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REGULAR ARTICLE

Functional nitrergic innervation of smooth muscle structures in the mucosa of pig lower urinary tract

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Abstract

Neurally released nitric oxide (NO) functions as an inhibitory neurotransmitter of urethral but not detrusor smooth muscles while relaxing bladder vasculature and muscularis mucosae (MM). Here, the distribution of nitrergic nerves was examined in the mucosa of pig lower urinary tract using immunohistochemistry, and their vasodilatory functions were studied by measuring arteriolar diameter changes. Properties of smooth muscle cells in the lamina propria (SMC-LP) of urethra and trigone were also investigated using florescence Ca^{2+} imaging. In the bladder mucosa, neuronal nitric oxide synthase (nNOS)–immunoreactive nitrergic fibres projected to suburothelial arterioles and venules. Perivascular nitrergic nerves were intermingled with but distinct from tyrosine hydroxylase (TH)–immunoreactive sympathetic or calcitonin gene–related peptide (CGRP)–immunoreactive afferent nerves. MM receive a nitrergic but not sympathetic or afferent innervation. In the mucosa of urethra and trigone, nitrergic nerves were in close apposition with sympathetic or afferent nerves around suburothelial vasculature but did not project to SMC-LP. In suburothelial arterioles of bladder and urethra, N ω-nitro-L-arginine (L-NA, 100 μM), an NOS inhibitor, enhanced electrical field stimulation (EFS)–induced sympathetic vasoconstrictions, while tadalafil (10 nM), a phosphodiesterase type 5 (PDE5) inhibitor, suppressed the vasoconstrictions. SMC-LP developed asynchronous spontaneous Ca^{2+} transients without responding to EFS. The spontaneous Ca^{2+} transients were enhanced by acetylcholine (1 μM) and diminished by noradrenaline (1 μM) but not SIN-1 (10 μM), an NO donor. In the lower urinary tract mucosa, perivascular nitrergic nerves appear to counteract the sympathetic vasoconstriction to maintain the mucosal circulation. Bladder MM but not SMC-LP receive an inhibitory nitrergic innervation.

Keywords Nitrergic nerve · Urinary bladder · Urethra · Blood vessel · Muscularis mucosae

Abbreviations

α-SMA	α-Smooth muscle actin
ACh	Acetylcholine
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene–related peptide
DSM	Detrusor smooth muscle
EFS	Electrical field stimulation
LUTS	Lower urinary tract symptoms
L-NA	N ω-nitro-L-arginine
MM	Muscularis mucosae
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PDE5	Phosphodiesterase type 5
PSS	Physiological salt solution
SMC	Smooth muscle cell
SMC-LP	Smooth muscle cell in the lamina propria
TH	Tyrosine hydroxylase

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Introduction

In the lower urinary tract, neuronal nitric oxide synthase (nNOS)–immunoreactive nitrergic nerve fibres are abundant in smooth muscle layers of the urethra and bladder trigone of several species including human (Persson et al. 1993; Smet et al. 1996). Consistent with the dense nitrergic innervation, neurally released nitric oxide (NO) plays a predominant role in the relaxation of smooth muscle in the bladder neck and urethra (Persson and Andersson 1992; Werkström et al. 1995; Bustamante et al. 2010), indicating their roles in the opening of bladder outlet preceding the initiation of micturition. Nitrergic nerves are also distributed in the detrusor smooth muscle (DSM) layer of

the bladder, but neurally released NO has only a marginal role in the relaxation of DSM (Persson and Andersson 1992; Smet et al. 1996; Mitsui et al. 2020). DSM even respond poorly to exogenously applied NO (Persson and Andersson 1992; Filippi et al. 2007; Oger et al. 2010) as they do not express NO-sensitive guanylyl cyclase (Lies et al. 2013). Thus, the nitrergic nerves in the DSM layer may have roles other than a DSM relaxant. Nitrergic nerves projecting to smooth muscle layers in the lower urinary tract are considered to be originated from the major pelvic ganglia (Persson et al. 1998; Persson et al. 1998), while pudendal nerves may also include nitrergic fibres (Parlani et al. 1993).

Nitrergic nerves are distributed around the mucosal blood vessels of the lower urinary tract of several species including human (Keast and Kawatani 1994; Persson et al. 1995; Smet et al. 1996), although their projection to arterioles or venules has not been precisely defined. In the bladder mucosa of rats and mice, sympathetic vasoconstrictor fibres project to suburothelial arterioles and venules (Hashitani et al. 2011; Mitsui and Hashitani 2013; Shimizu et al. 2014; Mitsui et al. 2020), and therefore, it was envisaged that nitrergic nerves also innervate the blood vessels to counteract sympathetic vasoconstriction. This notion was supported by our recent study demonstrating the nitrergic vasodilatory transmission in suburothelial arterioles of rat bladder (Tanaka et al. 2021). In addition, nitrergic vasodilatory nerves functionally innervate the pre-capillary arterioles of the mouse bladder mucosa, where a sympathetic innervation is absent (Tanaka et al. 2021). Nevertheless, the distribution and function of nitrergic innervation in suburothelial microvasculature of the urethra or trigone remain to be further explored.

Despite the insignificant role of nitrergic nerves in regulating the contractility of bladder detrusor, electrical field stimulation (EFS) causes transient relaxations of muscularis mucosae (MM) of the pig bladder that are mediated by NO (Mitsui et al. 2020). MM are smooth muscle bundles that form a meshwork in the mucosa of human, pig and guinea pig bladders, but not mouse and rat (Dixon and Gosling 1983; Heppner et al. 2011; Mitsui et al. 2020) and develop spontaneous action potentials (Lee et al. 2018) and associated phasic contractions (Heppner et al. 2011; Isogai et al. 2016; Mitsui et al. 2020). Nevertheless, the nitrergic innervation to MM as a source of endogenous NO has not yet been morphologically demonstrated. In the urethral mucosa of rabbit or pig, sparsely distributed, longitudinally arranged smooth muscle cells (SMCs) have been described (Mattiasson et al. 1985; Brading 1999). We have also reported scatteredly distributed SMCs in the lamina propria (SMC-LP) of pig urethra and trigone (Mitsui et al. 2020). Because of the anatomical distribution of SMC-LP, they may be a cell population similar to bladder MM. However, it remains to be determined if the SMC-LP are spontaneously active and receives an inhibitory nitrergic innervation as the MM in the bladder.

