

Sildenafil, a phosphodiesterase type 5 inhibitor, augments sphincter bursting and bladder afferent activity to enhance storage function and voiding efficiency in mice

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Objectives

To investigate the influence of low-dose sildenafil, a phosphodiesterase type 5 inhibitor (PDE5-I), on the function of the mouse lower urinary tract (LUT).

Materials and Methods

Adult male mice were decerebrated and arterially perfused with a carbogenated Ringer's solution to establish the decerebrate arterially perfused mouse (DAPM). To allow distinction between central neural and peripheral actions of sildenafil, experiments were conducted in both the DAPM and in a 'pithed' DAPM, which has no functional brainstem or spinal cord. The action of systemic and intrathecal sildenafil on micturition was assessed in urethane-anaesthetised mice.

Results

In the DAPM, systemic perfusion of sildenafil (30 μM) decreased the voiding threshold pressure [to a mean (SEM) 84.7 (3.8)% of control] and increased bladder compliance [to a mean (SEM) 140.2 (8.3)% of control, an effect replicated in the pithed DAPM]. Sildenafil was without effect on most voiding variables but significantly increased the number of

bursts of the external urethral sphincter (EUS) per void in DAPM [to a mean (SEM) 130.1 (6.9)% of control at 30 μM] and in urethane-anaesthetised mice [to a mean (SEM) 117.5 (5.8)% of control at 14 ng/kg]. Sildenafil (10 and 30 μM) increased pelvic afferent activity during both bladder filling and the isovolumetric phase [to a mean (SEM) 205.4 (30.2)% of control at 30 μM]. Intrathecal application of sildenafil (5 μL of either 150 μM or 1.5 nM) did not alter cystometry and EUS-electromyography variables in urethane-anaesthetised mice.

Conclusions

Low-dose sildenafil increases bladder compliance, increases pelvic nerve afferent activity, and augments the bursting activity of the EUS. We propose that the novel actions on afferent traffic and sphincter control may contribute to its beneficial actions to restore storage and voiding efficiency in LUT dysfunction.

Keywords

phosphodiesterase type 5, Sildenafil, lower urinary tract, external sphincter function, mice

Introduction

Phosphodiesterases (PDEs) are enzymes that inactivate the second messenger molecules cyclic adenosine monophosphate and cyclic guanosine monophosphate (cGMP). Selective PDE type 5 inhibitors (PDE5-Is) increase cGMP levels by slowing its degradation, increasing nitric oxide (NO) production in peripheral tissues [1]. PDE5-Is are widely used to treat

erectile dysfunction and are increasingly used clinically to treat LUTS [2]. Several clinical studies have shown that PDE5-Is improve male LUTS, including both storage and voiding symptoms, without the impairment of sexual function seen with other BPH/LUTS treatments [3–7]. However, none of these studies showed an improvement in peak urinary flow rate, leaving open the question of how they act to improve LUTS [3,4,8].

Studies in rodents and humans suggested that PDE5 inhibition may influence several mechanisms implicated in the pathology of BPH/LUTS, including relaxation of detrusor smooth muscle [9], suppression of bladder afferent activity [10], reduced prostatic stromal cell proliferation [11], and an anti-inflammatory action on the prostate [12]. However, the effects of PDE5-Is on the motor control of the lower urinary tract (LUT) have not been determined. This is of interest as PDE5 is found in the CNS, including the grey matter of the lumbosacral spinal cord [13].

The present study aimed to investigate in detail the influence of the PDE5-I sildenafil on the function and control of the mouse LUT. To help achieve this aim, without the confounding effects of anaesthesia, we employed a recently described *in situ* mouse model, the decerebrate arterially perfused mouse (DAPM) [14]. This has many of the advantages of accessibility afforded by *in vitro* approaches but retains functional neural connectivity. Using this approach, we found that low-dose sildenafil exerts specific actions: increasing bladder compliance, increasing pelvic nerve afferent activity, and augmenting bursting activity of the external urethral sphincter (EUS). We propose these latter novel actions may underlie some of its beneficial actions in LUT dysfunction.

Materials and Methods

All experiments conformed to the UK Home Office guidelines and were approved by our Institutional Ethical Review Committee. Male CD1 mice ($n = 77$; 17 DAPM, 34 pithed DAPM, 20 *in vivo* and six *in vitro* experiments) were used in these experiments.

In situ DAPM Preparation

For the DAPM preparation [14], the mouse was deeply anaesthetised with isoflurane (2–3%) and the stomach and intestine were vascularly isolated and removed through a midline laparotomy. The ureters were cut bilaterally and ligated distally to prevent bladder filling. Next the mouse was cooled by immersion into modified Ringer's solution (5–6 °C on ice, composition below). After craniotomy, the mouse was decerebrated by aspiration, at the pre-collicular level and anaesthesia was discontinued. The preparation was skinned and then pinned to a dissecting dish on ice. The left phrenic nerve was freed from connective tissues and the lungs and diaphragm removed.

The preparation was moved to a recording chamber and a flushed double-lumen cannula (diameter 1.2 mm, Argyle™; Covidien Ltd., Dublin, Republic of Ireland) was inserted into the ascending aorta via the left ventricle. The right atrium and inferior vena cava were incised to prevent venous hypertension during arterial perfusion. The preparation was

perfused with carbogen-gassed Ringer's containing an osmotic agent, Ficoll-70 (1.25%; Sigma Aldrich, St. Louis, MO, USA). The heated perfusate (31 °C) was pumped (15–20 mL/min, Watson-Marlow pump 505D; Watson-Marlow Ltd., Falmouth, Cornwall, UK) from a reservoir flask and was recycled from the recording chamber back to the reservoir. Aortic perfusion pressure was monitored via the second lumen of the cannula. A glass suction electrode was used to record the left phrenic nerve activity, which was AC amplified (5–10 k), band pass filtered (100 Hz–3 kHz), and digitised at 10 kHz.

