Experimental Neurology 285 (2016) 110–125

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

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

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ARTICLE INFO

*Article history:*
Received 11 February 2016
Received in revised form 30 May 2016
Accepted 20 June 2016
Available online 21 June 2016

*Keywords:*
Postnatal development
Substance P
Neurokinin-1 receptor antagonist
Suburothelial nerve plexus

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 ($R^2 = 0.996–0.998$; $p \leq 0.01$) increases in Sub and CGRP expression in the urothelium and significantly ($p \leq 0.01$) 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 μg/ml), significantly ($p \leq 0.01$) 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.



## 1. 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

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http://dx.doi.org/10.1016/j.expneurol.2016.06.021
0014-4886/

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, 2000c, 2000d). 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.

## 2. Materials and methods

### 2.1. 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 ($p \leq 0.01$) 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.

### 2.2. 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 microgram of RNA per sample was used to synthesize complementary DNA using a mix of random hexamer and oligo dT primers with M-MLV reverse transcriptase (Promega Corp.) in a 25-μl final reaction volume. The quantitative PCR standards for all transcripts were prepared with the amplified cDNA products ligated directly into pCR2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen). The nucleotide sequences of the inserts were verified by automated fluorescent dideoxy dye terminator sequencing (Vermont Cancer Center DNA Analysis Facility). To estimate the relative expression of the receptor transcripts, 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., 2010) 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′-CGGTGCCAACGATGAT CTAAA-3′); reverse primer (5′-ACGCCTTCTTTCGTACTTCTG-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