Phosphodiesterase type 5 (PDE5) inhibitors, which enhance endogenous NO-cyclic guanosine monophosphate (cGMP) signalling, have been used for the treatment of lower urinary tract symptoms (LUTS) (Gacci et al. 2016), although their precise cellular target remains to be determined. We have recently reported that tadalafil, a PDE5 inhibitor, enhances the vasodilatory actions of neurally released NO in bladder arteries and arterioles of rats and mice (Tanaka et al. 2021), suggesting that perivascular nitrergic nerves can be a therapeutic target of PDE5 inhibitors. However, it remains to be determined if nitrergic fibres are similarly distributed and/or a target of PDE5 inhibitors in the bladder vasculature of larger animals such as pigs. Moreover, nitrergic innervations and their vasodilatory functions in the suburothelial vasculature of trigone or urethra need to be explored.

In the present study, the distribution of nitrergic nerves in the mucosa of pig bladder, trigone and urethra was compared using immunohistochemistry for nNOS. Co-staining of α-smooth muscle actin (α-SMA) was carried out to reveal nitrergic nerve fibres innervating suburothelial arterioles, venules or other smooth muscle elements, namely, MM in the bladder or SMC-LP in the trigone and urethra. Double-staining of sympathetic or primary afferent fibres with nitrergic fibres were also conducted to visualise their close apposition or co-localisation with nitrergic nerves. In suburothelial arterioles of the bladder and urethra as well as MM, the effects of the blockade of NOS or PDE5 with L-NA or with tadalafil, respectively, on EFS-evoked responses were also examined. In addition, the functional properties of SMC-LP and their innervation were investigated using intracellular Ca^{2+} imaging and immunohistochemistry.

Materials and methods

Ethical approval

Protocols used in the present study were approved by the animal experimentation ethics committee at Nagoya City University Graduate School of Medical Sciences.

Treatment of pig tissues

The bladder and proximal urethra of pigs of both sexes were obtained from a local abattoir. These tissues were immersed in physiological salt solution (PSS) at 4 °C and transported

to the laboratory. The composition of PSS was as follows: 137.5 mM Na^+, 5.9 mM K^+, 2.6 mM Ca^{2+}, 1.2 mM Mg^{2+}, 15.5 mM HCO_3^-, 1.2 mM $H_2PO_4^-$, 134.4 mM Cl^- and 11.5 mM glucose. Small pieces of bladder and urethra were dissected under a dissection microscope. Tissues used were immersed in phosphate buffered saline (PBS) at 4 °C for immunohistochemistry and in PSS for functional experiments.

Immunohistochemistry

Whole mount preparations of the lamina propria of pig bladder or urethra were prepared by removing the smooth muscle layers and the urothelial tissue under the dissection microscope using small scissors and tweezers. Judging from the images of section of pig lower urinary tract in our previous study (Mitsui et al. 2020), the thickness of lamina propria whole mounts of bladder, trigone and urethra are about 1 mm, 500 µm and 500 µm, respectively. Whole mount preparations of DSM layer were also used. Preparation was pinned flat and immersed in Zamboni fixative for 5 min. After removing the pins, preparations were immersed in the same fixative for 4 h at 4 °C, immersed in DMSO to remove picric acid and washed in PBS.

For making cryosections of bladder or urethral smooth muscle layer, the tissues were fixed in a similar way as whole mounts and immersed in PBS containing 30% sucrose for 1 h at 4 °C. They were embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan) and stored in a freezer at − 80 °C. A cryostat was used for making 14 µm sections; they were mounted on MAS-coated glass slide (Matsunami Glass Industry, Osaka, Japan) and dried using a fan.

Immunohistochemical staining was conducted using a protocol described previously (Mitsui and Hashitani 2013). Briefly, whole mount preparations or sections of pig bladder and urethra were immersed in PBS containing 2% bovine serum albumin (BSA) for 10 min, immersed in Block Ace for 20 min and incubated with primary antibodies for 4 days at 4 °C. Tissue was incubated with biotinylated swine anti-rabbit IgG antibody (1:300, Dako, Glostrup, Denmark) for 30 min when the rabbit antibody was used. Tissue was then incubated with two secondary antibodies or a secondary antibody and fluorescence-labelled streptavidin as well as the nuclear staining reagent Hoechst 33,342 (10 µg/ml, Molecular Probes) for 2 h. All specimens were observed using a confocal laser scanning microscope (FV3000; Olympus, Tokyo, Japan). Specimens of at least 3 pigs were used for describing each immunohistochemical characteristic in the present study.

Primary antibodies used in the present study were as follows: goat antibody for neuronal nitric oxide synthase (nNOS, 1:300, Merk Millipore, Darmstadt, Germany), mouse monoclonal antibody for α-smooth muscle actin (α-SMA, 1:200, clone 1A4, Sigma, St. Louis, MO, USA), rabbit antibody for tyrosine hydroxylase (TH, 1:500, Abcam, Cambridge, UK) and rabbit antibody for calcitonin gene–related peptide (CGRP, 1:1000, ImmunoStar, Hudson, WI, USA).

