The Developmental Maturation of Piezo1 and Piezo2 Mechanosensors in the Micturition Reflex in Mice

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Linna Lahmadi

The University of Vermont

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Defense Committee

Margaret A. Vizzard, Ph.D. (Advisor)

Jaeda Coutinho-Budd, Ph.D. (Chair)

Nathan Jebbett, Ph.D.

Béatrice Girard, Ph.D.

Abstract

A commonly overlooked neurophysiological danger neonates face is infantile bladder dysfunction. Abnormalities in the micturition reflex of newborns can cause a multitude of detrimental symptomatic diseases including early onset urinary incontinence, urinary tract infections, underactive bladder function, enuresis, and renal failure. The pathophysiological etiologies of these disorders have been associated with urinary bladder tissue-localized protein disruption owing to deviations in regulated tension-modulating protein expression. The presence of mechanosensory proteins Piezo1 and Piezo2 in non-specified bladder tissue of neonatal mice as well as their functional roles in adult murine models have been suggested. In this study I evaluated and quantitively determined the relative genetic transcript expression of Piezo1 and Piezo2 specifically in the urothelial and detrusor smooth muscle layers of the urinary bladder in postnatal mice. Piezo1 and Piezo2 relative mRNA expression was determined using quantitative polymerase chain reaction (Q-PCR) of complementary DNA (cDNA) transcripts. Piezo1 and Piezo2 plasticity in the mucosal tissue across postnatal development was ascertained. Heightened expression was observed in early stages of development with subsequent age-dependent decrease until complete subject maturation with statistical significance as follows: (1) Piezo1, postnatal day (P) 9 vs. P21, 28, and 36 ($P < 0.05$), (2) Piezo2, P6 vs. P28 ($P < 0.05$), P9 vs. P21 ($P < 0.05$), P9 vs. P28 ($P < 0.001$), and P9 vs. P36 ($P < 0.01$). Expression in the detrusor was discerned to be most prominent at the earliest stages of development and demonstrated diminishment by adulthood. Statistical significance was presented as follows: (1) Piezo1, P6 vs. P17 ($P < 0.05$), and P6 vs. P28 ($P < 0.01$), (2) Piezo2, no significance (NS). Elucidation of Piezo1 and Piezo2 presence transitionally across neonatal development provides a basis for further studies; my findings, optimistically, could precipitate future molecular and functional understandings of Piezo1 and Piezo2 relative to the maturation of the micturition reflex and the successive treatment and prevention of urinary abnormalities.

Introduction

Neurophysiology of the Lower Urinary Tract and Mature Voiding Function

The urinary bladder is composed of smooth muscle tissue and functions to hold and expel liquid waste from the kidneys; its predominant purpose is to clear the body of toxins (Yoshimura & Chancellor, 2002). The bladder wall is structurally composed of three layers: mucosal, muscular, and serosal. The mucosal layer is further comprised of two layers. The first is a layer of transitional epithelial cells (the urothelium), which line the lumen, and the second is the lamina propria. The urothelium lines the bladder and acts as an impermeable barrier. Additionally, the urothelium has a sensory component allowing it to sense sensory information and respond to it. The lamina propria (suburothelial layer) integrates information transmitted by the urothelial cells and the smooth muscle and in response maintain proper bladder function (Chancellor & Yoshimura, 2002). The muscular division of the urinary bladder is termed the detrusor, and is necessary for the expulsion (contraction) of urine into the urethra, or accommodating the filling of soluble waste (expansion). The serosa is a layer of connective tissue which articulate with the abdominal wall (Yoshimura & de Groat, 1997).

The lower urinary tract (LUT) contains a multitude of cell tissues and muscular structures including the urethra as well as the urinary bladder. It is under neural control and as such is innervated by three set of peripheral nerves found in both the autonomic and somatic nervous systems. Within the autonomic division of the nervous system, the pelvic parasympathetic nerves surface at the sacral level of the spinal cord and release the neurotransmitters acetylcholine and adenosine triphosphate (ATP), which bind to various muscarinic receptors and purinergic receptors, respectively, on the smooth muscle of the bladder, ultimately causing contractions (Yoshimura & de Groat, 1997). The hypogastric nerve contains sympathetic axons which ultimately innervate the base of the bladder, the bladder body, and the urethra. The nerves release the neurotransmitter norepinephrine which lead to contractions of the urethral smooth muscle and base of the bladder, while simultaneously relaxing the bladder body in order to accommodate bladder filling (Chancellor & Yoshimura, 2002). Somatically, the pudendal nerve exits the sacral spinal cord to innervate the external urethral sphincter muscle, which leads to contraction of the muscle to prevent leakage and facilitate bladder storage (Yoshimura & de Groat, 1997). The LUT relies heavily on afferent nerve communication, as well. The parasympathetic, sympathetic, and somatic nerves that transmit information from the spinal cord to the periphery (LUT) also contain afferent axons which convey information from the periphery back to the lumbosacral spinal cord (Yoshimura & Chancellor, 2002). These neurons send projections to several supraspinal micturition integration centers in the brain (Thor et al., 1989).