LUT Recordings

The pubic symphysis was cut in the midline to allow access to the EUS. A glass suction electrode (tip diameter ~200 μm) was placed on the proximal sphincter, slightly lateral to the midline and directly below the bladder neck to record EUS-electromyography (EMG). A 27-G needle was inserted into the bladder dome and connected via saline-filled tubing and a three-way tap to a pressure transducer and a syringe pump (GenieTouch™; Kent Scientific Corp., Torrington, CT, USA) for pressure monitoring and infusion of 0.9% saline, respectively. The standard infusion rate of saline into the bladder was 25 μL/min in the DAPM, as used in previous mouse urodynamic studies *in vivo* [15]. The following micturition variables were measured (averaged over at least three voiding cycles):

- Basal pressure was taken as the lowest bladder pressure reached after a void.
- Voiding threshold was the bladder pressure when the EUS-EMG started bursting, indicating the initiation of voiding.
- Micturition pressure was the absolute value of peak bladder pressure achieved during voiding (bursting phase of the EUS-EMG).
- Non-voiding contractions (NVCs) were identified as discrete increases in bladder pressure (>1 mmHg) seen during the filling phase in voiding preparations.
- In preparations without voiding (i.e., pithed DAPM), the small rhythmical pressure fluctuations with an amplitude > 0.4 mmHg were termed putative micromotions.
- Bladder compliance was defined as bladder capacity/ (threshold – basal pressure) (μL/ΔmmHg) during filling at a rate of 25 μL/min.

Systemic Administration of Sildenafil in DAPM

The effect of sildenafil on bladder and urethral function was analysed in DAPM. The bladder was filled at 25 μL/min and once a stable pattern of micturition was established, baseline cystometric variables were measured (after more than four micturition cycles). After obtaining baseline recordings of voiding cycles, sildenafil was added to the circulating

perfusate with a cumulative dose every four voiding cycles (10 and 30 μM).

Distinguishing Central and Peripheral Actions of Sildenafil in the DAPM

To investigate the peripheral effects of sildenafil on the bladder (and to exclude any drug effects on CNS), the DAPM was established and then the cord transected at the medullo-spinal junction to remove the brain stem; the spinal cord was then pithed with a blunt wire. The effectiveness of this procedure was confirmed by a loss of phrenic nerve activity and of respiratory/pinch-evoked movements. As expected, without central control, the micturition cycle was lost and the bladder became overflow incontinent. The urethra was clamped to allow measures of filling pressure and compliance. Saline was infused into the bladder (25 $\mu\text{L}/\text{min}$) to a maximum intravesical pressure of 15 mmHg, to avoid over-distension. After baseline control fills, sildenafil was added to the perfusate and the filling cycle was repeated.

The Effects of Sildenafil on Pelvic Afferent Nerve Activity

The pithed DAPM was set up with a polyethylene catheter (PE-50; Clay-Adams, Parsippany, NJ, USA) sutured into the dome of bladder. The pelvic nerve was identified, cut and recordings were made from the distal end with a bipolar glass suction electrode. Bladder distention-induced pelvic nerve activity increased exponentially over the normal range of pressures seen during the micturition cycle (<15 mmHg). Nerve activity was AC amplified (5–10 k), band pass filtered (100 Hz–3 kHz), and digitised at 10 kHz. Multifibre afferent nerve activity was quantified by thresholding to count the number of action potentials (Spike2; Cambridge Electronic Design [CED], Cambridge, UK). The threshold level for spike counting was set at the peak level of the smallest identifiable spikes in the baseline pelvic nerve recording (i.e., with an empty bladder). After clamping the urethra, saline was infused (25 $\mu\text{L}/\text{min}$) until the intravesical pressure reached 15 mmHg (filling phase) and then the infusion was stopped for 3 min (isovolumetric phase). At the end of each cycle, the bladder was emptied by aspiration.

The Effects of Systemic and Intrathecal Sildenafil *in vivo*

Sildenafil was administered either intraperitoneally (i.p.) or intrathecally to urethane-anaesthetised mice (1.0–1.2 mg/g, i.p.) to investigate its systemic and spinal actions on the LUT. For EUS-EMG recordings, stainless steel insulated wires (0.075 mm diameter) were inserted into the sphincter muscle percutaneously. The bladder was cannulated with a PE-50 catheter (Clay-Adams) to monitor intravesical pressure.

Systemic sildenafil at doses of 4.67, 14 and 140 ng/kg was made up to 50 μL with saline before i.p. administration (estimated final concentration of 10, 30 and 300 μM in body fluid, assumed to be distributed in total body water, i.e., 70% of the mouse body weight). For intrathecal access, the L4–L6 vertebral spines were exposed through a midline skin incision. Sildenafil (5 μL of 150 μM) was applied intrathecally, assuming the intrathecal cerebrospinal fluid volume was 20 μL , this resulted in an estimated final intrathecal concentration of 30 μM . Likewise, for a final intrathecal concentration of 300 μM we injected 5 μL of 1.5 nM sildenafil. Lidocaine (5 μL , 1%) was injected at the end of experiment to confirm intrathecal targeting by prompt cessation of voiding ($n = 3$).

Electrical Field Stimulation (EFS)-Induced Contractions of EUS *in vitro*

Mice were killed by cervical dislocation and the whole bladder outflow tract was removed through a midline laparotomy. The preparation (10 mm length, 3 mm diameter, containing urethral smooth muscle and the EUS) was tied to an isometric force transducer in a perfusion trough, and superfused with Ringer's solution at 37 °C. Nerve-mediated tetanic contractions were generated by EFS (0.1 ms pulses, 40 Hz, 3-s train). The peak force of contractions and area under the curve (AUC) were normalised for tissue preparation weight (mN/g). After sildenafil (10 and 30 μM) application, sodium nitroprusside (SNP, 100 μM) was administered as a positive control [16] and EFS repeated.

Drugs and Solutions

The composition of Ringer's was (in mM): NaCl (125), NaHCO₃ (24), KCl (3.0), CaCl₂ (2.5), MgSO₄ (1.25), KH₂PO₄ (1.25); Glucose (10); pH 7.35–7.4 with 95% O₂/5% CO₂. Ficoll-70 (1.25%) was added as an oncotic agent to the perfusate. Stock solutions of sildenafil (750 μM) and SNP (340 mM) were made in distilled water and kept frozen until the experiment, at which time they were diluted in Ringer's and perfused at the final concentrations. All salts and drugs were from Sigma.