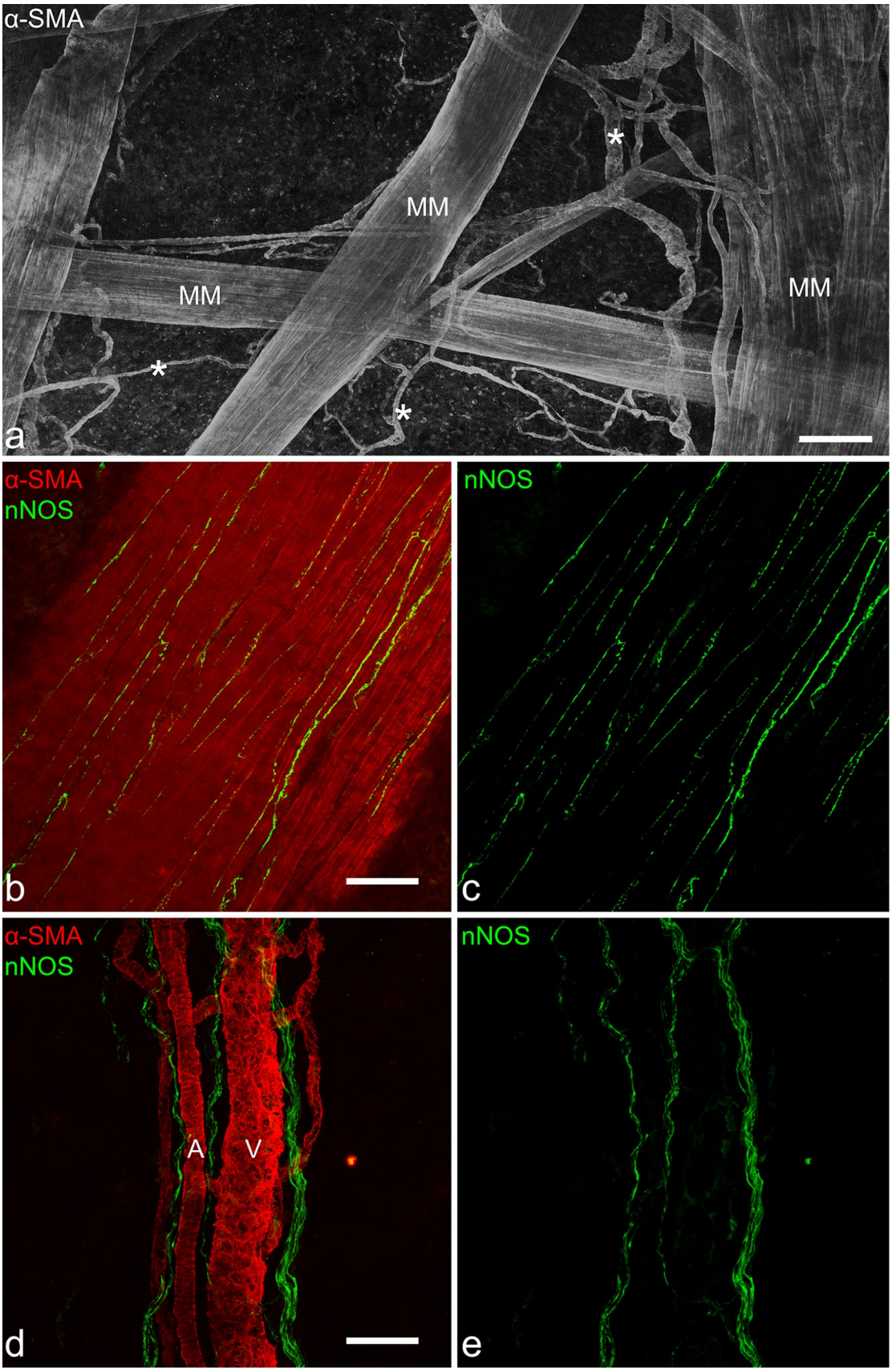
Secondary antibody and the streptavidin used were as follows: Alexa488-conjugated donkey anti-goat IgG antibody (1:500, Molecular Probes, Eugene, OR, USA), TRITC-conjugated rabbit anti-mouse IgG antibody (1:100, Dako), Cy3-conjugated streptavidin (1:200, Jackson ImmunoResearch, West Grove, PA, USA), Cy3-conjugated goat anti-mouse IgG antibody (2.5 µg/ml, Merck-Chemicon, Darmstadt, Germany) and ALEXA488-conjugated streptavidin (10 µg/ml, Molecular Probes, Eugene, OR, USA). TRITC-conjugated rabbit anti-mouse IgG antibody was incubated with rat serum at 10:1 ratio before its dilution to reduce non-specific binding.

Measurement of vascular diameter changes

A flat lamina propria preparation (approximately 5-mm square) of pig bladder containing mucosal microvessels was pinned on the silicon-coated bottom of recording chamber (volume, approximately 2 ml). Preparation was superfused with warmed (36 °C) PSS at a constant flow rate of 1.3 ml/min. Changes in the diameter of mucosal arterioles were monitored with a video camera and analysed in real-time using the edge-tracking software Diamtrak. Electrical field stimulation (EFS; 100 µs duration, 20 Hz for 2 s) was applied to the preparation every 3 min using a pair of platinum plate electrodes.

Contractile studies in MM

Lamina propria preparations of pig bladders containing MM were prepared by removing the DSM layer and urothelium and cut into small strips (about 2–3 mm × 8–10 mm). Silk threads were tied around both ends of the strips, and one thread was fixed at the bottom of organ bath, while the other was connected to an isometric force transducer. Preparations were perfused with warmed (36 °C) PSS bubbled with 95% O_2 and 5% CO_2. Nerve-mediated responses were evoked by EFS (50 µs duration, 20 Hz for 1 s), and neural selectivity of these stimuli was confirmed by their sensitivity to tetrodotoxin (1 µM).


α-SMA
MM
MM
MM
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*
a
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◂**Fig. 1** *Distribution of neuronal nitric oxide synthase (nNOS)–immunoreactive nitrergic nerve fibres in the mucosa of pig bladder.* A low magnification micrograph of pig bladder mucosa immunostained using α-smooth muscle actin (*α-SMA*) antibody is shown (**a**). Various sizes of smooth muscle bundles called muscularis mucosae (*MM*) and mucosal blood vessels (*asterisks*) were detected. Double immunostaining for *α-SMA* (*red*) and *nNOS* (*green*) revealed that varicose nitrergic nerve fibres ran parallel to the long axis of smooth muscle cells of MM (**b**, **c**). An arteriole (*A*) and venule (*V*) revealed by *α-SMA* immunohistochemistry (*red*) were surrounded by *nNOS*-immunoreactive (*green*) nitrergic nerve fibres in the pig bladder mucosa (**d**, **e**). The *scale bars* indicate 200 μm (a), 100 μm (b) and 50 μm (d)

Fluorescence intracellular Ca^{2+} imaging in SMC-LP

The protocol of Ca^{2+} imaging used in the present study was modified from a protocol previously described (Hashitani et al. 2018). Briefly, preparations of the urethral or trigonal lamina propria were incubated with low Ca^{2+} (0.5 mM) PSS containing Cal-520 AM (10 μM, AAT Bioquest, Sunnyvale, CA, USA) and 0.01% cremophor EL for 50 min at 35 °C. Preparations were set into a recording chamber and superfused at 2 ml/min with PSS at 36 °C gassed with 5% CO_2 in oxygen. Intracellular Ca^{2+}dynamics of SMC-LP were viewed using a water immersion objective (UMPlanFL × 10 or × 20; Olympus) and captured by a CCD camera attached to an upright fluorescence microscope (BX51WI, Olympus) and a high-speed scanning polychromatic light source (C7773; Hamamatsu Photonics). Tissues were illuminated at 490 nm, fluorescence emissions above 515 nm were captured through a barrier filter, and images were obtained using a micro photoluminescence measurement system (AQUACOSMOS, Hamamatsu Photonics). The relative amplitude of Ca^{2+} transients was shown as $\Delta F_t/F_0 = (F_t - F_0)/ F_0$ where F_t represents the fluorescence generated by an event, and F_0 indicates the basal fluorescence.

Drugs

Tadalafil, N ω-nitro-L-arginine (L-NA), acetylcholine (ACh), noradrenaline, atropine, propranolol, SIN-1, nifedipine, CPA and U46619 were purchased from Sigma. Tetrodotoxin (TTX) was from Wako Pure Chemical Industries (Osaka, Japan). Tadalafil, SIN-1, CPA and U46619 were dissolved in DMSO, and nifedipine was in ethanol. Other drugs were dissolved in distilled water. Since plasma concentration of tadalafil is maintained above 10 nM even few days after the drug application (single 20 mg dose) in healthy male subjects (Forgue et al. 2006), 10–100 nM can be considered as clinically relevant concentration and was used in the present study.

