Dysfunctional bladder neurophysiology in urofacial syndrome Hpse2 mutant mice

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Abstract

Aims: Urofacial syndrome (UFS) is an autosomal recessive disease characterized by detrusor contraction against an incompletely dilated outflow tract. This dysnergia causes dribbling incontinence and incomplete voiding. Around half of individuals with UFS have biallelic mutations of HPSE2 that encodes heparanase 2, a protein found in pelvic ganglia and bladder nerves. Homozygous Hpse2 mutant mice have abnormal patterns of nerves in the bladder body and outflow tract, and also have dysfunctional urinary voiding. We hypothesized that bladder neurophysiology is abnormal Hpse2 mutant mice.

Methods: Myography was used to study bladder bodies and outflow tracts isolated from juvenile mice. Myogenic function was analyzed after chemical stimulation or blockade of key receptors. Neurogenic function was assessed by electrical field stimulation (EFS). Muscarinic receptor expression was semi-quantified by Western blot analysis.

Results: Nitrergic nerve-mediated relaxation of precontracted mutant outflow tracts was significantly decreased vs littermate controls. The contractile ability of mutant outflow tracts was normal as assessed by KCl and the α1-adrenoceptor agonist phenylephrine. EFS of mutant bladder bodies induced significantly weaker contractions than controls. Conversely, the muscarinic agonist carbachol induced significantly stronger contractions of bladder body than controls.

Conclusions: The Hpse2 model of UFS features aberrant bladder neuromuscular physiology. Further work is required to determine whether similar aberrations occur in patients with UFS.

KEYWORDS

bladder dysfunction, genetic disease, mouse model, neuropathy, urination

1 | INTRODUCTION

Several early onset diseases featuring functional urinary bladder outflow obstruction have genetic bases.1 One of these is urofacial, or Ochoa, syndrome (UFS), a rare but potentially devastating autosomal recessive disorder.2-4 UFS can present before birth with an enlarged bladder on ultrasound screening. In childhood, UFS features...
dribbling incontinence and incomplete voiding, attributed to detrusor sphincter dysynergia where the detrusor contracts against an inadequately dilated outflow tract. Subsequently, urosepsis can cause pyelonephritis and renal failure. Radiology and urodynamics show that these children have low capacity and poorly compliant bladders, with detrusor hypercontractility. Treatments aim to empty the bladder using catherization and to increase bladder capacity with muscarinic and α-adrenoreceptor antagonists. Associated features in UFS are a grimace upon smiling and constipation.

Most patients with UFS carry biallelic mutations of HPSE2, encoding heparanase 2, which inhibits the enzymatic activity of classical heparanase. Other UFS families carry mutations of LRRG2, encoding leucine rich repeats and immunoglobulin like domains 2, a protein that modulates growth factor signaling. Experimental knockdown of heparanase 2 in Xenopus embryos disorganizes peripheral motor nerves but the frog is not an ideal model for UFS because it has a rudimentary bladder, the cloaca.

Ochoa, who first described UFS, postulated that defects in the pontine micturition center and facial nerve nucleus explained bladder and facial phenotypes. Overt anatomical brainstem lesions, however, have not been described in UFS, and other studies point to bladder nerve involvement. Heparanase 2 and LRRG2 have been immunodetected in nerves located between smooth muscle bundles in the human fetal bladder and in mouse pelvic ganglia, structures sending post-ganglionic autonomic motor neurons to the bladder. Mice with biallelic Hpse2 or Lrig2 mutations have abnormal patterns of peripherin expressing bladder nerves; an overabundance was detected in the bladder body, while fewer nitric oxide synthase (nNOS) expressing nerves spanning pelvic ganglia flanking the outflow tract. In healthy mice, nitrergic neurons dilate the outflow tract. Moreover, both Hpse2 and Lrig2 mutant mouse bladders are depleted of Nos1, the transcript encoding nNOS, and both Hpse2 and Lrig2 mutant mouse models have urination defects similar to people with UFS.