Unlike other involuntary visceral processes, the expulsion of urine from the bladder, termed voiding, is collectively dependent on learned behavior, as it requires, in part, a voluntary mechanism. The pelvic, hypogastric, and pudendal peripheral innervation and secondary connections to the central nervous system are ultimately what control urinary continence and micturition. Urine storage reflexes follow complex mechanistic procedures which begin with low levels of neuronal firing from the periphery in response to the bladder distension induced mechanosensor activation. In response to this, afferent signals are mediated in the spinal cord. During the storage phase, the hypogastric and pudendal nerves activate the bladder base and the urethral sphincter to hold urine within the bladder and prevent involuntary urinary flow (Yoshimura & de Groat, 1997) (Fig. 1a). The hypogastric nerve simultaneously causes inhibition of the detrusor muscle, ceasing the muscular contractions that lead to voiding. In contrast, when bladder distention reaches threshold level, the mechanosensory pathway changes to release the held urine. The voiding reflex is initiated by parasympathetic activity, in which the neural firing activates the spinobulbospinal pathway (Fig. 1b). This pathway ultimately communicates with central nervous system neurons located in the pontine micturition center in the brainstem. These neurons then send axons back to the spinal cord and this descending information elicits bladder contractions. The hypogastric and pudendal nerves in this system act as inhibitors, withdrawing any associated activity, which in turn leads to voiding (Yoshimura & de Groat, 1997). The anatomical, neurochemical, and neurophysiological differences in murine and human LUTs that pertain to micturition reflexes are minimal; mice show great potential in reproducibility in human, translational bladder-focused research (Reis et al., 2011; Bugos, Bhide, & Zilka, 2009).

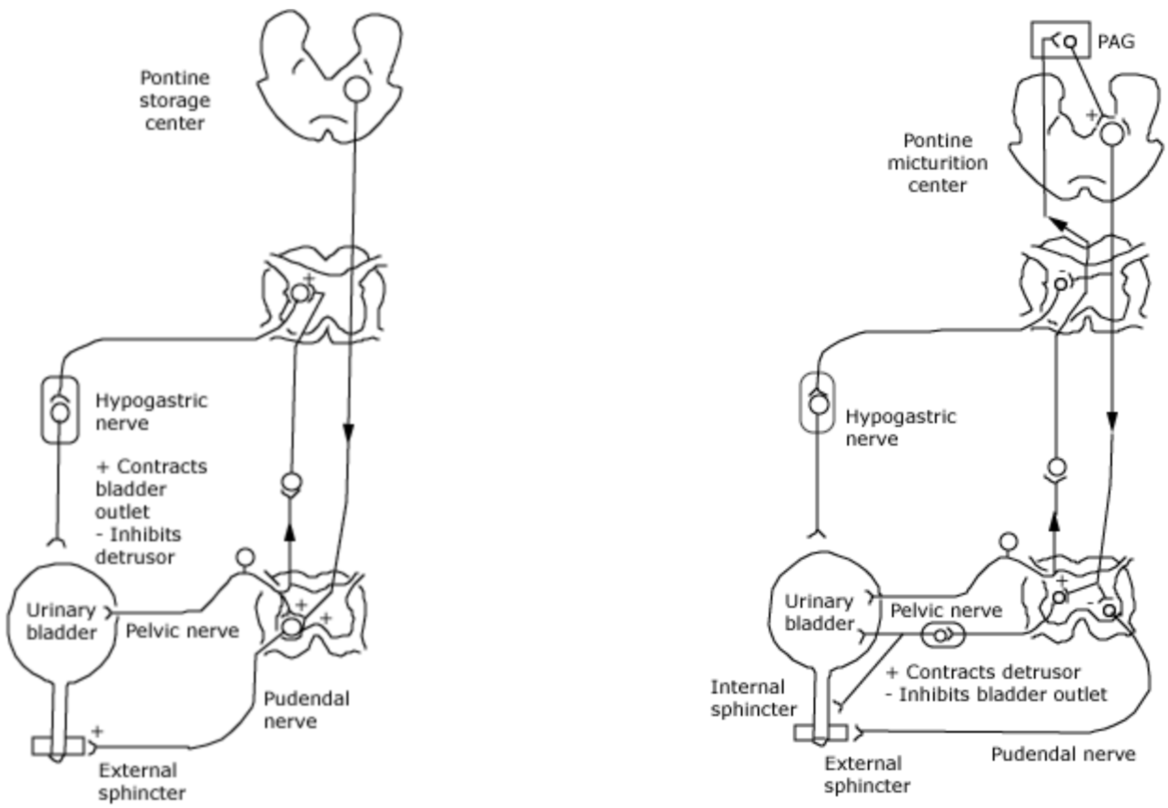

Figure 1. (a) Physiology of the urine storage reflex, (b) physiology of the voiding reflex, (Abrams et al., 2018).