Data analysis

Data in functional experiments are represented as mean ± SD. A normality test followed by two-tailed paired or unpaired Student's *t*-test (for two groups) or one-way repeated measures ANOVA followed by Bonferroni post hoc test (for multiple groups) was used for statistical analysis. $P < 0.05$ is statistically significant. The number of pigs used for each experiment was indicated as *n*.

Results

Distribution of nitrergic nerves in the lamina propria of pig bladder

In whole mount preparations of lamina propria of pig bladder (non-trigonal region), bundles of α-smooth muscle actin (α-SMA)–immunoreactive smooth muscle cells (SMCs), namely, the muscularis mucosae (MM), were distributed as previously reported (Mitsui et al. 2020) (Fig. 1a). Suburothelial arterioles and venules were also identified in the same layer by their α-SMA immunoreactivity in vascular SMCs (Fig. 1a). Arterioles and venules could readily be distinguished by the different morphology of the α-SMA-immunoreactive mural cells (Fig. 1d). Thus, arteriolar SMCs were circumferentially oriented and tightly packed, while venular SMCs extended several processes in various directions and were more sparsely distributed (Mitsui and Hashitani 2013; Hashitani et al. 2018). Double immunostaining for α-SMA and neuronal nitric oxide synthase (nNOS) revealed that varicose nNOS-positive nitrergic fibres innervated MM (Fig. 1b, c). Perivascular nitrergic nerve fibres immunoreactive to nNOS also projected to suburothelial arterioles and venules (Fig. 1d, e). In the pig bladder mucosa, intrinsic nNOS-positive nitrergic neurons reported in the mouse bladder mucosa (Tanaka et al. 2021) were not detected.

Nitrergic nerves intermingled with sympathetic and primary afferent nerves around bladder suburothelial vasculatures

In the bladder lamina propria double immunostaining for nNOS and TH, a sympathetic nerve marker, showed that perivascular nitrergic nerves were intermingled with sympathetic nerve fibres (Fig. 2a–d), while the neighbouring MM only received nitrergic nerve fibres (Fig. 2c). Varicose both nitrergic and sympathetic nerve fibres projected to the mucosal arterioles and venules (Fig. 2e–h). At higher magnification, the varicosities of perivascular nitrergic and

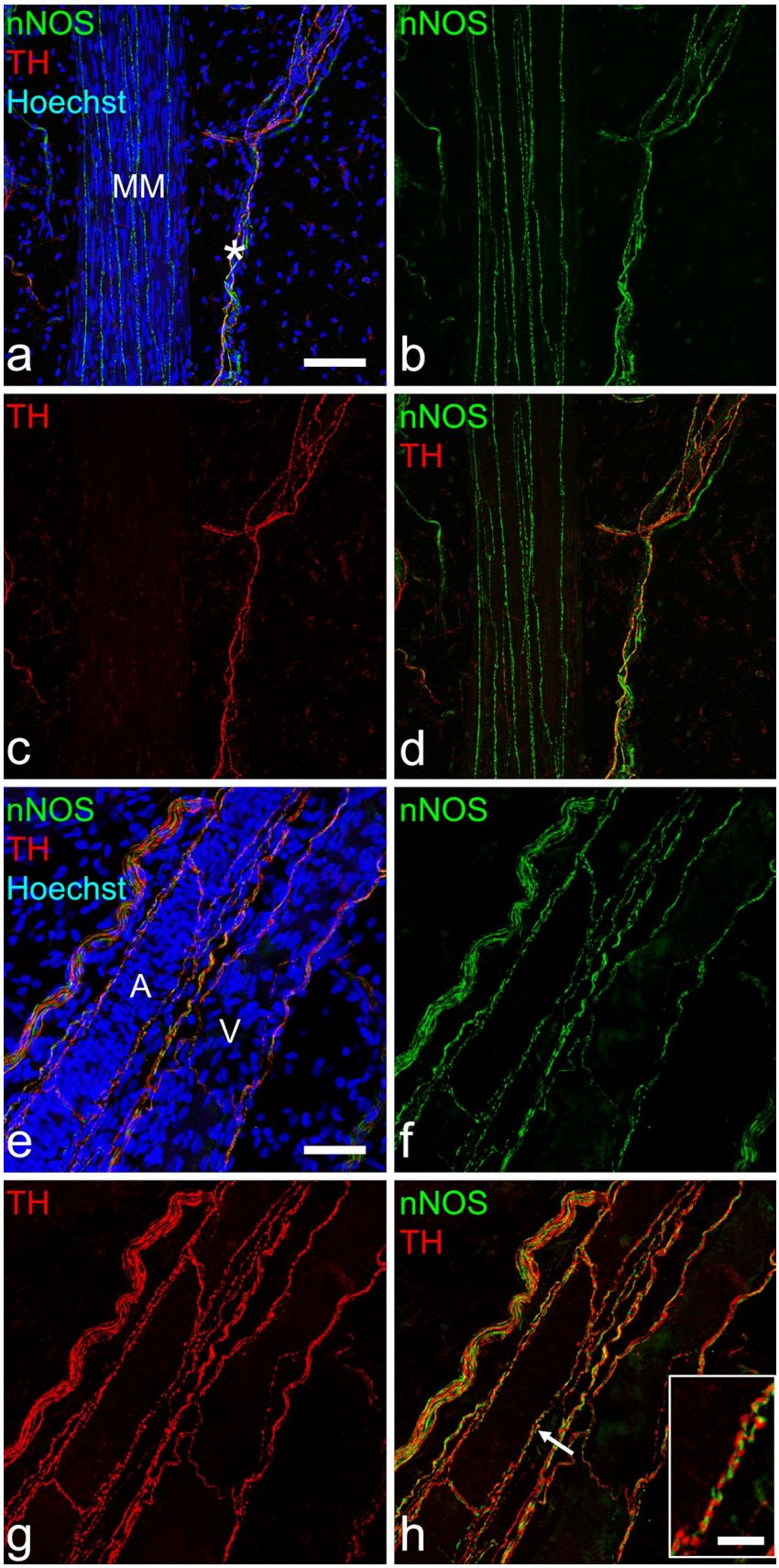

Fig. 2 *Comparison between projection patterns of nitrergic and sympathetic nerve fibres in the mucosa of pig bladder.* A mucosal whole mount preparation of pig bladder that contained the muscularis mucosae (*MM*) and blood vessel (*asterisk*) was immunostained for *nNOS* (*green*) and *TH* (*red*), while Hoechst 33342 was used for nuclear staining (*Hoechst*, *blue*) (**a**). In this specimen, varicose nitrergic nerve fibres projected to both MM and vasculatures, while sympathetic fibres immunoreactive for TH only projected to the vasculatures but not MM (**b**–**d**). In another specimen, a mucosal arteriole (*A*) and venule (*V*) have nitrergic and sympathetic innervations (**e**–**h**). An enlarged image of these two types of perivascular nerves indicated by an *arrow* in h showed that their varicosities were not colocalised (**h**, *inset*). Thus, these nitrergic fibres were distinct from sympathetic fibres. The *scale bars* indicate 100 µm (a, e) and 25 µm (inset of h)

sympathetic fibres were not colocalised (Fig. 2h inset), confirming that these nitrergic nerves are distinct from sympathetic fibres.

