Video Article

Bladder Smooth Muscle Strip Contractility as a Method to Evaluate Lower Urinary Tract Pharmacology

F. Aura Kullmann[1], Stephanie L. Daugherty[2], William C. de Groat[2], Lori A. Birder[1,2]

[1]Department of Medicine, Renal division, University of Pittsburgh School of Medicine

[2]Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine

Correspondence to: F. Aura Kullmann at aura.kullmann@gmail.com

URL: https://www.jove.com/video/51807
DOI: doi:10.3791/51807

Keywords: Medicine, Issue 90, Krebs, species differences, *in vitro*, smooth muscle contractility, neural stimulation

Date Published: 8/18/2014

Citation: Kullmann, F.A., Daugherty, S.L., de Groat, W.C., Birder, L.A. Bladder Smooth Muscle Strip Contractility as a Method to Evaluate Lower Urinary Tract Pharmacology. *J. Vis. Exp.* (90), e51807, doi:10.3791/51807 (2014).

Abstract

We describe an *in vitro* method to measure bladder smooth muscle contractility, and its use for investigating physiological and pharmacological properties of the smooth muscle as well as changes induced by pathology. This method provides critical information for understanding bladder function while overcoming major methodological difficulties encountered in *in vivo* experiments, such as surgical and pharmacological manipulations that affect stability and survival of the preparations, the use of human tissue, and/or the use of expensive chemicals. It also provides a way to investigate the properties of each bladder component (*i.e.* smooth muscle, mucosa, nerves) in healthy and pathological conditions.

The urinary bladder is removed from an anesthetized animal, placed in Krebs solution and cut into strips. Strips are placed into a chamber filled with warm Krebs solution. One end is attached to an isometric tension transducer to measure contraction force, the other end is attached to a fixed rod. Tissue is stimulated by directly adding compounds to the bath or by electric field stimulation electrodes that activate nerves, similar to triggering bladder contractions *in vivo*. We demonstrate the use of this method to evaluate spontaneous smooth muscle contractility during development and after an experimental spinal cord injury, the nature of neurotransmission (transmitters and receptors involved), factors involved in modulation of smooth muscle activity, the role of individual bladder components, and species and organ differences in response to pharmacological agents. Additionally, it could be used for investigating intracellular pathways involved in contraction and/or relaxation of the smooth muscle, drug structure-activity relationships and evaluation of transmitter release.

The *in vitro* smooth muscle contractility method has been used extensively for over 50 years, and has provided data that significantly contributed to our understanding of bladder function as well as to pharmaceutical development of compounds currently used clinically for bladder management.

Video Link

The video component of this article can be found at https://www.jove.com/video/51807/

Introduction

The bladder smooth muscle relaxes to allow urine storage, and contracts to elicit urine elimination. Relaxation is mediated by intrinsic smooth muscle properties and by tonic release of norepinephrine (NE) from the sympathetic nerves, which activates beta adrenergic receptors (β_3AR in human) in the detrusor. Voiding is achieved by inhibiting the sympathetic input and activating the parasympathetic nerves that release ACh/ATP to contract the bladder smooth muscle[1]. Numerous pathological conditions**,** including brain and/or spinal cord injury, neurodegenerative diseases, diabetes, bladder outlet obstruction or interstitial cystitis, can profoundly alter bladder function, with severe impact on the patient's quality of life[2]. These conditions alter the contractility of the smooth muscle by affecting one or more components of the bladder: the smooth muscle, the afferent or efferent nerves and/or the mucosa.

Several *in vivo* and *in vitro* methods to study bladder function have been developed. *In vivo*, cystometry is the primary measurement of bladder function. Though this is an intact preparation that allows collection of information under close to physiological conditions, there are a number of circumstances in which the use of smooth muscle strips is preferred. These include situations when surgical and/or pharmacological manipulations would affect the survival and stability of the *in vivo* preparation, or when the studies require the use of the human tissue or expensive chemicals. This method also facilitates an examination of the effects of drugs, age and pathology on each component of the bladder, *i.e.* smooth muscle, mucosa, afferent and efferent nerves.

Bladder strips have been employed over the years by many groups to answer a number of scientific questions. They were used to evaluate changes in myogenic spontaneous activity induced by pathology. This activity is believed to contribute to the urgency and frequency symptoms of overactive bladder (OAB), and is therefore a target for drugs being developed for OAB[3-9]. Bladder strips were also used to investigate myogenic and neuronal factors that modulate smooth muscle tone with the aim of discovering ion channels and/or receptors and/or intracellular

pathways that could be targeted to induce either relaxation or contraction of the smooth muscle[3,10-13]. Other studies have focused on the nature of neurotransmission, including transmitters and receptors involved and changes induced by pathology[14,15]. In addition, the method has been used for comparisons between tissues from different species[16-18], between organs[19-21], and evaluation of drug structure-activity relationships[22-24]. An extension of this method has been used to measure the effect of drugs on transmitter release from efferent nerves[25]. Furthermore, a variety of tissues (bladder, urethra, gastrointestinal tract, GI) harvested from animals or humans (from surgeries or organ donor tissue approved for research) and from a variety of animal models including spinal cord injury (SCI), bladder outlet obstruction (BOO), or interstitial cystitis (IC) can be investigated using this technique.

In this paper we illustrate the use of this method along with necessary experimental protocols, to address several scientific questions mentioned above.

Protocol

All procedures described here are approved by the IACUC committee at University of Pittsburgh.

1. Solutions

1. Prepare Krebs solution according to the recipe. Composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, dextrose 11.7.
2. Aerate Krebs with 95% O_2, 5% CO_2 and place it in a 37 ºC water bath to be used throughout the experiment. Place aside ~200 ml of aerated Krebs solution at room temperature to be used for tissue dissection.
3. Measure pH (~7.4) and osmolarity (~ 300 mOsm) of aerated Krebs.

2. Experimental Set-up (Schematic Figure 1A)

1. Fill aerated (95% O_2, 5% CO_2) chambers with 10 ml Krebs.
2. Start the circulating water pump to heat the chambers to 37 °C; turn on the necessary equipment: amplifier(s), stimulator(s) and recording software.
3. Calibrate transducers with a 1 g weight.

3. Tissue (Figure 1B)

Remove the bladder from an adult naïve female Sprague Dawley rat (200-250 g; ~10-12 weeks old) following these steps:

1. Prepare dissection area and necessary instruments: electric razors, forceps with teeth, scalpel blade, dissecting scissors, microscissors, two dissecting forceps (authors prefer Dumont forceps #3), tissue clips (or silk suture), a Sylgard coated dissection dish with Krebs and tissue dissection pins.
2. Anesthetize the animal with isoflurane inhalation (4% in O_2) in the induction chamber. Use veterinary ointment on eyes to prevent dryness while under anesthesia. Continuously monitor the level of anesthesia by observing the respiration rate, response to external stimuli, and loss of rear limb withdraw reflex.
3. When the animal is anesthetized shave the lower abdominal area. Expose the pelvic organs via a midline abdominal incision. Identify the bladder and urethra. Remove the bladder by cutting at the bladder neck close to proximal urethra. Place tissue immediately in the Sylgard coated dish filled with aerated Krebs solution.
4. If needed, remove additional tissue at this time: urethra, pieces of gastrointestinal (GI) tract and/or prostate, *etc.*
5. Sacrifice the animal using IACUC approved methods (*e.g.*, anesthetic overdose or CO_2 asphyxiation followed by a secondary method).
6. Insert tissue dissecting pins through the bladder dome, neck, and ureters, to stabilize the tissue for further dissection. Do not stretch the tissue. Remove fat, connective tissue, proximal urethra, and ureters if present.
7. Open the bladder from the base to dome to create a flat sheet, serosa side down/luminal side up (**Figure 1B**). Place dissecting pins on each corner of the tissue. Remove bladder dome and neck tissue.
8. If the purpose of the experiment is to determine the contribution of the mucosa (urothelium and lamina propria — see diagram **Figure 1C**) to the smooth muscle contraction, compare the properties of detrusor strips with and without the mucosa attached. For this, prior to cutting the tissue in strips, carefully remove the mucosal layer using iris spring scissors and fine forceps under a dissecting microscope. At the end of the experiment, fix the strips for H&E staining to confirm complete removal of the mucosa. Note that this procedure is easier in mouse bladder than in rat bladder.
9. Cut the tissue lengthwise from base to dome into strips of ~2 x 8 mm (**Figure 1B**). Tie or attach a tissue clip to both ends of each strip.
   NOTE: One rat bladder can usually be cut into 4 strips but the number of strips can increase or decrease depending on the animal/bladder size.
10. Transfer the strips to the experimental chambers. Attach one end of each strip to a force transducer, which measures the tissue contraction, and the other to a fixed glass/metal rod.
    NOTE: Tissue chambers vary in size (0.2 ml to 20 ml or larger). Typical chambers for rodent bladders are 5-20 ml, which provide sufficient height for the strips to be completely submersed in solution. Some chambers come with built-in stimulation electrodes, others not. Care should be taken to ensure that all connections of the electrodes are in good condition, otherwise electrical field stimulation is not reliable.
11. Apply a defined amount of force to each strip by gently stretching the tissue until baseline tension reaches 1 g (~10 mN). Initially the tissue tends to relax which is recorded as a decrease in baseline tension. Wash tissue approximately every 15 min using the warm aerated Krebs and adjust the baseline tension to 1 g after each wash. Allow tissue to equilibrate for ~1-2 hr or until baseline tension is stable (*i.e.* no further tissue relaxation).

