**Neuronal-derived Nitric Oxide Modulates the Activity of
Mouse Detrusor Smooth Muscle**

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ABSTRACT

Aims: We investigated the roles of neuronal-derived nitric oxide (NO) in the modulation of spontaneous activity of mouse detrusor smooth muscle. **Methods:** Detrusor smooth muscle strips were isolated from nNOS gene knock-out (nNOS^{-/-}) mice and their wild type siblings (nNOS^{+/+}). The properties of smooth muscle cells were assessed using intracellular electrophysiology and Ca²⁺ imaging by laser-scanning confocal microscopy. The effects of an nNOS inhibitor, 7-nitro indazole (7-NI) on electrically-evoked contractility were assessed using nNOS^{+/+} mouse detrusor strips. **Results:** In spontaneously active cells, the frequency of spontaneous action potentials (sAPs) and whole cell Ca²⁺ flashes in nNOS^{-/-} preparations was lower than that in the nNOS^{+/+} preparations. The frequency of sAPs was enhanced by a nitric oxide donor, diethylamine NONOate sodium salt (NONOate; 100 μM), both when used alone and when the cGMP pathway was blocked by 1H - [1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ, 10 μM). 7-NI (100 μM) significantly suppressed the electrically-evoked contraction of mouse detrusor strips. **Conclusions:** We suggest that neuronal-derived NO facilitates the generation of spontaneous activity via a cGMP-independent pathway, and consequently enhances the evoked contraction of detrusor. Dysregulation of nNOS containing nerves may underlie bladder pathologies.

INTRODUCTION

Detrusor smooth muscle cells (SMCs) of the urinary bladder exhibit spontaneous contractile activity during the storage phase. This contractile activity is poorly synchronised between cells; thereby, maintaining the shape of the bladder without elevating intravesical pressure.¹ It appears, however, that there may be alterations in contractile activity in certain bladder pathologies; overactive bladder, for example, is characterised by large amplitude, slow duration spontaneous contractions² that may arise from increased synchronization of spontaneous activity of SMCs.³

Neural activation of the bladder has been shown to be mediated by several neurotransmitters, but principally by acetylcholine and ATP with species variations in the proportions of cholinergic and purinergic control.⁴⁻⁵ Another important neurotransmitter, nitric oxide (NO), may be released from motor fibres⁶, intramural ganglia⁷, urothelium⁸, and from SMCs of the bladder.⁹ The application of NO donors reveals immunoreactivity for cyclic guanosine monophosphate (cGMP), a second messenger involved in NO signalling, in nerves, urothelium and interstitial cells of the bladder, but cGMP immunoreactivity was absent in detrusor smooth muscle¹⁰⁻¹¹ suggesting any NO effect is likely to be cGMP-independent. The role NO signalling plays in the functioning of the bladder is far from clear, with conflicting studies demonstrating relaxation¹² and enhancement of evoked contraction^{11,13}. Genetic disruption of neuronal-derived NO has been shown to evoke a dilated bladder and increased outflow resistance in male mice.¹⁴ Another study reported

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that there is no discernable cystometric effect in young female nNOS^{-/-} mice.¹⁵

In order to better understand the role of nNOS-derived NO on the spontaneous activity of detrusor SMCs, we have studied spontaneous electrical activity and Ca²⁺ dynamics in strips of detrusor dissected from the bladders of nNOS gene-disrupted mice and used drugs which enhance or block NO.

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MATERIALS AND METHODS

General

nNOS gene knock-out (nNOS^{-/-}) mice and their wild type siblings (nNOS^{+/+}) were obtained from a colony at the John Radcliffe Hospital (Oxford, UK) by backcrossing the nNOS^{-/-} (Jackson Laboratory, USA) on a C57BL/6 background. In order to confirm that the nNOS gene had been knocked out, mice were bred, chipped, and coded, and tail DNA samples were harvested for genotyping with polymerase chain reaction.¹⁶

Mice of either sex, weighing 20–39 g, were killed by head concussion followed by cervical dislocation. Efforts were made to minimise the number of animals used and their suffering; all experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and European Communities Council Directive 86/09/EEC and were approved by the Institutional Animal Care and Use Committee of the University of Oxford and National Defense Medical Center, Taipei, Taiwan pursuant to the Helsinki Declaration and internationally-accepted principles for the care and use of experimental animals. The urinary bladder was surgically removed and its ventral wall was opened longitudinally from the bladder neck to the top of the dome. The urothelium was either left intact (for microelectrode studies) or removed (for tension recording and Ca²⁺ imaging studies) by careful dissection (taking care not to stretch or damage the underlying detrusor muscle). Whole layer tissue strips, 6–8 mm long and 1–2 mm wide, which contained a few bundles of smooth muscle, were prepared as previously described.¹⁷