Developmental Maturation of the Micturition Reflex

Understanding postnatal development of the bladder and its associative micturition reflex is minimal. In many neonatal animals, afferent and efferent neural transmissions from the central and peripheral nervous systems have been identified to operate on the urinary bladder through the induction of a micturition reflex in similar manners. In early development, an exteroceptively actuated sacrospinal reflex comprised of somatic afferents (pudendal nerve) and parasympathetic efferents (pelvic nerve) is observed. The external stimulation is focal to the perineal area of the animal (perineal-to-bladder reflex). Reflex pathways comparable to the aforementioned one have been recognized in human infants. As nervous system maturation progresses, the perineal-to-bladder reflex becomes overshadowed by supraspinal region control and the development of the spinobulbospinal reflex pathway present in adults.

The spinobulbospinal micturition reflex pathway, pertinent to the adult involuntary voiding reflex, follows somatic stimulation of afferent fibers that ascend to the Pons (in the brainstem), synapse onto the periaqueduct gray followed by the pontine micturition center, and trigger excitatory responses transmitted in a descending parasympathetic manner to innervate the urinary bladder and induce the reflex (Fowler, Griffiths, & de Groat, 2008).

The transition from the somatovesical reflex to the spinobulbospinal reflex is thought to occur gradually during early postnatal development, up until the emergence of the brainstem-regulated pathway at approximately 3 weeks of age. In the cat, parentally-isolated subjects require perigential stimulation to evoke urination up until postnatal week 3, after which a vesicovesical reflex is seen (Thor, Blais, & de Groat, 1989). This timeline is further evinced by the rat model; rats require perineal somatic stimulation up until postnatal days 16-18 (Capek, & Jelinek, 1956; Maggi et al., 1986; Ng, de Groat, & Wu, 2007; Zvarova, & Zvara, 2012). It is likely that the brainstem mediated voiding pathway is present (albeit lacking functionality, or demonstrating inhibitory functions) in animals (Sugaya, Roppolo, Yoshimura, Card, & de Groat, 1997) and humans (Sillén, 2001), prior to complete maturation of the adult micturition reflex.

In humans, infantile bladder function differs from mature function, and undergoes stages of development across the first five years of life. Bladder capacity during the neonatal period averages around 10 milliliters, grows significantly (up to 50-60 milliliters) within the first year of life, up to a maximum capacity (120-150 milliliters) at around three years of age. Prior to maturation, it was suspected that that neonates underwent constant involuntary periods of detrusor contractions, which were stimulated by a spinal reflex (Sillén, 2001). However, it was observed through a series of urodynamic studies that non-neurogenic bladders experience sustained stability in the detrusor. Uninhibited (by the central nervous system) contractions should be regarded as abnormalities in young infants. In addition, it has recently been suggested that the brain presents neural pathway connectivity to the immature neonatal bladder leading to micturition, as opposed to the previously assumed involuntary voiding events which occurred in response to steady increase in bladder volume (Sillén, 2001). This study observed disturbances in neonatal patients as the micturition reflex functioned, implying that connections to the nervous system were in fact intact prior to complete maturation of the micturition reflex (Sillén, 2001). The development of the bladder and voiding ability matures as the central and peripheral nervous systems of neonates mature. With the maturation of the spinobulbospinal reflex pathway as well as neurological pathways within the brain intrinsic to specified target organs within the LUT and the spinal cord, the maturation of the micturition reflex follows.

Molecular plasticity in urinary bladder tissues could account for micturition maturation (Maggi et al., 1986). Upregulation of neuropeptides and their receptors is a necessary mechanistic predecessor (occurring during postnatal development) to the adult voiding reflex (Sann et al., 1997). Proteins responsible for mechanical force detection (mechanosensory proteins) and their roles in voiding function have recently been studied in urinary bladder urothelial cells (Birder & Andersson, 2013; Apodaca, Balestreire, & Birder, 2007). Mechanosensory expression in the bladder has been shown to be related to perceived bladder tension and volume by mediating and transmitting mechanical stimuli to supraspinal regions (Apodaca, Balestreire, & Birder, 2007). They have also been suggested to play a key role in the integration of nociception, mediation of gentle touch sensations, and the sustaining of proper chemical regulation (Nilius & Honoré, 2012; Coste et al., 2010; Coste et al., 2012).

Mechanosensation in the Urinary Bladder and Piezo1 and Piezo2 Mechanosensory Proteins

The urothelial lining of the bladder has classically been assumed as a passive barrier to water molecules, ionic compounds, and infections. In recent years, however, the urothelium has been deemed an important mechanosensory component of bladder function, its cells responding to physical (mechanical) and chemical stimuli (Birder & Andersson, 2013). A multitude of receptors and ion channels have been discovered and connected to the mechanoceptive and nociceptive sensory component of the urothelium. The sensory communication of the urothelium that results in receptor/ion channel moderated occurrences which transduce chemical mediation is known as the urothelial-associated sensory web (Apodaca, Balestreire, & Birder, 2007). Within the mechanosensory pathway involved in voiding, it has additionally been suspected that distention (stretch, fullness) of the bladder urothelium can be sensed by certain proteins termed stretch-activated channels (SACs). The opening of SACs is the first step in the sensory transduction pathway leading to micturition. In very recent years, the involvement of mechanosensory proteins Piezo1 and Piezo2 was deemed necessary in association with SACs and their overall function (Birder & Andersson, 2013; Nilius & Honoré, 2012).