12. Test tissue viability by adding KCl (80 mM) directly to the bath for ~5 min, or until a plateau response is reached. Responses to high concentrations of KCl can also be repeated during the experiment or at the end of the experiment and used for normalizing responses to other drugs or between strips (see normalization under data analysis section).
13. Wash tissue multiple times (3-5x) with the warm aerated Krebs to allow the tissue to return to pre-treatment conditions.

4. Stimulation Protocols

1. To investigate the effects of pathology on spontaneous myogenic activity or smooth muscle tone, use smooth muscle strips from different animal models such as SCI, BOO, or neonates. **Figure 2** illustrates the use of this method to investigate changes in bladder spontaneous activity during development and after SCI. In addition, pharmacological agents can be used to modulate spontaneous activity. **Figure 3** illustrates the effect of the KCNQ channel modulators, flupirtine and XE991, on spontaneous activity and smooth muscle tone.
2. For pharmacological smooth muscle stimulation construct concentration response curves by adding compounds from concentrated stock solutions directly to the bath at defined time intervals. Use drug and vehicle in parallel strips to account for vehicle and time effects.
    1. Make stock solutions of desired test compounds at 1000x the final working concentration. For carbachol (CCh), a muscarinic receptor agonist, prepare following stocks: 10^{-5} M, 3 x 10^{-5} M, 10^{-4} M, 3 x 10^{-4} M, 10^{-3} M, 3 x 10^{-3} M, 10^{-2} M. Final concentrations in the bath is 10^{-8} M to 10^{-5} M (**Figures 4C, D**). For neuromedin B (NMB), a bombesin receptor subtype 1 agonist, prepare following stocks: 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{-3} M and final concentrations in the bath are 10^{-11} M to 10^{-6} M. Both CCh and NMB are expected to increase tissue contractility.
    2. For a 10 ml tissue bath, add 10 µl of each CCh stock solution as soon as the response reaches a plateau (**Figure 4C, D**). In parallel strips add equal amounts of the vehicle (water). Similarly, add 10 µl of each neuromedin B stock solution every ~5 min.
    NOTE: Observe the excitatory effect of NMB and CCh on smooth muscle tone in strips from different species in **Figure 4**.
    3. Investigate the relaxation properties of the smooth muscles in pre-contracted tissue with an excitatory agent, usually CCh or KCl.
    4. To block an agonist response, pretreat the tissue for 10-20 min with the antagonist to allow tissue penetration, prior to agonist stimulation.

3. For neural stimulation of the smooth muscle, also called electric field stimulation (EFS) follow steps 1 to 3.13 and continue as described below. EFS is intended to selectively activate nerves versus smooth muscle. Parameters for stimulation should be carefully chosen to avoid direct smooth muscle stimulation.
    1. Establish stimulation parameters: type of stimulus (single pulses or trains), duration (pulse duration and train duration), frequency and intensity, as described in the steps below and illustrated in **Figures 5A, B**.
        1. For single pulse stimulation, set pulse duration, inter-stimulus interval and number of stimuli desired. Usual stimulation duration parameters are single pulses of 0.05-0.3 msec duration delivered at desired intervals (**Figure 5A**). Follow step 4.3.1.4 for stimulus intensity.
        2. For train stimulation, set the train duration and inter train interval. Typical values for bladder tissue are 3-10 sec delivered at least 1 min apart (**Figure 5B**). If tissue fatigue occurs (*i.e.* EFS contractions decrease during control period), increase the inter train interval.
        3. Establish the frequency of train stimuli (number of pulses in a train — **Figure 5B**). Run a frequency response curve ranging from 0.5-50 Hz. Typical frequencies for bladder are 10-20 Hz, which give reproducible and stable contractions mediated by ATP and ACh. Observe the frequency dependent responses to EFS stimulation in mouse bladder strips in **Figure 5** demonstrating how this method can be used to assess the contribution of cholinergic and purinergic mechanisms to neurotransmission.
        4. Establish intensity of the stimulus: systematically increase the intensity (voltage) of the stimulus until the amplitude of the contraction reaches a plateau (if using trains keep the frequency constant).
        5. Set the intensity of the stimulus depending on the aim of the experiment. If the aim is to increase the neurally-evoked contractions, then use submaximal intensity such that the amplitude of contraction is ~50% of maximal contraction. If the aim is to decrease the neurally-evoked contractions, then set the intensity to ~80% of maximal amplitude to avoid tissue fatigue.

    2. Once stimulation parameters (duration, frequency and intensity) are established, allow ~20-30 min for EFS- evoked contractions to stabilize prior to drug testing.
    NOTE: To verify the selectivity of EFS for neural transmission, block neural transmission with the Na^+ channel blocker, tetrodotoxin (TTX; 0.5-1 µM). Perform this step at the beginning of the experiment, as TTX washes off relatively easy. In addition, perform this at the end of the experiment (see step 4.3.5. below).
    3. Prepare stock solutions at 1,000x the final working concentrations for: alpha,beta-methylene ATP (ABMA; a purinergic receptor activator and desensitizer) 10^{-2} M, atropine (a muscarinic receptor antagonist) 10^{-3} M (**Figure 5C**). Observe other examples in **Figure 6**. The 5HT4 receptor agonist, cisapride (3 x 10^{-6} M, 10^{-6} M, 3 x 10^{-5} M, 10^{-5} M, 3 x 10^{-4} M, 10^{-4} M, 3 x 10^{-3} M, 10^{-3} M), increases tissue contractility and SB-203186 (3 x 10^{-3} M), a 5HT4 receptor antagonist, reverses cisapride's effects.
    4. To test the effects of ABMA and atropine on EFS (**Figure 5C**), perform two control frequency response curves. Add 10 µl of 10^{-2} M ABMA to the bath for a final concentration of 10 µM. This will contract the tissue due to direct stimulation of purinergic receptors in the smooth muscle. After the response returns to baseline, repeat frequency response curves. Add 10 µl of 10^{-3} M atropine for a final concentration of 1 µM. After ~10 min (needed for the atropine to block muscarinic receptors), repeat frequency response curves. In parallel strips add 10 µl of the vehicle, water, at each step.
    NOTE: For other examples in **Figure 6**, add 10 µl of each cisapride stock solution at defined time intervals (~ every 15 min; see discussion), followed by 10 µl of SB-203186 stock solution directly to the bath and monitor their effect on EFS-induced contraction. In parallel strips add 10 µl of the vehicle, DMSO. Observe the effects of cisapride, a 5HT4 receptor agonist, on EFS-evoked contractions in human bladder and ileum tissues in **Figure 6**. Additionally, observe the effect of DMSO, the vehicle for cisapride, on EFS-evoked contractions in human bladder and ileum tissues.
    5. At the end of the EFS protocol verify the selectivity of EFS by blocking neural transmission with the Na^+ channel blocker, tetrodotoxin (TTX; 0.5-1 µM). If TTX resistant contractions are still present, it is recommended to adjust the duration and intensity of the stimulus in subsequent experiments.