Following on from the above observations that Hpse2 mutant mice have both urination defects and disordered bladder nerves, we hypothesized that bladder neurophysiology would be perturbed in this animal model. Accordingly, we used myography to characterize bladder outflow and bladder body neurophysiology in Hpse2 mutant mice. The results of the study confirm that bladder neuromuscular physiology is aberrant in these mice. Further work is required to determine whether similar aberrations occur in individuals affected by UFS.

2 METHODS

2.1 Animals

Hpse2 mutant mice used had a gene-trap insertion into intron 6. Wild type and heterozygous (control) and homozygous (mutant) male mice on a C57BL/6 background were studied in the third and fourth postnatal weeks, because the homozygous mutant mice fail to thrive after weaning, and this is why juvenile mice were used in this study.

2.2 Myography

Animals were killed by cervical dislocation in accordance with Schedule 1 of the Animal (Scientific Procedures) Act, 1986. The bladder, outflow tract and external sphincter were removed and pinned to a Sylgard plate in ice-cold physiological salt solution (PSS; NaCl 122 mM, KC1 5 mM, HEPES 10 mM, KH2PO4 0.5 mM, Na2HPO4 0.5 mM, MgCl2 1 mM, glucose 5 mM, and CaCl2 1.8 mM; adjusted to pH 7.3 with NaOH). The outflow tract was isolated by cutting immediately below the bladder neck. On the distal end, a cut was made above the observed striated muscle. This resulted in an outflow tube 1.5 to 2.0 mm long, which was mounted on the closed pins of a small vessel myograph chamber (Danish Myo Technology, Hinnerup, Denmark) containing PSS. The pins were separated by 50 μm, a distance sufficient to hold the outflow in place while the chamber was secured in the myograph. Recording commenced immediately the tissue was mounted and PSS was continuously bubbled with air at 37°C. Once the outflow achieved a stable tension, the contractile response to 5 μM phenylephrine, a selective α1-adrenoceptor agonist, was determined. After washout, the pins were separated by a further 50 μm and phenylephrine reapplied. This procedure was repeated until the phenylephrine contraction reached maximum, indicating the optimal stretch for measuring contractile responses. There was no significant difference (P = .42) in stretch applied to control (144 μm ± 16, n = 9) and mutant (160 μm ± 9, n = 7) outflows. To measure nitrergic nerve-mediated relaxation, outflows were precontracted to a stable level with 5 μM phenylephrine. Electrical field stimulation (EFS) was then applied for 10 second at increasing frequencies (0.5–15.0 Hz), with 3 minute rest between each stimulation. Supramaximal pulses (80 V, 0.1 millisecond width) were delivered by a Grass (SD9) stimulator. The neurogenic nature of responses was confirmed by inhibition with 1 μM tetrodotoxin (TTX) or the NOS inhibitor N(ω)-nitro-l-arginine methyl ester (l-NAME; 200 μM). Relaxation was...
calculated as a percentage of the stable phenylephrine-mediated tension. The measured peak relaxation amplitude was used to construct a frequency-relaxation plot. In separate studies, phenylephrine was applied cumulatively at increasing concentrations (10 nM-100 μM) with resulting changes in tension used to construct a concentration-response curve. Graphpad PRISM 8 software was used to fit the data by least squares to the Hill equation:

\[ E = \frac{E_{\text{max}}}{1 + \left( \frac{E_{50}}{[A]} \right)^n}, \]

where \( E \) represents response, \( E_{\text{max}} \) the maximum response, \([A]\) the concentration of contractile agent, \( E_{50}\) the concentration producing half-maximum contraction and \( n \) is the Hill coefficient. The contractile response to 50 mM KCl was also tested.