Data Acquisition

Perfusion pressure, phrenic nerve activity, electrocardiogram, bladder pressure, pelvic nerve and EUS-EMG activity were recorded using custom-built AC amplifiers and transducers (built by Mr Jeff Croker, University of Bristol, Bristol, UK), and digitised using a micro1401 A–D interface (CED) to a computer running Spike2 software (version 7, CED).

Analysis

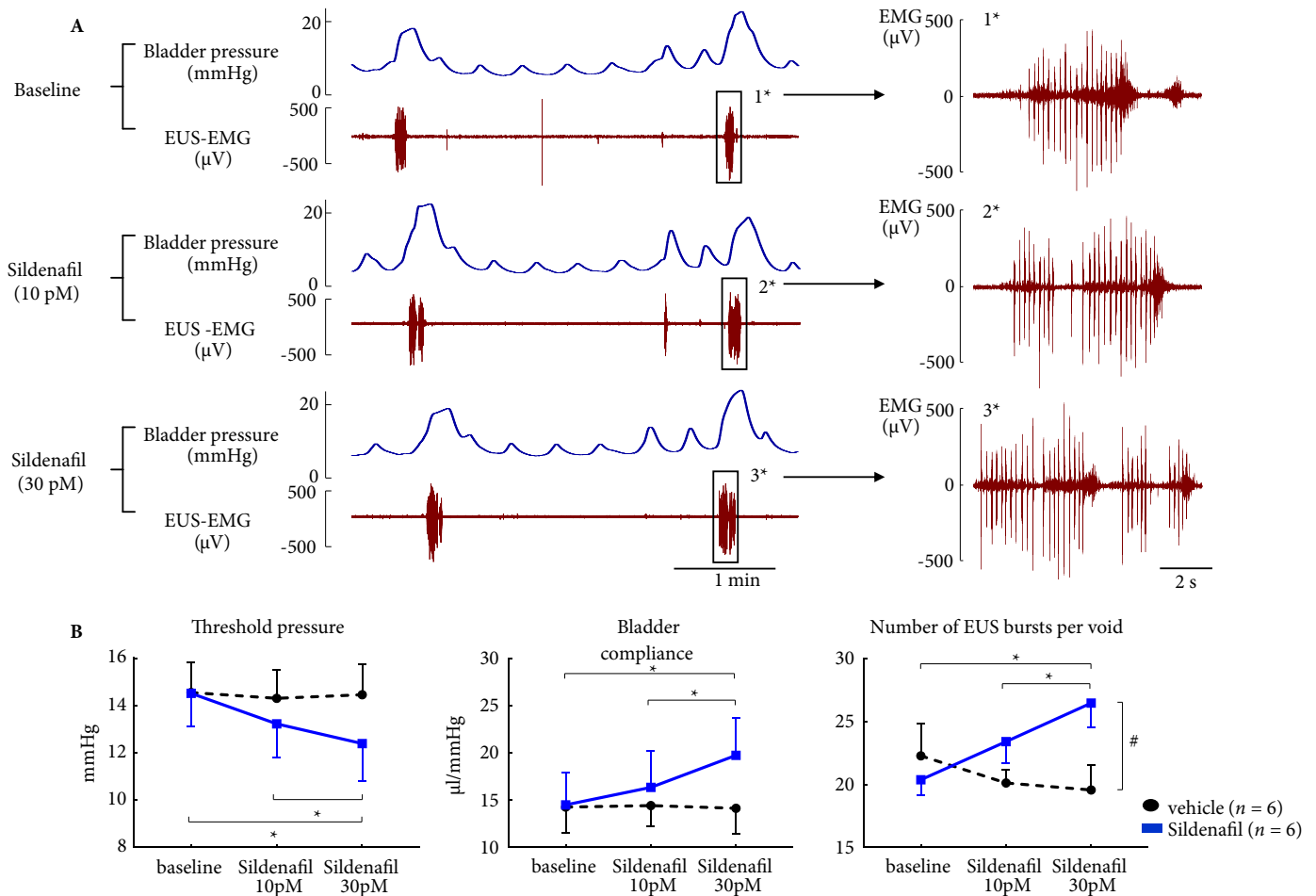
Analysis was conducted offline, using Spike2 software and the Statistical Package for Social Sciences (SPSS®), version 22

Table 1 Cystometry and perfusion pressure in DAPM before and after sildenafil.

Variable	Sildenafil (<i>n</i> = 6), mean (SEM)			Vehicle control (<i>n</i> = 6), mean (SEM)		
	Baseline	After sildenafil 10 pM	After sildenafil 30 pM	Baseline	After vehicle 1	After vehicle 2
Basal pressure mmHg	6.6 (0.7)	6.3 (0.8)	6.3 (0.8)	6.7 (0.4)	6.5 (0.4)	6.4 (0.4)
Threshold pressure, mmHg	14.5 (1.4)	13.2 (1.4)	12.4 (1.6)*	14.6 (1.3)	14.3 (1.2)	14.5 (1.3)
Micturition pressure, mmHg	23.1 (2.4)	22.1 (2.1)	23.4 (2.5)	22.6 (1.9)	23.0 (1.9)	23.1 (1.8)
Infused volume, μ L	100.4 (12.5)	92.5 (7.7)	98.6 (8.4)	99.8 (10.8)	100.6 (8.9)	90.1 (5.8)
Inter-void interval, s	241.1 (30.1)	221.9 (18.5)	236.7 (20.2)	239.6 (26.0)	241.1 (21.5)	237.6 (31.6)
Bladder compliance, μ L/ Δ mmHg	14.5 (3.4)	16.3 (3.9)	19.7 (4.0)*	14.3 (2.7)	14.4 (2.2)	14.1 (2.7)
Number of NVCs in 1 min	1.5 (0.7)	1.4 (0.5)	1.2 (0.4)	1.4 (0.3)	1.5 (0.3)	1.1 (0.3)
Average amplitude of NVC, mmHg	3.2 (0.7)	3.3 (0.4)	3.3 (0.4)	3.0 (0.7)	2.5 (0.4)	2.7 (0.4)
Perfusion pressure, mmHg	68.9 (4.3)	67.6 (3.9)	67.9 (4.5)	66.2 (3.0)	67.3 (3.0)	68.2 (2.7)