4. For determining the effects of drugs on pre or post-synaptic sites (**Figure 7A**) follow steps 1 to 4.3.2. Establish reproducible responses to carbachol and EFS, then add drug X.
5. At the end of the experiment, unclip or untie the strips, blot them gently on a piece of tissue paper to eliminate extra fluid and measure each strip's weight using a balance. Also measure the tissue length using a caliper to determine cross sectional area. This information is used for normalization of data (see section 5.4).

5. Data Analysis

Analyze data using adequate software (*e.g.*, Windaq, LabChart).

1. For spontaneous activity, select a window of at least 30 sec before and at the peak of the drug induced response and measure amplitude and frequency of myogenic activity (**Figure 3**).
    1. Use fast Fourier transformation analysis to determine the spectrum of frequencies contributing to contractile responses and whether there are differences between different parts of the bladder (*e.g.*, dome vs. neck) or with development, pathology, and drugs[8].
2. For effects on smooth muscle tone select a window of at least 10-30 sec before and at the peak of the drug induced response and measure amplitude of contraction.
3. For effects on neurally-evoked contractions measure amplitude, duration and area under the curve of contractions (at least 3) before and at the peak of the drug-induced response.
   NOTE: It is necessary to measure both the amplitude and area under the curve of EFS-induced contractions because purinergic and cholinergic components have different kinetics. The purinergic component is fast and transient (ATP activates purinergic ionotropic channels such as P2X1 that allow fast influx of calcium, then they desensitize), thus contributing more to the peak amplitude response and less to the area under the curve. The cholinergic component is slower and sustained (ACh activates metabotropic muscarinic receptors, which require more time to activate intracellular pathways that ultimately activate ion channels that depolarize the smooth muscle to induce a contraction). Thus, the muscarinic component is captured better by measuring the area under the curve.
4. Normalize the data to be able to compare results across strips and pharmacological treatments. The parameter chosen for normalization should not be influenced by the test compounds, pathological condition studied or experimental design. Among these parameters, use strip weight, cross sectional area, KCl responses (**Figure 4B**), % of the maximal response (**Figure 7B**) or % of the maximal response to another contractile agent (*e.g.*, CCh) or relaxing agents (*e.g.*, papaverine).

Representative Results

Spontaneous Myogenic Activity

Spontaneous myogenic activity is an important smooth muscle characteristic that undergoes changes with postnatal development[6-9] and pathology (*e.g.*, SCI, BOO)[3-5]. Because this activity is believed to contribute to the symptoms of overactive bladder (OAB)[2], an evaluation of receptors, intracellular pathways and pharmacological agents that modulate it, is of high interest for developing effective treatments for OAB and other smooth muscle dysfunctions. The method presented here can easily investigate these questions. **Figure 2** illustrates different patterns of myogenic spontaneous activity during development in neonatal (i), juvenile (ii) adult (iii) and spinal cord injured rats (SCI; iv). Strips from neonatal rats exhibit large amplitude, low frequency rhythmic contractions (**Figure 2Ai**), while strips from adult rats exhibit small amplitude, high frequency activity (**Figure 2Aii, iii**). After SCI the neonatal pattern re-emerges (**Figure 2Aiv**). In addition to using strips from animal models, various pharmacological agents can be used to induce spontaneous contractions in strips from naive animals, with the aim of understanding the mechanisms underlying the spontaneous contractions. Examples of suitable pharmacological agents include muscarinic receptor agonists (carbachol; CCh), compounds that increase ACh levels (such as acetylcholine esterase inhibitors), low concentrations of KCl (*e.g.*, 20 mM) or other experimental drugs. **Figures 3A-B**, illustrate modulation of spontaneous activity by pharmacological agents that act on KCNQ channels located on the smooth muscle. The KCNQ channel opener, flupirtine, decreases the amplitude and frequency of spontaneous activity in a concentration-dependent manner (**Figure 3Ai-iii**), while the KCNQ channel blocker, XE991, decreases the amplitude but increases the frequency of spontaneous activity (**Figure 3Bi-iii**).

Smooth Muscle Tone

Smooth muscle tone and contractility properties are important factors for proper function of the bladder during storage and voiding. This method can easily screen the effects of pharmacological agents on smooth muscle tone. **Figures 3Aiv** and **3Biv** show that flupirtine decreases basal tone, consistent with smooth muscle relaxation, while the XE991 increases smooth muscle tone. **Figure 4** illustrates concentration dependent increases in smooth muscle tone by activating bombesin receptors with neuromedin B (NMB; **Figure 4A, B**) or muscarinic receptors with carbachol (CCh; **Figures 4C, D**). Furthermore, intracellular pathways mediating these smooth muscle responses can be investigated using specific modulators (data not shown).

Neurally-mediated Responses and Modulation of Neurotransmission

Bladder contraction is achieved by the release of ACh/ATP from the parasympathetic efferent nerves. The contribution of muscarinic and purinergic systems to bladder contraction varies among species and pathological conditions, with predominant increase in purinergic contribution in pathologies such as interstitial cystitis, partial outlet obstruction, and overactive bladder[26]. **Figure 5C** demonstrates the use of this method to determine the contribution of muscarinic and purinergic components to neurotransmission in bladder strips from the mouse. The contribution of the cholinergic component was assessed using the muscarinic receptor antagonist, atropine. The contribution of the purinergic system was assessed using the purinergic receptor activator and desensitizer, alpha,beta-methylene ATP (ABMA). Additionally, the frequency dependent contribution of each component was assessed by varying the stimulation frequency from low to high frequencies (2-50 Hz).

The strength of the bladder contraction plays a significant role in voiding efficiently. Using this method, receptors and pathways that modulate neural transmission can be investigated as drug targets for voiding dysfunctions. The 5HT4 receptors are expressed pre-junctionally in

parasympathetic neurons and their activation increases ACh levels[27]. **Figure 6** illustrates the excitatory effect of the 5HT4 receptor agonist, cisapride, in human bladder and ileum strips.

Various experimental protocols can be employed to determine the site of action of a test compound. Diagram in **Figure 7A** illustrates a protocol used to assess pre- vs. post-junctional sites. If drug X reduces (or increases) the EFS response but has no effect on the CCh response, the most likely site of action is pre-junctional. If drug X alters both EFS and CCh response, then it may act on receptors located post-junctionally or both pre- and post-junctionally.

Role of Each Component: Smooth Muscle, Mucosa, and Neuronal

Different pathological conditions may affect various components of the bladder. For example interstitial cystitis (IC) affects primarily the urothelium, while OAB may result in altered smooth muscle contractility. Also, different receptors may be expressed in each bladder component and thus could be specifically targeted in a certain pathology. As opposed to *in vivo* methods, which measure a net effect of all bladder components, this *in vitro* method allows the investigation of particular components by using a combination of surgical and pharmacological procedures. To test smooth muscle contraction/relaxation in the absence of neuronal transmission, TTX (0.5-1 μM) can be added to the bath. In **Figure 4**, NMB and CCh were tested in the presence of TTX. To test the contribution of the mucosa (urothelium and lamina propria) to the smooth muscle contractility, strips with and without the mucosal layer are compared. **Figure 7B** shows that responses to CCh are reduced in the presence of the mucosa in the pig[28]. Similar results were reported in human bladder strips[29]. To test the role of nerve fibers, several approaches can be taken. One is to activate or inhibit specific fibers using pharmacological agents. For example, capsaicin activates a specific population of afferent nerves and causes species dependent smooth muscle contraction or relaxation[17,18]. Guanethidine inhibits the release of norepinephrine from sympathetic fibers, thus eliminating the contribution of these fibers. Another approach is to desensitize/eliminate specific fibers *in vivo* prior to the experiment. For example, systemic treatment of the animal with capsaicin desensitizes capsaicin sensitive afferent nerves. Other bladder components that can be studied in this preparation are interstitial cells or gap junctions by activating or blocking them with specific agents.