Double immunostaining for nNOS and calcitonin gene–related peptide (CGRP) showed that perivascular nitrergic nerves were intermingled with but distinct from CGRP-immunoreactive primary afferent nerve fibres (Fig. 3a–d). In contrast to MM in the guinea pig bladder, in which CGRP-containing nerve fibres are abundantly distributed (Lee et al. 2016), CGRP-immunoreactive afferent nerve fibres seldom projected to the MM in the pig bladder (Fig. 3c). Double immunostaining for α-SMA and CGRP showed that CGRP-immunoreactive afferent fibres were projected around both the mucosal arterioles and venules in the pig bladder (Fig. 3e, f).

Nitrergic nerve distribution in bladder DSM layer

The distribution of nitrergic nerves in the DSM layer was examined for a comparison. In whole mount preparations of DSM, nNOS-positive nitrergic nerve fibres including some varicose single fibres were distributed (Fig. 4a, b). In the sections of DSM, nitrergic nerves were found within the smooth muscle bundles as well as the septa (Fig. 4c, d).

Nitrergic nerve distribution in the urethral wall

In whole mount preparations of lamina propria of pig urethra, α-SMA-immunoreactive SMC-LP were scattered and predominantly arranged in the longitudinal direction (Fig. 5a), but smooth muscle bundles comparable to the bladder MM were virtually absent. In contrast to the bladder MM, SMC-LP did not receive a nitrergic innervation, while varicose nitrergic fibres projected to neighbouring vascular SMCs of suburothelial arterioles and venules (Fig. 5b). Consistent with the functional nitrergic innervation to urethral SMCs (see Introduction), nitrergic nerves were distributed within the smooth muscle bundles as well as the septa in the sections of urethral musculature (Fig. 5c, d).

Nitrergic nerves intermingled with sympathetic and primary afferent nerves in suburothelial vasculature of the urethra

Double immunostaining for nNOS and TH revealed that perivascular nitrergic nerves around suburothelial arterioles and venules of the urethra were intermingled with but distinct from varicose sympathetic fibres (Fig. 6a–d). Double immunostaining for nNOS and CGRP demonstrated that perivascular nitrergic nerves are in close proximity with CGRP-containing primary afferent nerves in both arterioles and venules of the urethra (Fig. 6e–h). Neither sympathetic nor afferent fibres projected to SMC-LP (not shown). Intrinsic nNOS-immunoreactive nitrergic neurons were not detected in the pig urethral mucosa.

Nitrergic nerve distribution in the trigonal wall

The distribution pattern of nitrergic nerve fibres in the lamina propria of pig trigone resembled that observed in the pig urethra. Perivascular nitrergic fibres of mucosal vasculature in the trigone were intermingled with but distinct from sympathetic fibres (Fig. 7a–d), while SMC-LP scattered in the mucosa of trigone did not receive a nitrergic innervation (Fig. 7e, f). In sections of the pig trigone, nitrergic fibres were distributed in the smooth muscle layer (Fig. 7g, h).

Functional nitrergic innervation to suburothelial arterioles of the bladder and urethra

In suburothelial arterioles of the pig bladder mucosa, EFS (100 μs duration, 20 Hz for 2 s) often failed to evoke a vasoconstriction; any EFS-induced vasoconstrictions were transient. L-NA (100 μM) unmasked or enhanced the EFS-induced vasoconstrictions ($n = 9$) (Fig. 8a). EFS-induced arteriolar constrictions were abolished by tetrodotoxin (1 μM, $n = 3$) or sympathetic nerve depletion with guanethidine (10 μM, $n = 3$), indicating they are mediated by sympathetic nerves.

In 6 bladder mucosa preparations in which EFS evoked consistent arteriolar vasoconstrictions without L-NA (17.2 ± 8.9% of resting diameter, n = 6), 10 nM tadalafil attenuated EFS-induced vasoconstrictions (6.5 ± 7.4% of resting diameter, $P < 0.05$) (Fig. 8b) without changing the resting diameter (46.9 ± 24.7 μm, ranging from 15.5 μm to 79.4 μm, 46.5 ± 22.6 μm in tadalafil). In 3 preparations, subsequent application of L-NA (100 μM) enlarged the EFS-evoked constrictions from 6.7 ± 10.4% ($n = 3$) to 23.8 ± 18.8% of the resting diameter (Fig. 8b).

In suburothelial arterioles of the urethral mucosa where EFS failed to evoke sympathetic vasoconstrictions, L-NA unmasked their generation (n =3, Fig. 8c). In 3 other preparations in which EFS evoked arteriolar vasoconstriction in the absence of L-NA, tadalafil (10 nM) suppressed the vasoconstrictions in a manner sensitive to L-NA (Fig. 8d). EFS-induced constrictions in the urethral arteriole were abolished by tetrodotoxin (1 μM, $n = 3$) and largely suppressed ($n = 3$) by guanethidine (10 μM), indicating that the response is predominantly mediated by sympathetic nerves.

