JPET Fast Forward. Published on September 5, 2013 as DOI: 10.1124/jpet.113.207340

Title Page

A functional analysis of the influence of β3-adrenoceptors on the rat micturition cycle.

Prajni Sadananda, Marcus J Drake, Julian FR Paton & Anthony E Pickering

PS, JFRP, AEP - School of Physiology & Pharmacology, Medical Sciences Building, University of Bristol, Bristol, United Kingdom;

PS (current address) - Department of Anatomy and Neuroscience, University of Melbourne, Grattan Street, Parkville, Victoria 3051, Australia

MJD - School of Clinical Sciences, University of Bristol, Bristol, United Kingdom.

JPET Fast Forward. Published on September 5, 2013 as DOI: 10.1124/jpet.113.207340
This article has not been copyedited and formatted. The final version may differ from this version.

Running title page

Role of β_3-adrenoceptors in rat micturition

Corresponding author:

Anthony E Pickering, School of Physiology and Pharmacology, University of Bristol, University Walk, Bristol BS8 1TD, UK.

Telephone: +44 117 3312311, Fax: +44 117 331 2288

Email: Tony.Pickering@bristol.ac.uk

Number of:

text pages - 33

tables - 1

figures - 7

references - 28

Number of words:

Abstract - 247

Introduction - 603

Discussion - 1399

A list of nonstandard abbreviations used in the paper.

β-AR - β-adrenoceptors; CNS – central nervous system; DAPR - decerebrate artificially perfused rat; EUS - external urinary sphincter; LUT – lower urinary tract; NMC – non micturition contraction; OAB – over-active bladder; PAN - pelvic afferent nerve

Recommended section assignment: - Neuropharmacology

JPET Fast Forward. Published on September 5, 2013 as DOI: 10.1124/jpet.113.207340
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Dysfunctions of the lower urinary tract such as overactive bladder syndrome and incontinence are the product of storage failure. Spontaneous regional bladder wall movements (non-micturition contractions, NMCs) are proposed to generate afferent activity that signals volume status to the CNS. The sympathetic nervous system, via activation of β-adrenoceptors (β-AR), causes bladder relaxation and promotes urine storage. We hypothesised that β-AR regulation of micturition is mediated by suppression of NMCs. We employed an un-anaesthetised decerebrate, artificially perfused rat preparation that allows simultaneous cystometry with external urethral sphincter and pelvic afferent nerve recordings. Systemic isoprenaline (10nM-1μM) increased inter-void interval and bladder compliance accompanied by a decrease in NMC amplitude, voiding pressure and voiding threshold. Isoprenaline also reduced arterial pressure and increased heart rate. The β_3-AR agonist mirabegron (10-100nM) increased inter-void interval and bladder compliance and reduced NMC amplitude yet preserved active voiding function and had no effect on arterial pressure or heart rate. All of these effects of mirabegron were blocked by the selective β_3-AR antagonist (L748,337), which alone shortened inter-void interval and decreased bladder compliance – suggesting the presence of a basal β_3-AR-mediated sympathetic tone. Similar effects of mirabegron were seen in an acetic acid-sensitised bladder preparation and in preparations after loss of spino-bulbar reflex bladder control. The β_3-AR-mediated increase in inter-void interval correlated with increased bladder compliance but not with the fall in NMC amplitude. These findings indicate that β_3-adrenoceptors have a selective effect to improve urine storage by increasing compliance without affecting the active components of voiding.

Introduction

Dysfunctions of the lower urinary tract (LUT) such as overactive bladder (OAB), urgency, incontinence and nocturia signify failures of urine storage (Abrams et al., 2002). The mechanisms by which the bladder signals its volume status to the central nervous system are key to understanding such failures of storage. During the storage phase of micturition a progressive increase in sympathetic activity releases noradrenaline that is believed to act on β-adrenoceptors (β-AR) on the bladder wall to produce smooth muscle relaxation. This increases bladder compliance and enables continued low pressure filling of the bladder (reviewed in Fowler et al., 2008). When a storage threshold is reached (and the environmental context is permissive), parasympathetic muscarinic receptor activation evokes detrusor contraction and emptying of bladder contents.

The major roles of β-AR and muscarinic receptors in regulating bladder function in health and disease have made them attractive drug targets for treatment of bladder pathophysiology (Andersson and Wein, 2004; Hood and Andersson, 2013). It is worth noting that voiding dysfunctions, such as poor stream and incomplete bladder emptying, can arise in conjunction with storage problems. Thus, the development of therapies for storage dysfunction needs to consider the potential adverse effect of impaired voiding function.

All of the known β-AR subtypes (1, 2 and 3) have been localised in human and rat bladders (Yamaguchi and Chapple, 2007) and detrusor relaxant actions have been described particularly for β_2- & β_3-AR, with some variation across species. Importantly, the relatively restricted distribution of the β_3-AR subtype on the bladder has led to the development and recent licencing of selective agonists as treatments for

OAB (Michel et al., 2011). However, it remains unclear how β_3-AR activation functionally improves urine storage – via direct relaxant effects on bladder smooth muscle (Takeda et al., 2000) or via changes in afferent transmission (Aizawa et al., 2012) with or without urothelial mediation.

We have developed and characterised a novel model for integrated functional physiological and pharmacological studies of the complete rat micturition cycle in an un-anaesthetised preparation – the decerebrate artificially perfused rat (DAPR (Pickering and Paton, 2006; Sadananda et al., 2011)), a variant of the working heart brainstem preparation (Paton, 1996). This has intact LUT-spinal-brainstem reflex loops and demonstrates autonomic tone whose presence is crucial for normal bladder function. It also facilitates both nerve and sphincter recordings and enables the precise control of the composition of the perfusate for pharmacological investigations – analogous to *in vitro* conditions.

We previously observed that loss of autonomic tone in the DAPR (both pharmacological with hexamethonium ganglion blockade and pathological with loss of brainstem function) enhanced non-micturition contraction (NMC) amplitude and reduced inter-void interval (Sadananda et al., 2011). This led us to hypothesise that the sympathetic drive acts to suppress NMCs – which are proposed to be part of a volume assessment reflex mechanism (Drake et al., 2001), and hence facilitate urine storage. We aimed to test this proposal by investigating the role of β-AR agonists on the micturition cycle with a focus on the functional effect of β_3-agonism, given the predominant role of the β3 subtype in mediating bladder relaxation (Igawa et al., 1999).

We show that both non-selective β-AR and selective β_3-AR agonism with mirabegron prolonged the inter-void interval and increased bladder compliance, while

suppressing NMC amplitude but not frequency. The β_3-AR-mediated effects promote storage without associated impairment of voiding function and are also seen in an acid sensitised bladder pathological model. However, counter to our original hypothesis, we show that the increase in the inter-void interval correlates with the degree of increase in bladder compliance produced by β_3-AR activation, but not with the reduction in NMC amplitude, nor with changed afferent sensitivity.