Species Differences

While most drug development is intended for the treatment of human disorders, basic research is primarily performed in animal tissue. Species differences exist in a number of receptors. For example, 5HT4 receptor agonists enhance neurally-evoked contractions in the human bladder but not in the rat bladder[19,30], EFS-induced contractions are almost exclusively atropine-sensitive in human and old-world monkey detrusor from stable bladders[31] but become partially atropine-resistant in human detrusor from patients with unstable bladder conditions (*e.g.*, neurogenic, obstructed bladders)[15,32,33], capsaicin elicits an excitatory response in rat and human bladder strips, no response in pig bladder strips and inhibitory response in guinea pig bladder strips[17,18]. **Figure 4** shows that bombesin receptor agonists have excitatory effects on rat bladder and no effects on mouse and pig bladder strips[16]. This information is critical for selecting the appropriate animal model for studying a specific receptor.

Comparison of Sensitivity across Organs

Drugs intended for the treatment of bladder disorders may also affect smooth muscle from other organs, such as the gastrointestinal tract, urethra, gallbladder, *etc.* This method allows estimation of organ selectivity and sensitivity to a pharmacological agent by comparing different tissues side by side. As illustrated in **Figure 6**, the 5HT4 receptor agonist, cisapride, has different efficacy and potency in human bladder vs. ileum tissue.

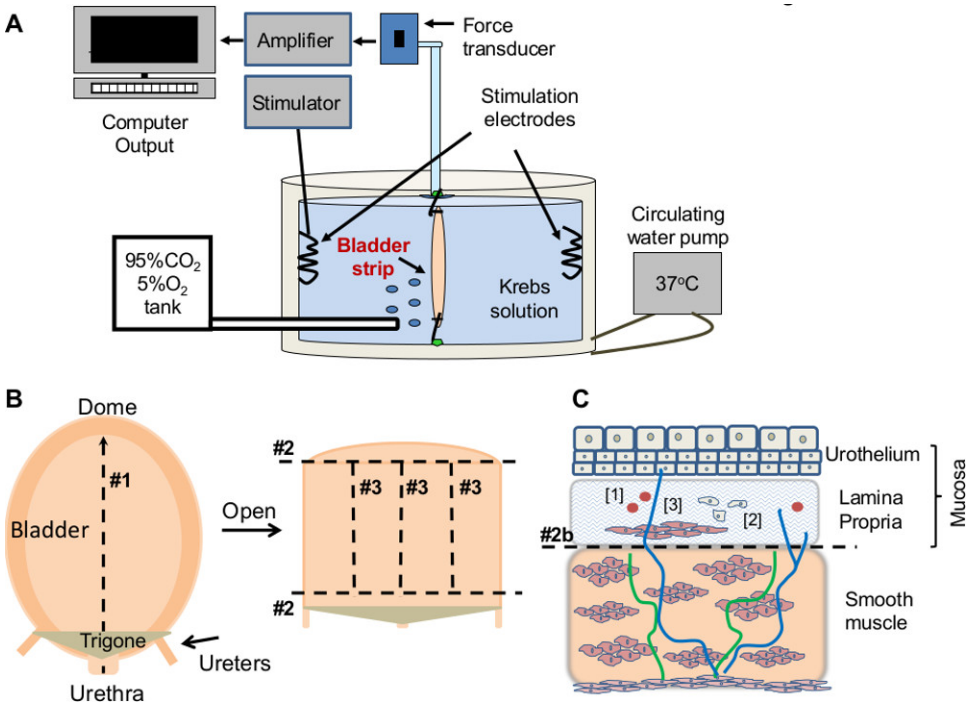

Figure 1. Experimental set-up and bladder strip preparation. A) Schematic of the experimental set-up. Bladder strips are submersed in tissue chambers filled with aerated Krebs solution kept at 37 °C via a circulating water pump. One end of the strip is attached to an isometric force transducer to measure tissue contractility, the other to a fixed rod. The force transducer is connected to an amplifier and computer for data recording. Electric field stimulation electrodes connected to a stimulator are placed in the chamber and used for evoking neurally-mediated bladder contractions. **B)** Preparation of tissue strips. The bladder is pinned down in a dish and the following procedures are performed: #1 vertical cut though ventral half of bladder from urethra to dome to open the bladder into a flat sheet. #2 horizontal cuts removing the dome and base of the bladder/proximal urethra. #3 vertical cuts dividing the mid bladder into equal strips (4 strips from a rat bladder). **C)** Schematic of strip components: smooth muscle and mucosa, both containing afferent (blue) and efferent (green) nerves. Mucosa consists of the urothelium and lamina propria. Lamina propria contains blood vessels [1], interstitial cells [2], and muscularis mucosae [3]. Dotted line labeled #2b indicates the procedure for removing mucosa layer. Please click here to view a larger version of this figure.

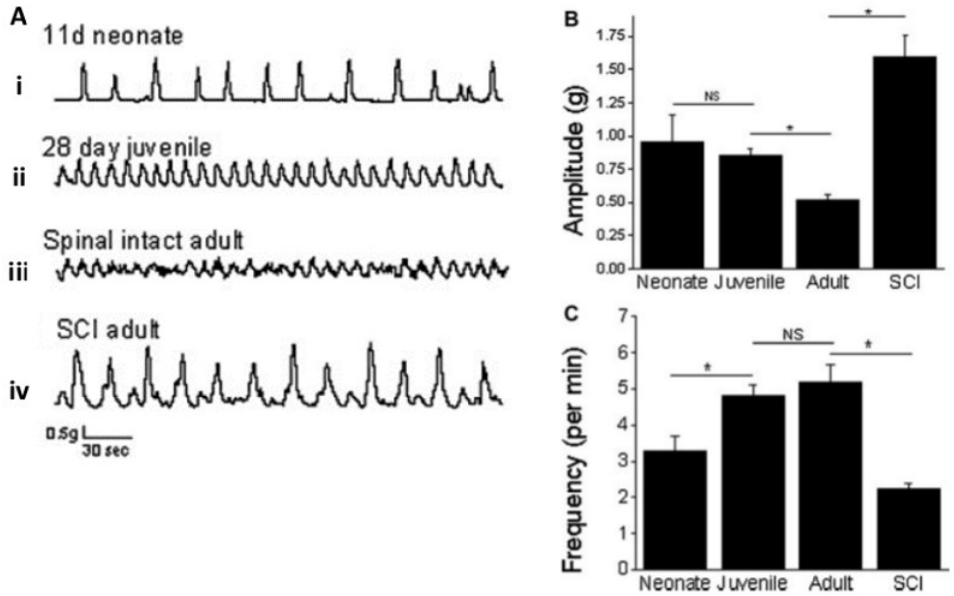

Figure 2. Myogenic spontaneous activity during development and after pathology. A) Examples of spontaneous activity in neonatal (i), juvenile (ii), spinal intact adult (iii) and spinal cord injured (SCI) adult (iv) rat bladder strips. The SCI rat was used at 4 weeks after surgery. **B**, **C)** Summary of amplitude (B) and frequency (C) of spontaneous contractions in the four groups investigated. (Reproduced with permission from Artim DE, Kullmann FA, Daugherty SL, Bupp E, Edwards CL, de Groat WC. Neurourol Urodyn. 2011 Nov;30(8):1666-74.) Please click here to view a larger version of this figure.