The bladder body was analyzed as follows. A transverse ring of tissue, 1.5 to 2.0 mm wide, was excised from the middle of the body. Each ring was mounted in a myograph chamber under 2 mN of applied tension and studied as for the outflow. Rings were stimulated by 50 mM KCl, increasing concentrations of carbachol (0.01-50 μM) or increasing frequency of EFS (0.5-15.0 Hz). Measured contractions were used to generate concentration-frequency-response plots. The former were fit to the Hill equation (Equation 1) to obtain \( E_{50}\) and \( E_{\text{max}}\) values. Rings were then incubated for 10 minute in 1 μM atropine to block muscarinic receptors, followed by EFS or 5 μM carbachol. Rings weighed at the end of each experiment did not differ significantly between controls (4.9 mg ± 0.25, n = 8) and mutants (4.1 mg ± 0.98, n = 6).

### 2.3 Western blot analysis

Whole bladder bodies were excised and stored at −80°C. Whole protein was extracted by mechanical disruption in ice-cold radioimmunoprecipitation assay buffer (600 μL) containing protease inhibitors, using Biopulveriser Lysing Matrix D beads on a Fast-Prep-24 (MP Biomedicals). Lysate was cleared by centrifugation at 14 000 g for 10 minutes at 4°C and quantified using a Pierce bicinchoninic acid assay. Samples were adjusted to 200 ng/μL and denatured in 5× Laemmli sample buffer (320 mM Tris pH 6.8, 5% sodium dodecyl sulfate, 25% glycerol, and 0.5% β-mercaptoethanol added fresh, in ddH2O). About 4 μg of protein was loaded per lane on a 10% Bio-Rad polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, which was blocked in 5% milk powder in phosphate-buffered saline (PBS)-Tween 0.1%. It was incubated overnight at 4°C with primary antibodies (rabbit anti-M3 (1:750, Abcam ab126168, epitope aa 286-461), or rabbit anti-M2 (1:1000, Abcam ab109226, epitope 150-250) predicted to be specific for those muscarinic receptors. Primary antibodies were detected with antirabbit-horseradish peroxidase (HRP; 1:2000, DAKO P0448). Membranes were washed, reblocked in 5% milk powder PBS-Tween and incubated in β-actin-HRP (Sigma A3854, 1:20 000) for 1 hour at room temperature. Bands were visualized on a ChemiDoc (Bio-Rad, Watford, UK) and quantified with the ImageJ software (https://imagej.nih.gov/ij).

### 2.4 Statistics

Data are reported as mean ± SEM of tissues from and were analyzed using GraphPad PRISM8. \( EC_{50}\) values were compared between control and mutant mice using the non-parametric Mann-Whitney test, while p\( EC_{50}\) and \( E_{\text{max}}\) data were compared by two-tailed, unpaired \( t \) tests. EFS frequency-response relationships of outflow relaxation and body contraction were analyzed by two-way, repeated measures analysis of variance, unless there were unplanned missing points in which case a mixed-effects model was used with the Geisser-Greenhouse correction, not assuming sphericity. Western blot band densities were compared between control and mutant mice by two-tailed, unpaired \( t \) test. In figures, *\( P < .05\), **\( P < .01\), and ***\( P < .001\).

### 3 RESULTS

#### 3.1 Contractility of Hpse2 mutant outflows

Although Hpse2 homozygous mutant mice do not gain weight as robustly as littermate controls in the first month of life, they are not overtly ill.\(^8,12\) In this study, as expected, controls were heavier than Hpse2 mutants (control: 10.5 ± 0.8 g, n = 14; mutant: 5.6 ± 0.4 g, n = 12; \( P < .0001\)). Average ages were similar (control 18.7 days ± 0.16, mutant 18.4 days ± 0.18). Mutants displayed apparently more frequent and smaller urinary voids than controls (Figure S1), consistent with a recent study.\(^12\)

