Control of lower urinary tract functions by parasympathetic preganglionic neurons

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Abstract

Micturition - the periodic evacuation of urine from the bladder - generally occurs voluntarily in adults but involuntarily in early childhood. The spino-bulbospinal micturition reflexes are initiated by excitation of pelvic afferents (considered to be a group of small myelinated Aδ afferents) which sense urinary bladder fullness by acting as stretch and tension receptors. This information is then relayed via the spinal cord to the pontine micturition center. The pontine micturition center, activated by urinary bladder afferent excitation through the spinal dorsal horn, excites parasympathetic preganglionic (PG) neurons in the lumbosacral spinal parasympathetic nucleus (SPN). PG neurons directly activate parasympathetic postganglionic neurons located near the urinary bladder to induce micturition. Therefore, PG neurons are the key spinal regulator for micturition. Recent studies have shown that the transient receptor potential vanilloid subfamily member TRPV1 has been proposed to play an important role in urinary bladder pain and urothelial signaling. However, relatively little is known about the role of synaptic inputs from urinary bladder C afferents on the spinal components of the micturition reflex circuit.

To elucidate how spinal synaptic inputs from urinary bladder afferents control the parasympathetic outflows for micturition. I developed *in vivo* patch-clamp (or

extracellular) recording technique from the SPN including PG neurons of urethane-anesthetized rats with simultaneous monitoring of intravesical pressure (IVP) and urethral perfusion pressure (UPP). Neuronal firing within the SPN, including PG neurons, induced micturition with an increase in IVP. Subthreshold oscillatory membrane depolarisations were essential for PG neuron excitation and were highly synchronized with urethra activity. Stable excitatory postsynaptic currents (EPSCs) could be also recorded from SPN neurons under voltage-clamp conditions. *In vivo* analysis in combination with spinal cord slice patch-clamp analysis revealed that SPN neurons showing tonic and phasic firing properties are likely to be PG neurons, which receive direct glutamatergic synaptic (monosynaptic) inputs mainly from C afferents, including capsaicin sensitive (TRPV1-expressing) afferents. Capsaicin also increased the frequency of miniature EPSCs (recorded in the presence of tetrodotoxin, TTX) in SPN neurons retrogradely labeled with DiI (a neurotracer) injected near the bladder. These results indicate the existence of a local spinal reflex circuit for micturition. Spinal application of capsaicin inhibited dorsal root stimulation evoked EPSCs in SPN neurons through one of the groups of C afferents, and decreased the inter-contraction interval of micturition with an increase in the IVP threshold for micturition, suggesting that capsaicin-sensitive C afferent spinal synaptic inputs play an important role in

setting the threshold for the normal and/or pathological micturition reflex.

I present newly developed *in vivo* approaches which allow a detailed characterization of the subthreshold integrative mechanisms of spinal PG neuronal excitation during the micturition cycle. In addition, this work proposes capsaicin treatment for the blockage of spinal synaptic inputs from TRPV1-expressing C afferents as an attractive target for the treatment of pathological urinary bladder function and also for its anti-nociceptive action on urinary bladder pain.

Introduction

The lower urinary tract (LUT) is composed of the urinary bladder and urethra, which function to store urine without leakage with periodic elimination of urine (micturition). The neural circuit underlying LUT function is complex and widely distributed from the periphery to the central nervous system. The storage/micturition cycle is dependent upon coordinated sympathetic and parasympathetic control of urinary bladder and urethra smooth muscle contraction/relaxation along with somatic control of the urinary sphincter. During the storage of urine, urinary bladder smooth muscle is relaxed by hypogastric nerve (sympathetic) activation and the sphincter is tightly closed by pudendal nerve (somatic) excitation. Urethral striated and smooth muscles are also contracted. These LUT responses during urine storage are driven and maintained by central sympathetic tone. On the other hand, micturition is regulated by central parasympathetic activity. The relaxation of urethral striated and smooth muscles is followed by contraction of urinary bladder smooth muscle mediated by pelvic nerve (parasympathetic) excitation, and finally sphincter relaxation for opening of the urinary bladder neck. The switch from the storage of urine to micturition is voluntarily controlled through the central spino-bulbospinal neuronal circuits including the pontine micturition center. Intense firing of primary afferents innervating the urinary bladder

wall as mechanical responses to urine fullness is considered to stimulate the pontine micturition center via spinal dorsal horn neurons and initiate the bulbospinal reflex for micturition.

Urinary bladder primary afferents consist of small myelinated Aδ and unmyelinated C afferents. Previous studies have shown that most urinary bladder Aδ afferents are mechano-sensitive for normal micturition; they respond to bladder distention in a stepwise fashion, greatly increasing their firing frequency around the intravesical pressure threshold for micturition. On the contrary, C afferents are believed to be insensitive to normal micturition, and therefore called ‘silent’ urinary bladder afferents. C afferents innervating the urinary bladder are excited primarily by noxious stimuli such as chemical irritant or cooling. Capsaicin, an ingredient of hot chili peppers, elicits a sensation of burning pain by activation of transient receptor potential vanilloid 1 (TRPV1), a non-selective cation channel expressed in somatic sensory primary C afferents. TRPV1 is also expressed in a subgroup of urinary bladder C afferents, and intravesical infusion of capsaicin elicits nociceptive behavior and increases the frequency of micturition. Desensitization of capsaicin-sensitive (TRPV1-expressing) C afferents by systemic capsaicin pretreatment has been also used to elucidate the functional role of TRPV1-expressing C afferents on micturition. It is

still unknown, however, how the urinary bladder afferents, including TRPV1-expressing afferents, send such urinary bladder information to the spino-bulbospinal neuronal circuit.