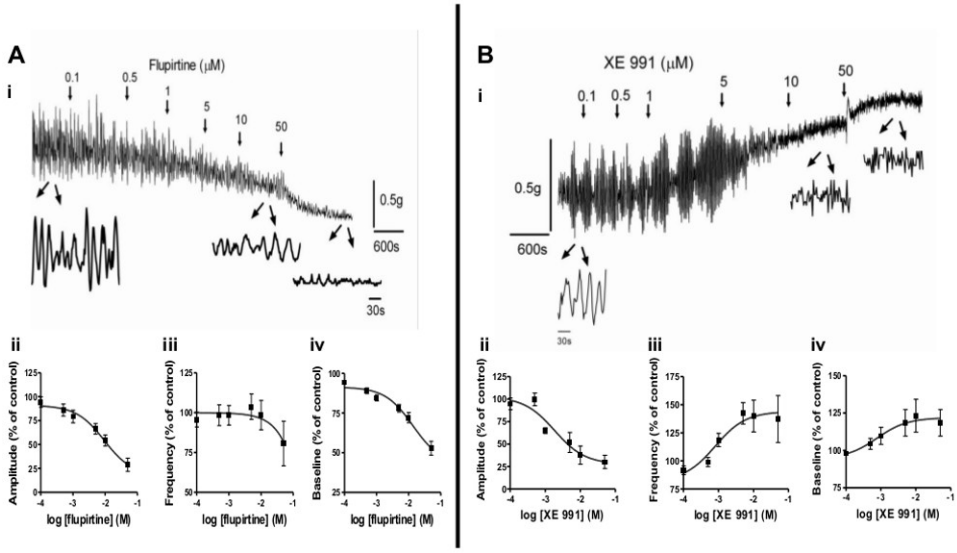

Figure 3. Modulation of myogenic spontaneous activity and smooth muscle tone. A) The effect of the KCNQ channel opener, flupirtine, on spontaneous activity and baseline tone in adult rat bladder strips. (i) Flupirtine was added in increasing concentrations (cumulative) at the times indicated by arrows. The enlargements under the trace show 4 min of strip activity during control and after application of 10 μM and 50 μM flupirtine. (ii-iv) Summary of effects of flupirtine (7 strips from 4 rats) on the amplitude (ii) and frequency (iii) of spontaneous activity and baseline tone (iv), expressed as % change from control (pre-drug) values, which were set to 100%. **B)** The effect of the KCNQ channel blocker, XE991, on spontaneous activity and baseline tone in adult rat bladder strips. (i) XE991 was added in increasing concentrations (cumulative) at

the times indicated by arrows. The enlargements under the trace show 2 min of strip activity during control and after application of 10 µM and 50 µM XE991. (ii-iv) Summary of effects of XE991 (9 strips from 4 rats) on the amplitude (ii) and frequency (iii) of spontaneous activity and baseline tone (iv), expressed as % change from control (pre-drug) values, which were set to 100%. Please click here to view a larger version of this figure.

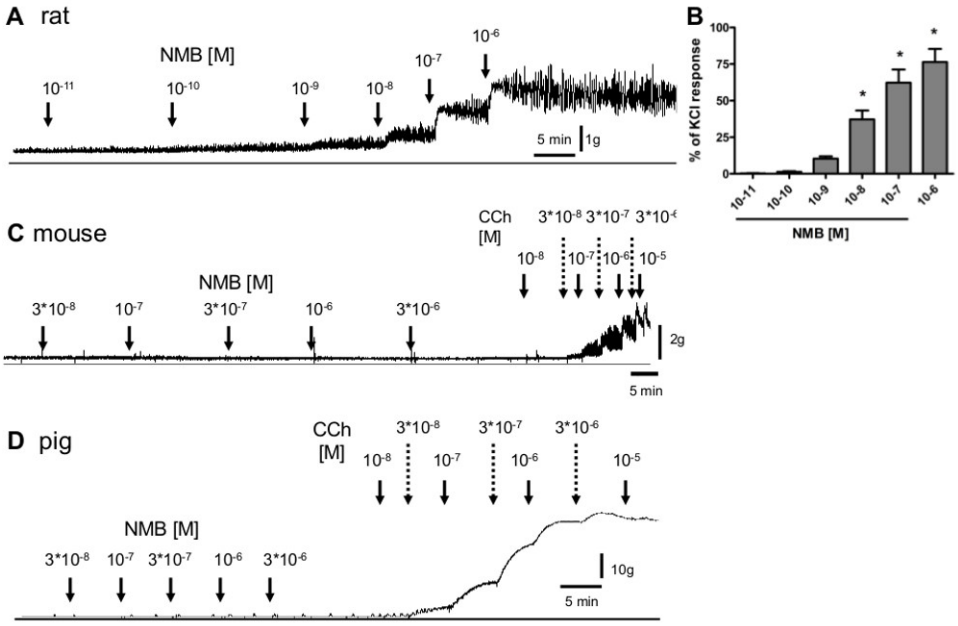

Figure 4. Species differences. A) Concentration dependent smooth muscle contractions in response to the bombesin receptor agonist, neuromedin B (NMB), in rat bladder strips. **B)** Summary of effects of NMB on smooth muscle contraction in the rat bladder strips. Data are normalized to the KCl (80 mM) response. **C**, **D)** Absence of responses to NMB in mouse (C) and pig (D) bladder strips. Carbachol (CCh) elicits strong concentration dependent contractions in both mouse and pig strips, indicating that the strips can respond to stimuli. TTX (0.5 µM) was present in the bath in all strips. (Reproduced with permission from Kullmann FA, McKenna D, Wells GI, Thor KB. Neuropeptides 2013 Oct;47(5):305-13.) Please click here to view a larger version of this figure.

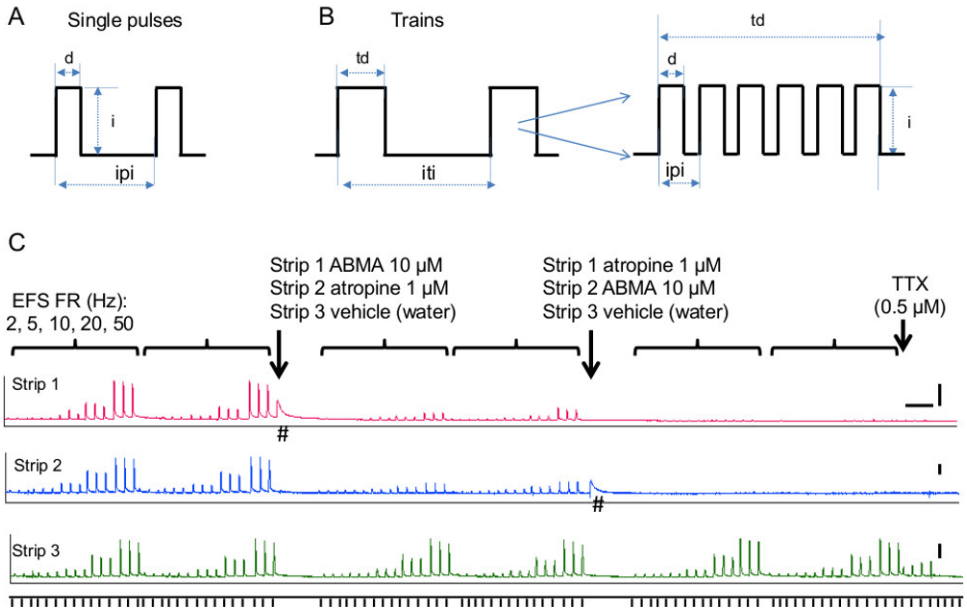

Figure 5. Electric field stimulation. A) Schematic of single pulse stimulation parameters. Abbreviations: d = duration of pulse, i = intensity of pulse, ipi = inter pulse interval. **B)** Schematic of train stimulation parameters. Abbreviations: td = train duration, i = intensity of pulse, iti = inter train interval. Inset shows the number of pulses in a train and the interval between them, which together with train duration determine the frequency of train stimulation. **C)** Contribution of purinegic and cholinergic components to neurally-evoked bladder contractions. EFS-FR represent stimulation frequencies, 2, 5, 10, 20, 50 Hz. Three stimuli delivered every 90 sec were tested for each frequency and each frequency series was repeated twice in control and twice after adding each compound. Alpha,beta-methylene ATP, abbreviated ABMA (strip 1), was used to desensitize purinergic receptors and atropine (strip 2) was used to block muscarinic receptors. Strip 3 served as control and was treated with the vehicle, water. Arrows indicate the time when each compound was added to each strip. Note that EFS-evoked contractions are strongly reduced by ABMA and atropine, while not affected by the vehicle. TTX was added at the end of the experiment while the EFS was delivered at 20 Hz. Note that remaining contractions observed in the control strip 3 were abolished by TTX, demonstrating their neural nature (*i.e.* initiated by

transmitter release from the intramural nerves). # indicates smooth muscle responses to ABMA in the absence of EFS. Scale bars are 5 min for x axis and 2 g for y axis. Please click here to view a larger version of this figure.

