Overexpression of NGF in mouse urothelium leads to neuronal hyperinnervation, pelvic sensitivity, and changes in urinary bladder function

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NGF IS A POTENT NEUROTROPHIN that exerts pleiotropic effects in the peripheral and central nervous system. It regulates sensory and sympathetic neuronal development and maintenance (41) and plays a role in painful somatic and visceral inflammation (3, 15, 32, 70, 84, 85). The effects of NGF are mediated through the TrkA and p75NTR receptors and are tissue specific. It is well documented that NGF plays an important role in inflammation of the urinary bladder, colon, and lung (21, 31, 93, 100). Although the contribution of NGF to urinary bladder function is unclear, it seems to play a role in urinary bladder hyperreflexia or overactivity (15, 16, 23, 34, 40, 78, 111). NGF administered intrathecally (107), intravesically (23), intramuscularly to the detrusor smooth muscle (111), or via adenovirus-mediated delivery in the urinary bladder (55) all induce rodent urinary bladder hyperreflexia and afferent neuronal hyperexcitability (107, 109). Conversely, sequestration of NGF or TrkA, as well as Trk inhibitors, reduce urinary bladder hyperreflexia in rodent models of experimentally induced urinary bladder inflammation (23, 40, 52). Elevated levels of neurotrophins have also been detected in the urine of women with interstitial cystitis (IC)/painful bladder syndrome (PBS) (79) and in the urothelium of individuals with IC/PBS or other painful bladder conditions (65). More recently, it was demonstrated that while 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) (49, 50, 59–64, 79, 106), they decreased in patients who responded to botulinum toxin-A or other medical treatments (60, 61, 63).

Chronic pathological conditions that cause tissue irritation or inflammation can alter the properties of sensory pathways, leading to a reduction in pain threshold and/or an amplification of painful sensation (hyperlgesia). Increased pain sensation can result from changes in the properties, density, and/or stimulation threshold of peripheral nociceptive afferents or from changes within the central pathways that process nociceptive inputs (89). NGF plays an important role in both somatosensory and visceral nociception (70, 84) and has been implicated in producing hyperalgesia by acting directly on sensory nerve endings or by indirectly increasing the expression of sensory neuropeptides, such as substance P and CGRP, neuropeptides known to mediate some of the peripheral and central effects of inflammation and to upregulate NGF expression. Consistent with this mechanism, exogenous delivery of NGF to the detrusor is associated with increased substance P and CGRP expression in central micturition pathways (111). NGF might also indirectly modulate nociceptive signaling by altering the expression of membrane ion channels such as TRPV1, P2X1, or voltage-gated sodium channels that are thought to play a central role in inflammation or tissue injury-induced pain and hypersensitivity (3, 84).

In the present study, we examined the role of NGF in urinary bladder dysfunction by generating a novel transgenic mouse model of urinary bladder hyperreflexia based on the hypothesis that chronic urothelial NGF overexpression would induce neu-
ronal hypersensitivity and increased urinary bladder reflex function. Chronic overexpression of NGF (NGF-OE) in the urothelium was achieved through the use of a highly urothelium-specific uroplakin II (UPII) promoter (56, 103, 104). Our findings revealed that urothelium-specific NGF overexpression in the urinary bladder of NGF-OE transgenic mice 1) stimulates neuronal sprouting or proliferation in the urinary bladder, 2) produces focal increases in mast cell infiltrates in the urinary bladder, 3) produces urinary bladder hyperreflexia, and 4) results in increased, referred somatic hypersensitivity.

MATERIALS AND METHODS

Generation of NGF-OE transgenic mice under the control of the uroplakin II promoter. To generate the UPII-NGFv2 transgene (Fig. 1A), a UPII promoter carrying vector was first generated by removing a LacZ-containing BamHI fragment positioned 3’ to the mouse UPII promoter in the plasmid pBluescript SKII+. The remaining pUPII vector contained a 3.6 kb 5’ upstream mouse UPII promoter fragment previously described (58). A 1579-bp BamHI-HindIII fragment containing a human β-globin polyA sequence was then subcloned from pBSKK-β-globin (17) into the BamHI-HindIII restriction sites 3’ of the UPII promoter in pUPI. Two NotI restriction sites were sequentially inserted by PCR at the 5’ end of the UPII promoter and at the 3’ end of the β-globin sequence (replacing the HindIII site), resulting in the pUPI-β-globin(N-N) vector containing a 5255 bp UPII-β-globin fragment flanked by NotI restriction sites. In parallel, an 817-bp fragment containing the full-length murine NGF cDNA, encoding the short transcript of NGF (27), was shuttled from pBSKK-mNGF (provided as a gift from Dr. Brian Davis, University of Pittsburgh) through pGEM-11Z (+) as an ApaI fragment. To generate the final UPII-NGFv2 transgene (Fig. 1A), the NGF coding sequence was excised as a BamHI-EcoRV fragment from the pGEM-11Z (+) shuttle vector and a BglII linker was ligated to the 3’ end of the fragment (New England Biolabs, Beverly, MA). The 820-bp BamHI-BglII NGF fragment was subcloned into the BamHI site of pUPI-β-globin(N-N). The UPII-NGF-β-globin transgene (UPII-NGFv2) was excised from NotI from the plasmid as a 6058 bp NotI fragment and fully sequenced to verify the sequence integrity. To generate NGF-OE transgenic founder (F0) mice, the UPII-NGFv2 fragment was purified and microinjected into the pronuclei of fertilized C57BL/6J (Jackson Laboratory, Bar Harbor, ME) embryos. All mice used in this study had the inbred genetic background C57BL/6J (Jackson Laboratories) and were derived from F2 to F4 generations maintained through a hemizygous backcross strategy to C57BL/6J wild-type (WT) mice. All animal use protocols were overseen and approved by the Roche Palo Alto Institutional Animal Care and Use Committee or by the University of Vermont Institutional Animal Care and Use Committee.