As soon as an outflow preparation was mounted in the myograph, before tension being applied, it began to develop spontaneous tone that reached a plateau over 20 minutes. There was no significant difference between final tensions of controls (2.9 ± 0.4 mN; n = 9) and mutants (3.2 ± 0.49 mN; n = 7). KCl (50 mM) induced contractions that were sustained until washout (Figure 1A), with similar responses in controls (0.23 ± 0.02 mN; n = 9) and mutants (0.22 ± 0.03 mN; n = 7) (Figure 1B).
Phenylephrine evoked concentration-dependent contractions in control and mutant outflows (Figure 1C). Contractions reached a peak within 1 minute; peak contractions developed with pEC$_{50}$ of 6.1 ± 0.2 (n = 8, EC$_{50}$ = 673 nM) in controls vs 6.6 ± 0.1 (n = 6, EC$_{50}$ = 250 nM) in mutants, with respective E$_{max}$ of 1.2 ± 0.2 mN and 1.0 ± 0.1 mN (both measures, P > .05) (Figure 1B). Neither measures differed significantly between the groups. Following the peak at 5 μM phenylephrine, tension declined over 5 minutes to a lower level, similar in controls (0.32 ± 0.04 mN, n = 9) and mutants (0.37 ± 0.06 mN, n = 7). Following this, level was reached, tension usually remained constant during phenylephrine exposure (eg, Figure 2C), although further gradual decline was sometimes apparent (eg, Figure 2A).

3.2 | Nitrergic nerve-mediated relaxation in the bladder outflow

EFS (0.5-15.0 Hz) in phenylephrine-contracted outflows produced frequency-dependent relaxations (Figure 2A). Relaxations were abolished by preincubation with the NOS inhibitor L-NAME (200 μM) (Figure 2C), or TTX (1 μM) to block neuronal action potentials (data not shown). Maximum relaxation in individual tissues was evoked by 4 to 8 Hz (Figure 2B) and was reduced by more than 50% in Hpse2 mutants vs controls. The frequency causing half-maximum relaxation in controls was 1.0 ± 0.2 Hz, compared with a significantly higher frequency of 3.0 ± 0.6 Hz in mutants (P = .003).

3.3 | Contractile properties of the bladder body

Rings of bladder body contracted in response to 50 mM KCl (Figure 3A). Mutants produced mean tension that was, on average, 50% larger than the control rings (Figure 3B). In bladder bodies from the control and mutant mice, the muscarinic agonist carbachol-evoked concentration-dependent contractions (Figure 3C) that peaked within 20 second. The carbachol pEC$_{50}$ in control bladders (6.1 ± 0.05, n = 8) was not significantly different
to that in the mutants (6.1 ± 0.1, n = 7) in mutant bladders. It is apparent from Figure 3D, however, that the maximum tension reached was around 56% higher in the mutants (P = .014).

EFS-induced frequency-dependent neurogenic contractions in controls and mutant rings (Figure 4A) that were abolished by 1 μM TTX (not shown). Mutants were less responsive to EFS at each frequency studied (Figure 4B); the largest contraction, observed at the highest frequency of 25 Hz, was reduced by 63% in mutants (control 2.4 ± 0.6 mN, n = 8; mutant 0.9 ± 0.2 mN, n = 7). The frequency evoking the half maximal response could not be calculated because maximum contraction was not reached at 25 Hz. Pretreatment with the muscarinic receptor antagonist atropine (1 μM) abolished carbachol-evoked contractions in both tissues (not shown). Atropine also reduced neurogenic contractions evoked by EFS (compare panels C and D in Figure 4 with panels A and B) but did not abolish them. The contraction evoked at 25 Hz was reduced by an average of 80% (pretropine 2.6 ± 0.8 mN, n = 6; postatropine 0.6 ± 0.1 mN, n = 5; P = .049) in controls and 94% (pretropine 1.0 ± 0.2 mN, n = 5; postatropine 0.06 ± 0.02 mN; P = .006) in mutants, with a significantly greater loss of contraction in the mutant (P = .006; Figure 4D).

3.4 | Western blot analysis

To investigate the mechanisms underpinning the apparent increased sensitivity of Hpse2 mutant bladders to carbachol, Western blot experiments were carried out to examine muscarinic receptor expression. Relative to the housekeeping protein β-actin (Figure 5), no significant difference was detected between controls and mutants for either M3 or M2 proteins.

