Functional properties and connective tissue content of pediatric human detrusor muscle

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CHILDHOOD ENURESIS may be refractory to conventional treatment, and those with continued symptoms may need treatments, including intravesical botulinum toxin (2, 8). Urodynamic observations include poor compliance, detrusor overactivity, and outflow abnormalities. Management of childhood lower urinary tract (LUT) dysfunction is extrapolated from adult therapies, but as the physiological properties of pediatric human detrusor tissue have not been investigated, such an assumption lacks justification.

In vitro contractile properties of adult human detrusor smooth muscle (DSM) have been well characterized from normal, overactive, and obstructed bladders. Atropine resistance of nerve-mediated contractions and functional denervation characterize pathological bladders; there is no evidence of diminished DSM function (9). As functional studies using tissue from neonatal or pediatric patients are lacking, it is unclear if the adult phenotype is present at birth. Autonomic innervation of the bladder is present at term (13), although there are no quantitative comparisons with adult tissue. The ratio of DSM to connective tissue (CT) is near unity in pediatric tissue but much greater in that from adults (17, 19). Theoretically, this would decrease unit contractile performance and increase pediatric bladder wall stiffness compared with adults, depending on the collagen types in CT. Studies with term fetal animals have suggested that Ca2+ regulation in detrusor myocytes is nearly complete (33), whereas tissue innervation remains incomplete (24, 31).

We hypothesized that the contractile and biomechanical properties of DSM from pediatric bladders are different from adults. This was tested by comparing these properties in vitro using samples from pediatric bladders with no obvious dysfunction and from normal adult bladders. This work represents the initial phase that examines changes to detrusor function in children with LUT congenital anomalies.

METHODOLOGY

Patient groups, tissue samples, and experimental preparations. Pediatric bladder samples (≈3 mm3) for histological and functional experiments were obtained from 16 children (3–48 mo, 10 male children and 6 female children) undergoing open bladder procedures [ureteric reimplantation (n = 13), open bladder stone removal (n = 1), or localized bladder tumor excision (n = 2)] and transported immediately to the laboratory in a nominally Ca2+-free solution. Three further small samples were obtained from older children undergoing ureteric reimplantation (110, 116, and 160 mo) where histological data alone were collected. No child had any recorded LUT functional disorder. Twenty adult samples (40–60 yr; 12 men and 8 women) were obtained from patients also with bladders without detrusor overactivity on filling cystometry and undergoing cystectomy. All samples were from the lateral bladder wall of the bladder dome and distant from any lesions. Ethical permission was obtained from the relevant research ethics committee and with written informed patient or guardian consent.

Solutions. Experiments were performed in Tyrode solution containing (in mM) 118 NaCl, 24 NaHCO3, 4.0 KCl, 1.0 MgCl2, 0.4 NaH2PO4, 1.8 CaCl2, 6.1 glucose, and 5.0 Na pyruvate at pH 7.6 with NaOH and gassed with 100% O2. Tissue dissociation to isolated cells used Ca2+-free solution with additional 20 mg/ml Worthington type II collagenase, 0.5 mg/ml hyaluronidase-LS, 0.5 mg/ml hyaluronidase-III, 0.9 mg/ml antitrypsin-IS, and 5.0 mg/ml BSA (11). All chemicals were from Sigma UK.

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Histology. Biopsy segments were placed in 10% formaldehyde and stored at 4°C. Samples were dehydrated with increasing ethanol solutions, placed in xylene, and paraffin mounted. Sections (5 μm) were mounted on 3-aminopropyltriethoxysilane-coated slides and stained with elastin van Gieson (collagen: red, muscle: yellow). Digital images (×20) were analysed by ImageJ to quantify the proportional areas of collagen and DSM using color filters; areas with no staining were not analyzed.