The pontine micturition center activated by urinary bladder afferent excitation through the spinal dorsal horn, excites parasympathetic preganglionic (PG) neurons located in the lumbosacral spinal parasympathetic preganglionic nucleus (SPN) of the intermediolateral grey matter (laminae V-VII). PG neurons are the key spinal regulator for micturition. Electrophysiological properties and synaptic responses of SPN neurons have been studied using spinal cord slice preparations. PG neurons were classified into two types, phasic and tonic, based on their firing properties. It is still unknown, however, how such PG neuron synaptic responses are evoked in response to urinary bladder distention and how PG neuron excitation itself induces bladder contraction for micturition.

In this study, I use newly developed methods to simultaneous record spinal neural activities and LUT function in anesthetized rats in order to understand the physiological role of spinal PG neurons on micturition. *In vivo* methods enabled us to analyze the correlation between PG neuron excitation and urinary bladder and urethral contractions. I found that oscillatory subthreshold membrane depolarisations evoked

in PG neurons were essential for their excitation, leading to urinary bladder contraction, and were highly synchronized with urethra activity. Furthermore, the physiological role of synaptic responses evoked in PG neurons was studied using *in vivo* methods in combination with spinal cord slice patch-clamp analysis. Unexpectedly, I detected direct synaptic inputs to SPN neurons from primary Aδ and C afferents. I reveal, in particular, that SPN neurons including PG neurons receive glutamatergic monosynaptic inputs mainly from C afferents, including capsaicin-sensitive (TRPV1-expressing) afferents, indicating the existence of a local spinal reflex circuit for micturition. In addition, our *in vivo* analyses show that capsaicin-sensitive C afferents play an important role in setting the threshold pressure for the normal micturition reflex. Furthermore, frequent urination induced by inflammation was inhibited by blockade of afferent signaling through capsaicin-sensitive afferents.

Materials and Methods

Animal

Sprague-Dawley female rats, 2-6 weeks old, were used in this study. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of National Institutes of Natural Sciences.

Labeling of parasympathetic preganglionic (PG) neurons

1,1'-Dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (FAST DiI, 20 μg/μl, Life Technologies Corp., Carlsbad, CA, USA) and fluorogold (FG) (Santa Cruz biotechnology, Santa Cruz, CA, USA) was dissolved in dimethyl sulfoxide (DMSO) and distilled water respectively. Two week old rats were anesthetized under 2-2.5% isoflurane. Five microliters of FAST DiI or FG solution was slowly injected into the ischiorectal fossa bilaterally and between the anus and the external urethral orifice 1-2 weeks before experiments.

Lumbosacral spinal cord slice preparation

Methods used for obtaining slice preparations of rat lumbosacral spinal cord were similar to those described previously (Miura et al., 2000). Female rats were deeply anesthetized with urethane (intraperitoneally, 1.2-1.5 g/kg). The spinal cord at spinal level of L1-S3 was removed and immediately transferred into a cold artificial cerebrospinal fluid (ACSF) high sucrose solution equilibrated with 95% O_2-5% CO_2. ACSF high sucrose solution contained (in mM): sucrose 252, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 2, NaH_2PO_4 1.25, glucose 10, $NaHCO_3$ 26. The spinal cord mounted on a vibratome (LinearSlicer PRO7, D.S.K., Kyoto, Japan) and then a transverse or horizontal slice (300 μm thickness for infrared differential interference contrast (IR-DIC) or 600 μm for blind patch-clamp) was made. The slice at L6-S1 level was placed in the recording chamber, then perfused with Krebs solution at a flow rate of 10-20 ml/min saturated with 95% O_2-5% CO_2 at 36±1℃. Krebs solution contained (in mM): NaCl 112, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, glucose 11, $NaHCO_3$ 25.

Whole cell patch-clamp recording

Neurons were visualized with an upright microscope equipped with Nomarski and fluorescence optics. PG neurons were identified as DiI-positive cells in the SPN of the

spinal cord. Whole cell patch-clamp recordings were made from neurons located in the SPN of rat spinal cord slices (3-6 weeks old) using 'blind' technique. The recording cells were validated to be same firing properties of DiI-positive cells, and the location of recorded cells was confirmed in selected instances by the intrasomatic injection of 0.4 % N(2-aminoethyl) biotinamide hydrochloride (Neurobiotin tracer, VECTOR lab, CA, USA). Orthodromic stimulation of the L6 dorsal root was performed with a suction electrode using a constant-current stimulator (SEN-7203, Nihon Koden, Tokyo, Japan). Whole cell currents were recorded from SPN neurons, including DiI-positive PG neurons, using Axoclamp 200B amplifier (Axon Instruments, Foster City, CA, USA). Patch pipettes were made from glass capillaries (TW150F-4; World Precision Instruments, Inc., Sarasota, FL, USA) and had a resistance of 5-12 MΩ when filled with internal solution contained (in mM): potassium gluconate 136, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, Mg-ATP 5, HEPES 5 (pH 7.2). The firing properties of SPN and PG neurons in response to prolonged depolarising current pulse were studied. In voltage clamp, the holding potential was -70 mV for recording of excitatory postsynaptic currents (EPSCs). Signals were digitized at 10 kHz and low pass filtered at 5 kHz (Digidata 1321A, Molecular Devices) for storage/analysis on a personal computer using a data acquisition program (Clampex version 10.3, Molecular Devices).

To evoke EPSCs, a stimulus (duration 100 μs) was given to an attached-L6 dorsal root (length, 5-10 mm) at a frequency of 0.2 Hz via a suction electrode. Aδ and C afferent-evoked responses evoked by dorsal root stimulation were distinguished on the basis of the conduction velocity of the afferents (C, < 0.8 m/s; Aδ, 2–11 m/s) (Nakatsuka et al., 1999) (Aizawa et al., 2010) (Briant et al., 2014). Aδ afferent-evoked EPSCs were considered to be monosynaptic in nature when the latency remained constant and there was no failure during repetitive stimulation at 20 Hz, whereas C afferent-evoked EPSCs were considered to be monosynaptic when failures did not occur during stimulation at 2 Hz (Nakatsuka et al., 1999) (Briant et al., 2014).