Methods

All experiments procedures conformed to the UK Animals (Scientific Procedures) Act 1986 and were approved by our institutional ethical review committee.

Preparation

The procedures for the DAPR preparation were as previously described (Pickering and Paton, 2006; Sadananda et al., 2011) and are outlined here in brief. Female Wistar rats (40–90g, P20–P28) were deeply anaesthetized with halothane until loss of paw withdrawal reflex. Following a midline laparotomy, the stomach, spleen, and free intestine were vascularly isolated with ligatures and removed. The animal was pre-collicularly decerebrated and the halothane anaesthesia was withdrawn. The preparation was chilled in ice-cold Ringer's solution and a midline sternotomy was performed. The lungs were removed and both atria were incised to avoid venous congestion during subsequent arterial perfusion. An incision at the apex of the heart allowed insertion of the perfusion cannula (Ø 1.25mm, DLR-4, Braintree Scientific, MA, USA) into the ascending aorta. The preparation was arterially perfused with carbogen-gassed Ringer solution (composition below), containing Ficoll-70 (1.25% Sigma), at 32ºC, at ~30ml/min. The arterial perfusion pressure was monitored via the second lumen of the cannula. Once perfusate flow was initiated the heart resumed beating and rhythmic respiratory muscle contractions were seen within minutes signalling the return of brainstem function (typically as arterial perfusion pressure reached 60-80 mmHg).

Recordings

Simultaneous recordings of phrenic nerve, bladder pressure, EMG of the external

urinary sphincter (EUS) and pelvic afferent nerve (PAN) activity were obtained. Rhythmic, ramping phrenic nerve activity gave a continuous physiological index of brainstem viability (Paton, 1996). The electrocardiogram (ECG) was visible on the phrenic nerve recording and heart rate was derived from the ECG. To allow EUS-EMG recording, the pubic symphysis was cut in the midline and a glass suction electrode (tip diameter 200μm) was placed slightly lateral to the midline directly below the bladder neck under visual control. The bladder afferent nerve was identified on the left and recorded proximal to the major pelvic ganglion, where it became the PAN. Both phrenic nerve and PAN were recorded with glass suction electrodes. A 30G needle was inserted into the bladder dome for pressure monitoring and fluid infusion.

Cystometry protocol

For cystometry both ureters were cut and ligated to stop natural filling from the kidneys. The bladder was filled by constant infusion with an artificial urine saline solution (Sadananda et al., 2011) at a flow rate of 20 μl/min - chosen to produce relatively frequent (every 3-4 minutes) and stable voids. For all drugs, at least four stable control voiding contractions (typically over a period of 20 minutes) were elicited before systemic administration of the drug. The following micturition parameters were measured from a minimum of 4 cycles under each condition (see figure 1):

1. *Baseline pressure*: The lowest bladder pressure at the start of the filling phase.

2. *Threshold pressure*: The bladder pressure at the point where the rhabdosphincter changes in activity from tonic to bursting, indicating the initiation of voiding (Maggi et al., 1986).

3. *Voiding pressure*: The peak bladder pressure achieved during the bursting phase of the rhabdosphincter.

4. *Voiding period*: determined from the duration of rhabdosphincter bursting.

5. *Inter-void interval*: time between voids.

6. *Compliance*: Obtained from the linear regression fit to the volume infused vs bladder pressure relationship during the filling phase from the end of one void to the initiation of the next (at the threshold point).

7. *NMCs:* The peak to trough amplitude and inter-event interval of successive bladder pressure waves that were not associated with rhabdosphincter bursting. The NMCs were identified (using a Spike2 function) by searching the inter-void regions for pressure waves of duration >5 sec and amplitude >0.1mmHg and were manually verified.

Acute, acetic acid bladder sensitisation was performed by switching to continuous intravesical infusion of acid at a pH of 5.5 ((Zhang et al., 2003; Yamaguchi and Chapple, 2007; Sadananda et al., 2009; Parsons and Drake, 2011)). The change in micturition parameters was observed within minutes of starting the infusion and the sensitisation continued for the remaining duration of the experiment.

Drugs and Solutions

The composition of the modified Ringer's solution used for arterial perfusion was (mM): NaCl (125); $NaHCO_3$ (24); KCl (3); $CaCl_2$ (2.5); $MgSO_4$ (1.25); KH_2PO_4 (1.25); dextrose (10); pH 7.3 after carbogenation (95% O_2/5% CO_2). All drugs and

salts were from Sigma unless otherwise stated. β-adrenergic agents were the non-selective agonist, isoprenaline (1nM – 1μM); the β_1 receptor selective antagonist - metoprolol (10nM); the β_3 receptor selective agonist mirabegron (1nM – 1μM, Astellas) and β_3 selective antagonist L748,337 (3μM, *N*-[[3-[(2*S*)-2-Hydroxy-3-[[2-[4-[(ph-enylsulfonyl)amino] phenyl]ethyl]amino]propoxy]phen-yl]methyl]-acetamide, Tocris UK). Isoprenaline (100uM stock) was dissolved with 1mM ascorbic acid to prevent oxidation (final pH 7.25 when diluted to 1μM in carbogenated modified Ringer's). Mirabegron (100uM stock) was dissolved in 20% 2-hydroxypropyl-beta-cyclodextrin (HBC) solvent. L748,337 (30mM stock) was dissolved in DMSO. For all other agents, stock solutions were prepared as aqueous solutions and diluted in Ringer's solution prior to administration. All drugs were added systemically to the perfusate at the final stated concentrations. Perfusion of Ringer's containing either HBC, DMSO or ascorbic acid alone (0.2%, 0.03% and 10μM, respectively) was without effect on micturition parameters.

Analysis

The effect of all of the agents was assayed against the micturition parameters (listed above) as well as on integrated rhabdosphincter EMG, pelvic afferent nerve and phrenic nerve activity alongside heart rate and arterial perfusion pressure (Figure 1). Results are expressed as mean ± SE, where normally distributed, or otherwise, as median [IQR]. Data were compared by paired t-test or repeated measures ANOVA with Dunnett's or Bonferroni's post hoc analysis and a significance threshold of $P<0.05$. The relationship between changes in NMC amplitude, compliance and inter-void intervals were explored using linear regression. All data analysis performed using Prism 5 (Graphpad software, La Jolla, USA)

JPET Fast Forward. Published on September 5, 2013 as DOI: 10.1124/jpet.113.207340
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Cystometry in the DAPR under control conditions (with a continuous infusion of artificial urine at 20µl/min) showed a baseline pressure of 2.9±0.4 mmHg with threshold and peak voiding pressures of 7.9±0.8 and 22.4±1.0 mmHg respectively (n=15). The inter-void interval was 181s [130 – 211], (n=18) and in time control experiments, these pressures and intervals remained stable for up to 3 hours (n=7).

