1 **Influence of sildenafil on the purinergic components of nerve-mediated and urothelial ATP**

2 **release from the bladder of normal and spinal cord injured mice**

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4 **Running title:** Sildenafil on detrusor contractile function and ATP release

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30 **Abstract**

31 **Background and Purpose:** PDE inhibitors such as sildenafil alleviate lower urinary tract

32 symptoms, however a complete understanding of their action on the bladder remains

33 unclear. We are investigating the effects of sildenafil, on post- and pre-ganglionic nerve-

34 mediated contractions of the mouse bladder, and neuronal and urothelial ATP release.

35 **Experimental Approach:** Bladders were used from young (12 weeks), aged (24 months), and

36 spinal cord transected (SCT), mice, for *in vitro* contractility experiments. An arterially-

37 perfused *in situ* whole mouse model was used to record bladder pressure. Nerve-mediated

38 contractions were generated by electrical field stimulation (EFS) of postganglionic nerve

39 terminals or the pelvic nerve. ATP release during EFS in intact detrusor strips, and during

40 stretch of isolated mucosa strips, was measured using a luciferin-luciferase assay.

41 **Key Results:** Sildenafil (20 μM) inhibited nerve-mediated contractions in young mice, with an

42 increase in $f_{1/2}$ values in force-frequency relationships, demonstrating a greater effect at low

43 frequencies. Sildenafil reduced the atropine-resistant, purinergic component of nerve-

44 mediated contractions, and suppressed neuronal ATP release upon EFS *in vitro*. Sildenafil

45 reduced the preganglionic pelvic nerve stimulated bladder pressure recordings *in situ*;

46 comparable to *in vitro* experiments. Sildenafil reduced stretch-induced urothelial ATP release.

47 Sildenafil also relaxed nerve-mediated contractions in aged and SCT mice.

48 **Conclusion and Implications:** Sildenafil has a greater effect on the low-frequency, purinergic-

49 mediated contractions, and suppresses neuronal ATP release. In addition, sildenafil reduces

50 stretch-induced urothelial ATP release. These results demonstrate a novel action of sildenafil

51 to selectively inhibit ATP release from nerve-terminals innervating detrusor smooth muscle

52 and the urothelium.

Keywords

Adenosine Triphosphate

Mice

Muscle, Smooth

Neuromuscular Junction

Urinary Bladder

Lower Urinary Tract Symptoms

Abbreviations

12-wk, 12 weeks; 24-mo, 24 months; ABMA, α,β-methylene ATP; CCh, carbachol; EFS, electrical field stimulation; IDO, idiopathic bladder overactivity; LUT, lower urinary tract; LUTS, lower urinary tract symptoms; NDO, neurogenic bladder overactivity; NO, nitric oxide; OAB, overactive bladder; PDE5, phosphodiesterase type 5; SCT, spinal cord transection; TTX, tetrodotoxin.

Bullet point summary

- Sildenafil alleviates lower urinary tract symptoms, however its action on the bladder is unclear.

- Sildenafil reduces purinergic-mediated contractions, and selectively and completely inhibits neuronal and urothelial ATP release.

- Sildenafil may target pathological pathways associated with enhanced purinergic motor and sensory pathways.

Introduction

Lower urinary tract symptoms (LUTS) associated with bladder pathologies, such as overactive bladder (OAB) and neurogenic bladder dysfunction, and also with ageing are highly prevalent and can have a severe effect on the quality of life of patients. Current treatments for LUTS have recognised limitations, including uncertain efficacy and adverse effects. PDE type 5 (PDE5) inhibitors such as sildenafil (Viagra®), which are used to treat erectile dysfunction (Hatzimouratidis et al., 2010) also alleviate LUTS (McVary et al., 2007). The entire lower urinary tract (LUT) expresses PDE5 (Filippi et al., 2007, Fibbi et al., 2010), and studies support the use of PDE5 inhibitors to reduce LUTS, utilising the nitric oxide (NO)/cGMP pathway on several potential targets. These include increasing LUT oxygenation, decreasing afferent nerve activity, negatively regulating proliferation of LUT stroma and LUTS-related inflammation (Gacci et al., 2016). The ability of sildenafil to relax smooth muscle directly has also been described, where sildenafil-induced relaxation of human detrusor strips is significantly decreased using a soluble GC inhibitor to block the formation of cGMP (Oger et al., 2010). However, a complete understanding of their actions on the peripheral control of bladder contractile function remains unclear. In this study, the effects of sildenafil on nerve-mediated contractions of isolated mouse detrusor strips, as well as on *in situ* bladder function were characterised in young-adult, aged and spinal cord injured mice. In particular, the action of sildenafil on ATP-mediated processes was investigated. ATP is co-released along with ACh in parasympathetic endings to detrusor. With human detrusor ATP is a functional transmitter only in overactive pathologies (Bayliss et al., 1999) and so regulation of its release would be a therapeutic target. Moreover, stretch-activated ATP release from the urothelium, proposed to finally activate bladder afferents, is also enhanced in overactive bladder pathologies (Cheng et al., 2014), thus providing a possible cause for urinary urgency. The effect on neuronal and

99   urothelial neurotransmitter release was also investigated. A comprehensive description of

100 how sildenafil reduces contractile function will give insight into the pathology of bladder

101 functional disorders and should suggest potential therapeutic mechanisms of PDE5 inhibitors

102 in their treatment.