Microelectrode recordings

Following bridge-balancing to compensate for the tip resistance (200–300 MΩ) of the microelectrode and neutralization of the tip capacitance, individual bladder smooth muscle cells in muscle bundles were impaled with microelectrodes from the serosal side. Membrane potential changes were recorded using a high input impedance amplifier (Axoclamp-2B; Axon Instruments, USA), and analysed as previously described.²²

Ca²⁺ imaging

Detrusor muscle strips were exposed to 10 μM Oregon Green 488 BAPTA-1 AM (Invitrogen, UK) in 1% DMSO/0.2% pluronic F-127 (Sigma, UK) in PSS for 70 min at 33 °C. Each strip was then rinsed in PSS for 10 min before transfer to a chamber mounted on the stage of a confocal microscope and continuously superfused with standard PSS (bath temperature 35 °C). Series of images were acquired and analysed as previously described.¹⁷

Tension Recording

Detrusor strips (approx. 5x1x1 mm; 2–4 mg) were tied at either end with suture (5/0; Pearsalls, UK) and mounted vertically in a 0.2 ml capacity organ bath between two recessed platinum electrodes through which electrical field stimulation (EFS) was delivered. The strips were stretched with an initial force of 1 g, and allowed to equilibrate for at least 1h prior to starting the experiments. The strips were constantly superfused with PSS (37 °C) at a rate of 1 ml.min⁻¹.

EFS was delivered from a stimulator (Grass S88; Grass Technologies, USA) at

parameters of 0.05 ms pulse width, 3 s train duration, 50 V intensity, at varying frequency. Isometric tension generated by the strips was measured using isometric force displacement transducers (Dynamometer UFI; Pioden Controls Ltd, or AD Instruments MLT050/D), amplified and analyzed using PowerLab 4/30 (ADInstruments) and Chart software (v.7).

Immunohistochemical study

OCT (0.1 ml) (R.A. Lamb, UK) was injected into the lumen of a whole bladder through the urethra. Bladders were mounted in OCT and snap-frozen in isopentane-chilled liquid nitrogen. Cryosections (10 μ m) were cut and thaw-mounted onto glass slides pretreated with Vectabond reagent (Vector Laboratories, UK) and air-dried for 30-60 mins. Sections were fixed with acetone for 10 mins and then incubated overnight in a moisture chamber with a primary rabbit polyclonal antibody (NOS1[R20]; Santa Cruz, USA), diluted 1:50 with phosphate buffered saline (PBS; Oxoid Ltd, UK) and 0.5 % bovine serum albumin (Sigma, UK). The next day, slides were washed three times with PBS and then incubated for 30 mins with secondary antibody (peroxidase conjugated swine anti-rabbit antibody; DAKO, UK) at a dilution of 1:50. After being washed, slides were incubated for another 30 mins with a tertiary antibody (peroxidase conjugated rabbit anti-swine antibody; DAKO, UK) at a dilution of 1:50. Slides were then washed again and incubated in substrate solution made with SigmafastTM-diaminodenzidine tablets (Sigma, UK) for 5 mins and then washed. Slides were then counterstained with filtered Harris's Hematoxylin (VWR International, UK) for 15 s, dehydrated with 100% alcohol, and treated with Histoclear (R.A. Lamb, UK). After

mounting, slides were dried overnight. Micrographs were obtained by use of a digital camera (Coolpix 950; Nikon, Japan) attached to a microscope (DMRB; Leica, UK).

Drugs and solutions

To produce stock solutions, diethylamine NONOate sodium salt (NONOate) and 7-nitroindazole (7-NI; Sigma, UK) were dissolved in distilled water; 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Tocris, UK) was dissolved in dimethyl sulfoxide. Stock solutions were stored at -20 °C. Drugs were delivered by substituting PSS with drug-containing solutions of the desired concentration for PSS. NONOate was stored in dark-coloured vessels in order to minimize light-induced degradation.