Actions of systemic β-AR agonists on the micturition cycle

Arterial infusion of isoprenaline (1nM to 1µM) caused a concentration dependent decrease in threshold pressure and voiding pressure at all doses from 100nM (Figure 2A, B, C; n=4). At the highest concentration (1µM; n=5), isoprenaline reduced threshold pressure by 28 ± 4.2 % ($P<0.02$) and voiding pressure by 21 ± 3.1 % ($P<0.008$). Isoprenaline increased both inter-void interval (by 231±63% of control) and bladder compliance (265±77% of control, $P=0.03$) but did not significantly change baseline bladder pressure. The duration of the voiding phase was reduced by 30 ± 8 % ($P=0.03$; n=6) but voiding remained complete. Notably, isoprenaline also caused a dose dependent fall in arterial perfusion pressure (figure 2D; from control of 70.8±3.6 to 54.0±3.1 mmHg at 100nM isoprenaline; $P=0.0006$) and an increase in heart rate (from 327±19 to 355±23 bpm; $P=0.008$; n=6).

In a further set of experiments, repetition of the same incrementing isoprenaline dose protocol in the presence of metoprolol (10nM; β_1AR antagonist) produced a similar profile of effects on bladder/micturition parameters in all 6 preparations including: reduced threshold pressure by 25 ± 6.3 % ($P<0.04$) and voiding pressure by 24 ±

JPET Fast Forward. Published on September 5, 2013 as DOI: 10.1124/jpet.113.207340
This article has not been copyedited and formatted. The final version may differ from this version.

7.1 % (P=0.02, n=6) and increased compliance (260±78% of control; P=0.04) and inter-void interval (180±42% of control; P<0.05) in all preparations. Isoprenaline still lowered perfusion pressure in the presence of metoprolol but no longer caused a significant change in HR providing proof of its antagonist activity.

The metoprolol-resistant effects of isoprenaline on micturition suggested that they may be mediated by a β_2- &/or β_3-AR mechanism so we tested the actions of incremental dosing with mirabegron - a β_3-selective agonist (1nM to 1uM). Mirabegron (100nM) increased inter-void interval (135±15%, P=0.04; n=7; Figure 3A,B), increased bladder compliance in all preparations (293±57%, P=0.01; n=7; Figure 3C) and produced a fall in threshold pressure (Figure 3D). However, at this concentration, there was no change in voiding pressure (Figure 3E), baseline pressure or duration of voiding phase nor in arterial perfusion pressure or heart rate. Only at the highest concentration (1uM) did mirabegron reduce arterial perfusion pressure (from 71.3±4.9 to 64.3±3.7mmHg; P=0.01 Figure 3A), although heart rate was still unchanged.

The actions of mirabegron (100nM) to prolong inter-void interval and increase compliance were blocked by the co-application of the β_3-AR antagonist L748,337 (3uM, n=3; Table 1) and mirabegron now appeared to shorten inter-void interval as well as increase voiding pressure. However, L748,337 alone was noted to reduce inter-void interval (from 177±44s to 105±28s, P<0.05) and increase voiding pressure (24.5±1.2 to 26.5±1.7mmHg, p<0.05) and baseline pressure (2.4±0.06 to 3.5±0.3mmHg, P<0.05) (Figure 3F). These findings indicate that there is ongoing sympathetic tone in the DAPR which is acting via β_3-ARs to promote urine storage. There was no significant additional effect of mirabegron (100nM) upon these changes evoked by β_3-AR-antagonism.

JPET Fast Forward. Published on September 5, 2013 as DOI: 10.1124/jpet.113.207340
This article has not been copyedited and formatted. The final version may differ from this version.

Actions of systemic β-AR agonists on non-micturition contractions

NMCs were readily apparent in most preparations with amplitude of 0.88±0.12 mmHg and inter-NMC interval of 25.9±5.4 s (n=17) in controls. Within preparations, NMC amplitude and frequency was maintained throughout the course of the experiment as long as brainstem control remained intact (as reflected by eupnoeic pattern of phrenic nerve activity; (Paton, 1996; Pickering and Paton, 2006)) but as we have noted previously (Sadananda et al., 2011) they showed a progressive increase in amplitude as bladder volume increased during the filling cycle (see figure 4A).

The amplitude of NMCs was reduced by both isoprenaline (-73±9%, P=0.02, n=6, 1μM, figure 4C) and mirabegron (-23±2.4%, P<0.0005, n=5, 100nM, figure 4A,D) in all preparations (Figure 4A). NMC frequency was not significantly altered in the presence of mirabegron (inter-NMC interval for control 21.0±4.7s vs mirabegron 23.8±8.3s, NS, figure 4D) or isoprenaline (Figure 4E). The β_3-AR antagonist, L748,337 increased NMC amplitude by 144±11%. (P=0.01; n=3) but had no effect on NMC frequency. The level of pelvic afferent nerve activity during NMCs and during voids was not altered by mirabegron (Figure 4B) with maintained afferent sensitivity to bladder pressure (0.12±0.02 control Vs 0.14±0.02 μV.s/mmHg in the presence of mirabegron, n=3, ns).

β_3-AR agonism in pathological models of micturition

We have previously noted that NMCs are enhanced when spinal-brainstem autonomic control of the lower urinary tract is lost (Sadananda et al., 2011) which mimics the situation after spinal injury or autonomic neuropathy. Under these conditions the NMCs changed in morphology with abnormally high amplitude (3.9 ± 0.4 mm Hg; n=6). In such preparations, mirabegron (100nM) still reduced NMC amplitude by -36± 5.5 % (n=4, P=0.003, see example in figure 5). The basal pelvic afferent nerve activity level diminished with the fall in baseline bladder pressure on mirabegron administration, but the amplitude of the bursts of increased pelvic nerve activity associated with each NMC was preserved (n=4, see figure 5).

Acute acetic acid sensitisation (intravesical application at pH 5.5, n=7) produced the anticipated increase in voiding frequency (181±27 to 129±25s, P=0.01, see figure 6A) accompanied by a decrease in compliance (32.9±9.0 to 23.0±9.7µl/mmHg, $p<0.05$). Acetic acid had no effect on NMC amplitude (control: 1.2±0.13 vs acid: 1.6±0.12mmHg) or frequency. Mirabegron (100nM, n=5 preparations, Figure 6B,C) reversed the acid-induced shortening of the inter-void interval (125±17s to 262±37s, P=0.01) and the decreased bladder compliance (25.2±12.8 to 85.4±13.8µl/mmHg), but had no effect upon NMC amplitude (Acid 1.6±0.1 vs 1.9±0.3mmHg, ns).

The restoration of inter-void interval by mirabegron in the acid sensitised preparation without an action on NMCs suggested that the β_3 receptor agonist might be exerting its main beneficial effect by increasing bladder compliance. Therefore, we performed correlation analysis on the dataset from naive preparations to look at

the relationships between mirabegron (100nM)-induced changes in inter-void interval, NMC amplitude and bladder compliance (Figure 7). A positive correlation was found between the inter-void interval and compliance (linear regression; R^2 0.45; $P<0.05$), but there was no evident relationship to NMC amplitude. Furthermore, there appeared to be no significant relationship between compliance and NMC amplitude suggesting that they may be independently regulated.

