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Involvement of TRPM4 in detrusor overactivity following spinal cord transection in mice

F. Aura Kullmann¹, Jonathan M. Beckel², Bronagh McDonnell¹, Christian Gauthier³, Andrew M. Lynn¹, Amanda Wolf-Johnston¹, Anthony Kanai^{1,2}, Irina V. Zabbarova¹, Youko Ikeda¹, William C. de Groat², and Lori A. Birder^{1,2}

¹University of Pittsburgh School of Medicine, Department of Medicine, Pittsburgh, PA 15261, USA

²University of Pittsburgh School of Medicine, Department of Pharmacology and Chemical Biology, Pittsburgh, PA 15261, USA

³University of Pittsburgh, Department of Biological Sciences, Pittsburgh, PA 15260, USA

Abstract

Purpose: Transient receptor potential cation channel subfamily M member 4 (TRPM4) has been shown to play a key role in detrusor contractility under physiological conditions. In this study we investigated the potential role of TRPM4 in detrusor overactivity following spinal cord transection (SCT) in mice.

Methods: TRPM4 expression and function were evaluated in bladder tissue with or without the mucosa from spinal intact (SI) and SCT female mice (T8-T9 vertebra; 1–28 days post SCT) using PCR, western blots, immunohistochemistry and muscle strip contractility techniques.

Results: TRPM4 was expressed in the urothelium (UT) and detrusor smooth muscle (DSM) and was upregulated after SCT. Expression levels peaked 3–7 days post SCT in both the UT and DSM. Pharmacological block of TRPM4 with the antagonist, 9-Phenanthrol (30 μ M) greatly reduced spontaneous phasic activity that developed after SCT, regardless of the presence or absence of the mucosa.

Conclusions: Detrusor overactivity following spinal cord injury leads to incontinence and/or renal impairment, and represents a major health problem for which current treatments are not satisfactory. Augmented TRPM4 expression in the bladder after chronic SCT supports the hypothesis that TRPM4 channels play a role in DSM overactivity following SCT. Inhibition of TRPM4 may be beneficial for improving detrusor overactivity in SCI.

Corresponding author: F. Aura Kullmann, Tel: 412 383 8711, University of Pittsburgh School of Medicine, Department of Medicine/ Renal and Electrolyte Division, Pittsburgh, PA 15261, USA., orcid.org/0000-0001-6625-2946, aurak@pitt.edu, aura.kullmann@gmail.com.

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Keywords

bladder strips; 9-Phenanthrol; isoproterenol; neurogenic bladder

Introduction

Neurogenic bladder characterized by detrusor overactivity as a result of spinal cord injury (SCI) is a life-long condition that poses substantial health risks to affected individuals, significantly decreasing their quality of life. Patients experience incontinence, urinary tract infections, as well as high bladder pressure which puts the upper urinary tract at risk (Taweel and Seyam 2015). The mechanisms underlying detrusor overactivity following SCI are not well understood. Many studies have shown that in animals, SCI triggers a cascade of events resulting in morphological and physiological changes in all components of the bladder (urothelium, smooth muscle, nerves) (Birder 2006; de Groat et al. 2015; de Groat and Yoshimura 2012). For example, the urothelium (UT), a stratified epithelium lining the lumen of the bladder, undergoes desquamation, partially losing the superficial umbrella cell layer, as early as 2–24h post SCT in rodents. This is followed by rapid proliferation which in mice peaks around 3 days post SCT. However, even at 28 days post SCT, the UT retains abnormal features such as incomplete differentiation and altered cytokeratin markers (Apodaca et al. 2003; Birder 2006; Kullmann et al. 2017a; Mimata et al. 1993; Shunmugavel et al. 2010; van Velzen et al. 1995). The detrusor smooth muscle (DSM) undergoes hypertrophy and sensitization to various agonists (e.g. purinergic; cholinergic) and ultimately becomes overactive. This is associated with increased spontaneous activity, which can give rise to bladder overactivity (Artim et al. 2011; de Groat et al. 2015; de Groat and Yoshimura 2010; de Groat and Yoshimura 2012; Horst et al. 2013; Johnston et al. 2012; Mimata et al. 1993; Ruffion et al. 2013; Seth et al. 2013; Yoshiyama et al. 1999; Yu et al. 2013). While not well understood, the mechanisms underlying this abnormal spontaneous activity include changes in ion channels that control DSM excitability (Andersson and Arner 2004; Andersson and Wein 2004; Hristov et al. 2013; Hristov et al. 2011; Hristov et al. 2016; Parajuli et al. 2012; Petkov 2011; Petkov et al. 2001; Thorneloe and Nelson 2004). Among these, different families of K⁺ channels contribute to the maintenance of DSM resting membrane potential, generation, depolarization and repolarization phases of DSM action potentials (Petkov 2011), and changes in their activity correlate with increased spontaneous myogenic activity. For example, studies in neurogenic human detrusor have found a decrease in the expression and/or function of large conductance Ca²⁺-activated K⁺ channels (BK) (Hristov et al. 2013) and voltage gated K⁺ channels (Kv2.1, Kv2.2) (Gan et al. 2008), alterations in the adenosine triphosphate-sensitive potassium (K_{ATP}) channels and small conductance Ca²⁺-activated K⁺ channels (SK) (Oger et al. 2011). K_{ATP} channel openers, such as ZD6169, ZD0947, or WAY-133537 are effective in reducing DSM hyperreflexia after SCI in rats (Abdel-Karim et al. 2002; Elzayat et al. 2006). Although less understood in the spinal cord injury model, changes in stretch activated K⁺ channels (K2P) (Pineda et al. 2017), or changes in the ligand-activated purinergic channels (P2X) (Rapp et al. 2005) may also contribute to abnormal DMS excitability. Additionally, factors released by the UT (e.g. ATP, NO, PGE₂, ACh and others) (Birder and Andersson 2013) may influence DSM excitability.