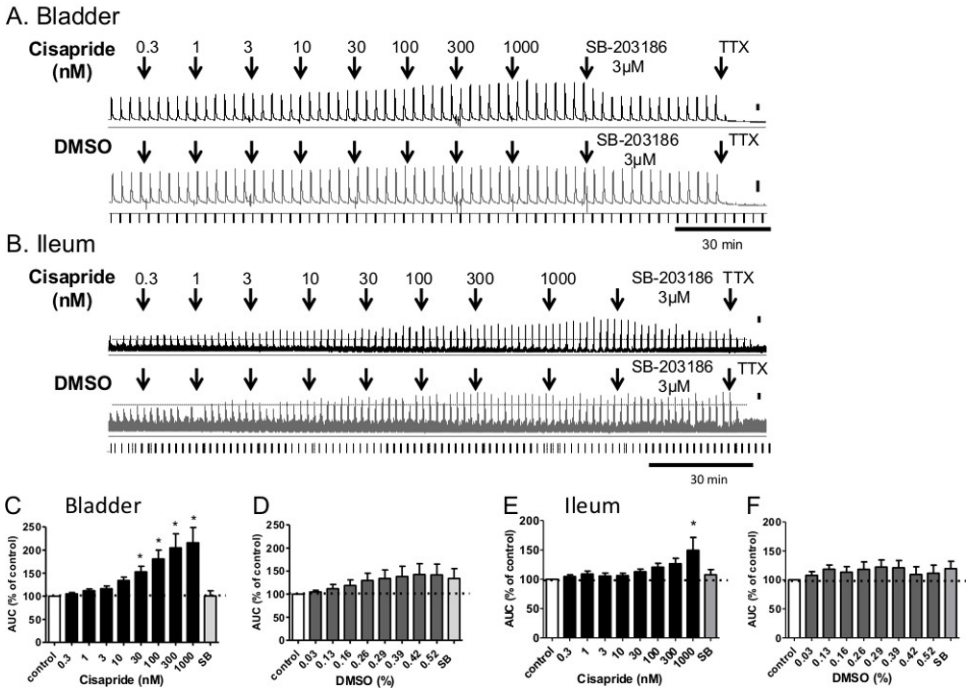

Figure 6. Modulation of neurally-evoked bladder contractions. A, **B)** Examples of the enhancement of the neurally-evoked contractions by the 5HT4 receptor agonist, cisapride in human bladder (A) and ileum strips (B). Cisapride (black records) or DMSO (grey records) was added in a concentration dependent manner at the times indicated by arrows. Black bars below the records in each panel represent EFS, which consisted of 10 sec trains delivered at 20 Hz every 120 sec. Vertical scale bars are 1 g for all examples. TTX concentration was 0.5 μM. **C-F)** Summary of the area under the curve (AUC) of EFS-evoked contractions in response to cisapride (black bars) or DMSO (grey bars) in bladder strips (C, D) and ileum strips (E, F). In C-F, SB stands for SB-203186, representing a summary of data obtained after the addition of the 5HT4 receptor antagonist. Dotted lines are set to 100% and represent control. (Reproduced with permission from Kullmann FA, Kurihara R, Ye L, Wells GI, McKenna DG, Burgard EC, Thor KB. Auton Neurosci. 2013 Jun;176(1-2):70-7.) Please click here to view a larger version of this figure.

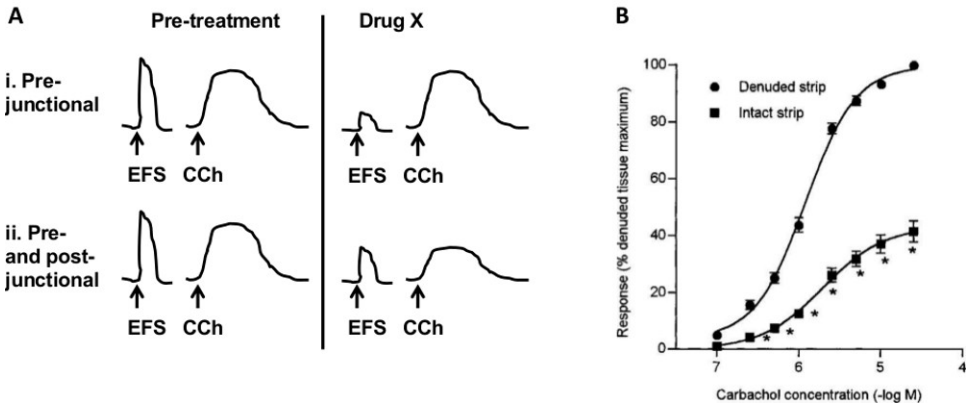

Figure 7. Sites of action of drugs and role of different components of bladder. **A)** Schematic of protocol for identifying the site of action of a drug. Strips are stimulated with ESF and carbachol (CCh). In i drug X reduces the EFS response but not the CCh response, indicating a pre-junctional site of action. In ii, drug X alters both responses, indicating an action on post-junctional or both pre- and post-junctional receptors. **B)** Influence of mucosa on smooth muscle contraction. Effects of carbachol are diminished in strips with the mucosa present (intact) compared to strips with the mucosa removed (denuded). (B is reproduced with permission from Hawthorn MH, Chapple CR, Cock M, Chess-Williams R. Br J Pharmacol. 2000 Feb;129(3):416-9). Please click here to view a larger version of this figure.

Discussion

In this paper we described a simple *in vitro* smooth muscle contractility method that can be used to address a number of important scientific questions related to bladder physiology and pathology, as well as aiding the discovery of new drugs to treat bladder dysfunctions. We have illustrated the use of this method for assessing developmental, pathological and pharmacological properties of bladder smooth muscle contractility (**Figures 2-4**), neurotransmission modulation (**Figures 5-7A**), species differences (**Figure 4**), organ differences (**Figure 6**) and relevance of specific bladder components (*e.g.*, mucosa, **Figure 7B**). Additional applications not illustrated here include evaluation of intracellular

pathways using pharmacological agents[3,10,11], structure-activity relationships of various drugs[22-24], or evaluation/quantification of transmitter release after neural stimulation[25].

While bladder function may ultimately be assessed *in vivo*, this *in vitro* method overcomes many situations that are problematic *in vivo*. These include situations when surgical and pharmacological manipulations would reduce the viability and/or survival of the organ or the animal, the use of human tissue, the need to identify and characterize responses from specific components (*e.g.*, smooth muscle vs. epithelium vs. nerves) or the use of expensive chemicals. The method allows systematic investigation of the effects of various pharmacological agents as well as pathology on contractile activity of the smooth muscle and in a well-controlled fashion and environment.

The method provides a plethora of information; however, care should be taken when interpreting and extrapolating this information. This is an *in vitro* method of a reduced preparation, disconnected from its normal environment and neural control. The experimental conditions are not physiologic, thus the data may not entirely reflect *in vivo* physiological situations. For example, the method cannot account for changes in blood flow, hormones, humoral substances, external mechanical forces, or extrinsic neural control. Tissue is acutely decentralized, thus injury and ischemia related responses need to be evaluated and taken into account. Pathological changes occurring in the brain or spinal cord cannot be tested using this method unless they have already altered afferent, smooth muscle, mucosa or intramural nerve function (*i.e.* cellular plasticity). Electric field stimulation (EFS) allows the evaluation of neurally mediated responses. However, it excites indiscriminately all nerves in the strip (*e.g.*, sympathetic, parasympathetic, afferents), as opposed to *in vivo* situation where the micturition reflex activates only particular pathways. One way to overcome this situation is to combine EFS with specific antagonists that selectively block different pathways. For example, guanethidine could be used to block norepinephrine release when studying contraction properties, or atropine could be used to block muscarinic receptors to prevent bladder contractions when studying relaxation properties. Finally, viability of the tissue is limited to a certain number of hours. In general, most components of bladder tissue are viable and stable (*i.e.* responding to EFS without deteriorating responses) over a period of 6-8h or longer. However, other tissues may be more sensitive (*e.g.*, ileum lasts ~6 hr or less; author's personal experience).