Discussion

We have undertaken a detailed examination of the mechanism of action of β-AR agonists on micturition in a functionally preserved, non-anaesthetised rat preparation. We have shown that systemic administration of non-selective β-AR and β_3-AR selective agents promote storage by increasing bladder capacity and hence prolongation of inter-void interval. This action was also seen following acute acetic acid bladder sensitisation. These β-AR mediated effects are associated with increased bladder compliance and a decrease in the amplitude of non-micturition contractions. The selective β_3-AR agonist mirabegron (Takasu et al., 2007) produced these effects on filling without changing active voiding parameters (unlike the non-selective β-AR agonist) or producing haemodynamic disturbances (see also (Takeda et al., 2000)). Selective β_3-AR antagonism reduced bladder capacity and inter-void interval indicating that there is a basal sympathetic tone acting via β_3-AR to promote urine storage.

These findings are consistent with an existing body of literature indicating that sympathoactivation via β-AR promotes urine storage both in rodents and in man (reviews: (Furuta et al., 2006; Yamaguchi and Chapple, 2007; Michel et al., 2011)). These studies have shown roles for both β_2- and β_3-AR mediated effects on the detrusor (Longhurst and Levendusky, 1999; Morita et al., 2000; Michel and Vrydag, 2006), on signalling from the urothelium (Otsuka et al., 2008; Deba et al., 2009; Masunaga et al., 2010; Kullmann et al., 2011) and latterly, afferent nerve signalling (Kanai et al., 2011; Aizawa et al., 2012). Much of these data have been gathered from *in vitro* studies or *in vivo* studies without intact micturition cycles and, as such, the

importance of each mechanism in the regulation of the micturition cycle has been difficult to ascertain.

We have taken advantage of the decerebrate, arterially perfused rat preparation (Pickering and Paton, 2006; Sadananda et al., 2011) which allows cystometry to assess both filling and voiding parameters while enabling *in vitro*-like control over dosing of β-AR agonists and antagonists. In particular, we have been able to focus on the differential effects of these agents on bladder compliance, contractility, NMCs and afferent signalling, and how these changes influence volume sensing and voiding frequency.

Alongside increasing inter-void interval, we observed that β-AR agonists consistently reduced NMC amplitude (but not frequency). Such spontaneous bladder motions may form part of a detection mechanism that signals bladder fullness to the CNS (Drake et al., 2001). Based on our previous observations that NMC amplitude was increased following loss of autonomic control (pharmacological or pathological; (Sadananda et al., 2011)), we hypothesised that β-AR sympathomimetic agents would decrease NMC amplitude and thereby promote urine storage. We observed that β-AR agonists both reduced NMC amplitude and increased inter-void interval. However, contrary to our original hypothesis, the increase in inter-void interval was not correlated with the degree of change in amplitude of NMCs, suggesting that these mechanisms are likely to be independent. The β-AR agonist-evoked increase in inter-void interval was, instead, correlated with an increase in bladder compliance. In the case of β_3-AR agonism, this increase in compliance was not accompanied by an alteration in the active voiding parameters, suggesting it did not produce a change in detrusor contractility (unlike the non-selective β-AR agonists). This result indicates that the effects of β_3-AR agonism are not achieved by directly opposing

parasympathetic actions. Thus, the mirabegron-induced change in compliance is mediated independently of an action on active contractility.

An important caveat to these conclusions regarding the mechanism of action of β_3-AR on urine storage should be noted; namely that the intravesical pressure change of the NMCs may not be the physiologically relevant feature of the event. It may be that it is the movement-induced localised change in bladder wall stretch that is the relevant signal to the bladder afferents (Drake et al., 2001) – indeed afferent nerve recordings in the presence of mirabegron suggested that this afferent signalling was preserved even though the amplitude of the NMCs was smaller.

It is also worth noting that the threshold pressure for the initiation of voids was lowered by the β_3-AR agonists, suggesting that there was no attenuation in the afferent signalling of bladder pressure. This was supported by our pelvic afferent nerve recordings that showed preserved afferent signalling in response to dynamic changes in bladder pressure (both NMCs and during voiding contractions) in the presence of β_3-AR agonist. This appears to contrast with recent reports from *in vitro* (Kanai et al., 2011) and *in vivo* (Aizawa et al., 2012) studies showing a decrease in afferent nerve activities by β_3-AR agonists. This disparity will require further investigation but our findings may be explained by the presence of dynamic bladder motions seen in our preparation with intact micturition that may provide a more physiological afferent activation.

The selective β_3-AR agonist, mirabegron improved urine storage and bladder compliance without attenuating voiding pressure, unlike the non-selective β-agonist isoprenaline. Thus, it is plausible that the effect of isoprenaline on voiding pressure is mediated via β_1/β_2-AR receptors to oppose parasympathetic contractile responses. In addition to the reduced voiding pressure we also noted that isoprenaline shortened the

duration of the voiding phase (unlike mirabegron). Although we did not directly study urethral outflow function, this shortening of the voiding phase may be consistent with activation of β_2-adrenoceptors that have a dilator function in the rat urethra (unlike β_3) (Michel and Vrydag, 2006) – such dilation may allow more rapid evacuation of bladder contents. This lowered LUT outflow resistance could in itself account for the lowered voiding pressure in the presence of isoprenaline rather than a direct action on the detrusor but this will require further experimental verification. Nonetheless, the features of selective β_3-AR agonism are clinically relevant to OAB as it suggests that storage function can be improved without affecting voiding characteristics. This is an important aspect, as storage disorders can co-exist with impaired voiding function.

Given this profile of action under physiological conditions, we next investigated the effect of β_3-AR agonism in pathological models. First, mirabegron reversed the abnormally high amplitude of NMCs seen after loss of brainstem control in the DAPR preparation. Second, following acid sensitisation to mimic acute bladder over-activity (Zhang et al., 2003), mirabegron increased inter-void interval and increased compliance without an effect on NMC amplitude. This observation again implies that the effects of β_3-AR agonism on storage are not mediated through a reduction in NMC amplitude. Although it has previously been noted in chronic models of lower urinary tract dysfunction such as bladder outlet obstruction (Hood and Andersson, 2013) that β_3-AR agonism suppresses NMCs it has not yet been demonstrated whether this action produces an improvement in urine storage in this pathological model.

Technical aspects of the use of the DAPR

An advantage of the DAPR illustrated in this study is the ability to assay the systemic effects of pharmacological agents on multiple organ systems (relatively independent of preparation viability) and thus detect potential side effects. This was illustrated in the current investigation where isoprenaline was shown to increase heart rate (via β_1-AR) and reduce arterial perfusion pressure. These actions were not seen with mirabegron, except at the highest dose where it caused a mild hypotensive effect.