The existing treatments for detrusor overactivity are not satisfactory. Available treatments include the antimuscarinic agents, which have limited benefit due to their side effects, the newer beta-3 adrenergic agonists, whose efficacy is not yet completely known, or injections of botulinum toxin into the bladder wall, which are invasive, require repeated treatments and pose safety concerns (Cameron 2016; Kuo and Kuo 2013; Oefelein 2011). Therefore there is a critical need for new and efficacious pharmacotherapy.

Recent studies have shown that transient receptor potential melastatin 4 (TRPM4), a non-selective cation channel member of the TRP family, is expressed in the bladder smooth muscle and plays a key role in regulating DSM excitability and contractility in several species including human (Hristov et al. 2016; Parajuli et al. 2013; Smith et al. 2013a; Smith et al. 2013b). This channel is permeable to Na^+ and K^+ but not to Ca^{2+} ; however it is activated by increases in intracellular Ca^{2+} and decreases in ATP. When the channel opens, it allows Na^+ influx into the cell, contributing to membrane depolarization.

Electrophysiological recordings from freshly-isolated DSM cells (from rat, guinea pig and human) that exhibit transient spontaneous inward currents demonstrated that an antagonist of TRPM4, 9-phenanthrol (9-Ph) significantly attenuates these currents and produces a hyperpolarizing shift in membrane potential, suggesting that TRPM4 is active at rest and regulates cell excitability. Furthermore, in bladder smooth muscle strips, 9-Ph reduced spontaneous contractions as well as contractions elicited by carbachol, KCl and nerve stimulation, indicating that TRPM4 contributes to contractile activity (Parajuli et al. 2013; Smith et al. 2013a; Smith et al. 2013b). These studies suggest that TRPM4 is a potential therapeutic target for detrusor overactivity. However, whether TRPM4 is involved in bladder dysfunctions has not been examined.

Given that TRPM4 is involved in regulation of cellular excitability in the DSM and that pathology-induced increases in TRPM4 expression are associated with cell/organ dysfunction in spinal cord and other organs (Gerzanich et al. 2009; Gorse et al. 2018; Simard et al. 2013), (Guinamard et al. 2015; Kruse and Pongs 2014), we examined the role of TRPM4 in detrusor overactivity following SCT. The data obtained suggest that TRPM4 is an important target for the development of new pharmacological therapies to treat detrusor overactivity. Combined with recent studies that have begun identifying potent and selective small molecule inhibitors of TRPM4 (Ozhathil et al. 2018), our pre-clinical data provide proof of concept for further investigating this target and related pharmacological agents for detrusor overactivity associated with neurogenic bladder.

Materials and methods

Animals:

All procedures were conducted in accordance with Institutional Animal Care and Use Committee at the University of Pittsburgh, which follows NIH Guidelines for the Care and Use of Laboratory Animals. Adult female C57BL/6 mice (20–25g, 5–6 weeks; n= 78) were purchased from Envigo (Indianapolis, IN). Females were used in this study because spinal cord transection requires manual bladder expression twice per day. This procedure is more difficult to perform in the males than in females, due to the male urethral anatomy and the presence of detrusor sphincter dyssynergia, thus increasing the risk of death from bladder

rupture and infections. Future studies will be performed in males. Mice were maintained under controlled conditions ($20 \pm 1^\circ\text{C}$; humidity 30–70%), housed 4 – 5 per cage on a 12 h light-dark cycle, and fed with standard chow. After surgical procedures mice were single housed with enhanced enrichment.

Spinal cord transection (SCT):

SCT was performed using our previously described methods (Kullmann et al. 2017a; Kullmann et al. 2017b). Briefly, mice were anesthetized using isoflurane (1.5–2% v/v in O_2), and a laminectomy was performed at the T8-T9 vertebral level. The spinal cord was completely transected and gelfoam (Pharmacia and Upjohn Company, Kalamazoo, MI) was placed between the two segments. The muscle and skin overlying the vertebrae were closed and the animals treated with prophylactic antibiotic (Polyflex, 100 mg/kg s.c. 5–7 days; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri) and analgesic (ketoprofen, 5 mg/kg s.c.; 3–5 days; Zoetis, Kalamazoo, MI) per veterinarian recommendation. All surgeries were performed between 9am-12pm and mice were allowed to recover on warm pads for 2–3 hours prior to returning to the animal facility. The bladder was manually emptied twice per day until the spinal micturition reflex recovered (~10–14 days) and once per day thereafter. Animals were sacrificed at different time points (1, 3, 7, 14 and 28 days post SCT) and bladder tissue was collected for molecular or functional assays as described below. Spinal cord intact (SI) mice served as controls. To reduce the number of animals, whenever possible, tissue from one animal was used for multiple assays (i.e. bladder was divided in half and used for western blot and strip studies).