Although the method is technically feasible and with good reproducibility, there are several critical steps necessary to ensure its success. First, tissue preparation should be performed carefully to ensure viability by making necessary changes to the dissection procedure (avoid stretching the tissue while preparing the strips) and/or media if needed for different tissue types or species. Another critical step is setting-up neuronal stimulation parameters, such that ceiling effects are avoided. As described in the method section, this depends on the type of the experiment performed and expected mechanism of action of the test compound. For example, for testing effects of cisapride, a 5HT4 receptor agonist, on bladder strips (**Figure 6**), we set the amplitude of EFS-evoked contraction to ~50% of the maximal. This was based on the known mechanism of action of 5HT4 receptor agonists, namely enhancing ACh release from the pre-junctional parasympathetic nerves[27], which in turn is expected to increase EFS-evoked contractions. Stimulation of muscle vs. nerves should be tested using TTX, which inhibits neural transmission and thus should inhibit EFS-evoked contractions. Adequate controls for vehicle and time must be performed during the drug testing to account for deterioration of the tissue with time and for any possible effects of the vehicle. For example, many drugs are dissolved in DMSO or ethanol. Our data (**Figure 6**) show that DMSO (0.1% and higher) can increase neurally-evoked contractions, an effect which needs to be subtracted from the effect of the test drug. Similarly, ethanol (up to 1%) reduces the spontaneous smooth muscle contractions but has no effect on neurally-evoked contractions[34,35]. If using genetically engineered animals or surgical models (*e.g.*, spinal cord injury or ovariectomy), controls should include tissue from the appropriate background mouse strain or sham operated animals, respectively. In addition, some tissues, such as human, mouse and guinea pig bladders contain intramural ganglia. When working with these tissues, protocol selection and data interpretation must take into account effects of drugs or EFS on intramural neurons that further stimulate the smooth muscle.

Designing the experimental protocol, choosing the right parameters (for EFS, for drug stimulation) and concentrations to be tested are critical to ensure meaningful data. While parameters should be adjusted for individual tissues and drugs, general principles/guidelines outlined below are applicable. Cumulative concentration response curves are desirable, however this is not possible for all compounds. Drugs targeting receptors that desensitize, such as the purineric ionotropic receptors (P2X), or drugs that are metabolized quickly in the tissue (example ACh), cannot be reliably tested using cumulative concentration response curves in the same tissue. In these cases, single concentrations are tested in different groups of tissue. To evaluate desensitization, it is recommended to compare the magnitude of response elicited by a single highest concentration to that achieved at the end of a cumulative concentration response curve.

For accurate fitting of the data obtained from concentration response curves, it is desirable to test half log concentrations (example CCh in **Figure 6**). However, log concentrations (example NMB in **Figure 4A**) are acceptable when tissue viability may be limited or other constraints may be in place.

To select a concentration range for a novel compound, in preliminary experiments, it is useful to consider the binding affinity of the compound and test two power of 10 above and below that concentration. In subsequent experiments, the protocol is refined to determine a starting point where no effect of the drug is observed and an end point where either the response is maximal or the concentration tested is no longer specific for the intended target.

The time interval for applying a drug should be chosen taking into consideration several factors: a) Time for a drug to have an effect. In general drugs targeting membrane receptors have a relatively fast response (seconds to minutes), whereas drugs for intracellular targets (*e.g.*, forskolin and other enzyme inhibitors[36], botulinum toxin[37]) require additional incubation time (30 min – 3 hr). Additionally, tissue thickness may play a role. b) Duration and mechanism of action of drug. For cases when the drug effect reaches a plateau that is sustained, such as NMB in **Figure 4A** and cisapride in **Figure 6**, time intervals of 5-15 min between drug applications are adequate for collecting sufficient data. This is not possible with drugs having a much shorter duration of action or different mechanism of action (ATP, CCh). For example the effect of CCh in **Figure 4C or 4D**, reaches a plateau rapidly but the tissue tension tends to return to baseline. In this case, the time intervals need to be adjusted accordingly, usually adding the next concentration when the first response reaches a maximum.

Data analysis, particularly normalization of data to allow comparisons between strips is a very important step. Different studies use different parameters for normalization, including strip weight[38], strip cross sectional area[39], KCl response[12], % of maximal response[28] or % of the maximal response to another contractile agent (*e.g.*, CCh[38]). The normalization parameter should be chosen depending on the purpose of the experiment, such that the parameter is not influenced by the test compounds, pathology or experimental design. For example, normalization to KCl response eliminates the weight and other dimensions of the strips, and thus could be used to compare responses in tissues where pathological condition

may increase the weight of the strips (*e.g.*, diabetes increases bladder mass). In addition, the response to KCl is not influenced by the removal of the mucosa/urothelium[29], thus could be used in experiments evaluating different components of the bladder (*e.g.*, mucosa vs. smooth muscles).

In summary, this contractility method provides a fast, easy and very powerful approach to assess bladder (and other organ) physiology and pharmacology. When used properly, it provides the ability to manipulate tissue in a reduced and well controlled environment. In the study of the urinary bladder function, this method was instrumental in the discovery and testing of compounds currently used for OAB management, such as the antimuscarinics and newly developed β_3AR agonists.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

This study was supported by NIH R37 DK54824 and R01 DK57284 grants to LB.