4 | DISCUSSION

Our results show that in Hpse2 mutants, functional neurophysiological defects are present in the bladder body and outflow. Specifically, there is reduced nitrergic nerve-mediated relaxation in the Hpse2 mutant mouse outflow tract, as well as reduced cholinergic and noncholinergic...
nerve-mediated contraction in the bladder body. The reduced sensitivity of the body to nerve stimulation is accompanied by hypersensitivity to direct stimulation of muscarinic receptors with carbachol and to stimulation with KCl.

4.1 Attenuated outflow relaxation in Hpse2 mutants

Release of NO from nitrergic nerves is considered the primary mechanism of outflow relaxation in several mammals. The reduced nitrergic nerve-mediated relaxation in Hpse2 mutant outflow tracts may help to explain why patients with UFS have a functional bladder outflow obstruction and voiding dysynergia. In health, spontaneous myogenic tone in the outflow tract may contribute to continence, at least in pigs. Our mouse outflows developed spontaneous tone in the absence of stretch or pharmacological stimulation and reached similar levels in controls and mutants. Contraction of the outflow in response to α1-adrenoceptor stimulation or KCl was also unchanged in Hpse2 mutants, suggesting that the reduced responsiveness of mutant outflows to nerve stimulation was not due to impaired altered contractility per se. This could be investigated further by comparing the effects of direct activators of the NO-cGMP signaling pathway on mutant and control mice. Consistent with the loss of EFS-induced relaxation, however, Hpse2 mutant mice have fewer nerves in their outflow tracts and reduced Nos1 transcripts. Therefore fewer nitrergic nerves may be the simplest explanation for impaired outflow relaxation upon nerve stimulation. It remains to be determined, however, whether each nitrergic neuron also has defective nNOS production or if there are dysfunctional structural/functional synapses with smooth muscle cells.

Other nonadrenergic, noncholinergic signaling molecules, including carbon monoxide and ATP, may mediate outflow relaxation in pigs and guinea pigs. Nevertheless, the outflow tract of the Nos1 knockout mouse is unable to relax and in the current study there was complete ablation of EFS-induced relaxation following l-NAME application. It is possible that mice rely exclusively on nitrergic nerves for outflow relaxation; other putative relaxation mediators were not investigated in the present study. Another important question is whether the phenylephrine-activated contraction we employed to study nitrergic relaxation is analogous to the tonic tension that mediates continence in vivo. All the mice we studied were males; in men, the bladder proximal outflow and neck generate increased tension during ejaculation to prevent retrograde flow of seminal fluids into the bladder. So, while EFS-evoked relaxation of phenylephrine- contracted outflow tracts could model normal voiding, it may
have implications for other functions of the outflow tract. Finally, we did not study the external skeletal muscle sphincter, which also helps to maintain continence, at least in humans.