Genomic characterization of NGF-OE transgenic mice. Genotype confirmation of F0 and F1-F3 breeders was carried out by Southern blot analysis. For all other animals, genotyping was performed by PCR. Southern blot analysis was used to estimate the number of integration events, the orientation of the integrated transgene fragments, and the transgene copy number for each transgenic F0 line. Genomic DNA (3–5 μg) was digested with BamHI or Pst I and hybridized with a 350 bp NGF-specific probe generated from the purified transgene DNA fragment. The endogenous NGF gene and transgene integrations were characterized using the unique BamHI site within the transgene. Probing BamHI-digested DNA identified a 3.0-kb BamHI band indicative of the endogenous NGF gene and additional bands indicative of multiple transgene integration events (Fig. 1B). Transgene copy number was estimated using a Pst I digest that identified a 1.6-kb band indicative of the endogenous NGF gene and a 1.0-kb band indicative of the integrated transgene (Fig. 1B). Copy number was determined by densitometric scanning (Image Master, Amersham Biosciences, Piscataway, NJ) and comparing the calculated signal intensities of the 1.6- and 1.0-kb bands. The signal intensity of the 1.6-kb endogenous NGF band was defined as 1 for a single copy gene. For Southern blot analysis, DNA was electrophoresed through 1% agarose and transferred to Hybond N+ (Amersham Biosciences, Piscataway, NJ) and exposed to Kodak XOMAT film.

Genotyping of genomic tail DNA was performed using PCR as follows: 3 min at 94°C, 45 min at 94°C, 45 min at 60°C, 45 min at 72°C, 7 min at 72°C for 40 cycles. For NGF-OE mice, a 1103-bp transgene-specific fragment was amplified using the primers: RP41-NGF-S 5’-CAA GGC GTT GAC AAC AGA TG-3’; βEx3-AS 5’-CAG GCC ATT AGC ACC ACC CA-3’ (Fig. 1A). The 5’
primer is located within the NGF sequence and the 3’ primer is located within exon 3 of the β-globin sequence. Reverse transcriptase PCR analysis of RNA. Total RNA was extracted from the bladders of NGF-OE transgenic mice and WT littermate controls (n = 5 each) with RNA/mRNA STAT-60 isolation reagent (Tel-Test ‘B’, Friendswood, TX) and reverse-transcribed, as described previously (13). Briefly, 2 μg of total RNA was used to synthesize first-strand complementary DNA (cDNA) using SuperScript reverse transcriptase and oligo(dT20) primers with the SuperScript II Preamplification Kit (Invitrogen, Carlsbad, CA). Amplification of cDNA was performed with AmpliTaq DNA polymerase (Applied Biosystems, Norwalk, CT) and oligonucleotide primers specific for mouse NGF (13), according to the following parameters: initial denaturation and enzyme activation at 94°C for 10 min, denaturation at 94°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 45 s with varying cycles (30–35 cycles), and final extension at 72°C for 5 min. PCR products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Routine controls included cDNA synthesis in the absence of either RNA or reverse transcriptase, as well as amplification with omission of template or primers. Measurement of urinary bladder NGF by ELISA. NGF content in the urinary bladder of NGF-OE transgenic mice and WT littermate controls at different ages was determined using an ELISA, as previously described (13). Microtiter plates (R&D Systems, Minneapolis, MN) were coated with a mouse anti-rat NGF antibody (R&D Systems). Sample and standard solutions were run in duplicate. A horse-radish peroxidase-streptavidin conjugate was used to detect the antibody complex. Tetramethyl benzidine was the substrate, and the radish peroxidase-streptavidin conjugate was used to detect the antibodies, respectively. For whole-mount immunohistochemistry of the urinary bladder of NGF-OE transgenic mice and WT littermate controls. (10, 40, 111) on 12- to 16-wk-old anesthetized mice (n = 10) of both sexes. The urinary bladder was exposed through a lower midline abdominal incision under general anesthesia (isoflurane 2.5–3.5%). A saline-filled PE-10 cannula with the end flared by heat was inserted into the abdomen and connected to one port of a pressure transducer, the other port of the pressure transducer was connected to a syringe pump. Room-temperature saline was infused at a rate of 5 μl/min to elicit repetitive urinary bladder urothelium, whole bladders were isolated and opened from the base to dome. Tissue was stretched by pinning onto balsa wood with small dissection pins (Watkins, Doncaster, UK), while keeping it wet with PBS. The pinned tissue was fixed in 10% buffered formalin for 4 h at 4°C. After fixation, the urothelium was carefully separated from the underlying smooth muscle layer. The tissue was blocked with PBS 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 5% normal goat serum, 0.2% Triton X-100 overnight at 4°C. The following primary antibodies were used: a rabbit protein gene product (PGP) 9.5 antibody (1:6,000) (Ultraclone, UK), a rabbit calbindin gene-related peptide (CGRP) antibody (1:500) (Sigma-Aldrich, St. Louis, MO), 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 containing 0.1% BSA, 0.1% Triton-X-100, 4× for 15 min each at room temperature. The secondary antibody used for all whole-mount preparations was a goat anti-rabbit Alexa Fluor 488 antibody (1:200) (Molecular Probes) incubated for 4 h at room temperature in the same solution as the primary antibody. The tissue was washed 3× for 10 min each in PBS and mounted with mounting medium (Polysciences). Immunostaining was analyzed with a Nikon Microphot SA fluorescence microscope, and all scoring of immunoreactivity was performed in a blinded manner. Measurement of urinary bladder NGF by ELISA. NGF content in the urinary bladder of NGF-OE transgenic mice and WT littermate controls at different ages was determined using an ELISA, as previously described (13). Microtiter plates (R&D Systems, Minneapolis, MN) were coated with a mouse anti-rat NGF antibody (R&D Systems). Sample and standard solutions were run in duplicate. A horse-radish peroxidase-streptavidin conjugate was used to detect the antibody complex. Tetramethyl benzidine was the substrate, and the radish peroxidase-streptavidin conjugate was used to detect the antibodies, respectively. For whole-mount immunohistochemistry of the urinary bladder of NGF-OE transgenic mice and WT littermate controls.
bladder contractions. At least four reproducible micturition cycles were recorded after an initial stabilization period of 25 to 30 min. Voided saline was collected to determine void volume. After each void volume was collected, the infusion was stopped, and residual volume was determined by withdrawing the residual saline through the intravesical catheter. Intercontraction interval, maximal voiding pressure, pressure threshold for voiding, and baseline resting pressure were measured (68). The number of nonvoiding urinary bladder contractions (NVC) per voiding cycle, maximal NVC pressure, and frequency of NVC were assessed. For these studies, NVC were defined as rhythmic intravesical pressure rises (greater than 5 cm H2O from baseline pressure) without a release of fluid from the urethra. Mice were excluded from studies when adverse events occurred such as ≥20% reduction in body weight postsurgery, a significant postoperative adverse event, lethargy, pain, or distress not relieved by our IACUC-approved regimen of postoperative analgesics. In the present study, one WT and one NGF-OE transgenic mouse were excluded from the study or from analysis because of postsurgical lethargy. Behavioral movements such as grooming, standing, ambulation, and defecation also revealed bladder pressure of von Frey hairs to the indicated areas until the hair bent slightly. The following behaviors were considered positive responses to pelvic region stimulation: sharp retraction of the abdomen, jumping, or immediate licking or scratching of the pelvic area (90). A positive response to hindpaw stimulation was sharp withdrawal of the paw or licking of the tested hindpaw (90). All somatic testing was performed in a blinded manner with respect to treatment and mouse strain. The groups were decoded after data analysis.