The PSS used had the following composition (mM): NaCl, 120; KCl, 5.9; MgCl₂, 1.2; CaCl₂ 2.5; NaHCO₃, 15.5; NaH₂PO₄, 1.2 and Glucose, 11.5.¹⁷

Analysis of data

The normality and homogeneity of variance was tested prior to statistical analysis (Excel, Microsoft and Graph Pad Prism 5) using Kolmogorov-Smirnov and Levene's tests, respectively (SPSS v16; SPSS Inc., USA). Where data sets did not vary significantly from chance (i.e. $P > 0.05$) for both tests, they are presented as mean $\pm$ S.E.M. and the Student's t test was used for comparison unless otherwise stated. In cases where data are non-parametric, the presentation method and statistical test are stated. The null hypothesis was rejected if $P < 0.05$. The paired t test or Wilcoxon signed rank test was used to compare pre- and post-treatment values of frequency and amplitude of sAPs generated

from individual detrusor SMCs. The term n_a refers to the number of animals; n_{smc} , number of smooth muscle cells; n_{ap} , number of spontaneous action potentials. Square brackets are used to denote range.

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RESULTS

Immunohistochemical identification of nNOS

In order to validate the nNOS^{-/-} strain, nNOS immunohistochemistry was performed. There was marked nNOS antibody staining of neurons in the suburothelial and detrusor layers of bladder wall and of nerve fibres traversing the detrusor layer in nNOS^{+/+} mice (*n*_a=3) (Fig. 1a). Large ganglia were most commonly found in the serosal layer (Fig. 1c). These structures were not stained in the bladder tissue from nNOS^{-/-} mice (Fig. 1b,d).

General electrical properties

The resting membrane potentials (RMPs) of the urothelium intact detrusor SMCs, determined at the most negative potential between each action potential, was -41 mV in both nNOS^{-/-} [range: -35 to -50 mV] and nNOS^{+/+} [-34 to -59 mV] preparations (*n*_a=11, *n*_{smc}=25 and *n*_a=18, *n*_{smc}=29, respectively; *P*=0.9). The membrane potentials were interrupted by spontaneous action potentials (sAPs) and frequent spontaneous depolarizations (Fig.2a). Many of the properties of sAPs¹⁷ were no different between nNOS^{-/-} and nNOS^{+/+} preparations (Table 1). There was, however, a slower rate of decay in sAPs of nNOS^{-/-} detrusor SMCs.

The frequency of spontaneous action potentials was lower in nNOS^{-/-} detrusor SMCs

The frequency of sAPs recorded from the urothelium intact nNOS^{-/-} detrusor SMCs (2.3 ±

0.38 min⁻¹, $n_a=13$, $n_{smc}=25$) was significantly lower in comparison to those recorded from the intact nNOS^{+/+} detrusor SMCs (4.3 ± 0.73 min⁻¹; $n_a=19$, $n_{smc}=29$; $P=0.025$, Fig. 2b).

Spontaneous Ca²⁺ dynamics of nNOS^{+/+} and nNOS^{-/-} detrusor SMCs

The frequency of whole cell Ca²⁺ flashes in nNOS^{-/-} preparations (median frequency: 0 min⁻¹ range: [0, 19], $n_a=7$, $n_{smc}=105$) was lower than that in the nNOS^{+/+} preparations (1.1 min⁻¹ [0, 58], $n_a=7$, $n_{smc}=152$) ($P < 0.0001$, Mann-Whitney U test; Fig. 2c).

Current-voltage relationship of mouse detrusor smooth muscle

Input resistance was lower in nNOS^{-/-} detrusor SMCs than in nNOS^{+/+} preparations (470 ± 16 M Ω vs. 580 ± 13 M Ω ; $P < 0.0001$; $n_a=4$, $n_{smc}=10$ for nNOS^{-/-}; $n_a=5$, $n_{smc}=12$ for nNOS^{+/+}) (Fig. 3).

Spontaneous depolarizations recorded from detrusor SMCs

The frequency of spontaneous depolarizations (SDs) was no different in nNOS^{-/-} (8.7 ± 1.6 min⁻¹, $n_a=7$, $n_{smc}=9$) or nNOS^{+/+} preparations (9.2 ± 1.2 min⁻¹, $n_a=10$, $n_{smc}=10$) (Figure 4a).

The shape of SDs did not differ (Table 1), but average SD amplitude was larger in nNOS^{-/-} detrusor SMCs (Figure 4b, c).