4.2 | Dysfunction of the bladder body of Hpse2 mutant mice

It has been reported that there is an overabundance of nerves in the bladder body of the Hpse2 mutant mouse. Moreover, in children with UFS, the bladder has been described as hypercontractile. These observations led us to speculate that isolated Hpse2 mutant mouse bladder body rings might be hypercontractile; although this was observed, it was not neural in origin. KCl and carbachol each produced 50% larger contractions of Hpse2 mutant bladder bodies, despite the agents working through different mechanisms. KCl evokes membrane depolarization, leading to Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. In contrast, carbachol binds to muscarinic receptors on the smooth muscle cell membrane to activate G-proteins and their downstream signaling cascades. M3 receptors are the predominant mediators of muscarinic stimulation in mouse and human bladders. These receptors couple to G\(_q/G_{11}\), stimulating phospholipase C to produce IP\(_3\) and diacylglycerol. This causes Ca\(^{2+}\) release from the sarcoplasmic reticulum via IP\(_3\) receptors and sensitization of the contractile proteins to Ca\(^{2+}\) as a result of protein kinase C activation and inhibition of myosin phosphatase. M2 receptors couple to G\(_i/G_o\) and inhibit signaling via cAMP; they play a minor role in healthy bladders but can indirectly enhance M3-mediated contraction by inhibiting cAMP-dependent relaxation. M3 and M2 protein expression was similar in control and Hpse2 mutant bladder bodies. Combined with the finding that carbachol sensitivity was also unchanged in excised Hpse2 mutant rings, this implies that their enhanced response to carbachol was not due to altered muscarinic receptors levels, but may be due to changes in downstream signaling modalities. Increased responsiveness to cytoplasmic Ca\(^{2+}\) concentration could account for hypercontractility to both carbachol and KCl and could be tested by directly measuring the Ca\(^{2+}\)-dependence of contraction in skinned bladder preparations. We postulate that hypercontractility is a compensatory mechanism that counteracts the loss of parasympathetic inputs. In vivo, non-neuronal sources of acetylcholine may play a role in aberrant UFS detrusor function, and this remains to be studied.
Contraction of the bladder body is primarily driven by parasympathetic nerves releasing acetylcholine to activate M3 muscarinic receptors. Small nerve-mediated contractions were recorded from Hpse2 mutant than control bladders, despite their stronger contractions to muscarinic receptor activation. The effective loss of neural input was therefore probably greater than the 60% measured here. The neural deficit was not restricted to cholinergic transmission, because the residual nerve-evoked contraction, measured after muscarinic receptor blockade with atropine, was also suppressed in Hpse2 mutants. Parasympathetic nerves release ATP as a cotransmitter with acetylcholine and part of the nerve-evoked contraction of mouse (but not normally human) bladder is mediated by ATP acting at purinergic P2X1 receptors. The loss of both cholinergic and purinergic transmission implies that there is either a loss of parasympathetic nerves innervating the bladder wall, or the nerves present are impaired or make inappropriate connections with the smooth muscle.

Roberts et al reported an increased density of nerves in the Hpse2 bladder body. In the current study, however, we found that EFS elicits a weaker contraction in Hpse2 bladder body rings. In reconciling these observations, we postulate that, although there are abundant nerves in the body of the mutant bladder, they do not function properly. Accordingly, future studies should be directed to seeking ultrastructural defects of axons and synaptic contacts with detrusor smooth muscle cells, and also to localize changes in neurotransmitter levels. Leading on from this, we hypothesize that the observed increased response of the Hpse2 bladder to exogenous muscarinic chemical stimulation may arise as a secondary phenomenon, rather than being intrinsic to the Hpse2 mutation itself. Again, this requires further study of detrusor smooth muscle and urothelial cells in the mutant mice.

**FIGURE 5** Western blot quantification of muscarinic receptors in control and Hpse2 mutant bladders. A, M2 receptor protein (left panel) was detected as a band just below 50 kDa. M3 receptor protein (right panel) was detected as a band at 75 kDa: nonspecific bands were also detected running at around 37 and 100 kDa. β-actin from the same samples ran between 37 and 50 kDa. Each lane represents lysate from an individual mouse bladder (C = control, M = Hpse2 mutant). B, Quantification of the data in A: densities of bands representing M2 (left panel) or M3 (right panel) receptor protein expressed relative to β-actin for control (n = 5) and Hpse2 mutant (n = 5) bladders. Neither M2 nor M3 protein levels were significantly different between the control and mutant bladders.

5 | CONCLUSIONS

This study provides direct evidence that Hpse2 mutant mice have functional neurophysiological bladder defects. Perhaps most importantly, there is reduced nitricergic nerve-mediated relaxation in the Hpse2 mutant mouse outflow tract. This indirectly supports the hypothesis that voiding dysfunction in patients with UFS is neurologically obstructive, and due to impaired NO-associated urethral relaxation. However, studies using patient tissues would be needed to confirm these points. Further studies are also required to determine whether similar physiological defects are present in juvenile Lrig2 mutant mouse bladder tissues, and whether similar defects are also manifest in adult mutant mice.
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CONFLICT OF INTERESTS
All the authors declare that there are no conflict of interests.

ETHICS STATEMENT
Experiments were approved by the University of Manchester Biological Services Ethical Review Committee and the United Kingdom Home Office (PPL 40/3550 and PAFFC144F) and were conducted according to Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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