**P* < 0.05, significant difference from baseline, repeated-measures ANOVA with Dunnett's test for multiple comparison.

Fig. 1 Sildenafil increases bladder compliance, reduces voiding threshold and increases EUS bursting. **(A)** Representative recordings of bladder pressure and EUS-EMG activity in the DAPM before and after administration of sildenafil (10 and 30 pM) to the perfusate. **(B)** Sildenafil significantly decreased the threshold pressure (*P* = 0.029) and increased bladder compliance (*P* = 0.011). Sildenafil significantly increased the number of bursts of EUS-EMG activity per void compared with baseline (*P* = 0.023) and vehicle control recordings (at 30 pM, *P* = 0.033). Repeated measures ANOVA with Dunnett's test for multiple comparison. **P* < 0.05 compared to baseline; #*P* < 0.05 compared to vehicle, unpaired *t*-test.



(SPSS Inc., Chicago, IL, USA). The distribution of the sampled variables was assessed using the Shapiro–Wilk normality test. Statistical testing was by repeated measures

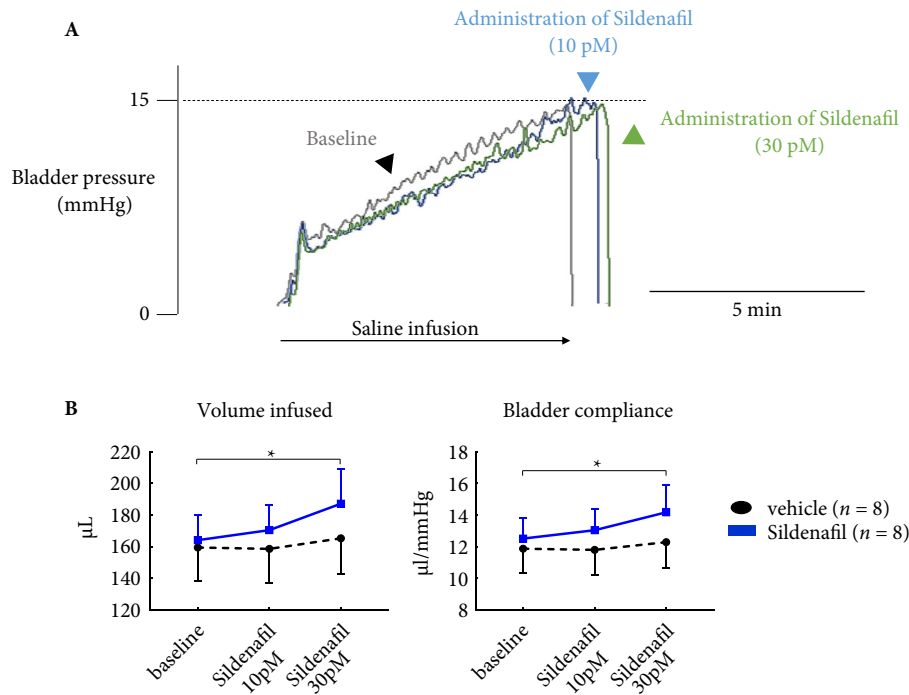
ANOVA with parametric (*t*-test) or non-parametric (Mann–Whitney *U*-test) tests as appropriate. All values are expressed as the mean (SEM) or median (interquartile range),

Table 2 EUS-EMG variables in DAPM before and after sildenafil.

Variable	Vehicle control (<i>n</i> = 6), mean (SEM)			Sildenafil (<i>n</i> = 6), mean (SEM)		
	Before	After vehicle 1	After vehicle 2	Before	After sildenafil 10 μ M	After sildenafil 30 μ M
Number of spike bursts in each void	23.4 (3.2)	20.1 (1.7)	19.6 (2.0)	20.4 (1.3)	23.4 (1.7)	26.4 (2.0) ^{*†}
Spike frequency within burst, Hz	4.7 (0.3)	5.0 (0.7)	4.6 (0.7)	4.7 (0.7)	4.6 (0.7)	4.6 (0.7)

**P* < 0.05 significant difference from before sildenafil administration, the repeated-measures Dunnett's test for multiple comparison. †*P* < 0.05 significant difference from vehicle of sildenafil, unpaired *t*-test.

Fig. 2 Peripheral actions of sildenafil. **(A)** Recordings of bladder pressure changes in response to saline infusion were made in the pithed DAPM. **(B)** Administration of sildenafil (30 μ M) to the perfusate increased bladder compliance, which led to a consequent increase in the volume infused before the maximum pressure threshold was reached (15 mmHg). Repeated-measures ANOVA with Dunnett's test for multiple comparisons **P* < 0.05: difference from baseline.



n = number of preparations. The null hypothesis was rejected at *P* < 0.05.

Results

Effects of Sildenafil on the Micturition Cycle and Urethral Sphincter Control in DAPM

Systemic administration of sildenafil significantly reduced perfusion pressure at 100 μ M in DAPM [to a mean (SEM) 85.8 (0.7)% of baseline, *n* = 5; *P* < 0.001, paired *t*-test]. Because perfusion pressure was monitored in the aorta in the DAPM (with maintained constant flow from the perfusion pump), this indicated that the 100 μ M dose of sildenafil was having a substantial biological effect to lower the vascular resistance. Therefore, to avoid this confounding consequence, sildenafil was used at 10 and 30 μ M in DAPM, which did not reduce perfusion pressure. The effect of sildenafil on

cystometric variables of the storage and voiding phases in DAPM was assessed (*n* = 6, Table 1). At 30 μ M concentration, sildenafil decreased the threshold pressure [to a mean (SEM) 84.7 (3.8)% of control, *P* = 0.029] and increased bladder compliance [to a mean (SEM) 140.2 (8.3)%, *P* = 0.011; Fig. 1A,B]. The number of bursts of EUS-EMG activity per void compared with baseline was also significantly increased [to a mean (SEM) 130.1 (6.9)% of control, *P* = 0.023; Table 2]. Sildenafil at 10 μ M, as well as vehicle (saline) controls, had no significant effects on these measures (Tables 1 and 2).