**Isolated detrusor smooth muscle contraction.** Isometric smooth muscle contraction was studied in urinary bladder detrusor strips, as described previously (69). Briefly, urinary bladders with intact mucosal lining were removed from NGF-OE transgenic mice and WT littermate controls and dissected into Krebs buffer. Tissues were mounted between two parallel plate electrodes in thermostatically controlled (37°C) organ baths (10 ml) containing Krebs buffer, gassed continuously with 95% O2 and 5% CO2. The composition of the buffer was (in mM): 118.2 NaCl, 4.6 KCl, 1.6 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 10 dextrose, and 24.8 NaHCO3. The detrusor strips were equilibrated at a resting tension of 0.5 g for 1 h with intermittent washing, followed by a KCl (67 mM) prime. KCl-induced contractions were used to normalize the data to control for differences in absolute contractile values resulting from differences in tissue size or health. Changes in isometric force were measured by Grass FTO3c transducers (Grass Instruments, Quincy, MA) and digitized using MacLab data acquisition software (ADInstruments, Colorado Springs, CO). To measure neurogenic-mediated contraction, a train of square electrical pulses was applied for 0.5 s with pulse frequency increasing in twofold increments (10 V with a pulse width of 0.5 ms). Pulses were delivered by a Grass S88 stimulator and divided across the tissue baths using a MedLab StimuSplitter (Grass Telefactor, West Warwick, RI). Electrically induced contractions were confirmed to be neurogenically mediated by their sensitivity to tetrodotoxin (100 nM). Neurogenic contractions were plotted by fitting a nonlinear equation. In experiments in which carbachol was used to induce contraction, cumulative addition of the drug was applied (99). The potency of carbachol was determined by fitting mean contractions for each concentration of the drug to the following four-parameter logistic equation, defined by response (percentage contraction to KCl) = bottom + (top-bottom)/(1 + 10(logEC50–[carbachol])/nH), where nH is the Hill Slope and EC50 is the concentration required for half the maximal effect.

**RESULTS**

**Generation and genomic characterization of NGF-OE transgenic mice.** NGF-OE transgenic mice were generated using the UPII-NGF2 construct shown in Fig. 1A with the aim of establishing urothelium-specific overexpression of NGF in the urinary bladder. To direct the expression of NGF in the urinary bladder, we used a 3.6-kb, 5-upstream promoter fragment from the mouse UPII gene (58). This promoter fragment has been shown to faithfully direct the expression of the LacZ gene to the urothelium, primarily to the apical umbrella and intermediate cells (58), with no expression detected in other tissues/organs including skin, lung, liver, intestine, kidney (excluding renal pelvis), spleen, heart, endothelium, or muscle (47, 58).

Multiple, independent F0 lines carrying the UPII-NGF2 transgene were established on the C57BL/6j background and a characterization of the genomic integration pattern and copy number for the NGF-OE transgenic F0 lines 6, 20, and 23 is shown in Fig. 1B. Transgene integration was evaluated by Southern blot analysis of BamHI-digested genomic DNA. Probing with an NGF-specific probe revealed a 3.0-kb BamHI fragment indicative of the endogenous NGF gene in both WT and NGF-OE transgenic lines (Fig. 1B). Additional bands representing unique transgene integration events were present in all transgenic F0 lines, with a major ~6.1-kb fragment, suggestive of a head-to-tail orientation of the integrated transgenes (Fig. 1B). Transgene copy number was estimated by probing Pst1-digested genomic DNA with an NGF-specific probe (Fig. 1B) and comparing the signal intensity of the 1.0-kb transgene-specific band with that of the 1.6-kb band representing the single copy endogenous NGF gene. The transgene copy number for NGF-OE lines 6, 20, and 23 was estimated to be 6.4, 1.0, and 5.8, respectively. All F0 mice transmitted transgenes to offspring in a Mendelian fashion. Mice derived from F0 line 23 were used for all of the studies presented with specific findings confirmed using mice derived from F0 line 6.