The discovery of Piezo1 and Piezo2 mechanosensitive cation channels in mammals was made by Coste et al. Their physiological and chemical properties closely resemble those of SACs, as their most important feature is their activation in response to pressure and other forms of mechanical force. They are a new family of mechanically-gated ion channels originally identified in the Neuro2A mouse cell line and are strictly based in higher eukaryotic cells. Piezo1 and its homolog Piezo2 are constructed very similarly, yet their kinetic properties differ slightly (Coste et al., 2010). Piezo2 is known to be inactivated at a much faster rate than Piezo1 and is also observed in somatosensory neurons. Piezo1 channels are composed of approximately 2,500 amino acids and carry 24-32 transmembrane domains. In comparison, Piezo2 proteins are 2,800 amino acids long and contain 16-18 transmembrane helices.

Both channels do not require additional proteins for activation; their activation is entirely dependent on sensing membrane tension (Gottlieb & Sachs, 2012).

Research by Coste et al. further identified that Piezo channels are expressed in the lung, colon, and bladder of mice, and more specifically, in the primary urothelial cells of the bladder (Coste et al., 2010). A study conducted by Miyamoto et al. found that the presence of Piezo1 in human and mouse urothelial cells had a functional role in mechanogated calcium influx, and immediate ATP release within the cells *in vitro*. A Piezo1-dependent increase in cytosolic calcium was observed in the urothelium, in association with ATP release, in response to stretch-activation (Coste et al., 2010).

A study conducted in 2019 presented alterations to Piezo1 expression in bladder mucosa of mouse subjects in response to restraint stress, showing neural connections between the mechanosensor and the bladder (Ihara et al., 2019). Another study demonstrated a significant increase in Piezo1 expression from day 3 to day 18 following partial bladder outlet obstruction in rat models. Piezo1 mRNA was found in suburothelial and detrusor levels – mechanistically speaking, inhibiting the expression of the protein may aid in the storage dysfunction aspect of bladder outlet obstructions (Michishita, Yano, Tomita, Matsuzaki, & Kasahara, 2016). In addition, knockout of Piezo1 was observed to be an embryonic lethal, due to its role in the vascular system - its expression in the endothelial tissues of blood vessels is necessary in the mediation of sensory information (Ranade et al., 2014; Li et al., 2014). Piezo1 overexpression in response to cystitis-associated bladder hyperactivity has also been noted, speculating the increase to be a compensative mechanism, which aided in the regulation of specific cells known to control contractile activity in the bladder (Hashitani & Lang, 2010).

The presence and importance of Piezo1 and Piezo2 in the adult urinary bladder is undisputed – their development postnatally, however, has yet to be demonstrated in specific bladder tissue layers. SAC presence and distribution determination across postnatal development may provide insight on the unexplored process. The transient receptor protein (TRP) ion channel superfamily holds a structural and functional identity similar to that of Piezo channels: non-specific with preferential Ca^{2+} permeability, and mechanosensitive. (Andersson et al., 2010; Araki, 2011; Eid, 2011) Proteins belonging to the TRP family (TRPA1, TRPV1, and TRPV4) have all exhibited marked increases in expression following postnatal maturation in the mouse (Merrill, Girard, May, & Vizzard, 2012). Ca^{2+}-activated big potassium (BK) channels have also demonstrated increase in expression across development in the neonatal rat (Ng, de Groat, & Wu, 2007).

Piezo presence, localization, and expression rates in postnatal urinary bladder structures could elucidate neuropeptide function in relation to micturition reflex maturation. In this paper I show Piezo1 and Piezo2 mechanosensor transcript expression measurements in the mouse urinary bladder ranging from postnatal day 6 to day 36 in hopes of furthering comprehension of the maturation process. I will use quantitative polymerase chain reaction (Q-PCR) to determine the expression of Piezo1 and Piezo2 messenger ribonucleic acid (mRNA) expression in the mouse bladder during postnatal development. Samples of the mRNA will be extracted during postnatal (P) development stages (P6-P36). These molecules will be reverse transcribed and converted into complementary DNA (cDNA) molecules. To find the relative quantities of the Piezo1 and Piezo2 proteins, their synthesized DNA sequences will then be amplified through the use of Q-PCR. The resulting samples will be analyzed through a sequence detection software.

Literature has suggested neural pathway maturation to underlie micturition reflex development from mechanistically primitive to cortically regulated (following P21). Upregulation of various stretch-activate neuropeptides has been demonstrated in the context of reflex transition, and mechanosensation is a requisite of adult voiding, as well. I hypothesize that a statistically significant increase in Piezo1 and Piezo2 mRNA expression will be observed in mouse urinary bladder throughout early postnatal development, as the micturition reflex matures.

Methodology

Animals

Male and female mice holding the inbred genetic identity C57BL/6 wild-type (WT) were used in this study. The population (n = 33) was bred and cared for under the University of Vermont's Office of Animal Care and Management's supervision, in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health guidelines prior to initiation of experiments. The mice were socially housed (group pairing up to 5 per cage; 12-hour light/dark cycle), and provisions were unregulated (unlimited access to food and water). Experimental protocols involving the animals were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC; PROTO201900020).