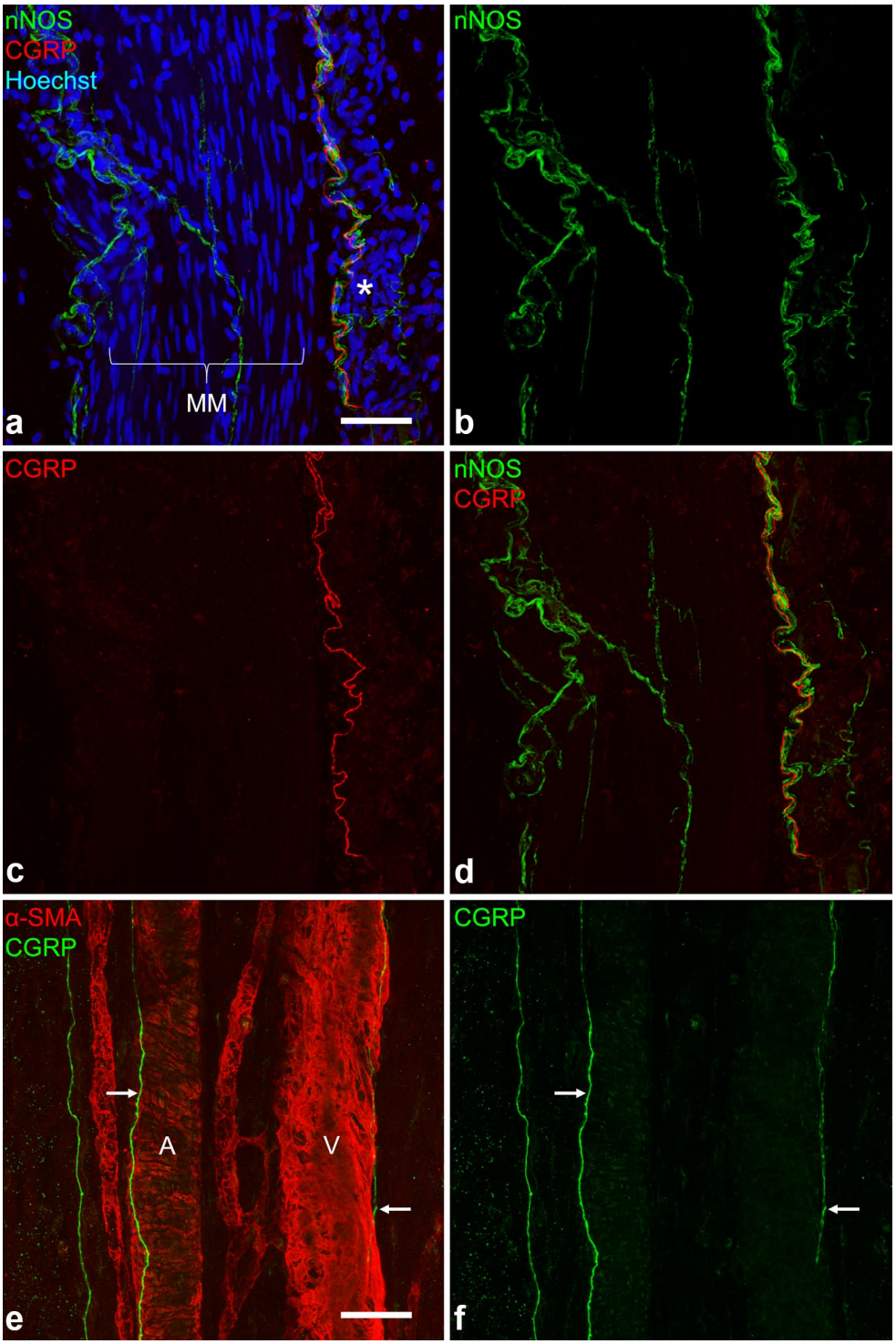

Fig. 3 *Comparison between projection patterns of nitrergic and afferent nerve fibres in the mucosa of pig bladder.* Double immunostaining for *nNOS* (*green*) and calcitonin gene–related peptide (*CGRP, red*) combined with nuclear staining (*Hoechst*, *blue*) was conducted using a mucosal whole mount of pig bladder (**a**–**d**). Nitrergic nerves projected to the muscularis mucosae (*MM*) and a blood vessel (*asterisk*), while CGRP-positive primary afferent nerve fibres only projected to the mucosal vasculature but not MM. Double immunostaining for *α-SMA* (*red*) and *CGRP* (*green*) showed that CGRP-positive afferent fibres (*arrows*) projected to both a mucosal arteriole (*A*) and venule (*V*) (**e**, **f**). *Scale bars* indicate 50 µm

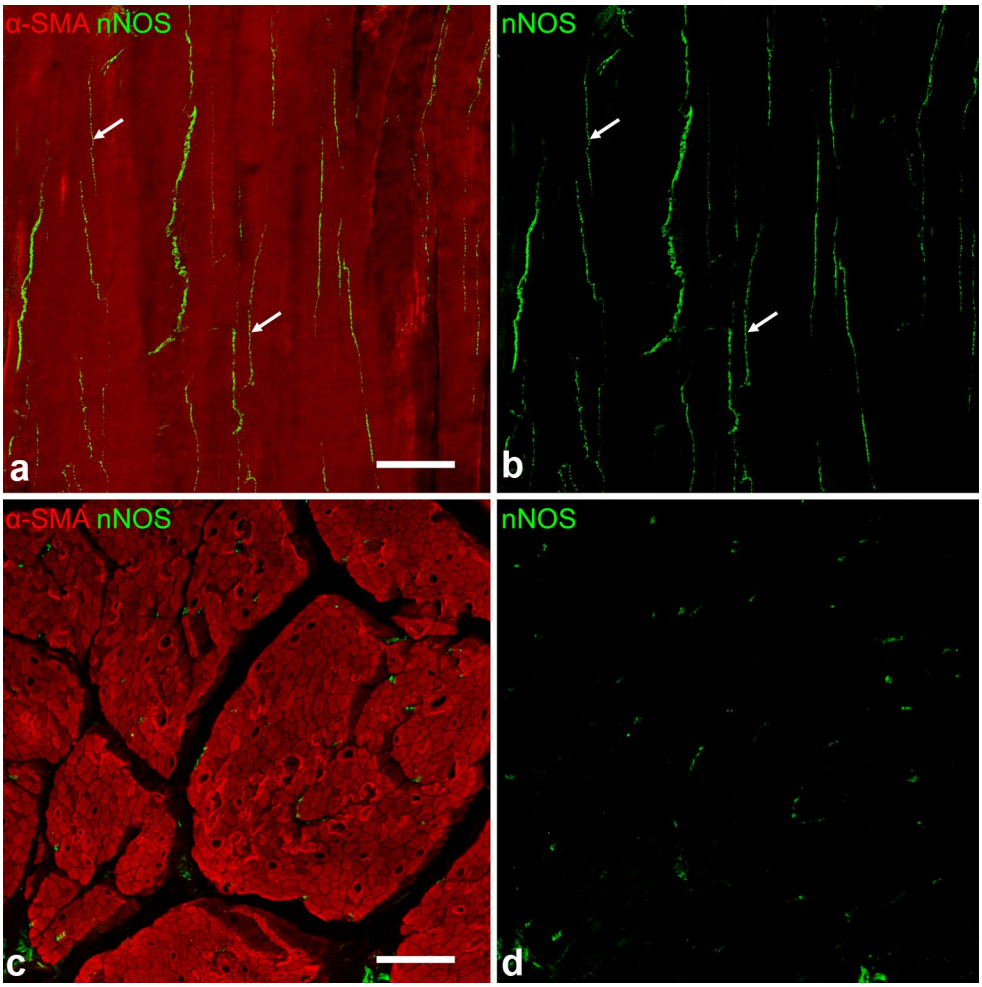

Fig. 4 *Distribution of nitrergic nerve fibres in the detrusor of pig bladder.* In a whole mount preparation of pig detrusor where smooth muscle cells were visualised by *α-SMA* immunoreactivity (*red*), *nNOS*-immunoreactive (*green*) nitrergic nerve fibres were detected (**a**, **b**). Some of them were varicose single nerve fibres (*arrows*). In a section of pig detrusor, nitrergic nerves were found within smooth muscle bundles as well as in space between the bundles (**c**, **d**). The *scale bars* indicate 100 µm (a) and 50 µm (c)

Roles of endogenous NO in modulating nerve-evoked changes in MM contractility

In MM strips that had been pre-treated with U46619 (100 nM) and atropine (1 µM), EFS evoked an NO-mediated transient relaxation as reported previously (Mitsui et al. 2020). Tadalafil (100 nM) prolonged the half-width of EFS-induced relaxations without increasing their amplitude ($n = 7$, Fig. 9a–d).