Direct Relaxant Action of Sildenafil on the Bladder

To test for a direct action of sildenafil on bladder function, the DAPM was pithed to ablate central neural control. With a fill to a vesical pressure of 15 mmHg, sildenafil (30 μ M)

significantly increased bladder capacity [to a mean (SEM) 112.5 (3.3)% of control, $P = 0.036$] and increased bladder compliance [to a mean (SEM) 111.9 (3.4)% of control, $P = 0.035$] compared with baseline ($n = 8$). Sildenafil at 10 μM had no significant effects (Fig. 2A,B, Table S1).

Sildenafil Augments Bladder Afferent Nerve Activity

Recordings were made from the pelvic nerve in the pithed DAPM to test for an action of sildenafil on bladder primary afferent terminals [14]. Under control conditions, intravesical saline infusion evoked an increase in pelvic afferent activity that ramped up with the increase of pressure [mean (SEM) firing rate 0.25 (0.1) Hz at 0 mmHg and 16.8 (3.0) Hz at 15 mmHg, $n = 18$ preparations). With a maintained bladder volume at 15 mmHg [mean (SEM) 80.8 (9.2) μL , $n = 18$], there was a partial reduction of afferent firing to a mean

(SEM) of 11.0 (2.2) Hz ($n = 18$). Sildenafil, at both 10 and 30 μM , significantly increased pelvic afferent activity during filling when referenced against both bladder pressure (Fig. 3A,Bi) and bladder volume (Fig. S1A) at doses of both 10 and 30 μM ; compared with the baseline values and also against vehicle controls (Fig. 3Bii and Fig. S1B). Sildenafil at 30 μM also significantly increased pelvic afferent activity during the isovolumetric phase [to a mean (SEM) 205.4 (30.2)% of control at 30 μM , $P = 0.002$; Fig. 3Ci,ii]. Vehicle treatment was without effect on the pelvic afferent activity (Fig. 3Ci,ii).

Systemic Sildenafil Increases EUS-EMG Bursting Activity per Void *in vivo*

Systemic application of sildenafil (4.67, 14 and 140 ng/kg) significantly increased the number of bursts of EUS-EMG

Fig. 3 Sildenafil increases bladder afferent activity. **(A)** Pelvic nerve recording of afferent activity showed a ramping increase with bladder filling and this declined to a plateau during an isovolumetric storage phase. Sildenafil (10 and 30 μM) augmented afferent activity during both phases. **(Bi)** Sildenafil (10 and 30 μM) increased pelvic afferent activity during the filling phase referenced against bladder pressure. **(Bii)** Proportionate change in afferent firing across bladder pressures caused by sildenafil compared to vehicle control, in each case normalised to the baseline bladder fill-evoked activity. **(Ci)** Sildenafil (10 and 30 μM) significantly increased pelvic afferent activity even after stopping saline infusion into the bladder and losing the dynamic component of bladder wall stretch ($P = 0.002$), and standardised values of afferent firing rate by baseline showed significant difference compared to vehicle group **(Cii)**. * $P < 0.05$, ** $P < 0.01$ difference from baseline, related-samples Friedman's one-way ANOVA by ranks. # $P < 0.05$, ## $P < 0.01$ difference from vehicle, Mann-Whitney U -test.