Tissue Harvest

Mice aged postnatal days 6 (P6) and 9 (P9) were euthanized via introduction to 5% isoflurane and subsequent decapitation. Total anesthetization was assured through the administration of a toe-pinch – no movement ensured complete desensitization and permitted initiation of the secondary method of euthanasia. Mice at postnatal ages of 17 (P17), 21 (P21), 28 (P28), and 36 (P36), were euthanized via primary exposure to 5% isoflurane and thoracotomy. Following euthanasia, access to the animals' abdominal cavities was achieved through a midline incision along the abdominal wall. The urinary bladder was removed from the abdominal cavity and pinned to a saline-filled Sylgard-coated dish for microdissection under RNAase-free conditions. An incision was made along the bladder's equatorial line exposing the urothelium (including the submucosa) and detrusor layers of the organ. The two layers underwent separation through the aid of forceps and iris scissors, and were individually placed in RNase-free nucleic acid isolation tubes (MP Biomedicals lysing Matrix D) for future mRNA analysis and cDNA creation (Guo et al. 2018). The samples were snap frozen and placed in a controlled -80˚C environment until further use (Arms, Girard, & Vizzard, 2010).

Quantification of Piezo1 and Piezo2 mRNA

Quantification of total observed RNA was completed through initial extraction with the use of the STAT-60 total TNA/mRNA reagent (Tel-Test "B", Friendswood, TX) (Arms, Girard, & Vizzard, 2010). Approximately one to two milligrams of RNA per sample were used to synthesize cDNA molecules through the utilization of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega), along with a mix of random hexamer and oligodeoxythymidylic acid (oligo((dT)) primers in a 25 μl final reaction volume. The synthetic cDNA templates were subjected to a 1:10 dilution to minimize the inhibitory effects of reverse transcription reaction components and were further assayed with SYBER Green I JumpStart *Taq I* ReadMix (Sigma, St. Louis, MO). A relative real-time quantitative polymerase chain reaction (Q-PCR) (Applied Biosystems 7500 Fast real-time PCR system, Foster City, CA) was completed through the sequence: serial heating at 94°C for 2 minutes, followed by amplification of product over 45 cycles at 94°C for 15 seconds, and 60°C for 30 seconds. The initial quantitative PCR criterion for each transcript was developed through direct insertion of the *Taq* polymerase-amplified cDNA products into the pCR2.1-TOPO (topoisomerase I) vector (TOPO TA Cloning, Invitrogen). The specific primer sequences utilized were developed in our lab, and are as follows: Piezo1 (Upper), ATCCTGCTGTATGGGCTGAC; Piezo1 (Lower), AAGGGTAGCGTGTGTGTTCC; Piezo2 (Upper), GATGGCATGGAGCATCACCTAC; Piezo2 (Lower), CCAGCAGCAGATTCGCATAGAC. Relative expression of the transcripts was approximated through administration of a 1:10 serial dilution of the stock vectors, establishing the vectors as standards for quantification. Data were analyzed after each assay with a sequence detection software (Sequence Detection Software, version 1.3.1; Applied Biosystems, Norwalk, CT). The assays involved plotting an arbitrary magnitude of SYBER I green fluorescence emission (ΔRn; default setting was used) against cycle number. The relative expression of the gene of interest was normalized to the relative quantity of the housekeeping gene L32, a ribosomal marker expressed in urothelial or detrusor tissue.

Statistical Analyses

Quantitative data are mean ± standard error of the mean (S.E.M.). Between-group statistical differentiation was determined through use of a one-way analysis of variance (ANVOA), followed by Tukey's multiple comparisons test compatible with GraphPad Prism software compatibilities (La Jolla, CA). Statistical significance was considered if $P < 0.05$.

Results

Piezo1 and Piezo2 expression in the urothelium.

Relative Piezo1 mRNA expression in the urothelial tissue layer of P9 mice was significantly higher than observed in P21, P28, and P36 mice ($P < 0.05$) (Fig. 1). A slight increase of 31.5% (NS) in mRNA expression from postanal days 6 to 9 was demonstrated, followed by a decrease in expression of the mechanosensor gene over the course of maturation and development. Percent change of P17 expression relative to P9 showed a slight change of -14.2% (NS), P21 relative to P17 produced a difference of -50.5% (NS), and P28 levels relative to P21were 20.9% (NS) lower. The levels of mRNA expression rose minimally from P28 to P36 by 33.2% (NS). Piezo2 expression in the urothelium/suburothelium followed the same general pattern of statistical change as Piezo1, with a slight 27.9% (NS) increase in presence from P6 to P9, followed by a gradual decrease continual with development (21.5% decrease from P9 to P17, 33.3% decrease from P17 to P21, 54.0% decrease from P21 to P28; NS) with an increase between stages P28 and P36 (73.8% increase; NS). Six-day postnatal mice expressed levels of the mRNA that were significantly higher than those of mice part of the P28 cohort ($P < 0.05$). Mice of the P9 subgroup expressed significantly more of the Piezo2 gene in comparison to P21 ($P < 0.05$), P28 ($P < 0.001$), and P36 ($P < 0.01$) subgroups (Fig. 2).