The DAPR approach requires the use of young rats typically less than 4 weeks of age. While there is some debate over the age at which rat micturition is fully mature (Yamaguchi and Chapple, 2007; Kanai et al., 2011; Zvarova and Zvara, 2012), we found coordinated and complete bladder emptying in all our preparations as described previously (Sadananda et al., 2011). It should be noted that for cystometry, we chose to use a bladder infusion rate (20µl/min) that produced a relatively short inter-void interval (typically less than 5 minutes) to allow drug responses to be assayed expeditiously, permitting repeated dosing studies with washouts.

Conclusions

Treatments for bladder storage dysfunctions such as OAB must possess a number of specific features: they must enhance bladder capacity and/or compliance while preserving the active voiding phase and they should be well tolerated systemically. Previous therapies have focused on direct relaxation of the detrusor muscle, resulting in improved storage, but potentially adversely affecting voiding function, when contraction is required. In our current study, we found β_3-AR agonism promoted urinary storage without impairing the voiding phase, and was relatively free of "off target" cardio-respiratory side effects. Intriguingly, we also demonstrate a basal tone of sympathetically mediated β_3 activation that regulates lower urinary tract function

and speculate that loss of such tone could play a role in the pathogenesis of conditions such as OAB.

Acknowledgements

The authors are grateful to Dr Cees Korstanje and Dr Frank Perabo for their insights and productive discussions. AEP is a Welcome Trust Senior Clinical Research Fellow.

Authorship Contributions

Participated in research design: Sadananda, Pickering, Paton, Drake

Conducted experiments: Sadananda

Performed data analysis: Sadananda & Pickering

Wrote or contributed to the writing of the manuscript: Sadananda, Pickering, Paton, Drake

References

Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U, van Kerrebroeck P, Victor A and Wein A (2002) The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. *Am J Obstet Gynecol* 187:116-126.

Aizawa N, Homma Y and Igawa Y (2012) Effects of Mirabegron, a Novel beta3-Adrenoceptor Agonist, on Primary Bladder Afferent Activity and Bladder Microcontractions in Rats Compared With the Effects of Oxybutynin. *European urology* 62:1165-1173.

Andersson KE and Wein AJ (2004) Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence. *Pharmacol Rev* 56:581-631.

Deba A, Palea S, Rouget C, Westfall TD and Lluel P (2009) Involvement of beta(3)-adrenoceptors in mouse urinary bladder function: role in detrusor muscle relaxation and micturition reflex. *Eur J Pharmacol* 618:76-83.

Drake MJ, Mills IW and Gillespie JI (2001) Model of peripheral autonomous modules and a myovesical plexus in normal and overactive bladder function. *Lancet* 358:401-403.

Fowler CJ, Griffiths D and de Groat WC (2008) The neural control of micturition. *Nat Rev Neurosci* 9:453-466.

Furuta A, Thomas CA, Higaki M, Chancellor MB, Yoshimura N and Yamaguchi O (2006) The promise of beta3-adrenoceptor agonists to treat the overactive bladder. *The Urologic clinics of North America* 33:539-543, x.

Hood B and Andersson KE (2013) Common theme for drugs effective in overactive bladder treatment: inhibition of afferent signaling from the bladder.

International journal of urology : official journal of the Japanese Urological Association 20:21-27.

Igawa Y, Yamazaki Y, Takeda H, Hayakawa K, Akahane M, Ajisawa Y, Yoneyama T, Nishizawa O and Andersson KE (1999) Functional and molecular biological evidence for a possible beta3-adrenoceptor in the human detrusor muscle. *Br J Pharmacol* 126:819-825.

Kanai A, Wyndaele JJ, Andersson KE, Fry C, Ikeda Y, Zabbarova I and De Wachter S (2011) Researching bladder afferents-determining the effects of beta(3) -adrenergic receptor agonists and botulinum toxin type-A. *Neurourol Urodyn* 30:684-691.

Kullmann FA, Downs TR, Artim DE, Limberg BJ, Shah M, Contract D, de Groat WC and Rosenbaum JS (2011) Urothelial beta-3 adrenergic receptors in the rat bladder. *Neurourol Urodyn* 30:144-150.

Longhurst PA and Levendusky M (1999) Pharmacological characterization of beta-adrenoceptors mediating relaxation of the rat urinary bladder in vitro. *Br J Pharmacol* 127:1744-1750.

Maggi CA, Giuliani S, Santicioli P and Meli A (1986) Analysis of factors involved in determining urinary bladder voiding cycle in urethan-anesthetized rats. *The American journal of physiology* 251:R250-257.

Masunaga K, Chapple CR, McKay NG, Yoshida M and Sellers DJ (2010) The beta3-adrenoceptor mediates the inhibitory effects of beta-adrenoceptor agonists via the urothelium in pig bladder dome. *Neurourol Urodyn* 29:1320-1325.

Michel MC, Ochodnicky P, Homma Y and Igawa Y (2011) beta-adrenoceptor agonist effects in experimental models of bladder dysfunction. *Pharmacol Ther* 131:40-49.

Michel MC and Vrydag W (2006) Alpha1-, alpha2- and beta-adrenoceptors in the urinary bladder, urethra and prostate. *Br J Pharmacol* 147 Suppl 2:S88-119.

Morita T, Iizuka H, Iwata T and Kondo S (2000) Function and distribution of beta3-adrenoceptors in rat, rabbit and human urinary bladder and external urethral sphincter. *Journal of smooth muscle research = Nihon Heikatsukin Gakkai kikanshi* 36:21-32.

Otsuka A, Shinbo H, Matsumoto R, Kurita Y and Ozono S (2008) Expression and functional role of beta-adrenoceptors in the human urinary bladder urothelium. *Naunyn Schmiedebergs Arch Pharmacol* 377:473-481.

Parsons BA and Drake MJ (2011) Animal models in overactive bladder research. *Handb Exp Pharmacol*:15-43.

Paton JF (1996) A working heart-brainstem preparation of the mouse. *J Neurosci Methods* 65:63-68.

Pickering AE and Paton JF (2006) A decerebrate, artificially-perfused in situ preparation of rat: Utility for the study of autonomic and nociceptive processing. *J Neurosci Methods* 155:260-271.

Sadananda P, Drake MJ, Paton JF and Pickering AE (2011) An exploration of the control of micturition using a novel in situ arterially perfused rat preparation. *Front Neurosci* 5:62-70.

Sadananda P, Shang F, Liu L, Mansfield KJ and Burcher E (2009) Release of ATP from rat urinary bladder mucosa: role of acid, vanilloids and stretch. *Br J Pharmacol* 158:1655-1662.