Methods

Tissue samples and ethics approval. All animal care and experimental procedures were in compliance with the University of Bristol Ethics Committee, in accordance with UK legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039), and in adherence to ARRIVE guidelines (Kilkenny et al., 2010) and *BJP* checklist for animal experimentation. Young (12 weeks (12-wk)) and aged (24 months (24-mo)) male C56Bl/7 mice (Harlan UK Ltd) were used for experiments.

Spinal cord transection. 12-wk male mice underwent complete spinal cord transection (SCT) at the T_8-T_9 level as previously described (Kadekawa et al., 2017, Ikeda et al., 2012, McCarthy et al., 2009). In brief, a laminectomy was performed at the T_8-T_9 spinal level under sterile surgical conditions. The spinal cord was transected and the cavity cleared to ensure complete transection, the space between cut ends was filled with a haemostatic sponge and muscle layers and overlying skin were closed with sutures. The surgery was performed under ketamine (70 mg/kg, i.p.) and medetomidine (0.5 mg/kg, i.p.) and upon recovery with atipamezole (1.0 mg/kg, i.p.) and buprenorphine (0.03 mg/kg, s.c.). Mice were also treated with ampicillin (0.10 mg/kg, s.c.) twice-daily for five days post-SCT to prevent urinary tract infections. Bladders of SCT mice were emptied by gentle abdominal compression and perineal stimulation twice a day for five days post-SCT. SCT mice were then euthanised with pentobarbital sodium (20-40 mg/mouse) and used for experiments.

Measurement of contractile function in vitro. Young (normal and SCT) and aged mice were euthanised by cervical dislocation and the bladder was removed through a midline laparotomy. The whole bladder was bisected and bladder strips from the bladder dome (detrusor with mucosa intact, 4-5 mm length, 1-2 mm width) were tied to a hook and an

isometric force transducer in a horizontal trough. Preparations were superfused with Tyrode's

solution at 37°C. Contractures were generated by exposure to carbachol (CCh, 1 μM) or high-

K$^+$ (80 mM) Tyrode's. Contractions generated by electrical field stimulation (EFS; 0.1 ms

pulses, 1-40 Hz, 3-s train every 90-s) that were inhibited by tetrodotoxin (TTX, 1 μM). With

some preparations TTX-resistant contractions were present, generated by direct muscle

stimulation, and were insensitive to atropine and α,β-methylene ATP (ABMA). Sildenafil (20

μM, see Results) was added to the superfusate and the effect on agonist- and nerve-mediated

contractions measured. These were subtracted from the EFS contraction, leaving a nerve-

mediated contraction. Tension amplitude (mN) was normalised to preparation weight

(mN.mg^{-1}). Nerve-mediated amplitude, $T(f)$, plotted as a function of stimulation frequency, f,

was fitted to equation 1a. The frequency-dependent percentage reduction of force, $R(f)$, by

an intervention was fitted to equation 1b. For frequency-dependent ATP release, $ATP(f)$, data

were fitted to equation 1c.

$$T(f) = \frac{T_{max}f^n}{f_{1/2}^n + f^n} \quad [1a]; \qquad R(f) = R_{Lf} - \left(\left(\frac{(R_{Lf}-R_{Hf}).f^m}{(f^m+k^m)}\right) + R_{Hf}\right)[1b];$$

$$ATP = ATP_b + \left(\frac{ATP_{max}f^n}{f_{1/2}^n + f^n}\right)[1c].$$

Where T_{max} is maximum tension at high frequencies, $f_{1/2}$ is the frequency to attain T_{max}/2, and

n is a constant (Pakzad et al., 2016): R_{Lf} and R_{Hf} are the maximum and minimum force

reductions respectively at low and high frequencies, m and k are constants. ATP_b is a

frequency-independent component of ATP release from detrusor preparations. Data fits were

performed with an iterative, least-squares algorithm (KaleidaGraph, v4.5, Synergy software,

CA, USA). Drug interventions were delivered via the superfusate, with appropriate vehicle and

time controls.

148 *Measurement of nerve-mediated ATP release in 12-wk normal and SCT mice.* Superfusate

149 samples (100 µl) were taken from a fixed point near the preparation, (two-thirds downstream

150 along the tissue length and 1mm lateral to the horizontally-mounted preparation) and with

151 minimal mechanical disturbance. Samples were taken before EFS and 2-seconds after the

152 initiation of EFS, the nerve-mediated release was taken as the difference in these two values.

153 Samples were stored on ice before assay of released ATP using a luciferin-luciferase assay

154 where the emitted light was proportional to the concentrations of ATP. The complete Sigma

155 ATP assay mix (FLAAM, Sigma-Aldrich, Dorset, UK) was diluted with the supplied assay buffer

156 as per manufacturer's instructions. Luminescence intensity was read using a luminometer

157 (Glomax 20/20, Promega) and calibrated with an ATP standard on the day of each experiment,

158 with luminescence as a linear function of concentration on a log-log plot over the range of

159 100 fM to 1 µM. Neuronal ATP release was inhibited by application of 1µM TTX. Appropriate

160 controls were carried out in background solutions using the solvents and chemicals tested.