In MM strips in which spontaneous phasic contractions were present, EFS evoked a phasic contraction that was predominantly mediated by neurally released ACh (see also Mitsui et al. 2020). Tadalafil (100 nM) did not affect either the amplitude or half-width of the EFS-induced contractions, while subsequent L-NA (10 µM) increased their amplitude and half-width ($n = 6$, Fig. 9e–i).

Properties of spontaneous Ca^{2+} transients in SMC-LP

SMC-LP in the urethra (Fig. 10a, supplementary video 1) and trigone (Fig. 10b, supplementary video 2) developed spontaneous Ca^{2+} transients. Spontaneous Ca^{2+} transients in individual SMC-LP were generated independently of each other, but displayed near synchronicity in two or three neighbouring SMC-LP. The mean amplitude, half-width or frequency of spontaneous Ca^{2+} transients in SMC-LP were not different between the urethra ($n = 21$) and trigone ($n = 24$) (Fig. 10c–e). In both urethra ($n = 5$) and trigone ($n = 6$), spontaneous Ca^{2+} transients in SMC-LP were abolished or largely suppressed by nifedipine (1 µM), an L-type voltage-dependent Ca^{2+} channel blocker. Residual spontaneous Ca^{2+} transients were abolished by CPA (10 µM), the blocker of sarco-endoplasmic reticulum Ca^{2+} ATPase, associated with

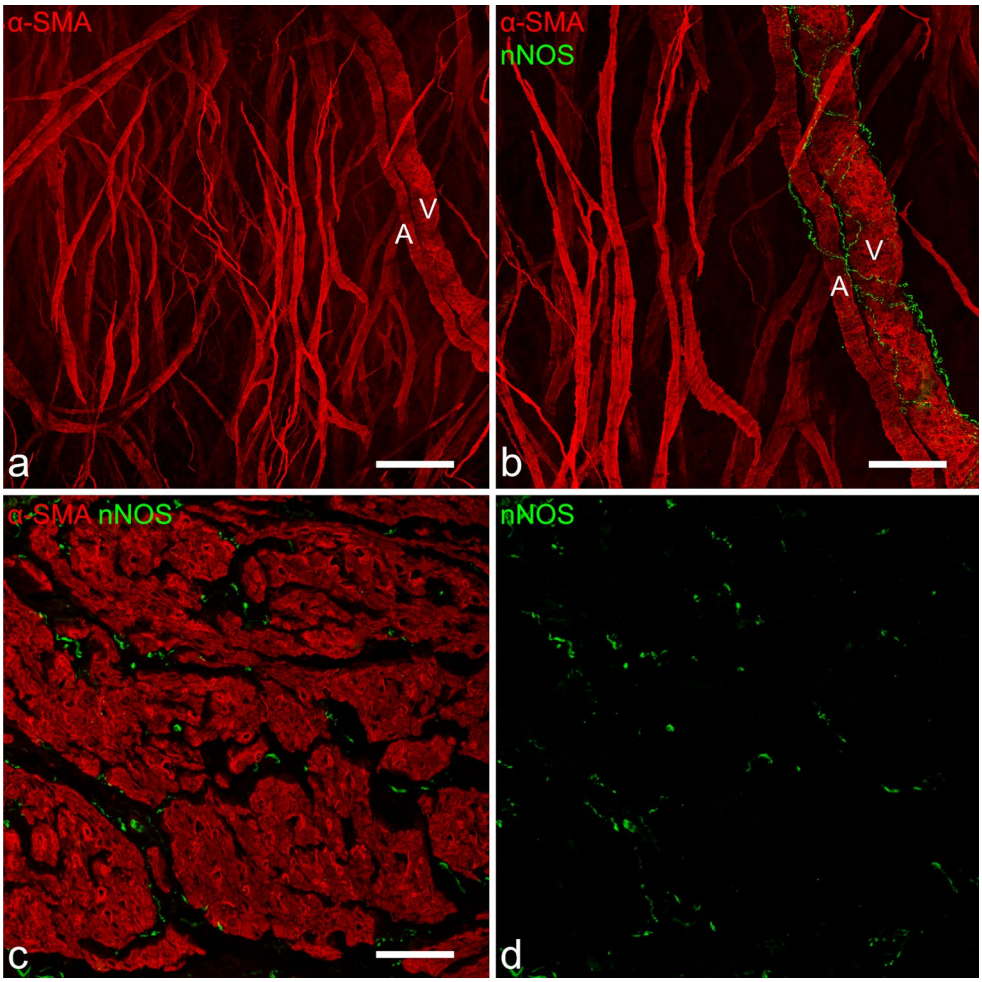

Fig. 5 *Distribution of nitrergic nerve fibres in the pig urethra.* In a whole mount specimen of the mucosa of pig urethra immunostained for *α-SMA*, scattered smooth muscle cells (SMCs) and vascular SMCs of arteriole (*A*) and venule (*V*) were detected (**a**). In contrast to the bladder, the muscularis mucosae, i.e., large SMC bundles, were not found. An enlarged image of the same preparation with *nNOS* immunoreactivities (*green*) is presented (**b**). Nitrergic nerve fibres projected to the vascular SMCs of arteriole (*A*) and venule (*V*) but not the scattered mucosal SMCs. In a section of smooth muscle layer of pig urethra, nitrergic fibres were detected (**c**, **d**). The *scale bars* indicate 200 µm (a), 100 µm (b) and 50 µm (c)

a rise in the basal Ca^{2+} level (urethra, $n=4$; trigone, $n=3$). In both urethra and trigone, SMC-LP did not respond to EFS (10 or 20 Hz, 1 s), while arteriolar SMCs developed EFS-induced Ca^{2+} transients, and thus, SMC-LP appear to lack a functional innervation.