Measurement of isometric tension. Similar preparations, tied between a static anchor and an isometric force transducer, recorded nerve-mediated or agonist-induced contractions. Nerve-mediated contractions were elicited with 3-s trains of electrical stimuli (100-μs

Figure 1. Connective tissue (CT) of human pediatric and adult detrusor samples. A: section of a pediatric bladder stained with elastin van Gieson (magnification: ×10). B: the smooth muscle (SM)-to-CT ratio (SM/CT) in pediatric and adult detrusor tissue. **P < 0.01 vs. adult tissue. C: dependence of SM/CT on age of the patient from whom pediatric samples were obtained (○). The median (25% and 75% interquartiles) value of SM/CT in adult detrusor tissue (●) is also shown; the shaded region delineates this range of adult values.

Table 1. Contractile variables of pediatric and adult detrusor smooth muscle

<table>
<thead>
<tr>
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<th>Adult</th>
<th>Pediatric</th>
<th>Corrected to Smooth Muscle Area</th>
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<tr>
<td>T&lt;sub&gt;carb&lt;/sub&gt;, mN/mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>56.3 (45.2, 86.4)</td>
<td>33.3 (8.2, 48.3)*</td>
<td>63.7 (51.1, 97.8)</td>
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<tr>
<td>n</td>
<td>20</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;, carbachol</td>
<td>6.15 (5.94, 6.36)</td>
<td>5.52 (5.46, 5.57)‡</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>15</td>
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<tr>
<td>T&lt;sub&gt;ABMA&lt;/sub&gt;, mN/mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>22.5 (17.7, 28.9)</td>
<td>9.37 (4.35, 14.2)†</td>
<td>25.4 (20.0, 32.7)</td>
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<tr>
<td>n</td>
<td>10</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;, mN/mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>48.8 (36.9, 59.4)</td>
<td>6.51 (3.09, 9.22)‡</td>
<td>55.2 (41.8, 67.2)</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>1/2&lt;sup&gt;Hz&lt;/sup&gt;</td>
<td>18.1 (15.2, 20.1)</td>
<td>15.1 (10.4, 17.3)*</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>15</td>
<td></td>
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<tr>
<td>Atropine resistance, %</td>
<td>0.2 (0.0, 4.1)</td>
<td>0.2 (0.0, 4.1)</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>17</td>
<td>12</td>
<td></td>
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<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;/T&lt;sub&gt;carb&lt;/sub&gt;</td>
<td>0.99 (0.76, 1.07)</td>
<td>0.21 (0.15, 0.54)‡</td>
<td></td>
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<tr>
<td>n</td>
<td>20</td>
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Data are medians (with 25% and 75% interquartiles); n, number of biopsy samples. T<sub>carb</sub>, maximum tension from a carbachol dose-response curve; T<sub>ABMA</sub>, tension from 1 μM α,β-methylene ATP (ABMA); T<sub>max</sub>, maximum tension from the force-frequency curve; 1/2<sup>Hz</sup> frequency to generate T<sub>max</sub>/2. Absolute values are also shown corrected for percent smooth muscle in the preparation cross section. *P < 0.05; †P < 0.01; ‡P < 0.001.
pulses, frequency: 1–40 Hz), and force-frequency relationships were generated. Tetrodotoxin (1 μM) completely abolished contractions. The estimated maximum tension at high frequencies (T_max) and frequency for Tmax/2 (f_{1/2}) were calculated as follows: T = (T_{max} × f)/f_{1/2}, where T is tension and f is frequency. Agonist-induced contractions were generated by addition to the superfusate using unstimulated preparations. Carbachol (muscarinic agonist) dose-response curves were generated with return to control after each concentration to avoid receptor desensitization. Contractions to the purinergic P2X receptor agonist α,β-methylene ATP (ABMA) were measured at a single maximal concentration (1 μM) as agonist washout was slow, precluding accurate dose-response determination.

Measurement of intracellular Ca^{2+} concentration. Intracellular Ca^{2+} concentration ([Ca^{2+}]_i) was measured in isolated cells by epifluorescence microscopy using the fluorochrome fura-2. Cells were incubated in fura-2 (5 μM) for 20 min at 4°C before being washed and placed in a superfusion chamber on an inverted microscope. Myocytes were alternately excited at 340 and 380 nm, and fluorescent light was collected between 410 and 510 nm. The system and calibration have been previously described (32).