161 The detection limit of the system was 100 fM ATP.

162 *Measurement of mucosa ATP release in 12-wk normal mice.* The mucosa was separated from

163 underlying detrusor by blunt dissection, mounted in a similar way to detrusor preparations to

164 an isometric force transducer in the horizontal trough and superfused with Tyrode's solution,

165 with or without sildenafil (20 µM). The hook was connected to a voltage-activated solenoid

166 that allowed rapid extension (within 1 s) of the tissue by 20% of the original length for 50 s

167 before restoration (within 1 s) to the original length. Samples were taken and assayed, as

168 above, before extension and at various time points upon extension, before and after drug

169 interventions.

170   *Measurement of nerve-mediated contractile function in situ.* Young mice were administered

171   heparin (50 IU, i.p.), and anaesthetised with isoflurane (2%) until loss of paw withdrawal reflex

172   for the following non-recovery procedure modified from previous studies with an *in situ*

173   arterially-perfused rat (Sadananda et al., 2011) and mouse (Ito et al., 2018, Ito et al., 2019).

174   The brain was removed, and the spinal cord pithed with a blunt wire before arterial perfusion

175   of the preparation. Both ureters were ligated to prevent natural bladder filling, the urethra

176   was clamped, and a catheter was inserted into the bladder lumen to record intravesical

177   pressure and maintain isovolumetric conditions. A glass suction electrode (tip diameter ~100

178   μm) was used to stimulate the left pelvic nerve once it had been dissected clear of underlying

179   tissue. Nerve-mediated bladder contractions were generated using an isolated stimulator

180   (Digitimer Ltd. UK; 0.1 ms pulses, 1-24 Hz, 3-s train every 90-s) and the pressure amplitude

181   (mmHg) was analysed. Female mice were used due to difficulties in successfully dissecting

182   and stimulating the pelvic nerve in male mice. Nerve-mediated whole bladder pressure

183   amplitude, P, as a function of frequency, f, was fitted to equation 1a, with peak pressure, P,

184   values substituting for tension, T, values. Drug interventions were delivered arterially.

185   *Data and statistical analysis.* Data are mean±SEM and differences between data sets were

186   tested with repeated measures two-way ANOVA followed by a parametric *post hoc* tests; the

187   null hypothesis was rejected at $p<0.05$. *n*-values refer to the number of preparations, one

188   each from separate animals. The data and statistical analysis comply with the

189   recommendation on experimental design and analysis in pharmacology {Curtis, 2015 #57} and

190   adhere to *BJP* guidelines. All statistical analyses were undertaken using GraphPad® Prism 7

191   (GraphPad Software Inc., CA, United States). The number of repeats in each control and

192   intervention set was based on a power calculation to reject the null hypothesis at p<0.05 and

193   a power of 80%, with variance of data based on previous experience data with these methods.

194   *Materials.* Tyrode's solution was composed of (mM): NaCl, 118; NaHCO$_3$, 24; KCl, 4.0;

195   NaH$_2$PO$_4$, 0.4; MgCl$_2$, 1.0; CaCl$_2$, 1.8; glucose, 6.1; Na pyruvate, 5.0; 5%CO$_2$, 95%O$_2$, pH 7.4.

196   The concentration of all stock solutions was between 1.0 and 10 mM. Sildenafil was dissolved

197   in DMSO. CCh, atropine and ABMA were dissolved in distilled water. Stock solutions were

198   diluted with Tyrode's solution to the final concentration as indicated. All chemicals were from

199   Sigma-Aldrich, Dorset, UK, except ABMA from Merck Millipore, Watford, UK.

200

201 **Results**

202 *Agonist-induced contractions in isolated detrusor: effect of sildenafil.* The peak force of the

203 CCh (1µM) contracture was significantly reduced by sildenafil at concentrations 1-100 µM. An

204 IC_{50} value was determined for each preparation, with a mean IC_{50} value of 5.4 ± 2.2 µM and

205 a residual force of 49.5 ± 4.7% (*n*=8: figure 1A). Sildenafil also reduced high-K^+ contracture,

206 with a mean IC_{50} value of 16.0 ± 9.6 µM and a residual force of 42.3 ± 13.6% (n=8: figure 1B).

207 The vehicle control, DMSO, had no effect on CCh and high K^+-contractures, except at

208 concentrations used for 30-100 µM test solutions when force was reduced by 9.6 ± 2.1%. A

209 test dose of 20 µM sildenafil was used for subsequent experiments with nerve-mediated

210 contractions.

211 *Nerve-mediated contractions in isolated detrusor: effect of sildenafil.* A combination of ABMA

212 (10 µM, to desensitise ionotropic purinergic receptors) and atropine (1 µM, to block

213 muscarinic receptors) abolished nerve-mediated contractions in detrusor from 12-wk mice.

214 Thus, nerve-mediated contractions are mediated by ACh and ATP release. Force-frequency

215 relationships (figure 2A) show atropine alone reduced contractions more at high frequencies,

216 and ABMA alone had a greater effect at low frequencies, data are shown for 12-wk mice. This

217 was quantified by calculation of $f_{1/2}$ values (see figure 2A control curve) that were reduced by

218 atropine and increased by ABMA (figure 2B). Thus, ATP as a neurotransmitter is released more

219 at low stimulation frequencies and ACh at higher frequencies. With detrusor from 24-mo mice

220 atropine and ABMA had similar effects on $f_{1/2}$ as in detrusor from young mice (figure 2Bii). In

221 addition, control T_{max} and $f_{1/2}$ values were not significantly different with young and aged mice

222 (young *vs* aged: T_{max}, control; 1.77±0.35 *vs* 0.97±0.37 mN.mm^{-2}; *n*=8,5).