Drugs.

The TRPM4 antagonist, 9-phenanthrol (9-Ph), was purchased from Tocris (Fisher Scientific) and dissolved in DMSO (stock solution, 100 mM). Further dilutions were made in Krebs (final DMSO concentration was $< 0.1\%$). 9-Ph has been shown to inhibit the TRPM4-mediated current in TRPM4-transfected HEK-293 and COS-7 cells (Grand et al. 2008; Woo et al. 2013). The reported IC_{50} of 9-Ph is ~15–20 μM and previous studies have shown that 30 μM almost completely blocks TRPM4 activity without altering the activity of several channels involved in vascular smooth muscle excitability (L-type voltage gated Ca^{2+} channels, large-conductance Ca^{2+} -activated K^+ channels - BKCa, voltage-gated K^+ channels, inward rectifying Kir channels) or TRP channels (TRPC3, TRPC6, TRPM5 and TRPM7) (Earley 2013; Gonzales et al. 2010; Grand et al. 2008; Guinamard et al. 2014; Kruse and Pongs 2014; Mathar et al. 2014). However, 9-Ph has been reported to block calcium dependent chloride channels TRPM16 with an IC_{50} of ~12 μM (Burris et al. 2015), activate the calcium-activated potassium channels KCa3.1 (also known as IK1, SK4 or KCNN4) in endothelial cells from rat mesenteric artery (Garland et al. 2015), and block transient outward, rapid delayed rectifier and inward rectifier K^+ currents, including the IK1 channel (KCa3.1) (at concentrations of 10 and 30 μM) in canine ventricular myocytes (Veress et al. 2018). More selective and potent TRPM4 antagonists are being identified and developed (Ozhathil et al. 2018). Currently there are no specific TRPM4 agonists commercially available. The muscarinic receptor agonist, oxotremorine-s (OxoS) and the beta adrenergic receptor agonist, isoproterenol (ISO) (Sigma), were dissolved in water at 100mM stock solutions and further dilutions made directly in the tissue bath.

Smooth muscle contractility studies

were performed as previously described (Kullmann et al. 2014; McDonnell et al. 2018). Animals were deeply anesthetized with isoflurane (4%) and euthanized by exsanguination. The bladder was removed and placed in aerated Krebs solution (composition in mM: NaCl 118.3, NaHCO₃ 24.9, KH₂PO₄ 1.2, glucose 11.7, KCl 4.7, CaCl₂ 1.9, MgSO₄ 1.2). The pH was maintained at 7.4 by oxygenation with 95% O₂ / 5% CO₂ and osmolarity was between 290–310 mOsm. Bladders were cut into longitudinal strips (~2–3 mm x 8–10 mm; ~3 strips from SI mice and ~5–6 strips of similar size from SCT mice (the size of the bladder is increased after SCT). In some strips (data presented in figure 4), the mucosa, defined as urothelium and lamina propria, was removed under a dissecting microscope. Strips were mounted into a vertical double jacketed organ bath (15 ml volume) (Radnoti, Monrovia, CA) containing aerated Krebs solution kept at 37° C via a circulating warm water bath. Tension was measured using a force displacement transducer (Grass, Astromed, RI) running under Windaq software (DataQ Instruments, Akron, OH) and analyzed offline using Windaq, Excel, Lab Chart (version 7; ADInstruments, Australia) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA). All tissues were washed several times during 1–2h and pre-equilibrated with a 1g tension. To determine whether TRPM4 is functional in SI mouse bladder strips, 9-Ph (30 µM) or vehicle (0.03% DMSO) were applied and effects on baseline tone and spontaneous activity recorded. Strips included in these experiments had the mucosa intact. In another series of experiments, tissue was first pre-contracted with the muscarinic receptor agonist oxotremorine S (OxoS; 1 µM), which increased baseline tone and augmented spontaneous activity. 9-Ph (30 µM) or vehicle (0.03% DMSO) were applied ~10–20 min after OxoS and strips monitored for another 20–30 min. Strips included in these experiments had the mucosa intact. Effects on baseline tone and spontaneous activity (quantified as area under the curve) were quantified by averaging data in 5 min windows before and approximately 10–15 minutes after 9-Ph or DMSO and expressed as a percentage change relative to pre-drug condition. To determine the effect of TRPM4 antagonist on spontaneous activity after SCT, spontaneous activity was recorded for ~3h until stable levels were attained, then 9-Ph (30 µM) or vehicle (0.03% DMSO) was applied for 20–30 min. These experiments included strips with and without the mucosa. Spontaneous activity was quantified as AUC in 10 min windows before and after 9-Ph and expressed as a percentage decrease relative to pre-drug condition. Baseline tone was also quantified in the same windows by averaging 3–5 data selections of 0.5–2 sec duration each, chosen when the baseline was at its nadir to avoid large amplitude spontaneous contractions. Data are expressed as percentage decrease relative to pre-drug condition.