Effect of a NO donor on nNOS^{+/+} and nNOS^{-/-} detrusor SMCs

A nitric oxide donor NONOate was added to nNOS^{-/-} preparations to determine whether the

lower sAP frequency of detrusor SMCs in nNOS^{-/-} could be restored to a frequency similar to nNOS^{+/+}. NONOate (100 μ M) significantly increased the frequency of sAPs in nNOS^{-/-} preparations (from $1.9 \pm 0.7 \text{ min}^{-1}$ to $9.4 \pm 3.3 \text{ min}^{-1}$, $n_a=8$, $n_{smc}=10$, $P=0.048$, i.e. to a greater frequency than in nNOS^{+/+}, which was $4.3 \pm 0.73 \text{ min}^{-1}$). NONOate also reduced the amplitude of sAPs (from $54 \pm 2.1 \text{ mV}$ to $50 \pm 2.3 \text{ mV}$, $P=0.017$) and depolarised the resting potential (from $-44 \pm 1.6 \text{ mV}$ to $-38 \pm 2.6 \text{ mV}$, $P=0.015$).

Effect of NO-sensitive guanylyl cyclase inhibition on nNOS^{+/+} detrusor SMCs

Soluble guanylyl cyclase is normally activated by NO to stimulate cGMP synthesis. A selective inhibitor of nitric oxide-sensitive guanylyl cyclase, ODQ, was added to nNOS^{+/+} preparations to block the synthesis of cGMP. ODQ (10 μ M) did not significantly affect the frequency (control: $1.6 \pm 0.7 \text{ min}^{-1}$ vs. ODQ: $3.3 \pm 0.7 \text{ min}^{-1}$, $P=0.09$) or amplitude (control: $56 \pm 0.5 \text{ mV}$ vs. ODQ: $55 \pm 0.3 \text{ mV}$, $P=0.29$) of sAPs in nNOS^{+/+} preparations ($n_a=5$, $n_{smc}=10$). To determine whether NO affects spontaneous electrical activity in the absence of cGMP signalling, NONOate (100 μ M), added following incubation of the tissue (20 mins) with ODQ (10 μ M), increased sAP frequency from $3.3 \pm 0.7 \text{ min}^{-1}$ to $8.6 \pm 1.9 \text{ min}^{-1}$ ($n_a=4$, $n_{smc}=5$, $P=0.012$; Fig. 5). Adding ODQ subsequently did not affect the frequency of sAPs (NONOate + ODQ: $8.6 \pm 1.9 \text{ min}^{-1}$ vs. NONOate: $6.3 \pm 2.4 \text{ min}^{-1}$, $n_a=4$, $n_{smc}=5$ for NONOate + ODQ, $n_a=6$, $n_{smc}=6$ for NONOate, $P=0.48$).

Effect of nNOS inhibition on EFS-evoked contraction of detrusor

In order to determine the effect of nNOS inhibition on EFS-evoked contractions, force-frequency relationships of nNOS^{+/+} detrusor were compared in the presence and absence of the nNOS inhibitor 7-NI. 7-NI (100 μ M) significantly reduced the amplitude of EFS-evoked contractions at all frequencies (e.g. control amplitude at 20 Hz: 1.39 ± 0.14 g.mg⁻¹ vs. 7-NI: 1.10 ± 0.12 g.mg⁻¹; $P < 0.0001$, $n_a = 15$) (Fig. 6a).

In order to determine whether there was an interaction between nNOS-derived NO and purinergic neurotransmission, force-frequency relationships were compared following purinoceptor desensitization by α,β -methylene ATP (α,β -mATP). α,β -mATP (3 μ M) significantly reduced the force-frequency relationship (e.g. control amplitude at 20 Hz: 1.01 ± 0.16 g.mg⁻¹ vs. α,β -mATP: 0.49 ± 0.07 g.mg⁻¹; repeated measures ANOVA, $P < 0.001$, $n_a = 12$). When detrusor strips were treated with 7-NI (100 μ M) in the presence of α,β -mATP (3 μ M), there was no additional reduction in the force-frequency relationship (e.g. α,β -mATP at 20 Hz: 0.49 ± 0.07 g.mg⁻¹ vs. 0.47 ± 0.07 g.mg⁻¹; $P = \text{NS}$) (Fig. 6b). On a strip-by-strip basis, this was a mean reduction of $7 \pm 6\%$ by α,β -mATP (3 μ M) plus 7-NI (100 μ M) compared to the effect of 7-NI (100 μ M).