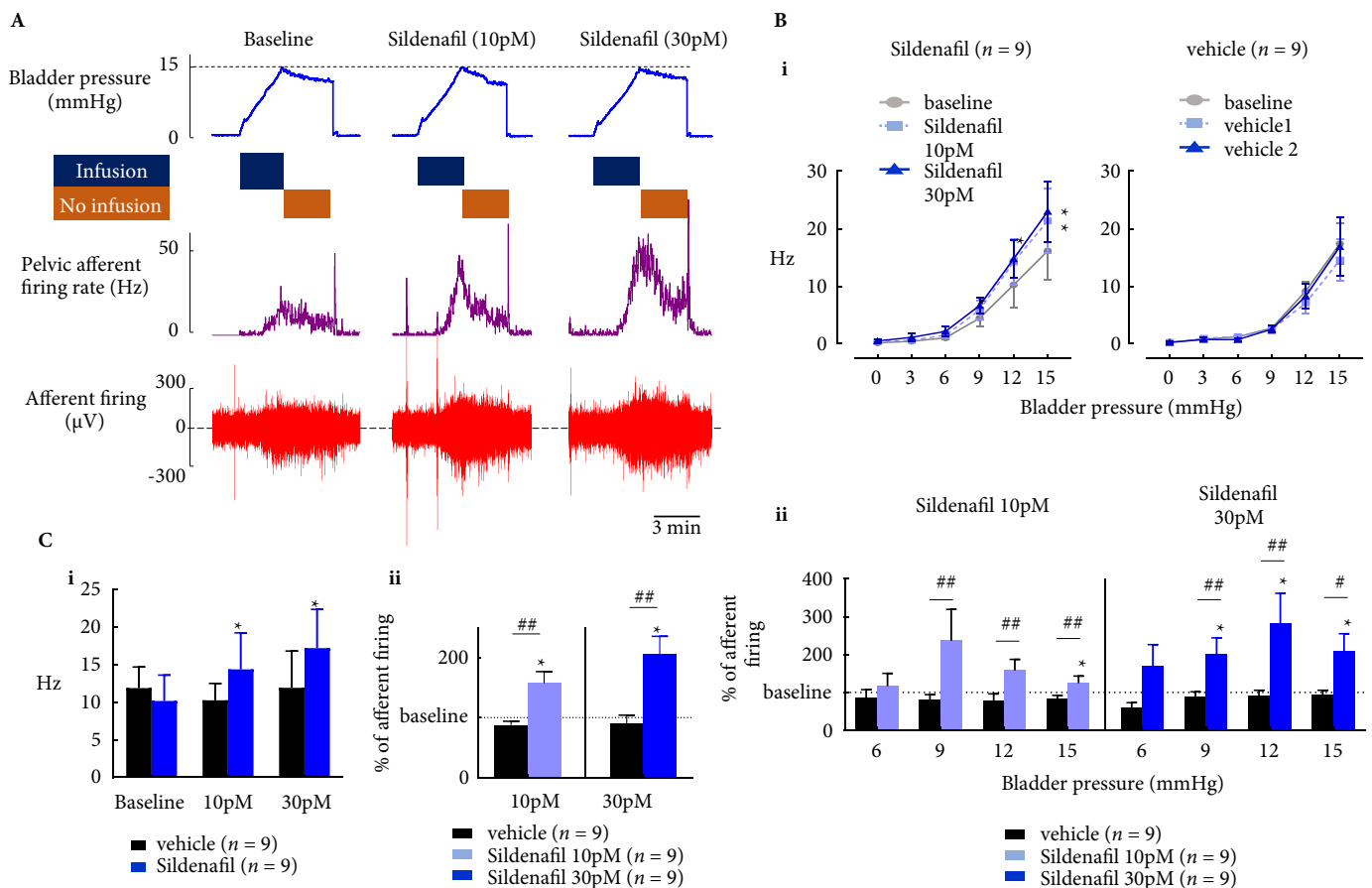


Table 3 EUS-EMG variables in urethane-anaesthetised mice before and after systemic sildenafil administration.

Variable	Systemic administration of vehicle (N = 7), mean (SEM)				Systemic administration of sildenafil (N = 7), mean (SEM)			
	Before	After vehicle 1	After vehicle 2	After vehicle 3	Before	After sildenafil 10 μM	After sildenafil 30 μM	After sildenafil 300 μM
Number of spike bursts in each void	21.8 (4.8)	22.3 (3.2)	22.1 (4.5)	22.1 (2.8)	23.0 (2.7)	25.2 (3.7)	27.0 (3.3)	27.1 (2.7)
Spike frequency within burst, Hz	6.8 (0.3)	7.1 (0.4)	7.1 (0.3)	7.1 (0.4)	7.0 (0.4)	6.9 (0.2)	7.1 (0.3)	7.2 (0.2)

activity per void compared with baseline [to a mean (SEM) 117.5 (5.8)% of control at 14 ng/kg, $P = 0.008$], as well as in comparison with vehicle control recordings ($P = 0.038$; Table 3, Fig. 4A,B). Cystometry showed no significant

changes after systemic application of sildenafil (Table 4). Intrathecal application of sildenafil (5 μL of 150 μM and 1.5 nM) produced no significant change in mouse cystometry (Table 5) or EUS-EMG variables (Table 6).

Fig. 4 Sildenafil increases EUS bursting *in vivo*, an effect not mediated by altered EUS contractility. **(A)** Representative recording of bladder pressure and EUS-EMG activity in urethane-anaesthetised mouse showing systemic (i.p.) administration of sildenafil 14 ng/kg (~30 μM) increasing the number of EUS bursts. **(B)** Pooled normalised data showing sildenafil significantly increased the EUS-EMG activity during voiding compared to both baseline and to vehicle group. **(C)** Representative recording of tetanic contractions of whole EUS induced by EFS after administration of sildenafil (10 and 30 μM) and the NO donor, SNP (100 μM) to the perfusate. Note that sildenafil had no effect on contractility, whereas it was markedly reduced by SNP. **(D)** Plots of maximum amplitude and AUC of EFS-induced contraction showed no change after sildenafil administration (10, 30 and 300 μM), and both were significantly reduced by SNP (100 μM). * $P < 0.05$, ** $P < 0.01$ difference from baseline, related-samples Friedman one-way ANOVA by ranks. # $P < 0.05$ difference from vehicle, Mann-Whitney U -test.