Piezo1 and Piezo2 expression in the detrusor.

Piezo1 mRNA expression in the detrusor muscle of the P6 mouse group was found to be significantly higher in comparison to P17 ($P < 0.05$), and P21 ($P < 0.01$) expression (Fig. 3). The gene was expressed in larger amounts earlier in the maturation process, as seen in P6 mice, with subsequent incremental decrease as follows: 20.9%, 15.6%, and 8.8% decrease from P6 to P9, P9 to P17, and P17 to P21, respectively. mRNA expression levels afterwards were observed to steadily increase from P21 up until end of observed stages of development, with a respective 14.0% and 13.2% increase from

P21 to P28, and P28 to P36, respectively. Presence of Piezo2 in the detrusor muscle of all postnatal mouse cohorts was not significantly differentiated, as seen following subjection to a one-way ANOVA (Fig. 4). A fluctuating, relative decrease in mRNA expression over the course of development was observed (NS), as listed: 20.6% decrease from P6 to P9, 14.2% decrease from P9 to P17, 3.5% increase from P17 to P21, 23.8% decrease from P21 to P28, and a 6.7% increase from P28 to P36.

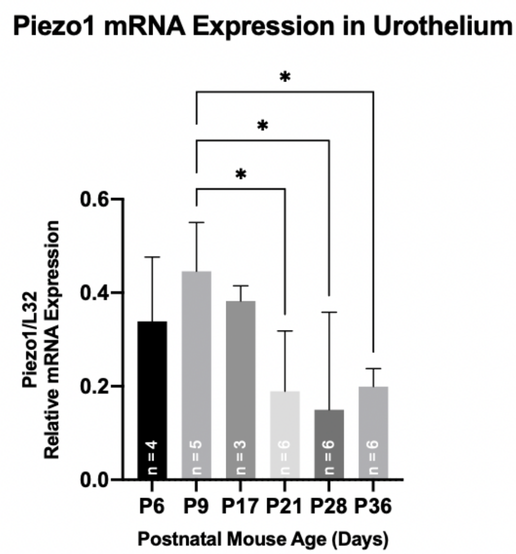

Figure 1. Piezo1 mRNA expression normalized against housekeeping gene L32 in urothelial bladder layer of littermate wild-type (WT) postnatal mice aged 6 (P6; n = 4), 9 (P9; n = 5), 17 (P17; n = 3), 21 (P21; n = 6), 28 (P28; n = 6), and 36 (P36; n = 6). Between-group comparisons show significant difference in expression in P9 vs. P21, 28, and 36 cohorts, with P9 urothelial Piezo1 presence holding larger quantities (one-way ANOVA; $P < 0.05$). Values are means + S.E.M. $*P < 0.05$.

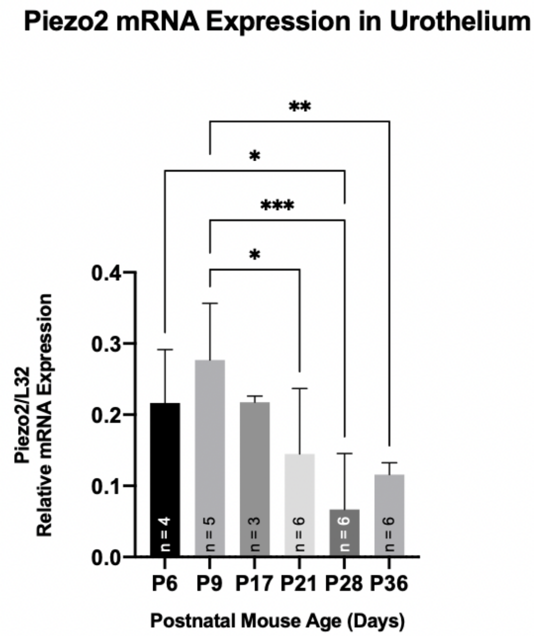

Figure 2. Piezo2 mRNA expression normalized against L32 in urothelial bladder layer of littermate wild-type (WT) postnatal mice aged 6 (P6; $n = 4$), 9 (P9; $n = 5$), 17 (P17; $n = 3$), 21 (P21; $n = 6$), 28 (P28; $n = 6$), and 36 (P36; $n = 6$). Between-group comparisons show significant differences in expression as follows: P6 vs. P28 ($P < 0.05$), P9 vs. P21 ($P < 0.05$), P9 vs. P28 ($P < 0.001$), and P9 vs. P36 ($P < 0.01$) (one-way ANOVA). Values are means + S.E.M. *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$