Biomechanical properties. After removal of the mucosa, muscle strips (diameter: ≤1 mm, length: ≤3 mm) were mounted in a superfusion bath for isometric tension recordings. The anchor was attached to a voltage-operated solenoid (308B Lever Arm System, Cambridge Technology) to move it in the horizontal plane and stretch/relax the preparation. A step voltage change applied across the solenoid altered preparation length in a 50-s loading and unloading cycle; tension changes were recorded. Voltage was adjusted to alter muscle length by 20% during the stretch phase (<10 μs). Tension changes were analyzed according to the standard linear model (30) described by a canonical equivalent of two elastic elements (E_1 and E_2) and a viscous component (η) with a time constant of E_2 stress relaxation (ηE_2) equal to ηE_2 (see Fig. 6).

Data presentation and statistics. Data are median values with 25% and 75% interquartiles, n is the number of patients contributing samples. Differences between data sets were tested using Wilcoxon’s signed-rank tests. The association between variables was tested by calculating Pearson’s correlation coefficient (r). The null hypothesis was rejected at P < 0.05. No sex-related differences were observed in

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Fig. 2. Carbachol dose-response curve for human pediatric and adult detrusor tissue. Data are expressed as percent tension compared with the estimated maximum value for each muscle sample.

![Graph showing carbachol dose-response curve for human pediatric and adult detrusor tissue.](image)

Fig. 3. Intracellular Ca^{2+} regulation human in pediatric and adult detrusor tissue. A: resting intracellular Ca^{2+} concentration ([Ca^{2+}]_i) in isolated myocytes from pediatric and adult bladders. B: Ca^{2+} transients generated by carbachol (left) and α,β-methylene ATP (ABMA; right) from pediatric bladder cells. C: Δ[Ca^{2+}]_i after exposure to carbachol, ABMA, high KCl solution, or caffeine in myocytes from pediatric (P) or adult (A) samples.

![Graph showing intracellular Ca^{2+} regulation in pediatric and adult detrusor tissue.](image)
pediatric or adult data sets for any variable, so data were analyzed independent of sex.

RESULTS

The DSM-to-CT ratio. This was determined from samples of 16 pediatric bladders (25 ± 15 mo, range: 3–48 mo, n = 16), which were also used for functional experiments; Fig. 1A shows a sample section. Data were also obtained from 20 similar adult biopsies (median: 51 yr, range: 47–56 yr, n = 20). The smooth muscle-to-CT ratio was significantly less in pediatric samples compared with adult samples (2.36 ± 1.04 vs. 7.60 ± 1.26; Fig. 1B). However, the smooth muscle-to-CT ratio increased significantly with age in pediatric samples (r = 0.507, P < 0.05, n = 16; Fig. 1C), although at 48 mo it did not reach the adult value. With three other small samples from older children (111, 116, and 160 mo), histology alone was possible and smooth muscle-to-CT ratios were 11.8, 4.4 and 13.2; these values were not included in the group analysis.

To determine if pediatric versus adult myocytes showed a sample section. Data were also obtained from 20 a slight difference in pediatric and adult tissues (Table 1). In intracellular Ca²⁺ responses to agonists were also measured to determine if similar normalized contractile responses in pediatric and adult samples were mirrored at a cellular level.

[Ca²⁺]i measurements. Resting [Ca²⁺]i was measured in one to three myocytes from each biopsy, and the average value for each biopsy was calculated. Values were 58 (50, 95) nM (n = 16) and 68 (46, 77) nM (n = 20) in pediatric versus adult cells (Fig. 3A) and were not significantly different. Figure 3B shows sample Ca²⁺ transients from pediatric myocytes in response to carbachol (left) or ABMA (right). The carbachol Ca²⁺ transient showed a temporary undershoot after the transient rise of [Ca²⁺]i, whereas the ABMA Ca²⁺ transient showed a monotonic decline. These morphologies are similar to those reported with adult human detrusor myocytes (32) and attest to different sources of Ca²⁺: the carbachol response from intracellular Ca²⁺ stores and the ABMA response from a transmembrane Ca²⁺ influx.