In order to determine whether there was an interaction between nNOS-derived NO and cholinergic neurotransmission, force frequency relationships were compared following muscarinic cholinergic receptor antagonism by atropine (1 μ M). Atropine (1 μ M) significantly reduced the force-frequency relationship (e.g. control amplitude at 20 Hz: 0.78

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$\pm 0.09 \text{ g.mg}^{-1}$ vs. atropine: $0.25 \pm 0.04 \text{ g.mg}^{-1}$; $P < 0.001$, $n_a = 18$). When detrusor strips were treated with 7-NI ($100 \mu\text{M}$) in the presence of atropine ($1 \mu\text{M}$), there was an additional reduction in force generated, of $38 \pm 8\%$ (to $0.16 \pm 0.04 \text{ g.mg}^{-1}$), relative to treatment by atropine alone ($0.25 \pm 0.04 \text{ g.mg}^{-1}$) ($0.1 > P > 0.05$, paired t test) (Fig. 6c).

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DISCUSSION

Spontaneous activity of the detrusor during the filling phase of the micturition cycle is of clinical relevance because bladder overactivity, a common and debilitating condition, is characterised by larger amplitude, fused spontaneous contractions of the detrusor.² By comparing nNOS knock-out and sibling control mice we demonstrate that neuronal-derived NO plays an important role in generating and modulating the spontaneous activity of the detrusor.

Confirmation of disruption of nNOS gene by lack of immunoreactivity

Genetically-modified mice were used in the present study. The nNOS^{-/-} strain used was originally developed by Huang *et al.*¹⁸, and has previously been used to investigate the role of NO in bladder function.¹⁴⁻¹⁵ Despite only the nNOS α splice variant being knocked-out in this nNOS^{-/-} strain (i.e. meaning that the β -, γ - and μ -variants may still be present) neuronal nNOS containing neurons were absent (Figure 1), consistent with previous studies using the same strain of nNOS gene-disrupted mice¹⁴⁻¹⁵ and validating the use of this strain as a true nNOS^{-/-}.

nNOS derived NO enhances the spontaneous activity of the detrusor

Detrusor SMCs generate sAPs caused by the opening of L-type Ca²⁺ channels and regulated by voltage-dependent mechanisms¹⁹. We used intracellular electrophysiology

and confocal imaging to detect the spontaneous activity of detrusor SMCs. The frequency of these two measures of cellular activity was significantly lower in the nNOS^{-/-} detrusor SMCs. After applying an NO donor (NONOate), the frequency of sAPs was augmented. This suggests that nNOS derived NO may play an excitatory role in the generation of sAPs and probably increase detrusor excitability; a finding that is consistent with previous research.²⁰

It has been demonstrated that NO, acting through S-nitrosylation pathways, acts as an excitatory agent in the regulation of electrical activity of excitable cells.²¹ Recently, Yoshida and colleagues²² presented a mechanism mediated by cysteine S-nitrosylation showing that NO may activate Transient Receptor Potential (TRP) channels and elicit entry of Ca²⁺ into human embryonic kidney cells, thereby causing excitation. Consistent with these studies, we demonstrated that NONOate enhanced the frequency of sAPs when the cGMP pathway was blocked by ODQ, suggesting NO may excite detrusor smooth muscle cells via a cGMP-independent pathway.

There were no significant differences in sAP waveform from nNOS^{-/-} and nNOS^{+/+} detrusor apart from a small but significant difference in rate of decay. This change is similar to the change in the shape of sAPs after applying a Ca²⁺-activated K⁺ channel blocker, charybdotoxin in guinea pig bladder SMCs¹⁹, suggesting that the lack of NO in nNOS^{-/-} mice may result in reduced function of the Ca²⁺-activated K⁺ channels²³ and/or Ca²⁺-mediated inactivation of the Ca²⁺ channels¹⁹, and consequently reduced the

frequency of sAPs.

Input resistance is negatively correlated with the degree of electrical connectivity of SMCs local to the site of impalement.²⁴ SMCs of nNOS^{-/-} preparations exhibited lower input resistance compared to nNOS^{+/+} preparations, indicating a greater degree of electrical coupling of SMCs in nNOS^{-/-} detrusor muscle. The functional implications are not currently clear; perhaps increased intercellular coupling helps generate additional voiding pressure.

