $\pm$ 0.87 $\times$ 10 <sup>-2</sup> *
5	M	P0	WT	0.011 $\pm$ 0.001	1.84 $\pm$ 0.34	5.91 $\times$ 10 <sup>-3</sup> $\pm$ 0.29 $\times$ 10 <sup>-2</sup>
5	M	P0	NGF-OE	0.033 $\pm$ 0.004*	1.91 $\pm$ 0.33	1.72 $\times$ 10 <sup>-2</sup> $\pm$ 1.21 $\times$ 10 <sup>-2</sup> *
5	F	P7	WT	0.019 $\pm$ 0.001	3.43 $\pm$ 0.25	5.50 $\times$ 10 <sup>-3</sup> $\pm$ 0.40 $\times$ 10 <sup>-2</sup>
5	F	P7	NGF-OE	0.057 $\pm$ 0.003*	3.45 $\pm$ 0.22	1.65 $\times$ 10 <sup>-2</sup> $\pm$ 1.36 $\times$ 10 <sup>-2</sup> *
5	M	P7	WT	0.020 $\pm$ 0.002	3.80 $\pm$ 0.19	5.30 $\times$ 10 <sup>-3</sup> $\pm$ 1.05 $\times$ 10 <sup>-2</sup>
5	M	P7	NGF-OE	0.061 $\pm$ 0.003*	3.51 $\pm$ 0.33	1.73 $\times$ 10 <sup>-2</sup> $\pm$ 0.09 $\times$ 10 <sup>-3</sup> *
5	F	P14	WT	0.021 $\pm$ 0.001	7.35 $\pm$ 0.24	2.90 $\times$ 10 <sup>-3</sup> $\pm$ 0.42 $\times$ 10 <sup>-2</sup>
5	F	P14	NGF-OE	0.063 $\pm$ 0.002*	7.24 $\pm$ 0.30	8.70 $\times$ 10 <sup>-3</sup> $\pm$ 0.67 $\times$ 10 <sup>-2</sup> *
5	M	P14	WT	0.022 $\pm$ 0.001	7.14 $\pm$ 0.35	3.11 $\times$ 10 <sup>-3</sup> $\pm$ 0.29 $\times$ 10 <sup>-2</sup>
5	M	P14	NGF-OE	0.073 $\pm$ 0.002*	7.66 $\pm$ 0.37	9.20 $\times$ 10 <sup>-3</sup> $\pm$ 0.54 $\times$ 10 <sup>-2</sup> *
5	F	P21	WT	0.022 $\pm$ 0.002	11.20 $\pm$ 0.24	1.97 $\times$ 10 <sup>-3</sup> $\pm$ 0.83 $\times$ 10 <sup>-2</sup>
5	F	P21	NGF-OE	0.066 $\pm$ 0.003*	11.22 $\pm$ 0.50	5.89 $\times$ 10 <sup>-3</sup> $\pm$ 0.60 $\times$ 10 <sup>-2</sup> *
5	M	P21	WT	0.021 $\pm$ 0.002	11.14 $\pm$ 0.40	1.89 $\times$ 10 <sup>-3</sup> $\pm$ 0.50 $\times$ 10 <sup>-2</sup>
5	M	P21	NGF-OE	0.069 $\pm$ 0.002*	11.35 $\pm$ 0.37	6.10 $\times$ 10 <sup>-3</sup> $\pm$ 0.54 $\times$ 10 <sup>-2</sup> *
5	F	P28	WT	0.026 $\pm$ 0.001	14.23 $\pm$ 0.24	1.80 $\times$ 10 <sup>-3</sup> $\pm$ 0.41 $\times$ 10 <sup>-2</sup>
5	F	P28	NGF-OE	0.078 $\pm$ 0.003*	14.92 $\pm$ 0.43	5.20 $\times$ 10 <sup>-3</sup> $\pm$ 0.69 $\times$ 10 <sup>-2</sup> *
5	M	P28	WT	0.028 $\pm$ 0.002	14.34 $\pm$ 0.33	1.95 $\times$ 10 <sup>-3</sup> $\pm$ 0.61 $\times$ 10 <sup>-2</sup>
5	M	P28	NGF-OE	0.089 $\pm$ 0.005*	14.95 $\pm$ 0.27	5.95 $\times$ 10 <sup>-3</sup> $\pm$ 1.85 $\times$ 10 <sup>-2</sup> *
5	F	P36	WT	0.028 $\pm$ 0.001	20.14 $\pm$ 0.24	1.39 $\times$ 10 <sup>-3</sup> $\pm$ 0.42 $\times$ 10 <sup>-2</sup>
5	F	P36	NGF-OE	0.084 $\pm$ 0.002*	20.12 $\pm$ 0.30	4.17 $\times$ 10 <sup>-3</sup> $\pm$ 0.67 $\times$ 10 <sup>-2</sup> *
5	M	P36	WT	0.030 $\pm$ 0.002	20.86 $\pm$ 0.34	1.44 $\times$ 10 <sup>-3</sup> $\pm$ 0.59 $\times$ 10 <sup>-2</sup>
5	M	P36	NGF-OE	0.099 $\pm$ 0.015*	20.25 $\pm$ 0.27	4.88 $\times$ 10 <sup>-3</sup> $\pm$ 0.56 $\times$ 10 <sup>-1</sup> *
5	F	A	WT	0.041 $\pm$ 0.001	25.30 $\pm$ 0.34	1.62 $\times$ 10 <sup>-3</sup> $\pm$ 0.29 $\times$ 10 <sup>-3</sup>
5	F	A	NGF-OE	0.127 $\pm$ 0.002*	25.92 $\pm$ 0.30	4.89 $\times$ 10 <sup>-3</sup> $\pm$ 0.67 $\times$ 10 <sup>-2</sup> *

N	Sex	Age	Genotype	Bladder Wt, g	Body Wt, g	Bladder Wt/Body Wt
5	M	A	WT	0.042 ± 0.002	25.14 ± 0.22	1.67 × 10 <sup>-3</sup> ± 0.91 × 10 <sup>-2</sup>
5	M	A	NGF-OE	0.126 ± 0.004*	26.22 ± 0.37	4.81 × 10 <sup>-3</sup> ± 0.11 × 10 <sup>-1</sup> *

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