Simultaneous recording of *in vivo* neuronal activity from PG neurons and bladder pressure

Female rats (6 weeks old) were anesthetized with urethane (intraperitoneally, 1.0-1.2 g/kg). The bladder was exposed via a midline incision in the abdomen and a polyethylene catheter (PE-50, Nihon Becton Dickinson, Tokyo, Japan), which was heated to create a collar, or double lumen catheter, was inserted into the urinary bladder via the top of the bladder dome. The catheter was then connected through a three-way

connector to a pressure transducer (DX-100, NIHON KOHDEN, Tokyo, Japan) with a pressure amplifier (AB-641G, NIHON KOHDEN) for measurement of bladder pressure, and to syringe infusion pump (TE-331S, Terumo, Tokyo, Japan) for continuous infusion of saline at rate of 0.1 ml/min into the bladder. Capsaicin (15 μM) and prostaglandin E2 (100 μM) were intravesically infused at the same infusion rate. Lumber laminectomy was performed at level of L5-S2 and the animal was then placed in stereotaxic apparatus. After the dura mater was opened, the pia-arachnoid membrane was cut to make a small window to allow the patch electrode to enter into the SPN. The surface of the spinal cord was irrigated with 95% O_2-5% CO_2 equilibrated Krebs solution at 38 ± 1℃. A tungsten microelectrode (impedance, 1 MΩ; A-M systems, Sequim, WA, USA) or patch electrode was placed into the L6 spinal cord. Patch electrodes were fabricated from thin-walled borosilicate glass capillaries, and had resistances of 5-15 MΩ when filled with internal solution. The patch electrode was advanced into the vicinity of the SPN of L6-S1 spinal levels at an angle of 45º from the horizontal using a micromanipulator according to set coordinates: I identified the area as containing urinary bladder related PG neurons based on distribution of cells expressing c-fos in response to urinary bladder contraction and also from the location of PG neurons retrogradely-labelled with neuronal tracer (see *Results*). Gigaohm seals

were formed using the blind patch-clamp technique (Furue et al., 2007) (Sugiyama et al., 2012) (Funai et al., 2014). The recorded data were analysed with MiniAnalysis software (Synaptosoft, Fort Lee, NJ).

Identification of c-fos positive spinal neurons in response to micturition

Female rats (6 weeks old) were anesthetized with urethane (intraperitoneally, 1.0-1.2 g/kg). A polyethylene catheter (PE-50) was inserted into the bladder via the urethral orifice. After the animals were placed in Bollman cages, micturition was induced by infusion of saline or 1% acetic acid at a flow rate of 0.05 ml/min via the catheter for 4 h. Animals were then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The fixed lumbosacral spinal cord was sliced into 40μm-thickness transverse sections. The sections were incubated for 3 days at 4℃ with anti-c-fos rabbit polyclonal antibody (1:2000; Ab-5(4-17), Merk Millipore, Darmstadt, Germany), followed by biotinylated secondary antibody (1:500, 2 h). The c-fos expressing cells were visualized with diaminobenzidine.

Immunocytochemistry

The spinal cord was fixed overnight with 4% paraformaldehyde with glutaraldehyde in 0.1 M phosphate buffer at 4℃. The fixed spinal cord was cut transversely at 20 μm thickness with vibratome. The sections were processed to visualize neurons that contain choline acetyl-transferase (ChAT), TRPV1 and FG. Each section was exposed to combination of goat anti-ChAT (1/100; Chemicon AB144P; Temecula, CA) and rabbit anti-TRPV1 (1/25000; NEUROMICS GT15129; Edina, MN), and incubated at 4℃. This was followed by a combination of donkey anti-goat or anti-rabbit conjugated Alexa Fluor 488 or 574 (1:500, 18-24 h; Life Technologies).

Drug application

Drugs were applied to the recording chamber or the spinal surface via perfusate. Drugs used in this work were capsaicin, tetrodotoxin (TTX; Nacalai tesque, Kyoto, Japan) and 6-cyano-7-nitroquinoxaline-2,3 -dione (CNQX; Sigma, St. Louis, MO, USA). Capsaicin and TTX were first dissolved in ethanol or distilled water, respectively, as stocks of 1 mM; CNQX was first dissolved in DMSO at a concentration of 20 mM. Stocks were then diluted to the final concentration in the perfusion solution immediately before use.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined as $p < 0.05$ using the two-tailed unpaired *t*-test.

Results

Identification of parasympathetic PG neurons in the lumbosacral spinal cord

First, I identified the location of PG neurons by c-fos expression in response to saline infusion-induced micturition and high frequency micturition (frequent urination) with acetic acid (AA) infusion. Saline infusion into the urinary bladder spontaneously induced a transient increase of intravesical pressure (IVP), indicating saline infusion induced contraction of the urinary bladder followed by saline elimination through the urethra (micturition). When AA was infused, an increase in frequency of spontaneous micturition was observed, but the maximum IVP did not differ between saline and AA infusions. The inter-contraction interval of micturition was significantly shortened by AA infusion. The distributions of c-fos expression at lumbosacral spinal levels in control (a catheter was inserted into the urinary bladder but saline was not infused), after saline infusion-induced micturition for 4 h and after AA infusion-induced frequent urination for 4 h. c-Fos positive neurons were detected in the spinal dorsal horn mainly at the L5-S1 spinal levels, in particular in the superficial area, SPN in the IML area, and dorsal commissure after saline-induced micturition. The detection of c-fos positive neurons was markedly enhanced after AA infusion but displayed a similar

anatomical distribution to that seen with saline infusion.