In a previous study we demonstrated that SDs, or spontaneous excitatory junctional potentials, occur when the magnitude of the current influx through P2X1 is insufficient to trigger a sAP.²⁵ It follows that the observation that nNOS^{-/-} detrusor SMCs exhibit higher amplitude SDs and less frequent sAPs in nNOS^{-/-} compared to nNOS^{+/+} suggests that the size of nerve-released ATP packets in nNOS^{-/-} is reduced. NO and ATP have been recognized as co-transmitters in the non-adrenergic non-cholinergic nerves.²⁶ Therefore, absence of neuronal-derived NO may possibly reduce the amount of ATP in synaptic vesicles, reducing sAP frequency and consequently suppress detrusor contraction. This theory is supported by our tension recording experiments, in which a nNOS inhibitor, 7-NI, suppressed EFS-evoked contraction. No additional reduction of detrusor contractility was observed when 7-NI was subsequently applied in the presence of blockers of purinergic or cholinergic neurotransmission, implying that neuronal-derived NO may modulate the evoked contractility of detrusor pre- rather than post-junctionally.

A previous study has found that there are only interstitial cells and nerve fibres in the

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3 mouse bladder wall respond to NO.²⁷ Fujiwara and colleagues demonstrated that EFS and
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5 the NO donor SIN-1 failed to induce relaxation of pre-contracted mouse detrusor
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7 preparations. In addition, SIN-1 and sodium nitroprusside were found to increase cGMP
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9 levels in the bladder tissue, but this increase was not correlated to smooth muscle
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11 relaxation.¹¹ Therefore it is unlikely that NO causes relaxation of mouse detrusor smooth
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13 muscle.^{11,28} Our data are in line with these studies and further demonstrated that
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15 nerve-derived NO plays an excitatory role in modulating mouse detrusor activity.
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26 **CONCLUSION**

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28 The present study suggests that neuronal-derived NO facilitates the generation of
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30 spontaneous activity via a cGMP-independent pathway, and contributes to the evoked
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Table 1. Descriptive statistics of the properties of sAPs and SDs recorded from nNOS^{+/+} and nNOS^{-/-} detrusor SMCs.

	nNOS ^{+/+}	nNOS ^{-/-}	Significance (P)
<i>Shape¹⁷</i>			
<i>Spike-type (%)</i>	26	20	0.4
<i>Pacemaker-type (%)</i>	74	80	
<i>sAPs</i>			
<i>Amplitude (mV)</i>	54 ± 1.0	53 ± 1.0	0.5
<i>Half-width (ms)</i>	7 ± 1.0	8 ± 0.3	0.17
<i>Rise time (ms)</i>	33 ± 3.2	38 ± 3.4	0.17
<i>Rising slope (mV.ms⁻¹)</i>	8.5 ± 0.5	7.3 ± 0.4	0.08
<i>Rate of decay (mV.ms⁻¹)</i>	13 ± 0.5	11 ± 0.6	0.017
<i>After-hyperpolarization amplitude (mV)</i>	9.1 ± 0.7	8.8 ± 0.5	0.69
<i>n_a</i>	19	13	
<i>n_{smc}</i>	29	25	
<i>SDs</i>			
<i>Amplitude (mV)</i>	6.2 ± 0.5	7.7 ± 0.3	0.016
<i>Half-width (ms)</i>	51 ± 5.7	64 ± 4.9	0.13
<i>Rise-time (ms)</i>	32 ± 2.5	29 ± 19	0.43
<i>Time constant of decay (ms)</i>	46 ± 5.2	55 ± 4.5	0.22
<i>n_{smc}</i>	10	9	

FIGURE LEGENDS

Figure 1. nNOS immunoreactivity was absent in nNOS^{-/-} mouse bladder tissue. (a)

nNOS positive ganglia (arrow) are seen in the serosal and detrusor layers in nNOS^{+/+}, but (b) not in nNOS^{-/-} bladder tissue. (c) At higher magnification nNOS positive staining of a ganglion (asterisk) and of nerve fibres (arrow head) was seen near the serosa of nNOS^{+/+} bladder, but (d) not in nNOS^{-/-}, but not in bladder tissue.

Figure 2. Spontaneous action potentials and whole cell Ca²⁺ flashes were less frequent in nNOS^{-/-} than