Western Blotting:

Bladder mucosa (defined as urothelium and lamina propria) and smooth muscle from 8–10 mice per time point were surgically separated and samples were homogenized using Lysing Matrix D in a FastPrep 24 instrument (MP Biomedicals, Solon, OH) in HBSS (5 mM KCl, 0.3 mM KH₂PO₄, 138 mM NaCl, 4 mM NaHCO₃, 0.3 mM Na₂HCO₃, 0.3 mM Na₂HPO₄, 5.6 mM glucose and 10 mM Hepes, pH 7.4 containing complete protease inhibitor cocktail (1 tablet/10 ml, Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma, 1:100). After centrifugation (13000 rpm; 15 min at 4°C), the membrane protein fraction was prepared by suspending the membrane pellets in lysis buffer containing 0.3 M NaCl, 50 mM

Tris-HCl (pH 7.6) and 0.5% Triton X-100 and the same concentration of protease inhibitors as above. The suspensions were incubated on ice and centrifuged (13000 rpm; 15 min at 4°C). The protein concentrations of the combined supernatants were determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL). After protein denaturation (100°C for 5 min) in the presence of Laemmli sample buffer, 25 µg lysate from each sample was separated on a 4–15% TGX Stain-Free SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA). As a loading control, total protein per sample was determined using Bio-Rad Stain Free SDS-PAGE gel technology. UV-activated protein fluorescence was imaged on a ChemiDoc MP (Bio-Rad). After proteins were transferred to polyvinylidene fluoride membranes, the membranes were incubated in 5% (w/v) dried milk dissolved in TBS-T (20 mM Trizma, 137 mM NaCl, 0.1% Tween-20, pH 7.6), rinsed with TBS-T, and incubated overnight at 4°C with primary antibody (anti-TRPM4 Alomone labs Cat # ACC-044, 1:5000) diluted in TBS-T containing 5% (w/v) milk. After washing in TBS-T, the membranes were incubated with secondary antibody (Donkey anti-rabbit HRP; 1: 5000; OE Healthcare, Pittsburgh, PA) for 1 hour in 5% (w/v) Milk TBS-T, washed, and incubated in WesternBright Quantum (Advansta, Menlo Park, CA) and then imaged on a ChemiDoc MP (BioRad). Optical density of each protein species was determined and normalized to total protein using Image Lab software (Bio-Rad).

RT-PCR:

Total RNA was extracted from mucosa and smooth muscle tissues obtained from SI mice (n=4). Following homogenization with a Tissue Tearor Rotor-Stator homogenizer, total RNA was extracted according to the manufacturer's published instructions for Trizol. RNA purity and concentration were determined using UV spectrophotometry. cDNA was created using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) and a PTC-100 Thermal Cycler (MJ Research). PCR products for the TRPM4 or the housekeeping gene β -actin channels were then amplified from the cDNA using the HotStarTaq Plus Master Mix Kit (Qiagen) using subtype specific primers. Primers were designed in-house using the publicly available RNA sequences from NCBI and Primer3 online software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The primers used (with NCBI accession numbers) are as follows: TRPM4 (NM_175130.4) F: TCTTCAGGCCAGGTAGAGA, R: AGTTCACCTTTGGGCGATGTC; β -actin (NM_007393.3) F: TTGCTGACAGGATGCAGAAG, R: ACATCTGCTGGAAGGTGGAC. The temperature program utilized to amplify PCR products was 95°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds; then 72°C for 10 minutes. PCR products were electrophoresed on a 1.8% agarose gel in TBE buffer and stained with ethidium bromide. Expected PCR product sizes were: TRPM4: 141bp; β -actin: 201bp. Products were visualized and recorded using a ChemiDoc XRS+ System with Image Lab Software (Bio-Rad).

Immunohistochemistry.

Bladder tissue from SI mice (n=8) was fixed with 4% PFA for 2h at room temperature (RT), embedded in mounting media (Tissue-Tek OCT, Sakura Finetek, Torrance, CA), cut in a cryostat (10 µm sections) and mounted on glass slides (Fisher Scientific, Pittsburgh, PA). Sections were washed in PBS and incubated with permeabilizing/blocking solution (0.5%

Triton X-100 and 5% donkey serum, 2 hours at room temperature - RT) followed by anti-TRPM4 primary antibody (1:1000; 24h at 4°C; Alomone labs, Israel; Cat # ACC-044). This antibody has been shown to label TRPM4 in the bladder smooth muscle in previous studies (Hristov et al. 2016). Some sections were first incubated with the TRPM4 blocking peptide (2h at RT; Alomone labs; Cat # ACC-044) followed by anti-TRPM4 primary antibody overnight. The second day, slides were washed and incubated with secondary FITC-conjugated donkey anti-rabbit (1:1000, 2h RT, Invitrogen). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:2500; 2h RT). Control experiments included omission of 1st antibody and exposure to the blocking peptide. Images consisting of single optical sections were taken with a BX-62 Olympus upright microscope equipped with a 40X NA:1.00 oil objective and running under HCSImaging software (Hamamatsu Photonics, Bridgewater, NJ). Composite images were assembled using Adobe Illustrator CS6 (Adobe Systems, San Jose, CA).