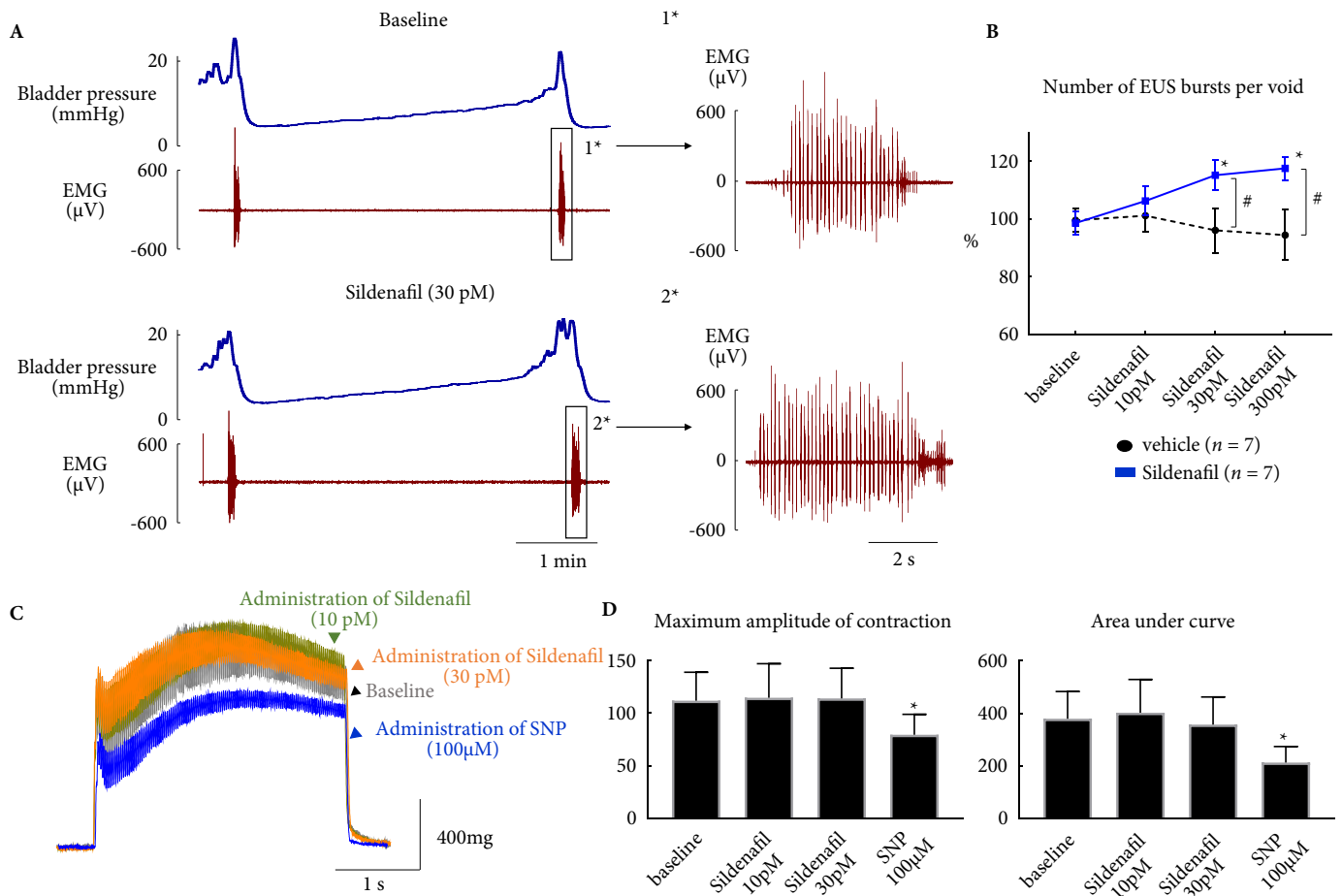


Table 4 Cystometry in urethane-anaesthetised mice before and after systemic sildenafil.

Variable	Systemic administration of vehicle (n = 7), mean (SEM)			Systemic administration of sildenafil (n = 7), mean (SEM)		
	Before	After vehicle 1	After vehicle 2	Before	After sildenafil 10 µM	After sildenafil 30 µM
Basal pressure, mmHg	3.2 (0.5)	3.4 (0.4)	3.6 (0.4)	2.7 (0.3)	2.7 (0.2)	2.7 (0.2)
Threshold pressure, mmHg	7.0 (0.6)	7.1 (0.2)	7.0 (0.4)	7.4 (0.2)	7.5 (0.5)	7.1 (0.7)
Micturition pressure, mmHg	16.8 (2.0)	16.0 (1.7)	16.4 (1.8)	16.3 (1.1)	15.5 (1.0)	15.4 (1.0)
Infused volume, µL	113.3 (23.9)	108.9 (18.8)	107.3 (17.8)	113.3 (11.0)	117.0 (11.2)	111.5 (9.9)
Inter-void interval, s	208.7 (31.1)	201.7 (21.9)	199.2 (20.3)	247.4 (11.3)	254.8 (3.1)	245.7 (18.0)
Bladder compliance, µL/ΔmmHg	32.1 (4.8)	31.7 (4.4)	32.4 (3.8)	25.2 (3.9)	26.6 (4.0)	28.8 (1.7)
Number of NVCs in 1 min	1.0 (0.2)	0.7 (0.1)	0.7 (0.1)	0.3 (0.1)	0.2 (0.1)	0.1 (0.1)
Average amplitude of NVC, mmHg	3.6 (0.9)	2.3 (0.3)	2.4 (0.4)	2.0 (0.5)	1.1 (0.4)	1.2 (0.4)

Sildenafil does not Modulate the Contractility of the EUS *in vitro*

Sildenafil (10 and 30 µM) did not alter the maximum amplitude [mean (SEM) 124.5 (28.2) mN/g at baseline] or AUC [mean (SEM) 424.4 (110.1) mN/g.s at baseline] of the tetanic EUS contractions generated by EFS ($n = 6$, Fig. 4C). By contrast, a positive control experiment with the NO donor SNP (100 µM) reduced the magnitude and AUC of the tetanic EUS contraction (by a mean (SEM) of 66.1 (6.3)% and 53.9 (9.1)%, respectively, $n = 6$, $P = 0.014$; Fig. 4D).

Discussion

Several mechanisms have been proposed by which PDE5-Is can improve LUTS including relaxation of the bladder and prostate smooth muscle, increased peripheral oxygenation, and decreased afferent activity [2]. However, the precise mechanism(s) that provide the clinical benefit remain to be elucidated.

In the present study, systemic sildenafil decreased the threshold pressure for voiding and increased bladder compliance, with effects at a relatively low concentration of 30 µM [17]. The effect on bladder compliance was independent of central neural control, as it was also observed in the 'pithed' DAPM preparation. Previous *in vitro* organ-bath studies with rabbit and human detrusor strips showed sildenafil relaxed detrusor strips at 0.1–1 µM [9,18,19] and thus were about three orders of magnitude higher than that used in the present study (10 and 30 µM). Attempts to use higher systemic doses of sildenafil (≥ 100 µM) in the DAPM were compromised by a significant fall in vascular resistance (a known effect of PDE5-Is on vascular smooth muscle) indicating that the effects reported in the present study were within a functional systemic range for the drug. Thus, the observed effects of sildenafil on LUT function at very low concentrations may be clinically relevant and indicates the presence of mechanisms that do not rely solely on direct smooth muscle relaxation.

Systemic (but not intrathecal) sildenafil increased the number of EUS-EMG bursts in each void without a direct effect on EUS muscle contractility. These data indicate that the effects of sildenafil on EUS-EMG bursting arise outside the spinal cord but not within the sphincter itself. We propose that this bursting activity is important for efficient voiding in mice [14] and rats [20,21], and that the increase of bursting will facilitate voiding. There are no previous studies measuring the effects of PDE5-Is on the electrical activity of EUS. A previous immunocytochemical study identified PDE5 expression in the striated muscle of rat EUS, suggesting that pelvic striated muscles are possibly regulated by PDE5 [16]. However, the present study did not demonstrate any effect of sildenafil on the contractile function of isolated mouse EUS

Table 5 Cystometry in urethane-anaesthetised mice before and after intrathecal sildenafil.