Takasu T, Ukai M, Sato S, Matsui T, Nagase I, Maruyama T, Sasamata M, Miyata K, Uchida H and Yamaguchi O (2007) Effect of (R)-2-(2-aminothiazol-4-yl)-4'-{2-[(2-hydroxy-2-phenylethyl)amino]ethyl} acetanilide (YM178), a novel

selective beta3-adrenoceptor agonist, on bladder function. *J Pharmacol Exp Ther* 321:642-647.

Takeda H, Yamazaki Y, Akahane M, Igawa Y, Ajisawa Y and Nishizawa O (2000) Role of the beta(3)-adrenoceptor in urine storage in the rat: comparison between the selective beta(3)-adrenoceptor agonist, CL316, 243, and various smooth muscle relaxants. *J Pharmacol Exp Ther* 293:939-945.

Yamaguchi O and Chapple CR (2007) Beta3-adrenoceptors in urinary bladder. *Neurourol Urodyn* 26:752-756.

Zhang X, Igawa Y, Ishizuka O, Nishizawa O and Andersson KE (2003) Effects of resiniferatoxin desensitization of capsaicin-sensitive afferents on detrusor over-activity induced by intravesical capsaicin, acetic acid or ATP in conscious rats. *Naunyn Schmiedebergs Arch Pharmacol* 367:473-479.

Zvarova K and Zvara P (2012) Urinary bladder function in conscious rat pups: a developmental study. *American journal of physiology Renal physiology* 302:F1563-1568.

Footnotes

- Astellas Pharma Inc. provided unrestricted grant funding for research [AG2009/00508]. AEP is in receipt of a Wellcome Senior Clinical Research Fellowship (ref 088373).

JPET Fast Forward. Published on September 5, 2013 as DOI: 10.1124/jpet.113.207340
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Figure 1. *Micturition parameters recorded in DAPR preparation.*

(A) Cystometrogram showing two voiding contractions under control conditions (continuous infusion at 20µl/min). The start of voiding is signalled by the onset of EUS bursting (seen in B on an expanded time-base) from which the threshold pressure is derived. The inter-void interval is measured between the threshold points of successive voids. Voiding pressure is the maximal pressure during EUS bursting phase. The baseline pressure is the trough pressure after a void once the bladder has relaxed allowing filling to recommence. During the filling phase the bladder pressure gradually ramps, with superimposed non-micturition contractions whose peak-trough amplitude and period were quantified. The pelvic nerve recording showed characteristic responses during the filling and voiding phases, with bursts of activity corresponding to the NMCs, correlated with changes in bladder pressure and also with EUS activity. Note the preparation also showed increases in heart rate, arterial perfusion pressure and phrenic nerve activity during each void, indicating global autonomic changes during micturition. (EUS, Phrenic and Pelvic nerve recordings rectified and integrated with a time constant of 200ms)

Figure 2. *Dose-response to systemic isoprenaline infusion.*

(A) Incremental dosing with isoprenaline (10nM-1uM in the perfusate) prolonged inter-void interval, reduced voiding pressure and lowered arterial perfusion pressure. The pooled data showed significant reductions in voiding pressure (B) at isoprenaline

≥100nM, threshold pressure (C) isoprenaline ≥100nM and arterial perfusion pressure (D) at isoprenaline ≥10nM.

(Repeated measures ANOVA with Dunnett's post hoc tests, * - P<0.05, ** - P<0.01, *** - P<0.001)

Figure 3. *Influence of* β_3*-adrenoceptors on storage and voiding.*

(A) Incremental dosing with mirabegron in the perfusate (steps from 10nM to 1μM shown) increased (B) inter-void interval (100nM, pooled data in red, n=7, P<0.05), (C) bladder compliance (100nM; pooled data in red, n=7, P<0.01) and (D) threshold pressure without changing the (E) voiding pressure. (F) Application of the β_3-adrenoceptor antagonist L748,337 (3μM) alone had the opposite effect - shortening intervoid interval - suggesting the presence of a basal sympathetic tone to the bladder. (Repeated measures ANOVA with Dunnett's post hoc tests, * - P<0.05, ** P<0.01, P<0.001)

Figure 4. *Non-micturition contractions are attenuated by* β_3*-adrenoceptor agonists.*

(A) Application of mirabegron (100nM) increased the inter-void interval and decreased the amplitude of NMCs. (Pelvic nerve activity was rectified and integrated - time constant 200ms (purple) and 2s (black)). (B) Pelvic afferent nerve activity retained the same sensitivity to bladder pressure in the presence of mirabegron showing marked increases in activity during NMCs and during voids. (Correlation analysis using rectified and integrated pelvic nerve activity with time constant 500ms)

JPET Fast Forward. Published on September 5, 2013 as DOI: 10.1124/jpet.113.207340
This article has not been copyedited and formatted. The final version may differ from this version.

Pooled data showed that both isoprenaline (C, 1μM, n=4, P<0.01) and mirabegron (D, 100nM, n=5, P<0.01) significantly reduced NMC amplitude (individual paired data in black and means in red) without an action on the frequency of occurrence of the NMCs (E, F). (Paired t-test, * - P<0.05, ** P<0.01)

Figure 5. *Pathologically augmented non-micturition contractions are suppressed by* β_3*-adrenoceptor agonist.*

After loss of brainstem-spinal control, micturition ceased and the amplitude and morphology of NMCs and pelvic afferent activity was pathologically increased (shown on left, bladder at constant volume). Systemic infusion of mirabegron (100nM) decreased both the amplitude of the NMCs and reduced the pelvic afferent nerve activity (mirroring the reduction in bladder pressure) without a change in bladder volume. Note that the smaller NMCs in the presence of mirabegron still evoked similar sized bursts of afferent discharge. Pelvic nerve activity was rectified and integrated – shown here with two time constants 200ms (purple) and 2s (black).

Figure 6. *Intravesical acetic acid-sensitisation of the lower urinary tract is reversed by* β_3*-adrenoceptor agonist.*

(A) Intravesical infusion of acetic acid (pH 5.5) shortened inter-void interval and decreased bladder compliance. Systemic infusion of mirabegron (10 and 100nM), in the continued presence of intravesical acetic acid, prolonged the inter-void interval

((B) ≥10nM, P<0.05, n=7) and increased bladder compliance ((C) 100nM, P<0.01, n=7) without significantly changing NMC amplitude. (Paired t-test, * - P<0.05 and Repeated measures ANOVA with Dunnett's multiple comparison test versus acid sensitised condition, # - P<0.05, ## - P<0.01)

Figure 7. *Mirabegron-induced increase in the inter-void interval is correlated with the degree of increase in compliance.*

(A) Linear regression analysis showed a positive correlation between the change in compliance and the degree of increase in inter-void interval for each preparation (R^2 0.45; P<0.05; n=7). (B) In contrast there was no significant correlation between the change in NMC amplitude and inter-void interval (n=5). (C) The NMC amplitude change was also independent of the mirabegron-induced changes in bladder compliance (n=5).