223   Sildenafil (20 µM) reduced nerve-mediated contractions in isolated detrusor preparations

224   from 12-wk (figure 2C) and 24-mo mice. Figure 2D plots the percentage reduction of force by

225   sildenafil at each frequency in 12-wk and 24-mo mice, with a greater reduction of force at

226   lower stimulation frequencies compared to that at higher frequencies. This was corroborated

227   by an increase of $f_{1/2}$ value in both the 12-wk and 24-mo mice data (figure 2Bi, 2Bii), where

228   values were increased to those similar to that in ABMA. The difference between the

229   contraction suppression by sildenafil at high and low frequencies (ΔR_{Lf}-R_{Hf}, eq 1b) is a measure

230   of the purinergic component of the contraction if it blocks ATP release during nerve-

231   stimulation (see fig 3A below). With detrusor from 24-mo mice this was significantly greater

232   than with 12-wk mice (64.9±7.6 vs 41.3±7.6%, p=0.05, n=5,8 respectively), suggesting a more

233   significant contribution from purinergic transmission in 24-mo mice. Thus, the action of

234   sildenafil may be due either to a reduction of ATP release during nerve-stimulation, or a

235   selective inhibition of the action of ATP on detrusor. However, the latter is unlikely as

236   sildenafil also attenuated contractures mediated by carbachol (figure 1A). Accordingly, the

237   first hypothesis was tested by investigating the mode of action of sildenafil with detrusor from

238   12-wk mice.

239   *Nerve-mediated and stretch-induced ATP release: effect of sildenafil*. Nerve-mediated ATP

240   release was measured directly by measuring superfusate [ATP] near the preparation. In

241   control conditions, a frequency-dependent release of [ATP] was superimposed on a

242   background fraction (*ATP*$_b$, figure 3A); data were analysed between 1-16 Hz. With subsequent

243   addition of 20 µM sildenafil *ATP*$_b$ was not significantly different (0.54 *vs* 0.43 pmol.µl^{-1}, n=7),

244   however, the frequency-dependent component was completely abolished.

245   Previous studies have shown that the mucosa is also a source of ATP, especially when

246   stretched and it was of interest to determine if this also is affected by sildenafil. Rapid stretch

247   by 20% of the resting length for 50 s was followed by restoration of the original length.

248   Superfusate samples were collected prior to stretch, during the stretch and at 0.5, 1.0, 2.0,

249   5.0 and 10.0 min after restoration. In control conditions, ATP release peaked at 2 min after

250   restoration to nearly 60-fold increased over pre-stretch levels and remained elevated after

251   10 minutes. Sildenafil (20 μM) did not affect pre-stretch levels but significantly attenuated

252   the post-stretch increase (figure 3B).

253   *Bladder contractions evoked by preganglionic pelvic nerve stimulation: effect of sildenafil.* The

254   preganglionic pelvic nerve was dissected as much as possible from adhering tissue and

255   stimulated *in situ* in 12-wk mice (figure 4A) to generate intravesical bladder pressure

256   transients (figure 4B). The frequency-dependence was identical to *in vitro* contractions

257   through postganglionic nerve-stimulation (figure 4C). Sildenafil (20 μM) also reduced the

258   amplitude of the pressure transients with an action greater at lower frequencies (figure 4D),

259   as also observed *in vitro*, figure 2D. Thus, as above, the $f_{1/2}$ value was increased by sildenafil

260   (3.7±0.4 *vs* 6.0±0.6 Hz respectively, *n*=5, *p*<0.05).

261   *Nerve-mediated contractions and ATP release in spinal cord-transected mice: effect of*

262   *sildenafil.* Sildenafil (20 μM) reduced nerve-mediated contractions in SCT 12-wk mice, with a

263   greater effect at low stimulation frequencies as observed in young and aged mice with an

264   intact spinal cord (figure 5A). The inset shows that $f_{1/2}$ values were significantly increased with

265   sildenafil. Nerve-mediated ATP release was also measured that was also abolished by

266   sildenafil, and superimposed on a background release (ATP_b, figure 5B). When compared with

267   12-wk intact mice the frequency-dependent release of ATP was however significantly smaller:

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268   for example at 8 Hz stimulation the frequency-dependent ATP release was 2.63±0.62 *vs*

269   0.54±0.12 pmol.g^{-1} (*n*=8,5; *p*<0.05) in normal and SCT 12-wk mice.

Discussion and Conclusions

270

271 *Actions of sildenafil on detrusor and efferent nerve activity*. PDE5 inhibitors, such as sildenafil

272 and tadalafil are successfully used to manage erectile dysfunction, and it has also been

273 observed they relieve lower urinary tract symptoms, especially in men with benign prostatic

274 hyperplasia, although there is no consensus about their principal modes of action (Gacci et

275 al., 2016; Andersson, 2018). This study, using mice, confirmed a direct relaxant effect of

276 sildenafil on detrusor smooth muscle, with a similar potency to that with human detrusor

277 (Oger et al., 2010) where it was proposed to act by altering cyclic nucleotide levels and

278 modulating K^+ channel activity, such as K_{ATP} and BK channels. However, there has been less

279 investigation of their action on nerve-mediated contractions and this study reports a

280 substantial and frequency-dependent action. It cannot be excluded that the concentration

281 chosen for interventions (i.e. 20 µM) had an effect on other PDEs, however this concentration

282 was that which produced a half-maximal relaxation in *in vitro* experiments.