Statistics.

Data were analyzed with GraphPad Prism 6 and the Student's t-test, and one or two way ANOVA followed by appropriate post-hoc tests as mentioned in text and figure legends. $p < 0.05$ was considered significant. Results are expressed as mean $\pm$ SD.

Results

TRPM4 is expressed in the bladder smooth muscle and urothelium of the SI mouse

To determine whether TRPM4 is present in the mouse bladder, expression of TRPM4 was evaluated using PCR, WB and immunofluorescence. TRPM4 mRNA and protein were detected in the SI mouse bladder in the mucosa and DSM (Fig. 1a, 3; $n = 8-10$ bladders/assay). TRPM4 immunoreactivity was observed in the UT and DSM, and was mostly absent in the lamina propria (Fig. 1b, c). Staining was not detected in tissue treated with the TRPM4 blocking peptide or in the absence of the primary antibody (Fig. 1d, e).

TRPM4 is functional in the bladder of the SI mouse

To determine whether TRPM4 is functional, we examined changes in the contractile activity of bladder strips with mucosa intact, after application of the TRPM4 antagonist, 9-Phenanthrol (9-Ph; 30 μ M). Previous studies indicate that 9-Ph blocks TRPM4 channels with an IC₅₀ of $\sim 15-20$ μ M (Mathar et al. 2014). 9-Ph had no significant effect on smooth muscle tone (reduction by $\sim 5\%$; paired t-test, $p > 0.05$), but significantly decreased spontaneous activity by $\sim 20\%$ (paired t-test, $p < 0.05$) (Fig. 2a-c). Isoproterenol (10 μ M) used as a positive control, decreased baseline tension and reduced spontaneous activity.

To determine if TRPM4 channels also affect agonist evoked contractile activity of DSM, bladder strips were pre-contracted with muscarinic receptor agonists, oxotremorine-s (OxoS, 1 μ M; Fig. 2d-f) which increased smooth muscle tone and augmented spontaneous activity. 9-Ph (30 μ M) significantly reduced both OxoS-augmented DSM tone (by $\sim 16\%$; Fig. 2e; paired t-test, $p < 0.05$) and OxoS-augmented spontaneous activity (by $\sim 35\%$; Fig. 2f; paired t-test, $p < 0.05$). DMSO vehicle did not alter DSM tone or spontaneous activity (gray bars in Figs. 2b, c, e, f). Together, these results indicate that TRPM4 is expressed and functional in

the female mouse bladder and can contribute to DSM spontaneous phasic activity and muscarinic agonist augmented phasic activity.

TRPM4 expression is upregulated in mouse bladders after SCT

To investigate if TRPM4 plays a role in the plasticity occurring in the bladder after SCT, we first analyzed changes in the protein expression of TRPM4 in the mucosa and DSM from SCT mouse bladder at different time points after transection. SCT increased the expression of TRPM4 protein in both the mucosa and DSM in a temporal manner (Fig. 3). Protein expression levels peaked at 3 and 7 days after SCT in the mucosa and DSM and was not statistically different from control at other time points of the study (Fig. 3; n=8–10 tissues per time point).

Spontaneous activity that develops after SCT is reduced by blocking TRPM4

Previous studies in various species have shown that following SCT, the detrusor develops abnormal spontaneous activity (Artim et al. 2011; Fry et al. 2012; Seth et al. 2013). Increased spontaneous activity was also evident in our experiments at all time points investigated (Fig. 4). Bladder strips with and without the mucosa were assessed at 3–7 days post SCT when levels of TRPM4 were at their peak, and at 14 days post SCT, when the levels of TRPM4 were declining. Spontaneous activity, measured as area under the curve (AUC), increased significantly after SCT (by ~300% compared to the SI group; Fig. 4 e, f, black and gray bars; one way ANOVA followed by Tukey's multiple comparisons test, $p < 0.05$ for both groups when compared to SI). No significant differences were found between 3–7 and 14 days SCT groups (unpaired t-test, $p > 0.05$). Similar to previous studies (Ikeda and Kanai 2008; McDonnell et al. 2018), strips without the mucosa, from both SI and SCT mice had on average lower levels of spontaneous activity than those of strips with the UT, although this did not reach statistical significance (compare black bars with gray bars in Fig. 4e, f; one way ANOVA followed by Tukey's multiple comparisons test $p > 0.05$ for all groups).