Ca²⁺ transients were generated by exposure to several agonists: carbachol (10 μM), ABMA (1 μM), raised extracellular K⁺ concentration (4–40 mM) to depolarize myocytes, and caffeine (10 mM) to release Ca²⁺ from intracellular stores. All interventions generated Ca²⁺ transients of similar magnitude. Moreover, there were no significant differences between Ca²⁺ transient magnitudes from pediatric or adult myocytes with any intervention (Fig. 3C). These data and the above contractile responses indicate that pediatric detrusor tissue has similar contractile properties and intracellular Ca²⁺ regulation processes to adult tissue.

Nerve-mediated contractions. Electrical field stimulation of detrusor preparations from adult and pediatric samples generated nerve-mediated (tetrodotoxin-sensitive) contractions with increased magnitude as stimulation frequency was raised. Tmax and f₁/₂ were calculated from force-frequency curves. The Tmax value was significantly smaller for pediatric samples, even when normalized to both the cross-section area of the preparation and proportion of smooth muscle (Table 1). Figure 4A
shows force-frequency curves for pediatric and adult tissue, expressed as a percentage of \( T_{\text{max}} \) for each curve. The curve for pediatric tissue was shifted to the left, reflecting a significantly smaller \( f_{1/2} \) value (Table 1).

The substantially smaller \( T_{\text{max}} \) value with nerve-mediated stimulation in pediatric tissue may result from reduced functional innervation, as muscle contractility per se is not different compared with adult tissue. This was tested with the ratio of \( T_{\text{max}} \) to \( T_{\text{carb}} \): a reduction would imply reduced functional innervation, as muscle contractility per se is not different compared with adult tissue (Fig. 4B). Functional innervation, as measured by normalized \( T_{\text{max}} \) values, significantly increased with age, but at 48 mo did not achieve the value in adult preparations \( (r = 0.807, P < 0.001, n = 15; \text{Fig. 4C}). \)

**Atropine resistance of nerve-mediated contractions.** In vitro, detrusor from human adult stable bladders is characterized by an absence of atropine-resistant nerve-mediated contractions and was also shown in these preparations (Table 1), implying that ACh is the predominant functional transmitter. Atropine (1 \( \mu \)M) was applied to 12 of the pediatric preparations, and significant atropine resistance was observed in all preparations (Fig. 5A and Table 1). These contractions were subsequently abolished with ABMA (1 \( \mu \)M) to desensitize purinergic P2X receptors, which implies that ACh and ATP are functional excitatory neurotransmitters in human pediatric detrusor tissue. The percentage of the atropine-resistant contraction significantly decreased \((r_s = -0.633, n = 13, P < 0.05)\) with age in pediatric samples between 3 and 48 mo but was not reduced to adult values over this range (Fig. 5B).

ATP is released at lower stimulation frequencies in sympathetic nerves compared with its cotransmitter norepinephrine (15). This differential profile of transmitter release was tested in the parasympathetic supply to these detrusor preparations. Thus, low-frequency contractions as a fraction of \( T_{\text{max}} \) should be larger in pediatric samples with atropine-resistant (ATP-dependent) contractions. This was tested by calculating the \( T_4\)-to-\( T_{\text{max}} \) ratio (where \( T_4 \) is tension at low-frequency stimulation, 4 Hz) and was significantly greater in pediatric samples (Fig. 5C). This observation also provides an explanation for the smaller \( f_{1/2} \) value in pediatric samples, as greater tension at low frequencies would shift the force-frequency curve to the left. Moreover, the proportion of atropine resistance should have a negative association with \( f_{1/2} \) values in pediatric samples or a positive association with the \( T_4\)-to-\( T_{\text{max}} \) ratio. Figure 5D indeed shows a significant negative association between the proportion of atropine resistance and \( f_{1/2} \) values \((r_s = -0.601, n = 13, P < 0.05)\). Corresponding data for atropine resistance and \( T_4\)-to-\( T_{\text{max}} \) ratios showed a significant positive association with atropine resistance \((r_s = 0.614, n = 13, P < 0.05)\).