**Increased NGF mRNA and protein expression in NGF-OE transgenic mice.** To determine whether ectopic NGF mRNA was selectively expressed in the bladder urothelium of transgenic mice, we measured total NGF mRNA by RT-PCR in multiple tissues of NGF-OE transgenic mice and WT littermate controls (Fig. 1C). Endogenous NGF mRNA was present in the urinary bladder and brain, but not the liver (Fig. 1C, data not shown), of both WT and transgenic mice. Although NGF mRNA was present at similar levels in the urothelium and detrusor smooth muscle of WT mice, the level of total NGF mRNA
mRNA was much higher in the urothelium compared with the detrusor smooth muscle of NGF-OE mice, consistent with our previous findings (13). PCR reactions performed without reverse transcriptase were negative, indicating that the amplicons detected were derived from cDNA and not contaminating genomic DNA (data not shown).

The amount of NGF protein in the whole urinary bladder was measured by ELISA in NGF-OE transgenic mice and WT littermate controls at several postnatal (P) ages, including days P7-P10, 5–6 wk, and 12–16 wk (Fig. 2). At each postnatal age examined, the amount of NGF in the urinary bladder NGF-OE mice was significantly \( P \leq 0.01 \) greater than that detected in WT mice (Fig. 2). In addition, the NGF content in the urinary bladders of NGF-OE transgenic mice increased progressively with age, as illustrated in Fig. 2.

We further evaluated NGF expression in the urinary bladder by immunolocalization to determine whether ectopic NGF was present in the UPII-expressing urothelial cells of NGF-OE transgenic mice. In the WT mice, NGF immunoreactivity was detected at low to moderate levels in the basal and intermediate cell layers of the urothelium but was undetectable in the apical urothelial umbrella cells (Fig. 3, A, C, E). In contrast, NGF immunoreactivity was present and appeared to be increased in all cell layers of the urothelium in the NGF-OE transgenic mice (Fig. 3B), including a subpopulation of intermediate-to-apical urothelial cells (Fig. 3, B, D, F, see arrows), where it was colocalized with UPII (Fig. 3, D and F). UPII immunostaining was most prominent in the apical cell layer of the bladder urothelium in both WT (Fig. 3, C and E) and transgenic (Fig. 3, D and F) mice, but it was also present in all suprabasal cell layers of the urothelium consistent with its known pattern of expression in the mouse (58, 73). Although we could detect NGF immunoreactivity in apical urothelial cells of transgenic mice at postnatal day 7 (Fig. 3), we were unable to detect ectopic NGF immunoreactivity in these cells in 8- to 10 wk-old transgenic mice (data not shown). The reasons for this discrepancy are unclear but could be due to limitations in the sensitivity of NGF immunolocalization in these tissues or to rapid uptake and retrograde transport of secreted NGF by suburothelial sensory afferents. It is also possible that, unlike uroplakin II proteins, which are stable after they are assembled in the umbrella cells, NGF may be less stable in the mature and highly specialized urothelial umbrella cells.

Pathophysiological changes in the urinary bladders of NGF-OE transgenic mice. NGF-OE transgenic mice from F0 lines 23 and 6 developed normally with no adverse clinical signs or altered behaviors once the breeding colonies were stabilized after \(-1\) year (see below). Blood chemistry and hematology profiles were also similar between 8- and 10-wk-old WT and transgenic mice. However, a consistent finding in male and female NGF-OE mice of both transgenic F0 lines was urinary bladder enlargement (Fig. 4, B and D), resulting in a significant increase in urinary bladder weight and a 2- to 3-fold increase in bladder-to-body weight ratios compared with WT mice (Table 1). Total milligrams of bladder protein was also significantly increased in transgenic compared with WT mice.
ranging from a 1.9-fold increase at age P7-P10 (means ± SE: 2,427.4 ± 120 vs. 1,312.4 ± 95 for transgenic and WT mice, respectively; \( n = 5–8, P \leq 0.001 \)) to a 1.5-fold increase at 12–16 wk of age (means ± SE: 3,715.2 ± 185 vs. 2,543 ± 175 for transgenic and WT mice, respectively; \( n = 5–8, P \leq 0.001 \)). H&E- (data not shown) and Masson Trichrome-stained (Fig. 4, A–D) tissues indicated that this urinary bladder enlargement was due to transmural nerve fiber hyperplasia/expansion, particularly in the suburothelial mucosa (Fig. 4B, arrows), although enlarged nerve bundles were also present within the detrusor smooth muscle tissue and on the serosal surface of the urinary bladder. There was no evidence of urothelial hyperplasia in the bladders of transgenic mice despite the expression of NGF by urethelial cells. In addition, although marked nerve fiber hyperplasia was present in the bladder, other lower urinary tract tissues lined by UPII-expressing urothelium, such as the ureters and renal pelvis were morphologically normal (data not shown).

As mentioned above, the NGF-OE transgenic colonies stabilized after 1 yr of breeding. During the first year, death was observed in a small proportion of mice (both sexes) from F0 line 23 (~12–14%) and F0 line 6 (15–16%) at ~2 mo of age compared with a death rate of ~1% for the WT littermates. Moribund NGF-OE transgenic mice had distended abdomens and severe bilateral hydronephrosis with renal pelvic dilation, an indication that the likely cause of death in these mice was obstructive renal failure (data not shown). Histological assessment of these mice revealed marked nerve fiber hyperplasia throughout the lower urinary tract, including the ureters, urinary bladder, and proximal urethra (data not shown), suggesting that these neuronal changes may have contributed to renal pelvic dilation. A likely explanation for these findings was that high levels of ectopic NGF were more broadly expressed throughout the urothelium of the lower urinary tract in some early transgenic animals, resulting in an exaggerated phenotype. This is not inconsistent with the expression pattern of endogenous UPII in the mouse, or of several transgenes expressed under the control of the 3.6-kb UPII promoter (12, 47, 57, 58, 73, 105). Unique insertional events or other epigenetic factors might have contributed to this heterogeneity of transgene expression with some mice expressing a higher level of NGF throughout the lower urinary tract. Since this resulted in a phenotype incompatible with survival, it is likely the colony underwent a process of natural selection yielding increasingly viable NGF-OE transgenic mice.