283 Parasympathetic postganglionic fibres activate detrusor by release of ACh and ATP and

284 abolition of contractions by atropine and ABMA implies that muscarinic and $P2X_1$ receptors

285 mediate this process, in contrast to the situation in guinea-pig detrusor (Kennedy et al, 2007).

286 In most animals and human OAB both transmitters are functionally active, although in normal

287 human bladders only ACh has a role due to high ectoATPase activity (Bayliss et al., 1999;

288 Harvey et al., 2002). Sildenafil suppressed nerve-mediated contractions but more at low

289 stimulation frequencies (i.e. 1-4 Hz) so it is unlikely this was due only to a direct effect on

290 smooth muscle. Release of noradrenaline and ATP in the sympathetic nervous system can be

291 separately regulated (Speirs et al., 2006). Differential release of ACh and ATP in detrusor

292 parasympathetic endings also occurs (Pakzad et al., 2016), where ATP release is reduced

16

293   selectively by A1 receptor agonists. The cellular pathways regulating separate release of ATP

294   and ACh remain to be established; however, it has potential implications for therapeutic

295   management of human OAB, as ATP is a functional transmitter in this condition. The ability

296   of sildenafil to regulate ATP release selectively was more directly shown by its abolition of

297   TTX-sensitive, frequency-dependent release of ATP. EFS may possibly result in urothelial ATP

298   release in these experimental conditions, either by direct stimulation (Sadananda et al., 2009)

299   or indirectly by the changes in muscle contraction. However, sildenafil reduced both neuronal

300   and urothelial ATP release in this study.

301   *In vitro* experiments with isolated detrusor preparations were mirrored by stimulation of

302   preganglionic fibres in an *in situ* model. The frequency-dependence of pressure transients

303   mirrored that of contractions *in vitro* and were similarly influenced by sildenafil. This implies

304   that the actions of sildenafil may be explained only by its action at the post-ganglionic junction

305   and on smooth muscle, with no separate action on pre-ganglionic fibres. The data also imply

306   that under these experimental conditions the pelvic ganglion does not modulate the gain of

307   pre- to post-ganglionic transmission.

308   *Sildenafil effects on mucosal ATP release and afferent signalling*. The mucosa exerts

309   considerable effects on detrusor contractile performance; it reduces contractility (Hawthorn

310   et al., 2000), but enhances spontaneous activity (Ikeda & Kanai, 2008). Mucosal influence over

311   spontaneous activity is in part due to stretch-mediated ATP release (Kushida & Fry, 2016) and

312   the observation that sildenafil greatly reduced such ATP release offers another route for the

313   PDE5 inhibitor to reduce detrusor contractile function. Of note, A1 receptor agonists and

314   sildenafil both reduce stretch-activated mucosal ATP release (Dunning-Davies et al., 2013), as

315   well as nerve-mediated release, suggesting a common regulator of ATP release in these two

316 tissues. Another proposed role for mucosal ATP release is suburothelial afferent nerve

317 activation to provide a transduction pathway between bladder wall stretch and sensation

318 (Vlaskovska et al., 2001). Sildenafil reduces bladder afferent activity (Behr-Roussel et al.,

319 2011), but it has to be determined if the action is a direct one on nerves, or indirectly through

320 modulation of mucosal ATP release.

321 *Sildenafil and the ageing bladder*. Ageing is associated with fibrosis and either a decline or

322 maintenance of detrusor contractile function in animal and human models (Lluel et al.,2000;

323 Fry et al., 2011; Kamei et al., 2018). More consistent is the increased significance or purinergic

324 neurotransmission with age (Yoshida et al., 2004), greater purinergic receptor expression

325 (Daly et al., 2014) and enhanced stretch-mediated mucosal ATP release (Sui et al., 2014). This

326 suggests a potential role for sildenafil to suppress neural and mucosal release of ATP that is

327 associated with OAB in humans. Data here (figure 2D) showed the frequency-dependent

328 reduction of nerve-mediated contractions by sildenafil was greater in aged animals,

329 suggesting an increased purinergic component in neuromuscular transmission.

330 *Sildenafil and bladder function with spinal cord injury*. A greater role for purinergic

331 transmission and mucosal release is also suggested in idiopathic and neurogenic bladder

332 overactivity (IDO, NDO). Atropine-resistance, suggesting functional purinergic transmission,

333 is a feature of IDO and NDO (Bayliss et al., 1999) but the data here showed nerve-mediated

334 ATP release was actually smaller in SCT mice. If this is extrapolated to the human condition

335 purinergic contractions can only be explained by reduced hydrolysis of ATP in the

336 neuromuscular junction, as indeed observed in detrusor from patients with IDO and NDO (Fry

337 et al., 2018). Mucosal ATP release is also reported in tissue isolated from OAB (Khera et al.,

338 2004; Salas et al., 2007; Smith et al., 2008) which will potentially increase afferent firing for a

339   given stretch of the bladder wall. Both targets are therefore useful for PDE5 inhibitors to

340   suppress, potentially to alleviate symptoms of OAB. This approach offers an advantage over

341   current therapeutic pathways to suppress OAB that use muscarinic receptor antagonists,

342   receptors that mediate the normal mode of bladder contraction.