JPET Fast Forward. Published on September 5, 2013 as DOI: 10.1124/jpet.113.207340
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1: Effect of L748,337.

n=3	Control	Mirabegron + L748,337
IVI	177±44	133±44*
Voiding Pressure	24.5±1.2	26.2±1.6*
Threshold Pressure	3.7±0.3	4.9±1.1
Baseline Pressure	2.4±0.06	3.5±0.3
Compliance	41.37±6.3	34.2±6.8

*P<0.05 paired t-test.

Figure 1

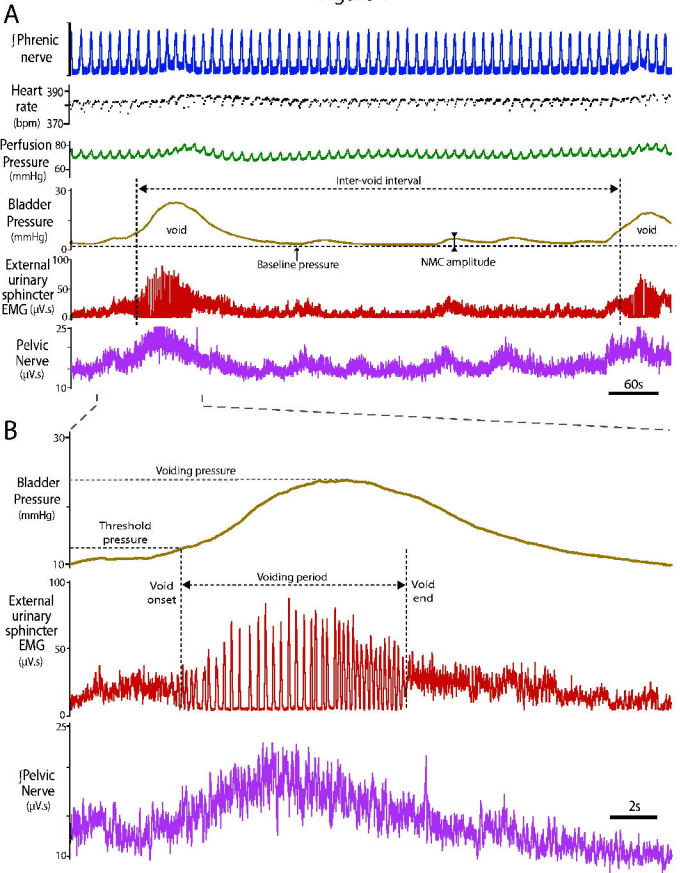

A
∫Phrenic nerve
Heart rate (bpm)
390
370
Perfusion Pressure (mmHg)
80
60
Inter-void interval
Bladder Pressure (mmHg)
30
0
void
void
Baseline pressure
NMC amplitude
External urinary sphincter EMG (μV.s)
100
50
0
Pelvic Nerve (μV.s)
25
10
60s
B
30
Voiding pressure
Bladder Pressure (mmHg)
Threshold pressure
10
100
Void onset
Voiding period
Void end
External urinary sphincter EMG (μV.s)
50
0
25
∫Pelvic Nerve (μV.s)
2s
10

Figure 2

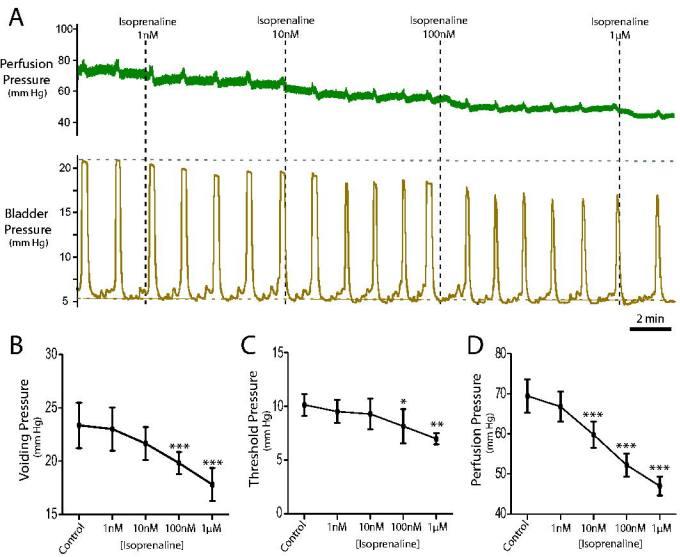

A
Isoprenaline 1nM
Isoprenaline 10nM
Isoprenaline 100nM
Isoprenaline 1μM
Perfusion Pressure (mm Hg)
Bladder Pressure (mm Hg)
2 min
B
Voiding Pressure (mm Hg)
C
Threshold Pressure (mm Hg)
D
Perfusion Pressure (mm Hg)
Control
1nM
10nM
100nM
1μM
[Isoprenaline]

Figure 3

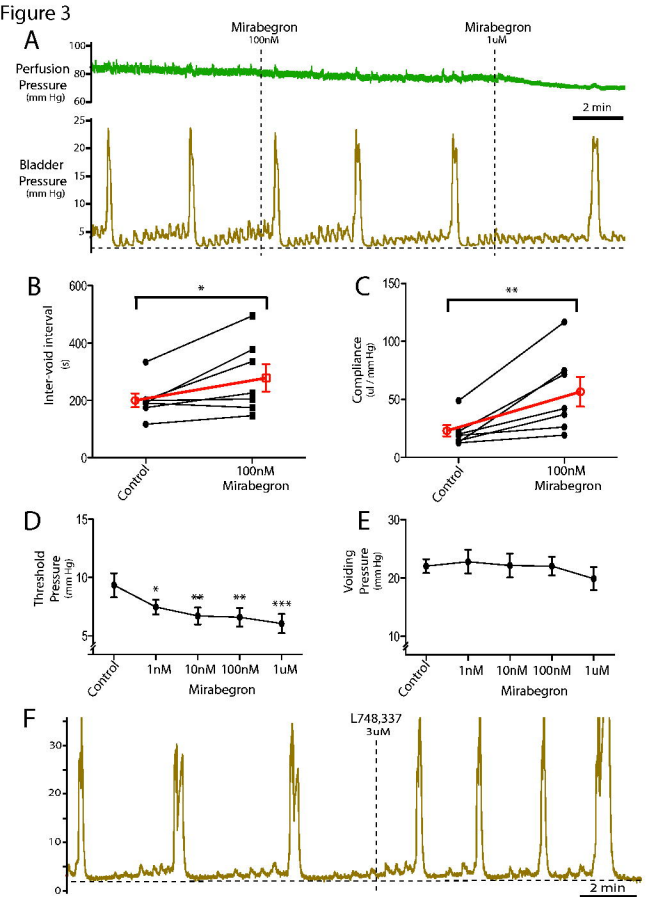

A
Mirabegron
100nM
Mirabegron
1uM
Perfusion Pressure (mm Hg)
100
80
60
2 min
Bladder Pressure (mm Hg)
25
20
15
10
5
B
Inter-void interval (s)
600
400
200
0
*
Control
100nM Mirabegron
C
Compliance (ul / mm Hg)
150
100
50
0
**
Control
100nM Mirabegron
D
Threshold Pressure (mm Hg)
15
10
5
*
**
**
***
Control
1nM
10nM
100nM
1uM
Mirabegron
E
Voiding Pressure (mm Hg)
30
20
10
Control
1nM
10nM
100nM
1uM
Mirabegron
F
L748,337
3uM
30
20
10
0
2 min