In vivo SPN firing during micturition

To record PG neuronal activity, I developed the techniques of *in vivo* extracellular and *in vivo* patch-clamp recording from the SPN of the lumbosacral spinal cord from urethane-anesthetized rats; IVP was simultaneously recorded from the animals to monitor the micturition cycle. Simultaneous *in vivo* extracellular recordings of spinal neural activity and IVP were made from 66 neurons in the SPN of the spinal cord. In 24 neurons out of them, spontaneous bursting of firing was observed coincident with a transient increase in IVP (micturition). The depth of the neurons showing burst firing with simultaneous micturition was 313 ± 18.9 μm from the surface of spinal cord, and located within the SPN. IVP was increased just after the onset of SPN neuronal burst firing and the firing lasted until IVP reached to maximum voiding pressure.

When urinary bladder infusion of saline was changed to AA, the inter-contraction interval of micturition was decreased, as mentioned above, together with a synchronous decrease in inter-bursting interval of SPN neurons. During the urine storage phase, spontaneous firing was frequently elicited after AA infusion). The

average firing frequency of SPN neurons during the storage phase (control, 0.1 ± 0.01 Hz, n = 3) was significantly increased after AA infusion (2.1 ± 1.0 Hz, n = 3; $p < 0.05$). After AA infusion, the maximum frequency of SPN neuron firing during micturition was also significantly increased at IVPs of 5-10 and 10-15 cmH_2O. During micturition, the average firing frequency of SPN neurons (control, 2.1 ± 0.3 Hz, n = 3) was further increased after AA infusion (6.6 ± 1.9 Hz, n = 3; $p < 0.05$).

Whole-cell current and voltage recordings of PG neuron activities *in vivo*

I was able to record spinal activity, IVP and urethral perfusion pressure (UPP) simultaneous by using *in vivo* 'blind' whole-cell patch-clamp method. Stable *in vivo* whole-cell voltage or current recordings were made from neurons located in the SPN lasting an average of 33 ± 13.9 min (n = 10) and for up to 2.5 h. These neurons had a resting membrane potential of -61.3 ± 2.7 mV. Spontaneous action potentials were recorded at a frequency of 0.11 ± 0.09 Hz. In response to current injection through the recording pipette, these neurons elicited action potentials: overshooting action potentials and a prominent transient rectification that is characteristic of PG neurons. Voltage clamp recordings of SPN neurons showed ongoing elicited excitatory postsynaptic

currents (EPSCs) with an amplitude of 23.1 ± 3.3 pA and a frequency of 15.7 ± 3.2 Hz (n=10, holding potential of -70 mV) that likely summate to drive the ongoing action potential discharge. The EPSC shown in an expanded time course showed the presence of populations with fast and slow kinetics, suggesting that these inputs are distinct.

PG neurons show burst discharge during micturition and sub-threshold membrane potential oscillations that are synchronous with UPP oscillation.

A subset of SPN neurons (4 out of 10 neurons recorded with *in vivo* patch-clamp) showed bursts of action potentials commencing at the initiation of bladder contraction during voiding. The number of action potentials in each burst was positively correlated with the magnitude of the IVP increase. On this basis, they were considered to be bladder PG neurons. As an example of the resolving power of this recording approach, I noted that at the peak of the burst of PG neuron firing, the subthreshold membrane potential showed an underlying rhythmical oscillatory behavior. This oscillation had a peak to trough amplitude of 10-12 mV, a duration of 17-38 ms and an inter-depolarisation interval of 136.8 ± 10.8 ms (obtained from 38 micturition cycles).

Interestingly, the oscillatory membrane depolarisation occurred in phase with the UPP oscillation, which corresponds to the known bursting of the external urethral sphincter producing a “squirting” voiding action. Importantly, this oscillation was only seen during voiding contractions that were associated with UPP oscillations and not with bladder contractions to an equivalent pressure. The threshold IVP at onset and the duration over which the membrane potential oscillations occurred was similar to that found for the UPP. The mean threshold IVP of the PG neuron membrane and UPP oscillations were 24.3 ± 1.5 and 30.2 ± 1.2 cmH_2O (n=7), respectively, the mean duration of these oscillations were 3.0 ± 0.4 and 2.7 ± 0.4 s (n=7), respectively, and the number of oscillatory deploarizations was 20.9 ± 2.3 and 18.6 ± 2.5 (n=7), respectively. Both the duration and number of oscillatory membrane depolarisation were strongly correlated with the UPP oscillations. This suggests that the membrane potential oscillations serve to synchronize the discharge of the PG neurons with the voiding phase of the urinary sphincter burst.

Monosynaptic responses elicited in SPN neurons by dorsal root stimulation

To investigate synaptic inputs to SPN neurons from urinary bladder afferents, I recorded

synaptic currents elicited in SPN neurons by dorsal root stimulation using a spinal cord slice preparation. In some instances, recorded neurons were stained with neurobiotin diluted in the recording internal solution to identify the location of the recorded neurons. Their morphological features were also similar to those of PG neurons which extend their dendrites dorsolaterally and medially and their axons to the ventral horn. PG neurons are previously reported to show tonic and phasic firing (Miura et al., 2000). Most of SPN neurons recorded in this study (33 out of 39 neurons) also showed similar tonic or phasic firing patterns. Tonic type neurons tested initiated action potentials in response to depolarising currents with a short delay of firing of 72.0 ± 11.1 ms (n = 6). On the other hand, phasic PG neurons tested elicited action potentials with a relatively long delay of 780.7 ± 73.5 ms (n = 20). Both types of SPN neurons had similar resting membrane potentials (tonic, -58.5 ± 0.9 mV; phasic, -57.1 ± 1.2 mV).