343   **Conclusions**. The PDE5 inhibitor sildenafil exerts a direct detrusor relaxant effect as previously

344   reported. The novel finding of this study is that sildenafil also selectively and completely

345   inhibits ATP release from motor nerves to detrusor, as well as stretch-activated release from

346   the mucosa. The particular pathways whereby this effect is mediated is yet to be determined

347   – for example, is this a NO/cGMP dependent effect, etc. This blockade was observed not just

348   in young animal animals but also in aged animals and those subjected to spinal cord injury.

349   The significance of this observation in aged and SCT animals is that in humans these conditions

350   are especially associated with OAB syndromes and enhanced purinergic motor and sensory

351   pathways. This offers the possibility that PDE5 inhibitors will be useful to suppress OAB

352   symptoms by suppressing pathological pathways in humans, rather than supressing normal

353   physiological pathways, as occurs at present with current therapeutic interventions.

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Figure legends

Figure 1. The effect of sildenafil (1–100 μM) on **(A)** carbachol (CCh, 1 μM, *n*=8) and **(B)** high-K$^+$ (80 mM, *n*=5) induced contractions in isolated detrusor from 12-wk mice. Data are mean±SEM. *p< 0.05. Data are fitted to equation 1B, Methods.
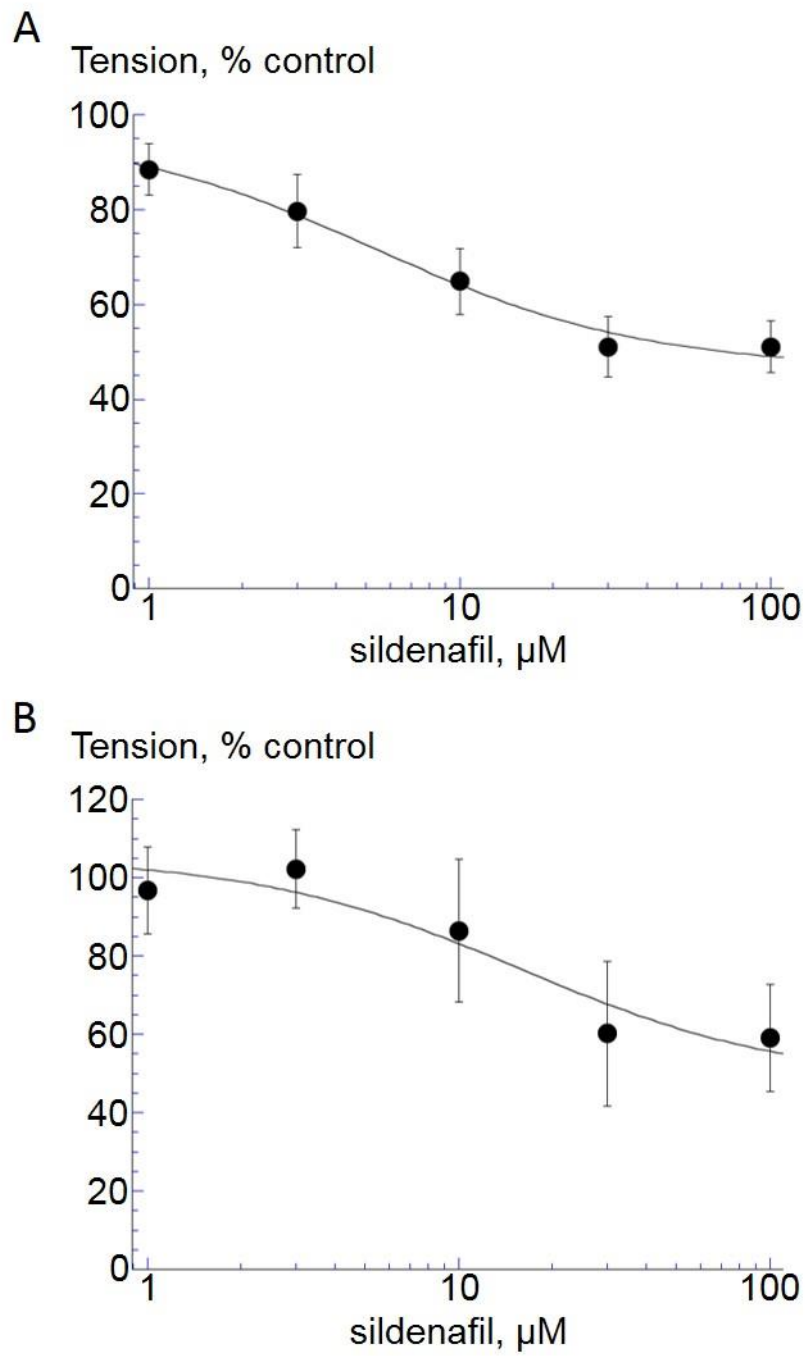
Figure 2. (A). Frequency-response curves for nerve-mediated contractions in control (*n*=8) and in the presence of α,β-methylene ATP (ABMA, 1 μM, *n*=6) or atropine (1 μM, *n*=6). Data are plotted as a percentage of the maximum estimated contraction under control conditions, $T_{max,control}$. The value of the $f_{1/2}$ for the control curve is shown, i.e. the frequency to achieve $T_{max/2}$. The fits are from equation 1a, Methods. **(B).** $f_{1/2}$ values for control conditions (*n*=8), and in the presence of ABMA (*n*=6) and atropine (*n*=6), the value in the presence of 20 μM sildenafil is also shown (*n*=8, 5). *p< 0.05; data are for 12-wk mice (**Bi**) and 24-mo mice (**Bii**). **(C).** Traces of nerve-mediated contractions under control conditions and with 20 μM sildenafil. The frequency values are those used to elicit nerve-mediated responses. **(D).** Percentage reduction of nerve-mediated contractions by sildenafil with preparations from 12-wk (left, *n*=8) and 24-mo (right, *n*=5) mice. Fits are from equation 1b, Methods. All group data are mean±SEM.