**Passive biomechanical properties of detrusor tissue.** Figure 6A shows the experimental protocol: a rapid tissue stretch of 0.5 mm (~20% resting length) followed by an equally rapid

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Fig. 5. Atropine resistance in pediatric and adult detrusor smooth muscle. A: percent atropine resistance of nerve-mediated contractions in pediatric and adult human detrusor tissue. B: relationship of atropine resistance and age of pediatric detrusor tissue. C: value for adult human detrusor tissue. D: value for adult human detrusor tissue. *P < 0.05. D: dependence of the frequency for \( T_{\text{max}}/2 \) \( (f_{1/2}) \) value for nerve-mediated contractions on the proportion of atropine resistance. Data are for pediatric samples (\( \square \)) and the combined data for adult detrusor samples (\( \bullet \)).
restoration after 50 s generated an increase of stress (tension) that partially relaxed followed by a mirrored undershoot and the eventual restoration of resting stress. Data were analyzed according to the physical model shown in the inset in Fig. 6A. Magnitudes of steady-state tension ($E_1$), transient tension ($E_2$), their ratio ($E_1/E_2$), and $\tau$ were all measured after the rapid stretch. Values of these variables were similar if calculated from the rapid relaxation phase after removal of the stretch; hence, these data are not reported separately.

$E_1$ was significantly greater in pediatric samples, but $E_2$ was not significantly different (Fig. 6B,i). The percentage of the viscoelastic component [$E_2/(E_1 + E_2)$] to overall stiffness was significantly less in pediatric samples (Fig. 6B,ii). $\tau$ was greater (i.e., slower) in pediatric samples (Fig. 6B,iii). Overall, compared with that from adult tissue, relaxed detrusor preparations from pediatric bladders had a greater stiffness coefficient and a proportionately smaller viscoelastic component.

**DISCUSSION**

These data showed measurable differences between detrusor phenotypes from pediatric and adult bladders. This was characterized by increased stiffness, with greater CT deposition, substantial purinergic atropine resistance, and decreased functional innervation.

The functional properties of DSM itself were similar in pediatric and adult tissue, as assessed by 1) contractile responses to carbachol and ABMA (normalized to muscle amount in the cross section) and 2) resting $[Ca^{2+}]$, and the magnitude of changes to $Ca^{2+}$ concentration in response to several antagonists. Therefore, different in vitro function between human detrusor tissues from adult and pediatric bladders cannot be explained by a variation of detrusor contractile activation per se. Similar agonist contractile responses have also been measured when fetal and adult animal detrusor tissues were compared and when human detrusor responses from elderly (>70 yr) or younger patients with either normal or overactive bladders were compared (9, 33). Thus, DSM function itself is similarly developed in pediatric and adult bladders and remains so even when aging bladder pathologies emerge. This concurs with the observation that fetal human bladders have fully differentiated smooth muscle cells (10).

In contrast, nerve-mediated contractions were smaller in pediatric samples, implying more limited functional innervation, at least up to 48 mo. However, over this timescale, while there was a significant increase of functional innervation, it did not attain the adult value. Innervation of human detrusor tissue is evident as early as 13–15 wk of gestation (10, 13). However, to our knowledge, there is no study of the density of nerve fibers to DSM during postnatal development. However, in adult human overactive or obstructed bladders, reduced functional innervation, i.e., reduction of $T_{max}$, is mirrored by reduced nerve density (6, 9, 11, 22), and so a similar association may be hypothesized in postnatal development.