nNOS^{+/+} siblings. (a) Intracellular recordings from a nNOS^{+/+} and a nNOS^{-/-} SMC. Resting membrane potentials were -40 mV for nNOS^{+/+} and -43 mV for nNOS^{-/-}. (b) The frequency of sAPs is lower in SMCs from nNOS^{-/-} than nNOS^{+/+} mice. The data shown are the mean and SEM. . denotes $P < 0.05$ ($n_a=18$, $n_{smc}=29$ for intact nNOS^{+/+}; $n_a=11$, $n_{smc}=25$ for intact nNOS^{-/-}). (c) The frequency of spontaneous whole cell flashes was greater in SMCs from nNOS^{+/+} mice ($n_a=7$, $n_{smc}=152$) than from nNOS^{-/-} mice ($n_a=5$, $n_{smc}=105$). There was, however, no significant difference in the whole cell flash amplitudes between the two groups ($\Delta F/F$ of 0.29, $n_{smc}=47$, in nNOS^{-/-} preparations and 0.27, $n_{smc}=110$, in nNOS^{+/+} preparations). Frequent Ca²⁺ waves and focal Ca²⁺ transients could also be seen within the individual smooth muscle cells, but their frequencies were not quantified as part of the present study. Data shown are frequencies and amplitudes per cell (symbols) and median per group (horizontal line). Statistical comparisons are Mann Whitney U test.

Figure 3. nNOS^{-/-} detrusor SMCs had a lower input resistance compared to nNOS^{+/+} detrusor SMCs.

The membrane properties of nNOS^{+/+} and nNOS^{-/-} mouse detrusor SMCs were examined by a series of brief

intracellular current injections (-0.1 to 0.1 nA for 100 ms). The current-voltage relationship for SMCs in nNOS^{+/+} and nNOS^{-/-} was linear before the threshold of generating an action potential was reached (value of membrane potential: 0 ± 2.2 mV for nNOS^{+/+} and -10 ± 2.2 mV for nNOS^{-/-}). The input resistances of the detrusor SMCs calculated from a slope of the linear current-voltage relationship is significantly lower in nNOS^{-/-} preparations. The time constants of decay of the resultant electrotonic potential in SMCs were not significantly different (7.9 ± 0.9 ms vs. 7.6 ± 0.7 ms, $P=0.8$). The fitted line above this threshold is a third-order polynomial. ($n_a=5$, $n_{smc}=12$ for nNOS^{+/+}; $n_a=4$, $n_{smc}=10$ for nNOS^{-/-}).

Figure 4. Larger amplitude spontaneous depolarizations (SDs) occurred more frequently in nNOS^{-/-} than nNOS^{+/+}. (a) The frequency of SDs occurred in detrusor SMCs was no different in nNOS^{-/-} mice ($n_a=7$, $n_{smc}=9$) compared to that in nNOS^{+/+} mice ($n_a=10$, $n_{smc}=10$) ($P=NS$). (b) However, the amplitude distribution was shifted to the right in nNOS^{-/-} mouse detrusor SMCs, as demonstrated (c) by the corresponding significant difference in median amplitude between nNOS^{+/+} and nNOS^{-/-} mouse detrusor SMCs.

Figure 5. NONOate enhanced the spontaneous activity of detrusor in the presence of a selective inhibitor of nitric oxide-sensitive guanylyl cyclase, ODQ (10 μ M), in the nNOS^{+/+} mouse. (a) In the presence of ODQ, NONOate (100 μ M) significantly increased the frequency of sAPs (b) ODQ (10 μ M) alone did not increase sAP frequency in nNOS^{+/+} detrusor SMCs ($n_a=4$). Statistical comparisons are one-way ANOVA followed by Tukey's multiple comparison test. ** denotes $P < 0.01$ *** denotes $P < 0.001$.

Figure 6. The nNOS inhibitor 7-NI reduced EFS-stimulated detrusor contracture but there was no additional reduction following purinergic desensitisation or cholinergic blockade. (a) 7-NI (100 μ M) suppressed the EFS evoked contraction of nNOS^{+/+} detrusor smooth muscle strips ($n_a=15$). (b) P2X₁ receptor

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3 desensitisation, by α,β -methylene, ATP (3 μ M) reduced EFS-evoked contracture but there was no additive
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6 reduction of contraction by 7-NI (100 μ M) in combination with α,β -methylene ATP (3 μ M) ($n_a=12$). (c) The
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9 force-frequency relationship in the presence of 7-NI (100 μ M) and atropine (1 μ M) and (cii) contracture at 20
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11 Hz exhibited a borderline significant reduced contracture compared to that in the presence of atropine alone
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14 ($n_a=18$). See text for details.
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Figure 1. nNOS immunoreactivity is absent in nNOS-/- mouse bladder tissue. (a,) nNOS positive ganglia (arrow) are seen in the serosal and detrusor layers in nNOS+/+, but not in nNOS-/- bladder tissue, (b). (c) At higher magnification nNOS positive staining of a ganglion (asterisk) and of nerve fibres (arrow head) was seen near the serosa of nNOS+/+ bladder, but not in nNOS-/- , but not in bladder tissue (d).
78x69mm (300 x 300 DPI)