9-Ph (30 μ M) reduced spontaneous activity in all groups. In strips from SI mice, where the spontaneous activity levels were lower compared to SCT, this reduction was ~20%. In strips from 3–7 days and 14 days post SCT, the reduction was significantly larger compared to SI, ~50–65%, regardless of the presence or absence of the mucosa (Figs. 4 e, f; two way ANOVA followed by Tukey's multiple comparisons test, $p < 0.05$). The DSM tone was also reduced by 9-Ph reaching significance in the 14 days post SCT group (Fig. 4d; ~8% reduction in the 3–7 days post SCT group, paired t-test, $p > 0.05$; and ~14% reduction in the 14 days post SCT group, paired t-test $p < 0.05$). DMSO vehicle had no significant effects on either baseline tone or spontaneous activity (3–5 strips tested per group).

Discussion

This study demonstrated that TRPM4 is expressed in the mouse bladder smooth muscle and urothelium (Fig. 1). Application of the TRPM4 channel antagonist, 9-Ph, decreased spontaneous activity and muscarinic agonists-evoked DSM contractions in SI preparations (Fig. 2). Following SCT, TRPM4 protein expression was upregulated at early time points (3–

7 days; Fig. 3). Pharmacological blockade of TRPM4 significantly reduced SCT-induced detrusor spontaneous activity (Fig. 4). Removal of the mucosa in SCT preparations did not significantly alter the inhibitory effect of 9-Ph on SCT-induced spontaneous activity (Fig. 4). These findings indicate that TRPM4 channels in the UT may not play a major role in DSM neurogenic overactivity and that by exclusion, TRPM4 channels in the DSM must have the major role in the putative tonic facilitatory effect of these channels on spontaneous DSM contractile activity. Altogether, our data support a role of TRPM4 in detrusor overactivity in SCT mouse bladder and thus TRPM4 antagonists may be useful in alleviating bladder dysfunction due to detrusor overactivity after SCI.

TRPM4 expression, function and role in smooth muscle contractility and abnormal spontaneous activity after SCT

Recent studies in human, rat and guinea pig bladder have identified a key role for TRPM4 in controlling the excitability of the bladder smooth muscle under physiological conditions (Hristov et al. 2016; Smith et al. 2013a; Smith et al. 2013b). They have shown that TRPM4 can regulate resting membrane potential and may be activated during smooth muscle contractions induced by different stimuli including agonists for muscarinic receptors, strong depolarizations elicited by high KCl concentrations or electric field stimulation that evokes transmitter release from the efferent nerves. They also highlighted a more prominent role of TRPM4 in human as compared to rodents (Hristov et al. 2016; Smith et al. 2013a; Smith et al. 2013b). We extended these studies by demonstrating TRPM4 expression in the mouse bladder smooth muscle and urothelium (Fig. 1). Similar to human, rat and guinea pig, TRPM4 antagonism in mouse bladder strips induced relaxation of the smooth muscle pre-contracted with muscarinic agonists, and reduced spontaneous activity (Fig. 2), supporting the involvement of this channel in DSM excitability in this species under physiological conditions.

We further demonstrate that following SCT, protein expression of TRPM4 increased significantly, in both the smooth muscle and urothelium (Fig. 3). Peak expression occurred at 3–7 days, and remained elevated compared to control for the remaining time points investigated, although not statistically significant (up to 28 days). Similar increases in TRPM4 expression and function were reported in other systems under pathological conditions and were associated with cellular and organ dysfunction (Gerzanich et al. 2009; Schattling et al. 2012; Simard et al. 2013; Simard et al. 2012). Although the mechanisms that underlie increased expression of TRPM4 in pathology are unknown, it seems possible that increased expression could significantly contribute to the development and/or maintenance of myogenic detrusor overactivity after SCT. Ours and previous studies demonstrated that the smooth muscle shows large increases in spontaneous activity and becomes hyper-responsive to muscarinic agonists after SCT (These publications did not report increased responsiveness to muscarinic agonists after SCT. Thus refs should be deleted) (Artim et al. 2011; Fry et al. 2012; Seth et al. 2013). A reason for increased spontaneous activity may be release of ACh from damaged nerves and/or other non-neuronal sources (e.g. urothelium (Hanna-Mitchell et al. 2007)). As the muscarinic M3 receptor subtypes expressed in the DSM are linked to the PLC-IP3 and calcium release from the endoplasmic reticulum, muscarinic receptor stimulation in SCT tissue may result in larger

increases in intracellular calcium concentration, which can then readily activate TRPM4. Once activated, the TRPM4 channel allows Na⁺ influx which further depolarizes the cell membrane, resulting in increased excitability. Overexpression of TRPM4 can further amplify DSM depolarization. Thus, it is possible that after SCT, different stimuli, (i.e. release of Ach or ATP from the urothelium), can cause prolonged DSM contractions and increased spontaneous myogenic activity by activating TRPM4.