Elevated mast cell counts in the urinary bladders of NGF-OE transgenic mice. In addition to its role as a neurotrophic factor, NGF is also involved in inflammatory processes and is a maturation factor for mast cells (56). To determine whether the histopathological changes in the urinary bladders of transgenic mice were associated with an increased local inflammatory response, we compared the number of mast cells in the bladders of WT and NGF-OE transgenic mice. As shown in Table 2, mast cells were significantly increased in the urinary bladders of the transgenic vs. WT mice (means ± SE: 27.3 ± 6.39 vs. 0.3 ± 0.29 per 4-μm bladder section for female and 40.0 ± 5.63 vs. 8.3 ± 4.84 per 4-μm bladder section for male transgenic and WT mice, respectively; \( n = 3–4 \) per group). Increased numbers of mast cells were observed in both the detrusor smooth muscle and suburothelial regions of transgenic mouse bladders, particularly in perivascular triad

### Table 1. Urinary bladder and body weights of WT and NGF-OE transgenic mice

<table>
<thead>
<tr>
<th>N</th>
<th>Sex</th>
<th>Genotype</th>
<th>Bladder Wt, g</th>
<th>Body Wt, g</th>
<th>Bladder Wt/ Body Wt</th>
<th>Fold Change*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>F</td>
<td>WT</td>
<td>0.019 ± 0.001</td>
<td>20.06 ± 0.54</td>
<td>9.47 × 10⁻⁴ ±0.41 × 10⁻⁴</td>
<td>2.2</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>NGF-OE</td>
<td>0.042 ± 0.002**</td>
<td>19.56 ± 0.50</td>
<td>21.32 × 10⁻⁴ ±1.49 × 10⁻⁴**</td>
<td>3.3</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>WT</td>
<td>0.026 ± 0.001</td>
<td>25.03 ± 0.50</td>
<td>10.28 × 10⁻⁴ ±0.59 × 10⁻⁴</td>
<td>3.3</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>NGF-OE</td>
<td>0.073 ± 0.015*</td>
<td>22.58 ± 0.67*</td>
<td>33.30 × 10⁻⁴ ±7.49 × 10⁻⁴**</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Values are mean ± SE for 8-wk-old WT and NGF-OE mice from F0 line 23. *P < 0.05. **P < 0.005. Statistical comparison of WT and NGF-OE mice using an unpaired t-test. *Fold change in bladder/body weight ratios between WT and NGF-OE mice.
areas containing veins, arteries, and nerves in the subepithelial, muscularis, and adventitial tissues. Mast cells were replete with cytoplasmic granules, indicating a lack of degranulation activity. There was also no evidence of interstitial edema, vasodilation, or secondary inflammatory cell infiltrate (data not shown). The absence of obvious edema or a generalized inflammatory cell infiltrate in the urinary bladders of transgenic animals (Fig. 4B, D and data not shown), and the presence of normal peripheral blood white blood cell counts (data not shown), suggests that NGF overexpression caused focal increases in urinary bladder mast cells in the absence of a more generalized local or systemic inflammatory response.

Sensory and sympathetic hyperinnervation in the urinary bladders of NGF-OE transgenic mice. To further characterize the subpopulations of neurons contributing to the generalized hyperinnervation observed in NGF-OE transgenic mice, we performed whole-mount immunostaining of bladder urothelium using a panel of neuronal markers. Using the pan-neuronal marker PGP 9.5, a marked increase in total nerve fiber density was evident in the urinary bladders of transgenic vs. WT mice (Fig. 5, A and B), consistent with the histology stains. This dense network was composed of CGRP- (Fig. 5, C and D) and substance P-positive (Fig. 5, E and F) unmyelinated C-fiber sensory afferents, NF200-positive myelinated sensory afferents (Fig. 5, G and H) and TH-positive, postganglionic sympathetic nerve fibers (Fig. 5, I and J). The increased nerve fiber density observed in the transgenic mouse bladders was evident in both the neck (Fig. 5) and the dome (data not shown) region 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 (33) in both WT and transgenic mice (data not shown). With CGRP immunostaining, we also observed CGRP-positive ganglia (5–9 CGRP-positive cells per ganglia) interspersed among CGRP-positive nerve fibers within the suburothelial plexus in transgenic mice (Fig. 5, K and L). These CGRP-positive ganglia were not observed in whole-mount preparations of WT urinary bladder (data not shown). A phenotypically similar increase in nerve fiber density was observed in transgenic mice from F0 line 6.

Altered urinary bladder function in NGF-OE transgenic mice. To determine whether the urinary bladder hyperinnervation observed in transgenic mice resulted in changes in urinary bladder function, we performed a series of urodynamic studies on NGF-OE transgenic mice and WT littermate controls. Figure 6 shows representative open voiding cystometrograms in conscious, unrestrained mice, in which voiding reflexes were measured in response to a continuous infusion of saline. In contrast to the WT littermate controls, in which normal micturition contractions were observed (Fig. 6A), the NGF-OE mice had urinary bladder hyperreflexia (Fig. 6B) with significantly reduced void volumes and intercontraction intervals compared with the WT mice (P ≤ 0.001, Fig. 7, A and B). The reductions in void volume and intercontraction interval were present in both male and female transgenic mice with a similar magnitude of change; thus, data from both sexes were pooled and analyzed together. No changes in baseline resting pressure, micturition threshold pressure, or maximal voiding pressure were observed between WT and NGF-OE mice (Fig. 7C). There were also no differences in the residual volume between WT and NGF-OE mice (means ± SE: 5 ± 3 μl vs. 4 ± 2.5 μl, respectively).
Nonvoiding urinary bladder contractions (NVC) were present in WT and NGF-OE mice under conscious cystometry conditions but were not detected with each voiding cycle for either group (Fig. 8 and data not shown). However, the number of NVCs/voiding cycle, the amplitude of NVCs, and the frequency of occurrence of NVCs/voiding cycle were significantly increased in transgenic compared with WT mice (P < 0.01; Fig. 9, A–C).