Figure 2. Spontaneous action potentials and whole cell Ca²⁺ flashes are less frequent in *nNOS*^{-/-} than *nNOS*^{+/+} siblings. (a) Intracellular recordings from a *nNOS*^{+/+} and a *nNOS*^{-/-} SMC. Resting membrane potentials were -40 mV for *nNOS*^{+/+} and -43 mV for *nNOS*^{-/-}. (b) The frequency of sAPs SMCs from *nNOS*^{-/-} mice was significantly lower compared to that from *nNOS*^{+/+} mice. The data shown are the mean and SEM. *denotes $P < 0.05$ ($n_a = 18$, $n_{smc} = 29$ for intact *nNOS*^{+/+}; $n_a = 11$, $n_{smc} = 25$ for intact *nNOS*^{-/-}). (c) The frequency of spontaneous whole cell flashes was greater in SMCs from *nNOS*^{+/+} mice ($n_a = 7$, $n_{smc} = 152$) than from *nNOS*^{-/-} mice. ***denotes $P < 0.001$ ($n_a = 5$, $n_{smc} = 105$). There was, however, no difference in the whole cell flash amplitudes between *nNOS*^{+/+} ($n_{smc} = 110$) and *nNOS*^{-/-} mice ($n_{smc} = 47$). Data shown are frequencies and amplitudes per cell (symbols) and median per group (horizontal line). Statistical comparisons are Mann Whitney U test.

215x574mm (300 x 300 DPI)

Figure 3. nNOS^{-/-} detrusor SMCs have a lower input resistance compared to nNOS^{+/+} detrusor SMCs. The current-voltage relationship for SMCs in nNOS^{+/+} and nNOS^{-/-} was linear before the threshold of generating an action potential was reached (value of membrane potential: 0 ± 2.2 mV for nNOS^{+/+} and -10 ± 2.2 mV for nNOS^{-/-}). The input resistances of the detrusor SMCs calculated from a slope of the linear current-voltage relationship is significantly lower in nNOS^{-/-} preparations. The fitted line above this threshold is a third-order polynomial. (na = 5, nsmc = 12 for nNOS^{+/+}; na = 4, nsmc = 10 for nNOS^{-/-}). 72x72mm (150 x 150 DPI)

Figure 4. Larger amplitude spontaneous depolarizations (SDs) occurred more frequently in nNOS^{-/-} than nNOS^{+/+}. (a) The frequency of SDs occurred in detrusor SMCs was no different in nNOS^{-/-} mice (na = 7, nsmc = 9) compared to that in nNOS^{+/+} mice (na = 10, nsmc = 10) ($P = \text{NS}$). (b) However, the amplitude distribution was shifted to the right in nNOS^{-/-} mouse detrusor SMCs, as demonstrated (c) by the corresponding significant difference in median amplitude between nNOS^{+/+} and nNOS^{-/-} mouse detrusor SMCs.

54x18mm (300 x 300 DPI)

Figure 5. NONOate enhanced the spontaneous activity of detrusor in the presence of a selective inhibitor of nitric oxide-sensitive guanylyl cyclase, ODQ (10 μ M), in the nNOS+/+ mouse. (a) In the presence of ODQ, NONOate (100 μ M) significantly increased the frequency of sAPs (b) ODQ (10 μ M) alone did not increase sAP frequency in nNOS+/+ detrusor SMCs (na = 4). Statistical comparisons are one-way ANOVA followed by Tukey's multiple comparison test. ** denotes $P < 0.01$, *** denotes $P < 0.001$. 73x117mm (150 x 150 DPI)

Figure 6. The nNOS inhibitor 7-NI reduced EFS-stimulated detrusor contracture but there was no additional reduction following purinergic desensitisation or cholinergic blockade. (a) 7-NI (100 μM) suppressed the EFS evoked contraction of nNOS+/+ detrusor smooth muscle strips (na=15). (b) P2X1 receptor desensitisation, by α,β-methylene, ATP (3 μM) reduced EFS-evoked contracture but there was no additive reduction of contraction by 7-NI (100 μM) in combination with α,β-methylene ATP (3 μM) (na=12). (c) The force-frequency relationship in the presence of 7-NI (100 μM) and atropine (1 μM) and (cii) contracture at 20 Hz exhibited a borderline significant reduced contracture compared to that in the presence of atropine alone (na=18). See text for details.

74x109mm (150 x 150 DPI)