Next, I recorded EPSCs from neurons in the SPN by electrical stimulation of dorsal root attached to slice preparations under voltage-clamp conditions at a holding potential of -70 mV. EPSCs were recorded in 39 out of 137 neurons in the SPN with latencies of 1.2-37.5 ms. The conduction velocity of the afferents evoking the EPSCs ranged from 0.16-6.67 m/s (calculated from the latency and length of the attached dorsal root), suggesting that SPN neurons receive synaptic inputs from slow conducting

afferent and were considered to be C and Aδ afferents (see *Materials and Methods* section). Then, I examined whether neurons showing tonic or phasic types in the SPN elicited C and Aδ afferent-evoked EPSCs. In 33 neurons showing either tonic or phasic firing types, C afferent-evoked EPSCs were detected in 31 neurons, and Aδ afferent-evoked EPSCs were detected in 9 neurons. Repetitive dorsal root electrical stimulation at 2 Hz (for C afferents) or 20 Hz (for Aδ afferents), reliably elicited evoked EPSCs with no failures and a constant latency in 26 out of the 31 neurons receiving C afferents, and 7 out of the 9 neurons receiving Aδ afferents. This suggests that most of tonic and phasic firing type neurons receive monosynaptic inputs, mostly from C afferent. Of the 26 neurons receiving monosynaptic C afferent-evoked EPSCs, 23 neurons were tonic firing and 3 neurons were phasic firing. Of the 7 neurons receiving monosynaptic Aδ afferent-evoked EPSCs, 6 were tonic firing and 1 was phasic firing. The amplitude of monosynaptic Aδ and C afferent-evoked EPSCs tested were 122.3 ± 38.3 (n = 4) and 101.8 ± 24.5 pA (n = 5). Bath application of CNQX (10 μM) completely inhibited monosynaptic Aδ and C afferent-evoked EPSCs (data not shown). The monosynaptic C afferent-evoked EPSCs were sufficiently strong to be able to elicit action potential discharge in 4 out of 10 cells tested.

Previous studies have shown that TRPV1 is expressed not only in the soma but

also in the axon of a subgroup of C afferents (Tominaga et al., 1998) (Guo et al., 1999) (Michael and Priestley, 1999) (Hwang et al., 2005). In addition, capsaicin application can completely inhibit C afferent-evoked EPSCs in dorsal horn neurons of lumber spinal cord, possibly due to a conduction block of action potential propagation by axonal depolarisation elicited by TRPV1 activation (Yang et al., 1999) (see *Discussion*). Therefore, I examined whether C afferent-evoked EPSCs in the SPN were sensitive to capsaicin. Monosynaptic C afferent-evoked EPSCs were suppressed by capsaicin (1 μM). The amplitude of C afferent-evoked EPSCs was significantly decreased (control, 101.8 ± 4.5 pA, n = 5; capsaicin, 30.0 ± 37.0 pA n = 5, $p < 0.05$). In contrast, monosynaptic Aδ afferent-evoked EPSCs were not inhibited by capsaicin (1 μM). The amplitude of Aδ afferent-evoked EPSCs were not changed (control, 122.3 ± 38.3 pA, n = 4; capsaicin, 108.5 ± 19.5 pA, $p > 0.05$).

TRPV1-expressing C afferent input to PG neurons in the SPN

Next I injected neuronal tracers, FG or DiI near the urinary bladder to identify PG neurons, and examined whether the tracer-positive PG neurons in the SPN received direct synaptic inputs from TRPV1-expressing afferents. The location and spinal level

of FG-positive neurons were almost identical to that of the c-fos expression detected in the SPN of the spinal cord. The FG-positive neurons were also immunopositive for anti-choline acetyl transferase (ChAT). Neuronal axons immunopositive for anti-TRPV1 were detected in the spinal dorsal horn. They passed laterally into the SPN through superficial dorsal horn. Whole cell patch-clamp recordings were made from DiI-positive neurons in the SPN of spinal cord slices. Under current clamp conditions, almost all DiI-positive PG neurons exhibited either tonic or phasic firing types. All neurons tested (n = 22) exhibited spontaneous EPSCs under voltage clamp conditions at a holding potential of -70 mV. In the presence of TTX (1 μM), capsaicin (1 μM) elicited a barrage of miniature EPSCs in 9 out of 22 neurons in total. A small inward current was also elicited (7 out of 9 DiI-postive neurons). Capsaicin shifted the cumulative histogram for inter-event interval of miniature EPSCs to the left by significantly increasing the frequency of miniature EPSCs (control, 1.75 ± 0.21 Hz; capsaicin, 25.50 ± 3.74 Hz; n = 9; $p < 0.05$). CNQX (10 μM) inhibited miniature EPSCs, and in the presence of CNQX (10 μM), capsaicin did not induce any additional mEPSCs. The capsaicin-induced inward current was still induced in the presence of CNQX (10 μM) (n = 13). These data suggest that capsaicin acts on the presynaptic terminals of TRPV1-expressing afferents to enhance glutamate release and is consistent

with the proposition that capsaicin-sensitive C afferents make functional glutamatergic synaptic contacts with PG neurons in the SPN.

Activation and inhibition of capsaicin-sensitive C afferent conduction during micturition

To analyze the functional role of capsaicin-sensitive C afferents on micturition, *in vivo* simultaneous recordings of PG neuronal firing and IVP were used. During the recordings, I perfused capsaicin into the urinary bladder to activate TRPV1-expresing C afferents. Intravesical capsaicin (15 μM) shortened the inter contraction interval; in other words capsaicin induced frequent urination. Bladder application of capsaicin also significantly decreased the voiding threshold pressure. A burst of PG firing followed by IVP increase with micturition was also detected during capsaicin-induced frequent urination; this bursting prolonged beyond the end of the increase in IVP into the relaxation phase. Spontaneous activity was also evident during the storage phase. These results indicate that intravesical capsaicin induces bursting and spontaneous action potential firing in spinal PG neurons independently of micturition.