Figure 3. (A). Frequency-dependent nerve-mediated ATP release from 12-wk mice under control conditions (*n*=8) and in the presence of 20 μM sildenafil (*n*=6). Data were fitted to equation 1B, Methods. **(B).** Stretch-activated ATP from the mucosa of from 12-wk mice under control conditions (*n*=6) and in the presence of 20 μM sildenafil (*n*=6). Samples were obtained 2-min prior, during and 0.5, 1.0, 2.0, 5.0 and 10.0 min after a 50 s stretch. All group data are mean±SEM. *p< 0.05.

Figure 4. (A). Schematic diagram of the arterially-perfused *in situ* mouse model used for preganglionic pelvic nerve stimulation (n=6). The pelvic nerve was dissected and stimulated.
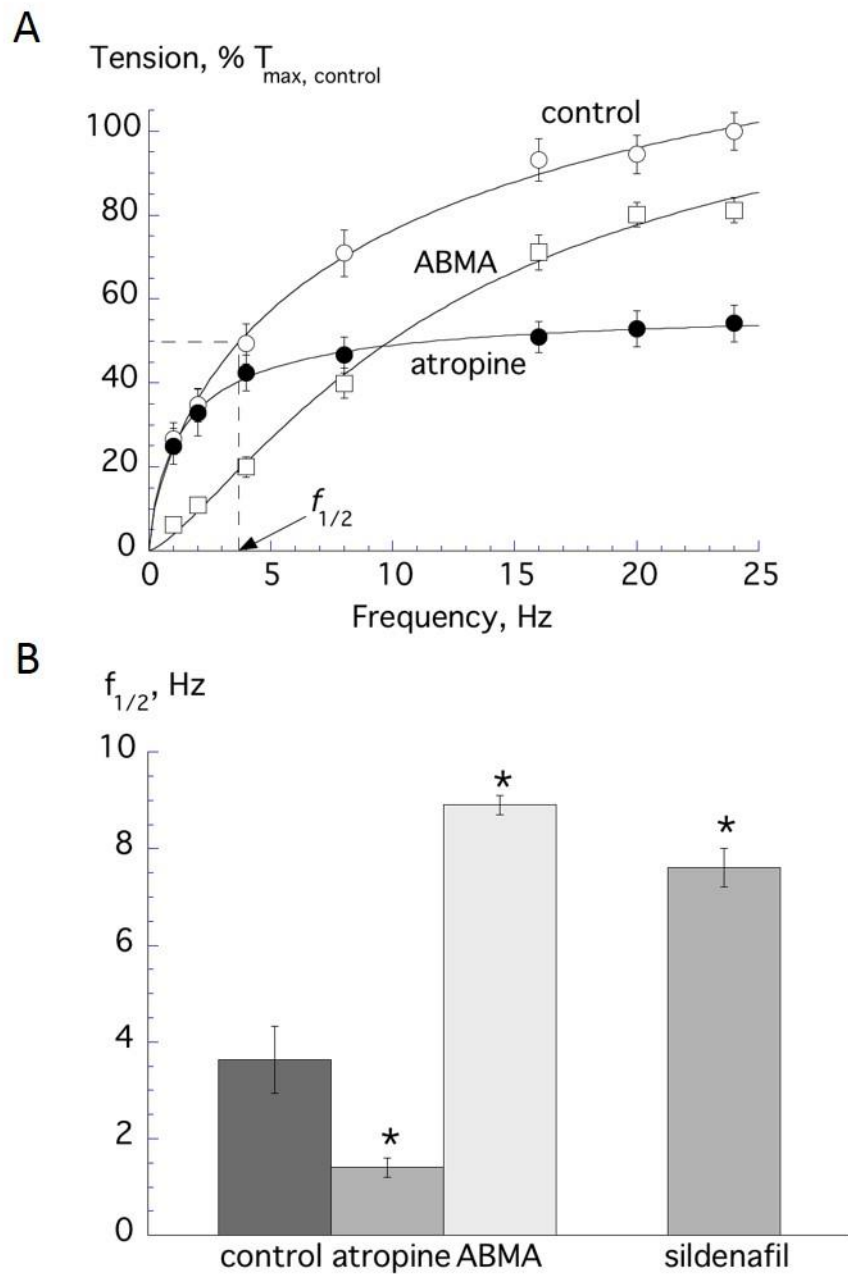
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486   **(B).** Traces of nerve-mediated whole bladder pressure contractions on control conditions and

487   in the presence of 20 µM sildenafil. **(C)**. Force- and pressure-frequency relationships with data

488   from the same 12-wk mice obtained *in situ* (P/P_{max}) and *in vitro* (T/T_{max}). Data fitted to

489   equation 1a, Methods. **(D)**. Percentage reduction of nerve-mediated pressure transients by

490   sildenafil with preparations from 12-wk mice (*n*=5), data fitted to equation 1b, Methods. All

491   group data are mean±SEM.