Figure 4

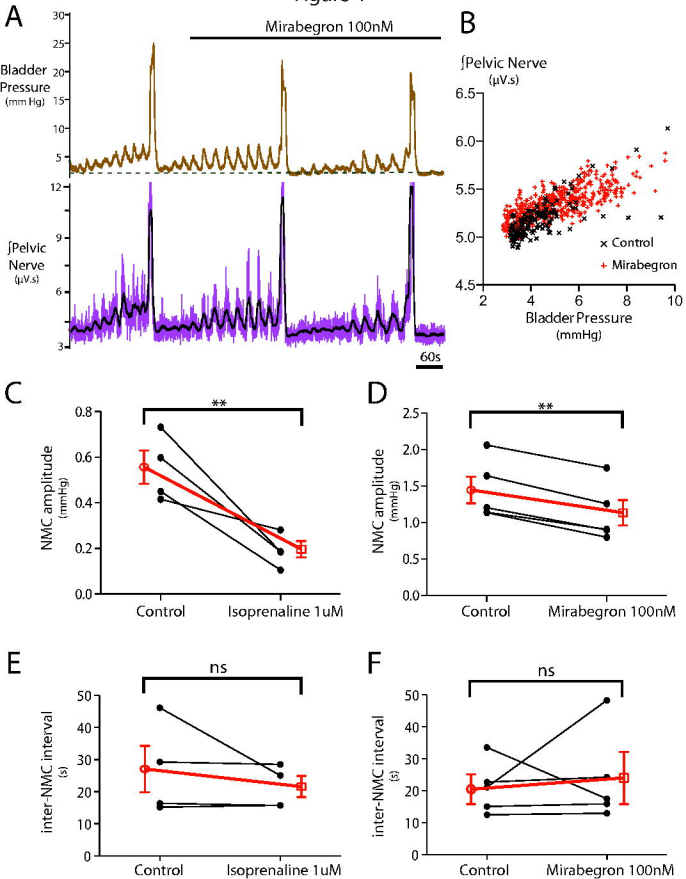

A
Mirabegron 100nM
Bladder Pressure (mmHg)
∫Pelvic Nerve (μV.s)
60s
B
∫Pelvic Nerve (μV.s)
Bladder Pressure (mmHg)
Control
Mirabegron
C
NMC amplitude (mmHg)
**
Control
Isoprenaline 1uM
D
NMC amplitude (mmHg)
**
Control
Mirabegron 100nM
E
inter-NMC interval (s)
ns
Control
Isoprenaline 1uM
F
inter-NMC interval (s)
ns
Control
Mirabegron 100nM

Figure 5

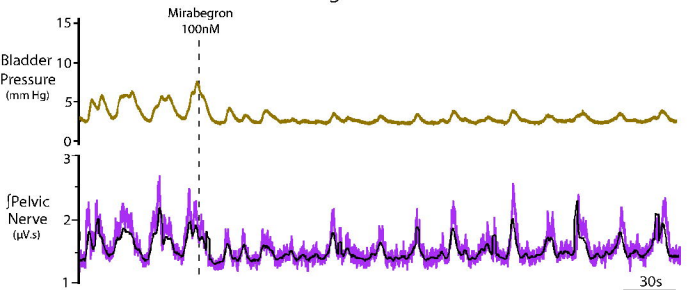

Mirabegron
100nM
Bladder
Pressure
(mm Hg)
15
10
5
0
∫Pelvic
Nerve
(μV.s)
3
2
1
30s

Figure 6

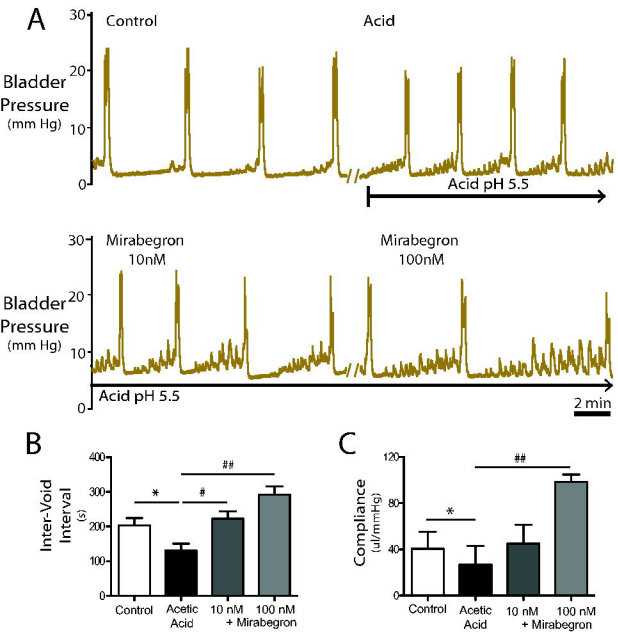
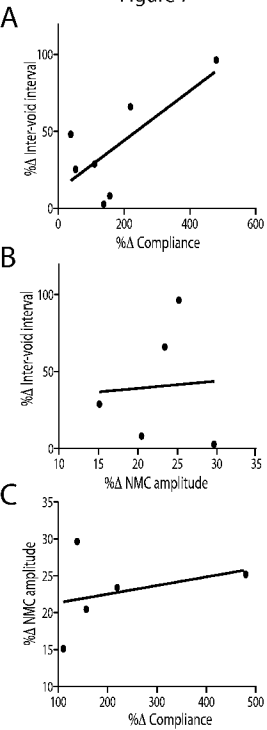

A
Control
Acid
Bladder Pressure (mm Hg)
30
20
10
0
Acid pH 5.5
Mirabegron 10nM
Mirabegron 100nM
Bladder Pressure (mm Hg)
Acid pH 5.5
2 min
B
Inter-Void Interval (s)
400
300
200
100
0
*
#
##
Control
Acetic Acid
10 nM + Mirabegron
100 nM + Mirabegron
C
Compliance (ul/mmHg)
120
80
40
0
*
##
Control
Acetic Acid
10 nM + Mirabegron
100 nM + Mirabegron

Figure 7


A
%Δ Inter-void interval
100
50
0
0
200
400
600
%Δ Compliance
B
%Δ Inter-void interval
100
50
0
10
15
20
25
30
35
%Δ NMC amplitude
C
%Δ NMC amplitude
35
30
25
20
15
10
100
200
300
400
500
%Δ Compliance