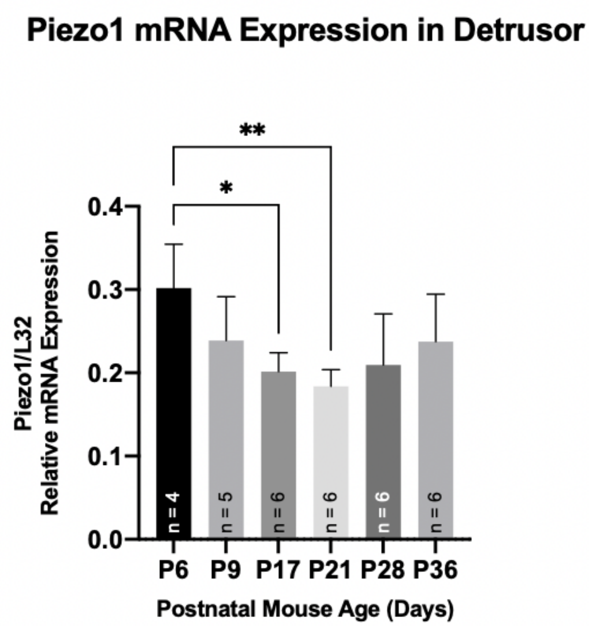

Figure 3. Piezo1 mRNA expression normalized against L32 in detrusor smooth muscle layer of littermate wild-type (WT) postnatal mice bladders aged 6 (P6; $n = 4$), 9 (P9; $n = 5$), 17 (P17; $n = 6$), 21 (P21; $n = 6$), 28 (P28; $n = 6$), and 36 (P36; $n = 6$). Between-group comparisons show significant difference in expression in P6 vs. P17 ($P < 0.05$), and P6 vs. P28 ($P < 0.01$) cohorts, with P6 Piezo1 presence holding larger quantities (one-way ANOVA). Values are means + S.E.M. *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$

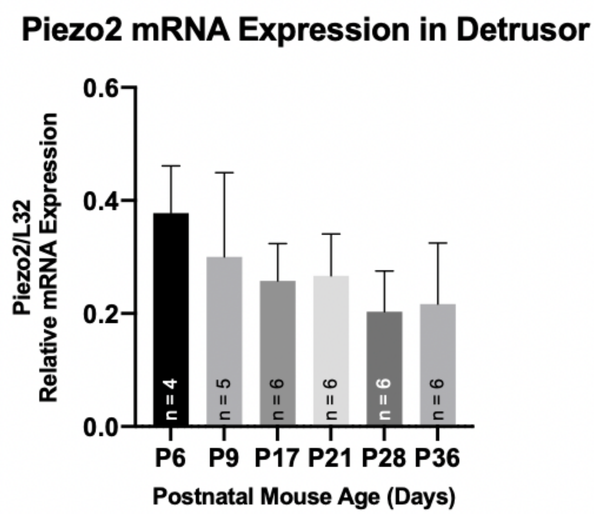

Figure 4. Piezo2 mRNA expression normalized against L32 in detrusor smooth muscle layer of littermate wild-type (WT) postnatal mice bladders aged 6 (P6; *n* = 4), 9 (P9; *n* = 5), 17 (P17; *n* = 6), 21 (P21; *n* = 6), 28 (P28; *n* = 6), and 36 (P36; *n* = 6). Between-group comparisons show no statistically significant differences in expression between any of the cohorts (one-way ANOVA). Values are means + S.E.M.

Discussion

This project focused on determining and establishing correlations between mechanosensory proteins Piezo1 and Piezo2 and the transitional development of the bladder and its functionality in postnatal mice. Previous studies have elucidated some neurophysiological features and functions of the Piezo1 (Ranade et al., 2014) and Piezo2 (Murthy et al., 2019) mechanosensors, specifically their roles in acute sensitization and modulation of stretch and pressure. Although the cation channels, following their morphological and anatomical discovery, have been heavily researched in the context of neonatal murine livelihood and bladder function (Sillén, 2001; de Groat & Araki, 1999; Etem et al., 2018), their contribution to bladder maturation postnatally remains elusive. I aimed at extending comprehension of the mechanosensors and clarifying their presence throughout murine development (up until neonatal day 36).

In this study, I provide evidence suggesting heightened Piezo1 and Piezo2 expression in the urothelium earlier in bladder development (P6 and P9, specifically), and contrastingly lower levels of expression as the bladder matures in postnatal mice, contradicting my original hypothesis. Piezo1 and Piezo2 were predominantly accounted for in the detrusor smooth muscle in P6 mice, and while Piezo1 levels diminished to significant levels relative to P6 prior to upregulation following postnatal day 21 up until day 36, Piezo2 expression held no between-group significance in comparison. Its expression level held a trend of decrease until P36.

The results remain solitary and novel in the urinary bladder field; as far as I know, only one other study has effectively provided expression levels of Piezo1 and Piezo2 throughout postnatal animal development (Etem et al., 2018). This study elucidated the genetic plasticity of the bladder as a whole and suggested a consistent increase in Piezo1 presence up until postnatal day 21 in mice, followed by a steady decrease, which was countered at P36, at which an increase in expression was observed again. Piezo2 expression was shown to fluctuate, with observable height allocated early in development, followed by a substantial decrease by two weeks postnatally, and subsequent increase until four weeks postnatally. Expression levels diminished following four weeks (Etem et al., 2018). My data are specific to differential bladder structures, preventing the opportunity for accurate comparisons. The fluctuation of increase and decrease mirrors my findings. More studies should aim at the verification of Piezo1 and Piezo2 expression levels in both detrusor and musical/submucosal bladder layers.