As mentioned above, TRPV1 receptors are expressed in the axons of C

afferents, and the activation of the receptors is known to suppress action potential conduction within C afferent. In my slice experiments, capsaicin selectively inhibited C afferent-evoked EPSCs without affecting Aδ afferent-evoked EPSCs in SPN neurons. I therefore made simultaneous recordings of SPN neuronal firings and IVP, and then applied capsaicin to the dorsal roots and surface of the spinal cord *in vivo* to block action potential conduction through TRPV1-expresing afferents. Spinal capsaicin (0.1 and 1 μM) applied to the dorsal roots and the surface of the spinal cord for 1 min, did not block micturition but significantly increased both the inter-contraction interval and threshold pressure for initiation of voiding normal micturition. In animals systemically pretreated with capsaicin for desensitization of capsaicin-sensitive (TRPV1-expressing) C afferents, spinal application of capsaicin did not change the inter-contraction interval. These data suggest that capsaicin-sensitive C afferents are activated during normal micturition. I further examined whether spinal capsaicin has any action on PGE2-induced frequent micturition. Following bladder sensitization with intravesical PGE2 (100 μM), spinal capsaicin also increased the inter-contraction interval and the threshold pressure.

Discussion

In this study, I developed a method of simultaneous recording from the SPN including PG neurons in the rat sacral spinal cord *in vivo* by patch-clamp (or extracellular) recording technique, and lower unitary tract activity by bladder and urethral pressure monitoring. This novel *in vivo* technique enabled us to analyze spinal subthreshold membrane potentials and action potentials under current-clamp conditions *in vivo* in addition to studying isolated excitatory synaptic currents under voltage-clamp conditions from autonomic central neurons. I have shown for the first time that bursts of action potential discharge along with oscillatory membrane depolarisations were synchronous with the urethral perfusion pressure oscillations and intravesical pressure increase during voiding.

Although urinary bladder C afferents are considered to be 'silent' afferents for micturition, my slice patch analyses showed that SPN neurons, including PG neurons, receive direct glutamatergic synaptic inputs mostly from slow-conducting C afferents, which are able to elicit action potential discharge. Capsaicin-sensitive afferents also made functional excitatory synaptic contact with PG neurons retrogradely labelled with neurotracers injected near the urinary bladder. These findings indicate that the spinal parasympathetic outflow from PG neurons can be controlled by a direct spinal drive

mediated through TRPV1-expressing C afferents (see below for further discussion). After spinal cord injury, the micturition reflex is reported to be lost for about a week, but then recovers over the following weeks. This indicates that in adult animals the spino-uninary feedforward loop may be strengthened to compensate if the pontine micturition center is unable to drive the micturition reflex. We further show that spinal application of capsaicin inhibited the evoked EPSCs in SPN neurons through capsaicin-sensitive C afferents, and decreased the inter-contraction interval of micturition with an increase of the IVP threshold for micturition. Although further studies are needed to elucidate the functional role of this feedforward excitation, this suggests that the spinal capsaicin-sensitive C afferent synaptic inputs play an important role in setting the threshold for a normal micturition reflex. Furthermore, frequent urination induced by PGE2 could be inhibited by spinal blockade of afferent signaling through capsaicin-sensitive afferents. Thus, the present *in vivo* approach has allowed a detailed characterization of the subthreshold integrative mechanisms of spinal PG excitation during the micturition cycle. In addition, it suggests that spinal capsaicin treatment for the blockage of spinal synaptic inputs from TRPV1-expressing C afferents is an attractive target for the treatment of pathological urinary bladder dysfunction and also for anti-nociceptive action on bladder pain.

Advantages of the present *in vivo* simultaneous recordings of spinal neural activity and lower urinary tract functions, and SPN neuronal activity *in vivo*.

It is well known that micturition involves peripheral neural components in a complex coordinated interplay between sympathetic, parasympathetic, somatic and visceral afferents (Fowler et al., 2008) (de Groat and Wickens, 2013), but much less has been known about the spinal cellular mechanisms during micturition. Previous attempts to define the cellular mechanisms driving the activity of the autonomic circuits controlling the urinary bladder by using 'sharp' microelectrodes to obtain intracellular recordings, have provided glimpses of the activity of sympathetic and parasympathetic preganglionic neurons (de Groat and Ryall, 1968a) (McLachlan and Hirst, 1980) (Dembowsky et al., 1986), as well as some insight into their synaptic inputs (Sasaki and Sato, 2013). However, it has not been possible to relate these recordings of intracellular activity to end organ function. Some of the limitations have recently been addressed in part by obtaining patch-clamp recordings from thoracic sympathetic preganglionic neurones through the use of the *in situ* approach in the working heart brainstem preparation (Briant et al., 2014) (Stalbovskiy et al., 2014). Although high fidelity recordings can be obtained using this approach, it requires spinal cord

transection to gain direct access to the afferent nerve, and it is less suited to the study of functions where a distal afferent-efferent loop is essential for triggering the behaviour (such as micturition). I have demonstrated that it is possible to obtain stable recordings from SPN neurones of the rat spinal cord. This enabled high fidelity recordings of membrane potential allowing observation of sub-threshold changes in membrane voltage and synaptic currents in a preparation with intact micturition. This method is therefore useful to quantitatively elucidate how PG neurons in the spinal cord control LUT functions.