492   **Figure 5. (A)**. Nerve-mediated contractions from spinal cord transected (SCT) 12-wk mice

493   under control conditions and in the presence of 20 µM sildenafil (*n*=5). Data were fitted to

494   equation 1a, Methods. The inset shows the respective $f_{1/2}$ values, *$p<0.05$. **(B)**. Frequency-

495   dependent nerve-mediated ATP release in 12-wk SCT mice under control conditions (*n*=5) and

496   in the presence of 20 µM sildenafil (*n*=5). Data were fitted to equation 1c, Methods. All data

497   are mean±SEM.

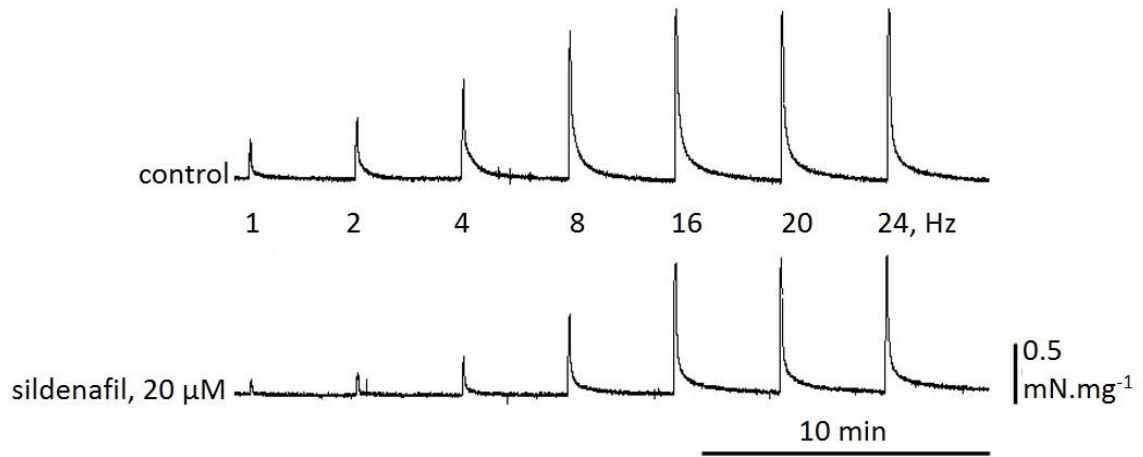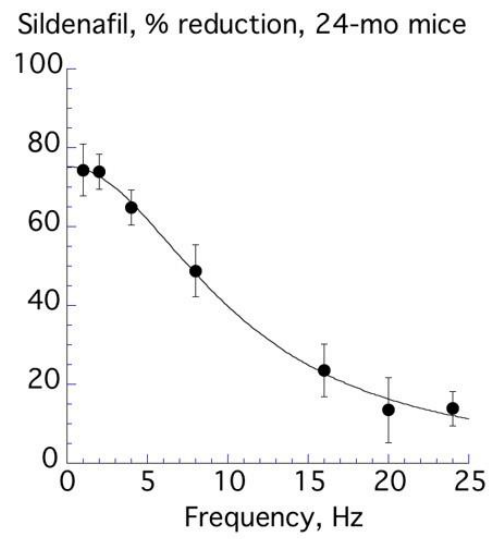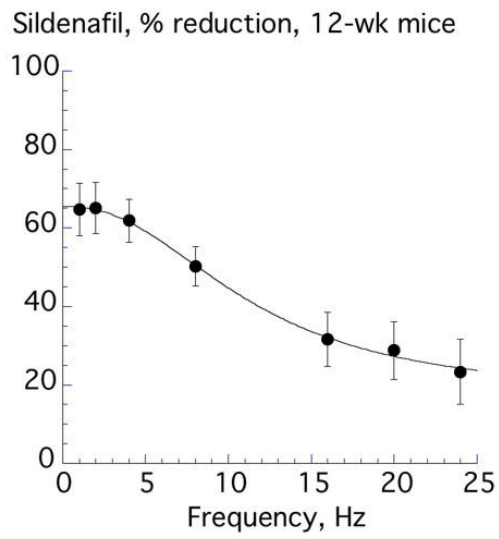498

499 **Figure 1.**

A

Tension, % $T_{max, control}$

B

$f_{1/2}$, Hz

500

501 **Figure 2.**

27

C

D

502

503 **Figure 3.**

A

Aorta
Heart

Perfusion
Tyrode's sol^n

Perfusion
pressure

Bladder
(saline 10μM)

Ligated ureter

Bladder
pressure

Pelvic nerve stim^n

Pubic bone
Urethral clamp

External urethral
sphincter

Tip of glass

Electrode

Pelvic nerve

(+)

(-)

Wire electrode

B

control

1   2   4   8   16   20   24, Hz

sildenafil, 20 μM

2 mmHg

10 min

504

Figure 4.

C

P/P$_{max}$ (○)
T/T$_{max}$ (●)

Frequency, Hz

D

Sildenafil, % reduction

Frequency, Hz

506

507   **Figure 4.**

508

509 **Figure 5.**