To determine whether the intrinsic contractile properties of the detrusor smooth muscle were also altered in transgenic mice, we measured cholinergic- and neurogenic-mediated contraction of detrusor smooth muscle strips in vitro in 8-wk-old NGF-OE transgenic mice and WT littermate controls. Similar concentration-dependent contractions to carbachol and similar neurogenic-mediated contractile responses to electrical field stimulation were observed between WT and NGF-OE mice (data not shown), suggesting that detrusor contractility and efferent neuronal sensitivity were unaltered in transgenic mice.

### Increased somatic hypersensitivity in the pelvic region of NGF-OE transgenic mice
Increased sensitivity to somatic stimuli, in the form of referred hyperalgesia and tactile allodynia, has been noted in the presence of visceral inflammation (34, 53, 54, 90). In separate groups of mice, we evaluated mechanical somatic sensitivity using a calibrated series of von Frey hairs on the plantar region of the hindpaw and the pelvic region overlying the urinary bladder in NGF-OE transgenic mice and WT littermate controls. Somatic sensitivity in the pelvic region, as evidenced by sharp retraction of the abdomen, jumping, or immediate licking or scratching of the pelvic area was significantly increased (P < 0.001) in transgenic mice with von Frey hairs of all forces tested (0.1–4 g) compared with WT controls (Fig. 10). In contrast, in the plantar region of the hindpaw, no differences in the paw pressure threshold that elicited a sharp withdrawal of the paw or licking of the tested hindpaw were seen between WT and transgenic mice (Fig. 10).
We describe here a transgenic mouse model of urothelium-specific NGF overexpression that represents a novel approach to exploring the role of NGF in urinary bladder inflammation and sensory function. NGF is a potent neurotrophic factor for sensory afferent and sympathetic nerve fibers and excitatory changes in these neurons underlie NGF-mediated hypersensitivity in a number of target tissues (22, 41, 70, 84, 88). Accordingly, the fundamental premise of our study was that chronic NGF overexpression in the urinary bladder would result in neuronal hyperinnervation and hypersensitivity, leading to changes in urinary bladder function. The major findings observed were that NGF overexpression led to marked hyperinnervation of the urinary bladder by sensory afferent and sympathetic nerve fibers, as well as an increased number of urinary bladder mast cells, consistent with the known survival and trophic actions of NGF on these cells (4, 56). Functionally, NGF-OE mice had urinary bladder hyperreflexia with frequent urination and the presence of nonvoiding bladder contractions, as well as referred somatic pelvic hypersensitivity. On the basis of these findings, we propose that the NGF-OE transgenic mice represent a novel animal model of urinary bladder hypersensitivity exhibiting many of the clinical features of urinary bladder dysfunction in man.

A notable feature of the NGF-OE mice is the highly urothelium-specific expression of ectopic NGF. Total NGF mRNA in the bladder urothelium/suburothelium of transgenic mice was significantly increased over that observed in WT mice, with no differences detected in the detrusor smooth muscle, consistent with our previous report (13). Bladder NGF was increased in transgenic mice from as early as postnatal days 7–10 through adulthood, consistent with the reported onset of expression of the mouse uroplakin II gene (44). Ectopic NGF was also detected mainly in the suprabasal cell layers of the bladder urothelium, consistent with the expression pattern of the endogenous mouse UPII protein (12, 58, 73). Although we were able to detect NGF immunoreactivity in apical urothelial cells of transgenic mice on days 7–10, we could not detect NGF in these cells in 8- to 10-wk-old transgenic mice. The reasons for this discrepancy are unclear, but one intriguing possibility is that ectopic NGF secreted by the urothelial cells underwent rapid uptake and retrograde transport by proximal TrkA-expressing nerve fibers—a process that might be markedly heightened in the context of the hyperinnervation present in the urinary bladders of NGF-OE mice. Given the potent neurotrophic actions of NGF, this could explain how the apparent low levels of ectopic NGF produced mainly by the urothelial cells in NGF-OE mice could cause such a striking nerve fiber proliferation within the suburothelial mesenchymal tissue layer.

In addition to the urinary bladder, the ureters, renal pelvis of the kidney, and proximal urethra are lined by UPII-expressing urothelium (57, 105). Expression of transgenes under the control of the UPII promoter has been noted in these tissues (12, 58, 73), and a similar pattern of NGF expression might be expected in the NGF-OE mice. Evidence of this was seen in...
CYP (52, 75, 100) or intravesical acrolein (35) also produced urinary bladder inflammation and hypersensitivity induced by pathological changes in bladder sensory and motor neurons (26). Transgenic mice had a significantly (*P ≤ 0.001) increased pelvic response frequency with all von Frey hairs (0.1–4 g) tested compared with WT mice. Hindpaw sensitivity testing with calibrated von Frey hairs was determined in WT and NGF-OE transgenic mice. No differences in the paw pressure threshold required to elicit a sharp withdrawal of the paw or licking of the tested hindpaw (90) were detected between WT and transgenic mice. Data represent the mean ± SE of n = 8 mice per group. All somatic testing and behavioral scoring were performed in a blinded manner with respect to treatment and mouse strain. Pts., points.