TRPM4 in the urothelium and alterations after SCT

The urothelium is thought to play an important role in bladder function by releasing factors, such as ATP, NO, PGE₂, ACh, and others, that alter DSM properties/function including excitability and contractility (Akino et al. 2008; Birder and Andersson 2013; Birder 2006; Fry and Vahabi 2016; Hawthorn et al. 2000; Murakami et al. 2007; Templeman et al. 2002). Our recent studies in the same mouse model of SCT (Kullmann et al. 2017a) as well as other studies in different species (Apodaca et al. 2003) have shown numerous time-dependent changes in the urothelial structure and function occurring after SCT. These include focal loss of umbrella cells which is evident as early as 1 day post SCT and may be associated with loss of barrier function (Apodaca et al. 2003). Structural recovery of the UT involves rapid proliferation of basal and intermediate cells which peaks at 1–3 days post SCT and is associated with increased number of UT cell layers. UT cell proliferation significantly decreases after 14 days post SCT; however, even though the structure (i.e., number of layers) of UT appears similar to control, the UT is not completely regenerated, displaying cells that exhibit aberrant expression of keratins and other differentiation markers (Kullmann et al. 2017a). Altered properties of these incompletely differentiated cells after SCT may involve altered calcium homeostasis, which can in turn activate TRPM4, overexpressed in these cells after SCT. Although the UT cells are not excitable, changes in the driving force for Na⁺ due to TRPM4 opening, combined with altered intracellular calcium, can influence ion channels, such as TRPs, stretch activated channels or ENaC, involved in transmitter release from the UT (Du et al. 2007; Olsen et al. 2011). Future studies are needed to determine whether changes in the TRPM4 expression in the UT after SCI play a role in detrusor overactivity.

Study limitations and future directions

Our functional data rely on the use of 9-Ph, currently the only commercially available TRPM4 antagonist. While this antagonist has proven to be a valuable tool for many pharmacological studies, its specificity limits the interpretation of results. Previous studies have shown that 9-Ph blocks TRPM4 channels without altering the activity of several channels involved in vascular (and presumably DSM) smooth muscle excitability. These include the L-type voltage gated Ca²⁺ channels, large-conductance Ca²⁺-activated K⁺ channels - BK, voltage-gated K⁺_v channels, inward rectifying Kir channels, and members of TRP channel family (TRPC3, TRPC6, TRPM5 and TRPM7) (Earley 2013; Gonzales et al. 2010; Grand et al. 2008; Guinamard et al. 2014; Kruse and Pongs 2014; Mathar et al. 2014). However, other studies have shown that 9-Ph can alter the activity of ion channels that could also be involved in DSM excitability. In rat arterial smooth muscle cells (myocytes), 9-Ph blocks the calcium dependent chloride channels TRPM16, with an IC₅₀ of ~12 μM (Burris et al. 2015). These channels are also expressed in the bladder (Ferrera et al. 2009), particularly in a subpopulation of vimentin positive cells in the mucosa and detrusor layers

(Bijos et al. 2014), thus 9-Ph effects on these channels may influence DSM spontaneous activity. In endothelial cells from mesenteric arteries, 9-Ph can activate the calcium-activated potassium channels KCa3.1 (also known as IK1, SK4 or KCNN4) (Garland et al. 2015), while in the canine ventricular myocytes 9-Ph (10, 30 μ M) can block transient outward, rapid delayed rectifier and inward rectifier K⁺ currents, including the IK1 channel (KCa3.1) (Veress et al. 2018). As these channels are expressed in the bladder (Ohya et al. 2000; Parajuli et al. 2012) part of 9-Ph's effect in our experiments could potentially be due to a nonspecific action on these channels. It is unknown whether the expression and function of these channels in the bladder are altered by SCI. There is evidence that the KCa3.1 channel expression is increased in the spinal cord tissue following SCI, and pharmacological block using TRAM34 improves locomotion after SCI (Bouhy et al. 2011). Additionally, alterations of BK and SK channels have been reported in pathological conditions associated with increased DSM activity (e.g. bladder outlet obstruction model (Kita et al. 2010; Li et al. 2008)). Future investigation using the newly developed TRPM4 antagonist (Ozhathil et al. 2018), and/or genetically engineered mice lacking expression of TRPM4 would help clarify the role of TRPM4 in DSM following SCI.

Conclusions:

Recent studies established a role of TRPM4 in DSM excitability and contractility under physiological conditions. Our study brings supporting evidence for a putative role of TRPM4 in detrusor overactivity after spinal cord injury. The results demonstrate increased expression of TRPM4 after SCT and significant reduction of SCT-induced spontaneous activity by pharmacological blockade of TRPM4. However due to lack of specificity, 9-Ph effect on spontaneous activity may involve other ion channels besides TRPM4. Nevertheless these data provide the first evidence for a potential role of TRPM4 in pathological conditions resulting in detrusor overactivity. This strongly supports further investigation of TRPM4 in pathologies associated with detrusor overactivity and further development of TRPM4 antagonists for controlling detrusor excitability. The importance of TRPM4 in pathology is not limited to the urinary bladder. In fact, the involvement of TRPM4 in the expansion of secondary injury in spinal cord injury (Gerzanich et al. 2009; Gorse et al. 2018; Simard et al. 2013), stroke (Chen et al. 2018), cardiac conduction deficits (Guinamard et al. 2015; Kruse and Pongs 2014) and other diseases, have sparked a search for potent and selective small molecule inhibitors which are currently being identified and optimized (Ozhathil et al. 2018). Such inhibitors may provide potential treatments for bladder dysfunction associated with detrusor overactivity.