Variable	Intrathecal administration of sildenafil (<i>n</i> = 6), mean (SEM)		
	Before	After sildenafil 30 μ M	After sildenafil 300 μ M
Basal pressure, mmHg	4.7 (0.7)	3.5 (0.3)	3.7 (0.5)
Threshold pressure, mmHg	10.0 (0.8)	8.9 (0.8)	9.2 (0.9)
Micturition pressure, mmHg	20.2 (3.4)	19.0 (3.2)	19.5 (3.1)
Infused volume, μ L	96.0 (13.9)	100.0 (13.8)	98.4 (14.5)
Inter-void interval, s	230.3 (33.5)	240.0 (33.2)	236.2 (34.8)
Bladder compliance, μ L/ Δ mmHg	19.4 (4.6)	19.1 (1.7)	18.9 (2.9)
Number of NVCs in 1 min	1.5 (0.8)	1.6 (0.5)	2.2 (1.1)
Average amplitude of NVC, mmHg	3.4 (0.8)	2.0 (0.1)	2.1 (0.3)

Table 6 EUS-EMG variables in urethane-anaesthetised mice before and after intrathecal sildenafil administration.

Variable	Intrathecal administration of sildenafil (<i>N</i> = 6), mean (SEM)		
	Before	After sildenafil 30 μ M	After sildenafil 300 μ M
Number of spike bursts in each void	26.1 (7.1)	26.6 (7.8)	27.9 (9.6)
Spike frequency within burst, Hz	6.6 (0.3)	6.4 (0.3)	6.2 (0.5)

preparations. In addition, sildenafil has been shown to relax urethral smooth muscle, but at much higher concentrations in mouse (0.1–10 μ M) [22] and rat (0.1–1 μ M) [16] isolated preparations. Such a relaxant effect of sildenafil using doses in the low micromolar range does not account for the increase in sphincter EMG bursting in our present study. We also note that previous clinical studies in man have not found PDE5-Is to increase urinary flow rate [3,4,8]. This suggests that a simple direct relaxant effect on the EUS cannot fully explain improvement of LUTS by sildenafil.

Direct recordings showed that sildenafil increased mouse pelvic afferent nerve activity (when assessed against both bladder pressure and distension, so controlling for the change in compliance). Several previous studies have reported that PDE5-Is and NO reduce mechanosensitive afferent activity of both A δ - and C-fibres evoked by bladder distension in the rat [10,23,24]. However, it is worth noting that these reports of inhibitory effects of PDE5-Is on bladder afferent discharge were found at higher bladder pressures (30 cmH₂O) than the voiding threshold in normal rats (about 10–15 cmH₂O [25,26]) possibly reflecting inhibitory effects in a noxious range (i.e., on nociceptors), rather than in the normal range of storage and threshold pressures. This increase in the afferent activity may account for the reduction in the threshold pressure for voiding produced by sildenafil.

We propose that the increased afferent activity produced by sildenafil in the normal range of micturition pressures and degrees of bladder distension facilitates better co-ordination of bladder and sphincter function. This is in contrast to the

previous report that higher doses of sildenafil (1–10 mg/kg) inhibit afferent transmission, proposed to reduce urgency [10]. Consistent with our proposal, it is known that EUS motor neurones can be activated via segmental inputs [27], and studies in rats [28] and cats [29] showed that electrical stimulation of pelvic afferents elicited reflex firing in pudendal nerve efferent or the EUS. Thus, the augmented bladder afferent activity observed in the present study might increase EUS-EMG bursting observed in mice after sildenafil. However, this will require further investigation to test this hypothesis (likely requiring pudendal nerve recording and assessment in pathophysiological animal models including spinal cord injury, BOO, and ageing).

Summary

Sildenafil, at picomolar concentrations, increased EUS activity during voiding, and in addition increased bladder compliance and decreased threshold pressure for micturition. Sildenafil increased bladder afferent activity in the normal range of bladder volumes and pressures, consistent with it increasing the mechanosensitive feedback from the bladder during the micturition cycle. We propose that the increased bladder afferent activity may drive increased EUS activity during voiding, as previous studies demonstrated links between bladder afferent activity and pudendal efferent/EUS activity. This novel finding, demonstrating increased sphincter EMG bursting, may provide an alternative mechanism for the beneficial effects of sildenafil in LUTS. Although sphincter bursting activity is not believed to be present in humans, the

increase in pelvic afferent traffic in the non-nociceptive range may improve LUTS symptoms in a proportion of patients.

Conclusions

Using the DAPM, we have identified a novel effect of sildenafil on the EUS and bladder afferents, which may mediate the clinically useful effects of PDE5-Is in the treatment of LUTS.

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Conflicts of Interest

Dr Drake reports a grant from National Institutes of Health, during the conduct of the study; and personal fees and non-financial support from Astellas and Ferring Pharmaceuticals unrelated to the content of the submitted article. Dr Pickering reports grants from National Institutes of Health, during the conduct of the study; personal fees from Lateral Pharma, outside the submitted work. All other authors have nothing to disclose.

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Abbreviations: AUC, area under the curve; CED, Cambridge Electronic Design; cGMP, cyclic guanosine monophosphate; DAPM, decerebrate arterially perfused mouse; EFS, electrical field stimulation; EMG, electromyography; i.p., intraperitoneal/ intraperitoneally; LUT, lower urinary tract; NO, nitric oxide; NVCs, non-voiding contractions; PDE5-I, PDE type 5 inhibitor; PDE,

phosphodiesterase; PE, polyethylene; SNP, sodium nitroprusside.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Sildenafil increases bladder afferent activity in the DAPM. (A) Sildenafil (30 μM) increased pelvic afferent activity during the filling phase when referenced against bladder volume. (B) Proportionate change in afferent firing against bladder volume caused by sildenafil compared to vehicle control (in each case normalised to the baseline bladder fill-evoked activity). * $P < 0.05$: difference from baseline, related-samples Friedman's one-way ANOVA by ranks. # $P < 0.05$, ## $P < 0.01$: difference from vehicle, Mann–Whitney U -test.

Table S1 Cystometry in pithed DAPM before and after sildenafil.