References

1. Fowler, C. J., Griffiths, D., & de Groat, W. C. The neural control of micturition. *Nat Rev Neurosci.* **9**, 453-466, doi:10.1038/nrn2401 (2008).
2. Andersson, K. E. Detrusor myocyte activity and afferent signaling. *Neurourol Urodyn.* **29**, 97-106, doi:10.1002/nau.20784 (2010).
3. Artim, D. E. *et al.* Developmental and spinal cord injury-induced changes in nitric oxide-mediated inhibition in rat urinary bladder. *Neurourology and urodynamics.* **30**, 1666-1674, doi:10.1002/nau.21143 (2011).
4. Kita, M. *et al.* Effects of bladder outlet obstruction on properties of Ca2+-activated K+ channels in rat bladder. *Am J Physiol Regul Integr Comp Physiol.* **298**, R1310-1319, doi:10.1152/ajpregu.00523.2009 (2010).
5. Barendrecht, M. M. *et al.* The effect of bladder outlet obstruction on alpha1- and beta-adrenoceptor expression and function. *Neurourol Urodyn.* **28**, 349-355, doi:10.1002/nau.20642 (2009).
6. Maggi, C. A., Santicioli, P., & Meli, A. Postnatal development of myogenic contractile activity and excitatory innervation of rat urinary bladder. *The American journal of physiology.* **247**, R972-978 (1984).
7. Ng, Y. K., de Groat, W. C., & Wu, H. Y. Smooth muscle and neural mechanisms contributing to the downregulation of neonatal rat spontaneous bladder contractions during postnatal development. *American journal of physiology. Regulatory, integrative and comparative physiology.* **292**, R2100-2112, doi:10.1152/ajpregu.00779.2006 (2007).
8. Szell, E. A., Somogyi, G. T., de Groat, W. C., & Szigeti, G. P. Developmental changes in spontaneous smooth muscle activity in the neonatal rat urinary bladder. *Am J Physiol Regul Integr Comp Physiol.* **285**, R809-816, doi:10.1152/ajpregu.00641.2002 (2003).
9. Szigeti, G. P., Somogyi, G. T., Csernoch, L., & Szell, E. A. Age-dependence of the spontaneous activity of the rat urinary bladder. *J Muscle Res Cell Motil.* **26**, 23-29, doi:10.1007/s10974-005-9003-z (2005).
10. Frazier, E. P., Braverman, A. S., Peters, S. L., Michel, M. C., & Ruggieri, M. R., Sr. Does phospholipase C mediate muscarinic receptor-induced rat urinary bladder contraction? *The Journal of pharmacology and experimental therapeutics.* **322**, 998-1002, doi:10.1124/jpet.107.125393 (2007).
11. Xin, W., Soder, R. P., Cheng, Q., Rovner, E. S., & Petkov, G. V. Selective inhibition of phosphodiesterase 1 relaxes urinary bladder smooth muscle: role for ryanodine receptor-mediated BK channel activation. *American journal of physiology. Cell physiology.* **303**, C1079-1089, doi:10.1152/ajpcell.00162.2012 (2012).
12. Frazier, E. P., Peters, S. L., Braverman, A. S., Ruggieri, M. R., Sr., & Michel, M. C. Signal transduction underlying the control of urinary bladder smooth muscle tone by muscarinic receptors and beta-adrenoceptors. *Naunyn-Schmiedeberg's archives of pharmacology.* **377**, 449-462, doi:10.1007/s00210-007-0208-0 (2008).
13. Svalo, J. *et al.* The novel beta3-adrenoceptor agonist mirabegron reduces carbachol-induced contractile activity in detrusor tissue from patients with bladder outflow obstruction with or without detrusor overactivity. *European journal of pharmacology.* **699**, 101-105, doi:10.1016/j.ejphar.2012.11.060 (2013).
14. Yokota, T., & Yamaguchi, O. Changes in cholinergic and purinergic neurotransmission in pathologic bladder of chronic spinal rabbit. *J Urol.* **156**, 1862-1866, doi:S0022-5347(01)65554-X [pii] (1996).
15. Bayliss, M., Wu, C., Newgreen, D., Mundy, A. R., & Fry, C. H. A quantitative study of atropine-resistant contractile responses in human detrusor smooth muscle, from stable, unstable and obstructed bladders. *J Urol.* **162**, 1833-1839 (1999).
16. Kullmann, F. A., McKenna, D., Wells, G. I., & Thor, K. B. Functional bombesin receptors in urinary tract of rats and human but not of pigs and mice, an *in vitro* study. *Neuropeptides.* **47**, 305-313, doi:10.1016/j.npep.2013.08.003 (2013).
17. Sadananda, P., Kao, F. C., Liu, L., Mansfield, K. J., & Burcher, E. Acid and stretch, but not capsaicin, are effective stimuli for ATP release in the porcine bladder mucosa: Are ASIC and TRPV1 receptors involved? *European journal of pharmacology.* **683**, 252-259, doi:10.1016/j.ejphar.2012.02.041 (2012).
18. Maggi, C. A. *et al.* Species-related variations in the effects of capsaicin on urinary bladder functions: relation to bladder content of substance P-like immunoreactivity. *Naunyn-Schmiedeberg's archives of pharmacology.* **336**, 546-555 (1987).
19. Kullmann, F. A. *et al.* Effects of the 5-HT4 receptor agonist, cisapride, on neuronally evoked responses in human bladder, urethra, and ileum. *Autonomic neuroscience : basic & clinical.* **176**, 70-77, doi:10.1016/j.autneu.2013.02.020 (2013).
20. Warner, F. J., Miller, R. C., & Burcher, E. Human tachykinin NK2 receptor: a comparative study of the colon and urinary bladder. *Clin Exp Pharmacol Physiol.* **30**, 632-639 (2003).
21. Zoubek, J., Somogyi, G. T., & De Groat, W. C. A comparison of inhibitory effects of neuropeptide Y on rat urinary bladder, urethra, and vas deferens. *The American journal of physiology.* **265**, R537-543 (1993).
22. Warner, F. J., Miller, R. C., & Burcher, E. Structure-activity relationship of neurokinin A(4-10) at the human tachykinin NK(2) receptor: the effect of amino acid substitutions on receptor affinity and function. *Biochem Pharmacol.* **63**, 2181-2186 (2002).

23. Warner, F. J., Mack, P., Comis, A., Miller, R. C., & Burcher, E. Structure-activity relationships of neurokinin A (4-10) at the human tachykinin NK(2) receptor: the role of natural residues and their chirality. *Biochem Pharmacol.* **61**, 55-60 (2001).
24. Dion, S. *et al.* Structure-activity study of neurokinins: antagonists for the neurokinin-2 receptor. *Pharmacology.* **41**, 184-194 (1990).
25. Somogyi, G. T., Zernova, G. V., Yoshiyama, M., Yamamoto, T., & de Groat, W. C. Frequency dependence of muscarinic facilitation of transmitter release in urinary bladder strips from neurally intact or chronic spinal cord transected rats. *British journal of pharmacology.* **125**, 241-246, doi:10.1038/sj.bjp.0702041 (1998).
26. Andersson, K. E., & Wein, A. J. Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence. *Pharmacological reviews.* **56**, 581-631, doi:10.1124/pr.56.4.4 (2004).
27. Agostino, G., Condino, A. M., Gallinari, P., Franceschetti, G. P., & Tonini, M. Characterization of prejunctional serotonin receptors modulating [3H]acetylcholine release in the human detrusor. *The Journal of pharmacology and experimental therapeutics.* **316**, 129-135, doi:10.1124/jpet.105.092551 (2006).
28. Hawthorn, M. H., Chapple, C. R., Cock, M., & Chess-Williams, R. Urothelium-derived inhibitory factor(s) influences on detrusor muscle contractility *in vitro*. *British journal of pharmacology.* **129**, 416-419, doi:10.1038/sj.bjp.0703068 (2000).
29. Chaiyaprasithi, B., Mang, C. F., Kilbinger, H., & Hohenfellner, M. Inhibition of human detrusor contraction by a urothelium derived factor. *J Urol.* **170**, 1897-1900, doi:10.1097/01.ju.0000091870.51841.ae (2003).
30. Testa, R. *et al.* Effect of different 5-hydroxytryptamine receptor subtype antagonists on the micturition reflex in rats. *BJU international.* **87**, 256-264 (2001).
31. Craggs, M. D., Rushton, D. N., & Stephenson, J. D. A putative non-cholinergic mechanism in urinary bladders of New but not Old World primates. *J Urol.* **136**, 1348-1350 (1986).
32. Fry, C. H., Bayliss, M., Young, J. S., & Hussain, M. Influence of age and bladder dysfunction on the contractile properties of isolated human detrusor smooth muscle. *BJU international.* **108**, E91-96, doi:10.1111/j.1464-410X.2010.09845.x (2011).
33. Kennedy, C., Tasker, P. N., Gallacher, G., & Westfall, T. D. Identification of atropine- and P2X1 receptor antagonist-resistant, neurogenic contractions of the urinary bladder. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* **27**, 845-851, doi:10.1523/JNEUROSCI.3115-06.2007 (2007).
34. Levin, R. M., Danek, M., Whitbeck, C., & Haugaard, N. Effect of ethanol on the response of the rat urinary bladder to *in vitro* ischemia: protective effect of alpha-lipoic acid. *Molecular and cellular biochemistry.* **271**, 133-138 (2005).
35. Malysz, J., Afeli, S. A., Provence, A., & Petkov, G. V. Ethanol-mediated relaxation of guinea pig urinary bladder smooth muscle: Involvement of BK and L-type Ca2+ channels. *American journal of physiology. Cell physiology.* **306**, C45-58, doi:10.1152/ajpcell.00047.2013 (2013).
36. Longhurst, P. A., Briscoe, J. A., Rosenberg, D. J., & Leggett, R. E. The role of cyclic nucleotides in guinea-pig bladder contractility. *British journal of pharmacology.* **121**, 1665-1672, doi:10.1038/sj.bjp.0701328 (1997).
37. Takahashi, R., Yunoki, T., Naito, S., & Yoshimura, N. Differential effects of botulinum neurotoxin A on bladder contractile responses to activation of efferent nerves, smooth muscles and afferent nerves in rats. *J Urol.* **188**, 1993-1999, doi:10.1016/j.juro.2012.07.001 (2012).
38. Sadananda, P., Chess-Williams, R., & Burcher, E. Contractile properties of the pig bladder mucosa in response to neurokinin A: a role for myofibroblasts? *British journal of pharmacology.* **153**, 1465-1473, doi:10.1038/bjp.2008.29 (2008).
39. Liu, G., & Daneshgari, F. Alterations in neurogenically mediated contractile responses of urinary bladder in rats with diabetes. *American journal of physiology. Renal physiology.* **288**, F1220-1226, doi:10.1152/ajprenal.00449.2004 (2005).