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Figure 1. TRPM4 is expressed in SI mouse urinary bladder:

a. mRNA expression of TRPM4 using RT-PCR in mucosa and detrusor smooth muscle (DSM) of the mouse bladder (representative images from n=4 SI mice). **b-e.** Immunofluorescence antibody staining for TRPM4 (green) in bladder sections from SI mouse (representative images from n=8 SI mice). Nuclei are stained blue. **b.** TRPM4 staining in the urothelium - UT (white line delimits the UT from the underlying lamina propria - LP). **c.** TRPM4 staining in the DSM. White lines delimit the DSM from LP and adventitia (Adv). **d.** Section in which the primary antibody was omitted. **e.** Section in which TRPM4 staining was performed in the presence of the blocking peptide. Scale (50 μ m) is the same for panels b-e and is illustrated in panel e.

Figure 2. TRPM4 is functional in SI mouse urinary bladder:

a. Examples traces showing the effects of the TRPM4 antagonist 9-Ph (30 μ M) (upper trace) and vehicle (DMSO 0.03%) on bladder strips from SI mice. Isoproterenol (ISO; 10 μ M), used as a positive control, reduced baseline tone. Gray dotted lines indicate the windows used for quantification of baseline tone and spontaneous activity (area under the curve). Inset **ai** shows enlargements of areas indicated by grey dots in **a**. **b.** Summary data showing percentage reduction in baseline tone by 9-Ph (30 μ M - black bars) or DMSO (gray bars), relative to pre-drug values. **c.** Summary data showing percentage reduction of spontaneous activity (AUC) by 9-Ph (30 μ M - black bars) or DMSO (gray bars), relative to pre-drug values. Number of strips tested is indicated in **b**, and were obtained from 8 SI mice. **d.** Examples traces showing the effects of 9-Ph (30 μ M) (upper trace) or vehicle (DMSO 0.03%, lower trace) on strips pre-contracted with OxoS (1 μ M). 9-Ph (30 μ M) reduced baseline tone and OxoS - augmented spontaneous activity. The vehicle, DMSO had no effect on either parameter. **e.** Summary data showing percentage reduction of baseline tone by 9-Ph (black bars) or DMSO (gray bars), expressed as a percentage reduction from the OxoS-induced plateau response. **f.** Summary data showing percentage reduction of spontaneous activity (AUC) by 9-Ph (black bars) or DMSO (gray bars), expressed as a percentage of the OxoS - augmented spontaneous activity. In all strips the mucosa was present. The number of strips tested is indicated in **e**, and were obtained from 4 SI mice. Asterisk (*) indicates

statistically significant differences tested with paired t-test before and after addition of 9-Ph ($p < 0.05$).

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Figure 3. TRPM4 protein expression is upregulated in bladder after SCT:

a, b. Protein expression of TRPM4 detected by Western Blot in the mucosa (**a**) and DSM (**b**) of mouse bladder in control (SI) and 1–28 days after SCT. Data are averages from $n = 8–10$ mice per time point. Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control (SI), tested using one way ANOVA followed by Tukey's multiple comparisons posthoc test. **c, d.** Examples of TRPM4 blots from the mucosa (**c**) and DSM (**d**) from mouse bladders obtained at different time points post SCT. Expected band size for TRPM4 is 136kDa.

Figure 4. 9-Ph reduces spontaneous activity in mouse bladder strips from SCT and SI mice:
a. Example of the effect of 9-Ph (30 μ M) on spontaneous activity in strips from SCT mice (upper trace; 14 days post SCT) and SI mice (lower trace). SCT strips show significant increase in spontaneous activity compared to SI strips. This activity is greatly reduced by 9-Ph. ISO, used as a positive control, further reduces spontaneous activity. Both strips in this example have the mucosa intact. **b, c.** Enlargements of areas indicated by grey dots in **a**, to show the pattern of spontaneous activity before and after 9-Ph. Y scale is the same as in **a**. **d.** Summary data showing reduction of baseline tone by 9-Ph (30 μ M - white bars) or DMSO (gray bars) in the 3-7 and 14 day post SCT groups. All strips had mucosa intact (5-8 strips as indicated in bar graphs in **d**, from 5-6 mice per group). For each group, asterisk (*) indicates statistically significant differences tested with paired t-test before and after addition of 9-Ph ($p < 0.05$). **e, f.** Summary data showing the effects of 9-Ph (30 μ M) on spontaneous activity in strips from SI and SCT strips with (**e**) and without (**f**) the mucosa present (n is 5-8 strips per group as indicated on the solid bar graphs, obtained from 5-6 mice per group). Spontaneous activity was measured as AUC in 10 min windows before and after 9-Ph. Asterisks (*) indicate statistically significant differences tested with two way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).