Some mice early in the development of our transgenic lines, where hyperinnervation of the ureters, urinary bladder, and proximal urethra was observed, possibly contributing to the renal failure and death in ~12–16% of these transgenic mice. This heterogeneous phenotype stabilized over time, and mice from the current transgenic colony (F0 line 23) have no increased mortality compared with WT littermate controls and have hyperinnervation predominantly in the urinary bladder; lower urinary tract organs, such as the ureters and kidneys, are morphologically normal. A possible explanation for this change is that NGF-OE mice underwent a process of natural selection, yielding increasingly viable mice with a reduced or more bladder-restricted expression of ectopic NGF. This would be consistent with the fact that bladder urothelium is known to express ~10 times more uroplakins than the ureteral urothelium (57, 105).

NGF-OE mice may represent a useful animal model for OAB or IC/PBS because the increased NGF content and other sensory and local mast cell changes observed in the urinary bladders of these mice are consistent with certain clinical changes observed in these lower urinary tract disorders. Altered NGF content is associated with urinary bladder inflammation and dysfunction in both rodents and humans in which it may underlie neurochemical (10, 51, 102, 112), organizational (101, 103), and electrophysiological (108) changes that can affect micturition reflex pathways. In rodents, urinary bladder inflammation induced by noxious chemical or mechanical stimuli increased NGF mRNA and/or protein in the urothelium or detrusor smooth muscle (9, 26, 78) and caused morphological changes in bladder sensory and motor neurons (26).

Urinary bladder inflammation and hypersensitivity induced by CYP (52, 75, 100) or intravesical acrolein (35) also produced changes in NGF or NGF receptor (TrkA and p75NTR) expression in micturition reflex pathways. NGF was also increased in hypertrophied bladders following spinal cord injury or BOO (26, 78, 94, 95, 100, 110), and in bladders of spontaneously hypertensive rats (16), in which it appears to correlate with neuronal hypertrophy (95) and persistent DO (16, 48). Clinically, increased NGF levels have been detected in the bladder urothelium of patients with IC/PBS (65), although one study found no association between increased urothelial NGF and DO (8). Elevated NGF levels were also detected in the urine of patients with IC/PBS or OAB symptoms associated with DO, stress urinary incontinence, or BOO (49, 50, 59–64, 79, 106), and patients with DO who responded to treatment had reduced urinary NGF levels (60, 61, 63).

Increased urinary bladder NGF content may underlie many of the sensory changes that occur in patients with OAB symptoms or IC/PBS, including irritative voiding symptoms and pain in the case of IC/PBS. IC/PBS is a chronic inflammatory bladder disease of unknown etiology characterized by urinary frequency, urgency, and suprapubic/pelvic pain (25, 91). Pain and altered bladder/visceral hypersensitivity in IC/PBS patients may involve organizational or functional changes in peripheral bladder afferents and central pathways, such that bladder afferent neurons become sensitized and hyperresponsive to normally innocuous stimuli, such as bladder filling (25, 91). 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 patients with IC/PBS (14, 38, 66, 80–82). Mast cells and other inflammatory mediators that are responsive to NGF (56), and increased in the urinary bladder of IC/PBS patients (67, 83, 92), might further sensitize bladder afferent neurons. In NGF-OE mice, chronic NGF overexpression resulted in marked hyperinnervation of the bladder by sensory afferent and sympathetic nerve fibers and an increase in bladder mast cells. These changes were associated with urinary bladder hyperreflexia, increased nonvoiding bladder contractions, and pelvic hypersensitivity without increased hindpaw sensitivity, consistent with the presence of ongoing, referred somatic hyperalgesia of the pelvic region in conjunction with bladder hyperreflexia. A common feature of visceral pain is that it is often referred to distant visceral or somatic sites and patients with IC/PBS often suffer from nongenitourinary chronic pain syndromes and experience increased mechanical hypersensitivity to somatic stimuli (1, 29, 76, 77). Thus, the morphological and functional features of NGF-OE transgenic mice reflect many of the changes observed in micturition reflex pathways in patients with OAB symptoms or IC/PBS.

Our findings support and extend many previous studies in rodents demonstrating the involvement of NGF in altered bladder sensory function and the development of referred hyperalgesia in response to bladder inflammation. Early studies established that intravesical NGF sensitized C-fiber and A8 bladder sensory afferents (22). Intrathecal NGF increased NGF expression in bladder afferents, increased bladder afferent hyperexcitability, and caused urinary bladder hyperreflexia, indicating that increased NGF levels in the afferent limb of the micturition reflex contributes to changes in bladder sensory function (107). Increased bladder NGF expression via adenoviral delivery (55) also increased voiding frequency, while chronic administration of NGF into the bladder wall (111)
increased voiding frequency, augmented central responses to bladder distension (increased c-Fos), and increased CGRP expression in the bladder and lumbosacral spinal cord, suggesting an NGF-mediated reorganization of micturition reflex pathways. In contrast, sequestration of NGF using a TrkA-IgG fusion protein (23) or the NGF scavenging agent REN1820 (40) reduced urinary bladder hyperreflexia (23, 40) and pain behaviors (40) following chemical irritant-induced inflammation. Urinary bladder inflammation and hyperactivity induced by CYP (34) or intravesical turpentine (42), acrolein (35), or NGF (34) also triggers somatic hyperalgesia referred to the hindpaw, and this is attenuated by blockade of NGF by TrkA-IgG (42) or immunoneutralization (35), or by blockade of Trk receptors using the nonselective tyrosine kinase inhibitor K252a (34, 35). In one recent study (7), urinary bladder NGF overexpression led to enhanced responses to colorectal distension in addition to somatic mechanical and thermal hyperalgesia referred to the hindpaw, suggesting a role for NGF in the development of referred visceral and somatic hyperalgesia.