Using the present approach, I have been able to record from neurones of the SPN and have identified PG neurons on the basis of their characteristic electrophysiology, and firing related to bladder contraction. Their firing was positively correlated with the magnitude of the increase in IVP during micturition. The intrinsic properties of the PG neurons are recognizable as similar to those reported from *in vitro* slice recordings of visualized PG neurons (Miura et al., 2000) (Miura et al., 2003). In contrast to the *in vitro* slice, however, I find that PG neurons receive a relatively high frequency and large amplitude EPSCs *in vivo* (15.7 ± 3.2 Hz, 23.1 ± 3.3 pA, n=10) compared with those recorded from spinal cord slices (9.6 ± 3.2 Hz, 10.6 ± 0.5 pA, n=12; $p<0.05$), suggesting that this stronger synaptic drive is a consequence of the intact

afferent and descending inputs to the parasympathetic neurons *in vivo*. A previous study using spinal cord slices has shown that PG neurons exhibit EPSCs in response to the electrical stimulation applied to the ipsilateral dorsolateral area of the white matter (Miura et al., 2001), suggesting that descending excitatory inputs that may originate from the pontine micturition centre, although a recent study suggested that the drive from the pontine micturition centre to PG neurons in cats is relatively weak and possibly multi-synaptic (Sasaki and Sato, 2013). My recordings of spontaneous synaptic responses under voltage-clamp conditions demonstrated that EPSCs with two different kinetics (fast and slow) can be found in the same neuron. This likely indicates that they are mediated by either distinct receptor subtypes or are found in different locations on the cell membrane. It remains to be determined whether these distinct EPSC profiles are functionally separated across afferent, interneuronal or descending control pathways.

I predict that this recording approach will allow these important subthreshold membrane mechanisms in SPN neurons in the sacral spinal cord to be better defined, including a resolution of the normal excitatory and inhibitory synaptic inputs mediating PG neuron excitation. I believe that this detailed understanding will enable the design of new therapeutic approaches to assist with LUT disorders. Further, this approach

could also be universally applied to investigations of the key circuits involved in the autonomic nervous control of the pelvic organs including the LUT.

Subthreshold membrane oscillation evoked in PG neurons during micturition and its synchronisation with urethral oscillation

As an example of the further advantage offered by this approach I have observed for the first time that PG neurons *in vivo* show a novel mechanism for synchronisation of their excitation, as they display oscillatory membrane depolarisations that entrain their action potential discharge. This subthreshold membrane mechanism would be undetectable from *in vivo* extracellular recordings which detect neuronal firing through field potentials. These membrane oscillations were synchronous with the oscillations in UPP (corresponding to the sphincter bursting that is characteristic of the rat) (Streng et al., 2004) (Sadananda et al., 2011). The oscillations coordinate the neural firing with the pumping of the sphincter; this mechanism is reported to facilitate the ejection of urine through the sphincter during synchronised urinary bladder contractions (Maggi and de Groat, 1993). There are several possible mechanisms that could underlie these oscillations including an inhibitory urethral sphincter afferent, interneuron and

parasympathetic preganglionic mechanism (de Groat and Wickens, 2013). It seems likely that descending fibers from the pontin micturition centre input not only to PG neurons in the SPN but also to Onuf's nucleus, which controls the urethral sphincter through interneuron activity. However, alternative mechanisms could involve a combination of intrinsic properties such as the plateau potential mechanism reported by Derjean *in vitro* (Derjean et al., 2005) or alternatively from recurrent inhibitory feedback via axon collaterals of PG neurons as described in the cat (de Groat and Ryall, 1968b). The detailed dissection of this mechanism is feasible using the whole cell recording approach *in vivo* with a combination of voltage-clamp, spinal pharmacological blockade (Furue et al., 2007) (Sugiyama et al., 2012) (Funai et al., 2014) and physiological afferent stimulation. I also noted that despite the persistence of the subthreshold oscillations and maintained depolarisation, the firing frequency of PG neurons reduced as the IVP increased during micturition. This was associated with a reduction in the rate of depolarisation during the rising phase of each oscillation. This may indicate a regulation of the membrane potential trajectory by an inhibitory feedback from the bladder (possibly mediated by supraspinal reflex). This effectively terminates the firing of the PG neurons despite a maintained depolarisation – likely to be important for the termination of active voiding contraction.

Direct Aδ and C afferent synaptic input to PG neurons in the spinal cord.

The present study reveals that monosynaptic evoked EPSCs mediated through Aδ and C afferents were elicited in PG neurons. Capsaicin suppressed C afferent-evoked EPSCs, possibly through an activation of capsaicin receptors, however Aδ afferent-evoked EPSCs were not affected. Although this inhibitory effect of capsaicin on excitatory transmission to substantia gelatinosa neurons has been previously reported (Yang et al., 1999), my results are the first to show this in visceral afferents. This suppression may be due to the inhibitory action of capsaicin on Ca^{2+} channels in nerve terminals, for example capsaicin depressed Ca^{2+} currents in cultured rat dorsal root ganglion neurons (Bleakman et al., 1990). Alternatively, depolarisation of C afferent terminals may result in the decrease of evoked transmitter release (Katz, 1969) or a conduction block of C afferents (Waddell and Lawson, 1989). Thus, capsaicin is a useful tool as a C afferent selective conduction blocker.