It is important to note the expression intensity in early stages of development and continual age-associated decrease in transcript expression of Piezo1 and Piezo2 in the urothelial layers of the neonatal mouse bladder. Murine bladder growth is intrinsically abrogated at the approximate postnatal age of five weeks (Jost, 1985), and cellular proliferation has been indicated to stop by postnatal day 14 (P14) (Erman et al., 2001). Arrest of cellular proliferation in urothelial cells by P14 could account for the steady observed decrease in Piezo1 and Piezo2 expression in urothelial-complex cells following the P9 cohort. Stabilization of the tissue layers' cellular components, which comprise the urothelium, and various suburothelial structures and related vasculature (three layers are fully established by P14) could account for the down-regulation and consequential decrease in gene expression.

Plastic changes to the synaptic aspect of the sacral parasympathetic pathways have been discovered as mediators of micturition reflex maturation (de Groat & Araki, 1999). Afferent fibers activated by tension (information derived from mechanosensors, including Piezo1 and Piezo2) in the bladder wall initiate autonomic communication with supraspinal micturition centers resulting in voiding. It has been theorized that maturation of spinal circuitry and nervous system plasticity precede the maturation of the micturition reflex (Sillén, 2001). A study suggested that the spinobulbospinal loop, which plays a significant role in mature micturition, does not fully develop until several weeks postnatally in rats (Schwaller, Kwok, & Fitzgerald, 2016). Age-dependent decrease in gene expression in the male and female bladder has been discovered, as well (Phillips, 1981). These determinations could provide substance as to an explanation for the age-dependent decrease in Piezo1 and Piezo2 transcript expression seen following P9 in the urothelial structures of mice, and following P6 in the detrusor, albeit expression changes are dependent on the two differentiating bladder structures.

It is understood that the micturition reflex follows a pathway determined by initial activation of SACs (Birder & Andersson, 2013). The contribution of stretch activated mechanosensory proteins Piezo1 and Piezo2 to the maturation of the reflex, as observed in my implemented neonatal murine model, is most significant early in development. Stretch-activated channel plasticity under the family of transient receptor proteins (TRP) across various stages of postnatal development in a rat model has previously been examined; results suggested increase in TRP transcript expression up until half of the neonatal life, followed by down-regulation until end of neonatal life (Merrill, Girard, May, & Vizzard, 2012).

Support of my findings is continually envisioned in the maturation of the murine circadian rhythm complex. A study presented that the nervous system nuclei that underlie circadian function in mouse models to develop gradually until completion at the postnatal infantile stage (deemed postnatal day 10) (Ansari, Agathagelidis, Lee, Korf, & von Gall, 2009). Clock gene expression findings (related to proper circadian rhythm establishment) in mouse models have recently linked Piezo1 presence in the urinary bladder to rhythm regulation (Ihara et al., 2018). Associations between these two papers could indicate that Piezo1 production in the nervous system increases earlier on in development of the circadian rhythm, reinforcing my results.

Future studies should continue evaluations on larger sample sizes of mice, separate the sex populations to study potential sex-dependent mRNA expression changes across development, and further their ventures by implementing a rat model (Bugos, Bhide, & Zilka, 2009; Merrill, Girard, May, & Vizzard, 2012). The six arbitrary days throughout development, though indicative of a direct pre- and post-spinobulbospinal reflex emergence date, did not correlate to days designated by other murine model-based studies. Mirrored postnatal days could aid in data comparison. Understanding of the direct contribution of the Piezo1 and Piezo2 mechanosensors on nervous system pathway development and micturition reflex maturation has not been completely achieved by my quantitatively observational study – mechanistic causations of their expression remain elusive. It should also be noted that Piezo1 and Piezo2 mRNA derived cDNA molecule expression was measured in my methodology, not the proteins themselves. Post-transcriptional modification should be account for, and translational changes not yet observed in my results should be acknowledged. Diagnostic protein assays including enzyme-linked immunoabsorbent assay (ELISA) (Gonzalez et al., 2015) and Western blot (immunoblot) (Merill, & Vizzard, 2014) should be used to combat this fault. Immunohistochemical assays should also be performed to provide qualitative data including Piezo1 and Piezo2 morphological differentiation across postnatal development and dispersion across urothelial tissue and detrusor smooth muscle tissue (Girard, Peterson, Malley, & Vizzard, 2016). The Piezo1 channel has been demonstrated to attenuate ATP signaling following stretched-evoked stimulation in adult mouse urothelial cells (Miyamoto et al., 2014). Immunocytochemical assays could provide insight on whether this response occurs during early development or after assumed synaptic reorganization owing to the emergence of the spinobulbospinal reflex (Miyamoto et al., 2014).

Recapitulation of the micturition reflex suggests that my findings could translate to human bladder models. My study is currently uncorroborated and focused solely on Piezo1 and Piezo2 contribution to urinary bladder maturation through their levels of presence over development. Future studies should focus on establishing the functional and molecular roles of Piezo1 and Piezo2 across development of the micturition reflex if the pathophysiological concerns dictated by neonatal bladder dysfunction are to be resolved.

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