A striking feature of the NGF-OE transgenic mice is the marked hyperinnervation of the urinary bladder by sensory afferent and sympathetic nerve fibers, likely resulting from the chronic overexpression of NGF starting from early postnatal development. This is consistent with the idea that NGF functions as a target-derived trophic factor capable of stimulating or modulating neuronal development, growth, and survival (41). It is also consistent with the findings from several different tissue-specific NGF transgenic mouse lines, in which NGF overexpression in pancreatic islets, pulmonary airways, skin, heart, brain, and smooth muscle resulted in the common phenotypic finding of increased sprouting by sympathetic efferent and sensory afferent nerve fibers to target organs and in many cases associated ganglion (2, 20, 27, 28, 36, 37, 39, 45, 46, 104).

It is reasonable to propose that the sensory and sympathetic hyperinnervation observed in NGF-OE mice contributed to the development of urinary bladder hyperreflexia and referred pelvic hypersensitivity, and several potential mechanisms could be proposed. The increased suburothelial innervation by substance P, CGRP, and NF200 immunoreactive nerve fibers suggests an increase in unmyelinated C-fiber and myelinated Aδ sensory afferents. Substance P and CGRP are released peripherally and centrally following the activation of sensory afferents and can initiate local neuroinflammatory responses and enhance sensory neuron excitability. Sensory information from the bladder is conveyed by pelvic and hypogastric/lumbar splanchnic afferents with cell bodies located in lumbosacral and thoracolumbar dorsal root ganglia (DRG), respectively (6). Both lumbosacral and thoracolumbar bladder afferents become sensitized under conditions of bladder/visceral inflammation and contribute to facilitation of the micturition reflex and visceral nociception (18, 19, 22, 70–72, 74). Interestingly, hypogastric/splanchnic afferents are reported to be more responsive to chemical stimuli than pelvic afferents (74), tend to be concentrated in the submucosa and bladder neck (98), and are largely (>90%) unmyelinated nociceptive-type C-fibers that express CGRP and bind the isolecint IB-4 (18). It remains to be determined whether NGF overexpression caused additional phenotypic or excitability changes in bladder sensory afferents that might contribute to the bladder hyperreflexia and pelvic hypersensitivity in NGF-OE mice. Growth factors, such as BDNF, and ion channels, such as TRPV1, P2X3, P2X2/3, or Nav1.8 (3, 84), can directly modulate pain and bladder/visceral sensory function and NGF-mediated changes in these mediators, either peripherally or centrally, could contribute to altered urinary bladder function in NGF-OE mice (30, 84, 97, 109). Studies investigating the transcriptional plasticity of these sensory mediators in micturition reflex pathways in NGF-OE mice are in progress.

Hyperinnervation by TH-positive, postganglionic, sympathetic efferents was also evident in the NGF-OE mice. A potential consequence of this hyperinnervation could be the establishment of sympathetic-sensory coupling either in the periphery or at the level of the DRG (43, 88). Sympathetic axon sprouting occurs in response to nerve/tissue injury or inflammation, and sympathetic-sensory coupling can lead to sensitization of peripheral sensory neurons (e.g., via sympathetically mediated neurogenic inflammation) (43). Aberrant sympathetic projections to primary sensory neurons may provide a mechanistic basis for the maintenance of chronic pain and hypersensitivity, and NGF is believed to play a critical role in promoting these changes (88). Anti-NGF treatment attenuated collateral sprouting into the DRG and pain-related behaviors in nerve injury-induced models of neuropathic pain (86). Transgenic mice overexpressing NGF in the skin (2, 20, 36) or glia (45, 104) develop a similar pattern of sympathetic sprouting into target tissues and sensory ganglia, and GFAP-NGF mice showed enhanced thermal and mechanical hypersensitivity, as well as sympathetic sprouting following neuropathic nerve injury (87). Thermal hyperalgesia induced by intradermal NGF in the hindpaw was also partially dependent on sympathetic postganglionic axons (5). We did not investigate whether sympathetic sprouting into DRG innervating the urinary bladder was present in NGF-OE mice; however, the dramatic hyperinnervation/sprouting into the bladders of these mice suggests this possibility. Future studies aimed at characterizing morphological and functional changes in the sensory ganglia of these mice will address this question. Sympathetic hyperinnervation in NGF-OE mice might also suggest an increase in bladder compliance, or urethral outlet resistance, as sympathetic efferents facilitate bladder storage via excitatory α-adrenergic receptors in the urethra and bladder neck and possibly via inhibitory β-adrenergic receptors in the bladder. We saw no evidence for these changes in NGF-OE mice, but additional studies to evaluate potential morphological/functional changes in the urethra would be of interest. Future studies should also address whether the observed bladder hyperinnervation in NGF-OE mice results from an increased number of neurons projecting to the bladder (via increased proliferation or rescue from apoptotic cell death early in development) or to enhanced sprouting of peripheral terminals, or both. Bladder afferent dye labeling and bladder nerve transection studies will also further elucidate the contribution of sensory afferent and sympathetic pathways to the NGF-mediated alterations in bladder sensory function in NGF-OE mice.

Perspectives and Significance

The NGF-OE transgenic mice described here represent a novel animal model of NGF-mediated urinary bladder dysfunction caused by chronic urothelial-specific overexpression of...
NGF OVEREXPRESSING MICE DEVELOP URINARY BLADDER HYPERACTIVITY

NGF. Our findings support and extend many previous studies in rodents demonstrating a role for NGF in mediating changes in urinary bladder function or referred hyperalgesia in response to inflammation or tissue injury. Although most rodent studies to date have focused on transient, exogenous administration of NGF into the bladder, or the use of NGF or NGF receptor blockade in models of chemical/irritant-induced bladder dysfunction, the NGF-OE transgenic mice described here offer a genetically stable model of chronic NGF overexpression from early postnatal development. The phenotype of these mice uniquely reflects many of the known roles of NGF in modulating the growth/maintenance of sensory and sympathetic nerve fibers, local mast cell responses, urinary bladder hyperreflexia, and referred hyperalgesia. Accordingly, the NGF-OE transgenic mouse model should afford considerable new opportunities for exploring the role of NGF in urinary bladder and visceral-somatic sensory function.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