There was a greater passive stiffness of pediatric than adult tissue, associated with the increased proportion of CT compared with smooth muscle. As the detrusor layer primarily determines bladder compliance (4), it is appropriate to measure changes to detrusor CT during development. A low smooth muscle-to-CT ratio in normal pediatric bladder has been previously measured (18). The age-dependent decline of CT toward the adult value could contribute to a similar age-dependent increase of urodynamic bladder compliance in children. Furthermore, this suggests that it is not possible to define a baseline value for bladder compliance in

![Fig. 6. Biomechanical properties of pediatric and adult detrusor tissue. A: experimental trace of tension changes upon a transient rapid stretch of a preparation by 0.5 mm from a pediatric tissue sample. Measured variables of steady-state tension ($E_1$), transient tension ($E_2$), and the time constant of $E_2$ stress relaxation ($\tau$) are shown. The inset shows the physical model used to analyze the data. $\eta$, viscous component. B: values of the elastic variables $E_1$ and $E_2$ (i), the proportion of the viscous component $[E_2/(E_1 + E_2)]$ (ii), and $\tau$ of viscoelastic relaxation (iii) in muscle strips from pediatric or adult samples. *$P < 0.05$ and **$P < 0.01$ vs. adult tissue.](image-url)
children. Compliance is positively associated with maximum bladder capacity, which increases into adolescence (23). Any condition-specific [e.g., bladder extrophy (26)] increase of CT deposition would further decrease compliance. After extrophy repair, bladder capacity and compliance increase (3); it is important to determine if this is accompanied by decreased CT content as well as specific changes that lower bladder compliance, e.g., increase of the collagen type III-to-type I ratio (5, 16) or elastin content (29).

Many children refractory to anticholinergic therapy show dysfunction on urodynamographic investigation (8). The altered phenotype described above in children is recapitulated in adults with detrusor overactivity (DO) and bladder outflow obstruction and mirrored in normal very young animals and those with DO induced by outflow obstruction (14). Voiding in infants occurs at high urodynamic detrusor pressure and an uncoordinated pattern, accompanied by urethral electromyographic activity (25, 27). Thus, the bladder phenotypes from young children and adult overactive bladders may have a common etiology. However, the similarity of isolated detrusor contractility in pediatric and adult tissue samples contrasts with the high micturition pressures recorded in young children, in particular boys with high-grade reflux (25, 27, 28). This suggests other causes for these high pressures, such as greater detrusor passive stiffness or raised outflow resistance.

Anticholinergic agents may treat LUT problems in children, but their effectiveness is variable (1, 20). The large proportion of atropline resistance in nerve-mediated contractions from pediatric bladders compared with adult bladders was perhaps the most striking observation. It may contribute to the variable effectiveness of anticholinergic therapy, and targeting the purinergic system may offer an alternative target.

Atropline resistance results from ATP acting as an additional excitatory neurotransmitter. This may result from reduced ATP breakdown at the neuromuscular junction by extracellular ATPases, as occurs in adult DO (12), or a greater functional detrusor expression of P2X1 receptors in pediatric versus adult tissue. Neither possibility was tested in this study, due to limitations of tissue availability, and would be the logical next steps in a functional characterization of pediatric detrusor tissue. However, the latter possibility is unlikely as the contractile response to an exogenous P2X1 agonist, ABMA, was similar in pediatric compared with adult tissue, as also observed in young mice (7). However, upregulation of other purinergic receptors (e.g., P2X3 receptors in afferent nerves) is observed in pediatric bladders (21). Whatever the reason, significant purinergic-mediated contractions in the pediatric bladder offer an additional drug target to regulate childhood bladder function and question the reliance on anticholinergic agents alone to manage bladder performance.

Conclusions. Detrusor tissue from pediatric patients with no evidence of LUT dysfunction shows fundamental differences to that from equivalent adult bladders. These differences are due to relative lack of innervation denervation and smooth muscle content of pediatric tissue but no change to detrusor contractile function. A large atropine-resistant component of contraction was also measured. These differences could underlie functional divergence in pediatric and adult bladders and suggest alternative therapeutic pathways to manage childhood bladder dysfunction.

Limitations and future work. This study had two limitations. First, pediatric tissue samples were from a heterogeneous group, although none had evidence of bladder dysfunction and it was assumed that their clinical condition did not have any impact on detrusor physiology or histology. Second, the relatively small sample number and tissue quantity in each sample limited the range of experiments; this precluded a comparison of many physiological and histological properties for the same preparation. Further experiments will characterize the molecular pathways underlying the above functional differences and provide a detailed analysis of connective tissue components and analysis of sex-related contributions.

REFERENCES