In my results, capsaicin also enhanced the frequency of miniature EPSCs in the presence of TTX, suggesting that C afferent terminals directly input to the PG neurons. Putative monosynaptic connections between primary afferents and SPN neurons have

been also previously postulated. Nerve endings identified with horse radish peroxidase applied to the pelvic nerve was detected in the SPN at the light microscopic level in the rat, cat and monkey (Morgan et al., 1981) (Nadelhaft et al., 1983) (Nadelhaft and Booth, 1984). Moreover, primary afferent terminals within the SPN have been demonstrated by anterograde labeling techniques (Nolan and Brown, 1981) (Nolan and Brown, 1984). Previous electrophysiological experiments performed *in vivo* with intact spinal cords also suggest that micturition reflex pathways are exclusively polysynaptic. Stimulation of afferents from the urinary bladder reveals long central delays (60 ms) in the micturition reflex. In the chronic spinally transected animals, however, short central delays (15 ms) that may be monosynaptic inputs are capable of initiating micturition (de Groat et al., 1981). It is known that these mechanisms are involved in the storage and elimination of urine change under pathological condition. Spinal transection at the level of the thoracic spinal cord caused an initial bladder areflexia, however the micturition reflex of spinal transected rats recovered after several weeks. It is speculated that changes in LUT afferent input to the spinal cord of spinal transected rats may have an influence on the reorganization and/or reconstruction of spinal reflex circuits (de Groat, 1995). Although de Groat et al. have suggested that the monosynaptic inputs observed in chronic spinal cats may be due to anatomical

reorganization of dorsal root afferent systems (de Groat et al., 1981) (de Groat et al., 1983), further quantitative experiments will be required to determine whether this anatomical reorganization occurs in the chronically transected animals or during development.

Functional role of capsaicin-sensitive C afferents on normal and pathological conditions

My results showed that the inter-contraction interval of micturition was prolonged by administration of capsaicin to the spinal surface under normal and pathological conditions with an increased IVP threshold for micturition. When capsaicin was administered for a longer time on the spinal surface, the micturition reflex was abolished completely (data not shown), suggesting that this is due to desensitization of C afferents. The inter-contraction interval of capsaicin pretreated rats was also previously shown to be increased under urethane anesthesia (Maggi et al., 1993) (Maggi and Conte, 1990). Furthermore, micturition was abolished in awake animals by intrathecal administration of capsaicin (Philippe and TL, 1988). The results of these previous studies have been explained by the depletion of neuropeptides within the

terminals of unmyelinated afferents (presumably TRPV1-expressing) caused by capsaicin stimulation and depolarisation. Therefore, capsaicin has been considered to cause a functional desensitization of unmyelinated afferents. As mentioned above, my slice experiments revealed that capsaicin inhibited C afferent-evoked EPSCs in SPN neurons and the capsaicin changed the IVP threshold for micturition. The bladder capacity of TRPV1-null mice is also increased in an anesthetized condition as compared to wild type mice (Birder et al., 2002).

Although C afferents are also insensitive to bladder filling under physiological conditions in the normal cat (Fowler et al., 2008). C afferent inputs begin driving a micturition reflex in the cat after chronic spinal cord injury. For example, it is known that infusion of cold water into the bladder of spinal injury patients induces reflex micturition but this does not occur in healthy individuals (Mannion, 1971) (Fall et al., 1990). These plastic changes in afferent input to the spinal cord in chronic conditions may have an influence on the reorganization and/or reconstruction of spinal reflex circuit and therefore warrant further experimental investigation.

Neurons positive for c-fos induced following activation of the LUT were located in four regions of lumbosacral spinal cord: superficial lateral dorsal horn, medial dorsal horn, dorsal commissure and SPN. Birder and de Groat have demonstrated that

distal urethral inputs project to the medial dorsal horn and that proximal urethral inputs project primarily to dorsal commissure; tension receptor afferents activated by distention of the bladder or by reflex bladder contractions preferentially activates neurons in the SPN and dorsal commissure (Birder and de Groat, 1992). I observed more c-fos positive neurons after chemical irritation than bladder distention by physiological saline in the same region. This suggests that nociceptive and non-nociceptive afferent pathways from the urinary bladder and urethra activate neurons in same regions of the spinal cord. Immunohistochemical staining of the spinal cord showed the location of these c-fos positive neurons also received projections from TRPV1-positive afferents. Therefore we can assume that TRPV1-expressing afferents contribute to nociceptive and non-nociceptive responses in the bladder.

The urinary bladder is rich with capsaicin-sensitive afferents, which are usually ineffective for initiating micturition in the normal adult. However, synthesis of several inflammatory mediators and neurotransmitters, such as prostaglandins (PGs) and nerve growth factor, which activate C afferents, are increased in the pathologic bladder (Maggi et al., 1988) (Schussler, 1990). It has been reported that PGs have an important role in regulating LUT function (Maggi, 1992). PGs (PGF1α, PGE1 and PGE2) are locally synthesized in the bladder by detrusor muscle stretch, bladder mucosa

damage, nerve stimulation and inflammatory mediators (Palea et al., 1998) (Meini et al., 1998) (Park et al., 1999). It is well known that intravesical administration of PGE2 evokes frequent urination in rats; this effect was prevented by systemic pretreatment with capsaicin (Ishizuka et al., 1995) (Maggi et al., 1988). In humans, PGE2 infusion into the bladder caused an urgency to urinate (Schussler, 1990). Therefore, PGE2 can contribute to decreasing thresholds necessary to trigger bladder contraction through the activation of capsaicin-sensitive afferents (Bultitude et al., 1976) (Maggi, 1992) (Park et al., 1999). My results demonstrated that PGE2-induced frequent urination was inhibited by supraspinal administration of capsaicin, an inhibitory effect that can be attributed to conduction block (Waddell and Lawson, 1989). PGE2 receptors are classified into four subtypes (Narumiya et al., 1999). Expression of EP1 receptors has been demonstrated in the rat bladder and also in dorsal root ganglion (DRG) neurons (Coleman et al., 1994). It is suggested that the EP1 receptor may function in afferent nerve terminals. Interaction between EP1 receptor and the TRPV1 channel, which is expressed not only in bladder afferents but also in submucosa and mucosa, has been reported (Birder et al., 2002), and EP1 receptor has been shown to facilitate the effects of capsaicin on DRG neurons (Moriyama et al., 2005). It is thought that the temperature threshold of TRPV1 activation is altered in the presence of PGE2

(Moriyama et al., 2005). Therefore activation of TRPV1 at normal body temperature could possibly lead to abnormal bladder sensation and urgency to urinate.