Effects of bath-applied neurotransmitters on spontaneous Ca^{2+} transients in urethral SMC-LP were examined. Bath-applied noradrenaline (1 µM) slowed and suppressed spontaneous Ca^{2+} transients or abolished their generation with a reduction in the basal Ca^{2+} level in a manner sensitive to propranolol, a β-adrenoceptor antagonist (1 µM, Fig. 11a). Effects of noradrenaline (1 µM) and subsequent propranolol (1 µM) on the amplitude and frequency of spontaneous Ca^{2+} transients were summarised (Fig. 11b, c). Bath-applied ACh (1 µM) transiently accelerated spontaneous Ca^{2+} transients and then ceased their generation with a sustained raise in the basal Ca^{2+} level in a manner sensitive to atropine, a muscarinic receptor antagonist (1 µM, Fig. 11d). Effects of ACh (1 µM) and subsequent atropine (1 µM) on the amplitude and frequency of spontaneous Ca^{2+} transients were summarised (Fig. 11e, f). Bath-applied SIN-1 (10 µM), an NO donor, failed to affect the amplitude (0.63 ± 0.15 in control, 0.63 ± 0.13 in tadalafil, $P > 0.05$, $n=7$) or frequency (4.3 ± 0.91 in control, 4.3 ± 0.95 in tadalafil, $P > 0.05$, $n=7$) of spontaneous Ca^{2+} transients.

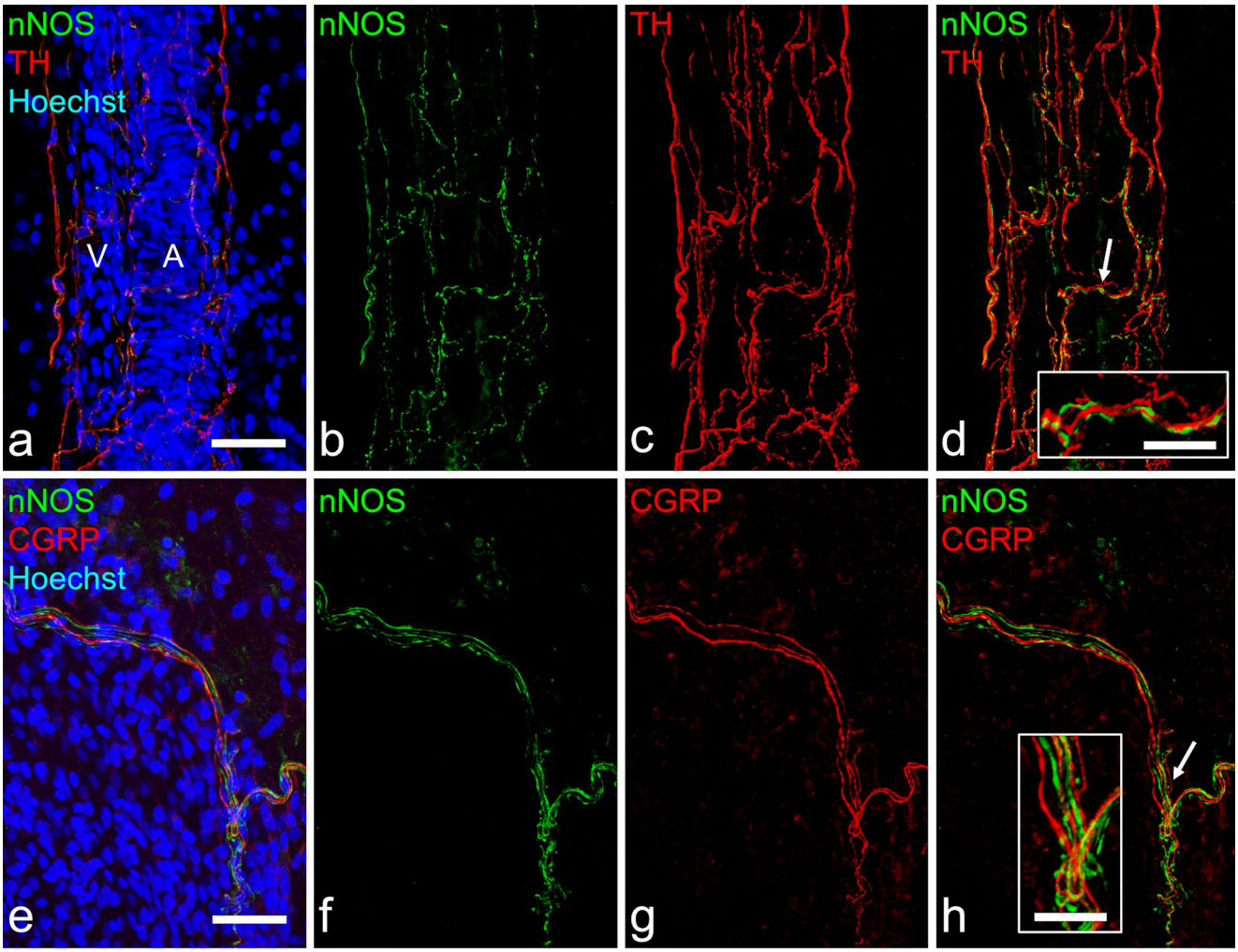

Fig. 6 *Perivascular nitrergic nerves running parallel to sympathetic and afferent nerves in the pig urethral mucosa.* In a whole mount preparation of pig urethral mucosa, *nNOS*-positive nitrergic nerves (*green*) and *TH*-positive sympathetic nerves (*red*) project to both the mucosal arteriole (*A*) and venule (*V*) (**a**–**d**). An area indicated by an *arrow* in d was enlarged to show that these two types of nerves are distinct from each other (**d**, *inset*). Perivascular calcitonin gene–related peptide (*CGRP, red*)–immunoreactive afferent fibres were intermingled with nitrergic fibres in the pig urethral mucosa (**e**–**h**). They are distinct types of nerves (**h**, *inset*). The *scale bars* indicate 50 µm (a, e), and 20 µm (insets of d and h)

Discussion

Nitrergic innervation to suburothelial vasculatures in pig bladder and urethra

In both bladder and urethra of pigs, varicose nitrergic nerve fibres were detected around the mucosal arterioles and venules (Fig. 12). These nNOS-positive perivascular nitrergic nerves were intermingled with but not colocalised with TH- or CGRP-positive perivascular nerves, indicating that nitrergic fibres are distinct from sympathetic or afferent fibres. This is consistent with a previous study demonstrating that chemical denervation of sympathetic nerves with 6-hydroxydopamine or primary afferent denervation with capsaicin has no effect on nerve-evoked, NO-mediated relaxations (Persson et al. 1997). Thus, it is unlikely that nNOS-containing nerves are in fact sympathetic or afferent nerves.

In the rat major pelvic ganglion, NOS-immunoreactive cell bodies also display choline acetyltransferase immunoreactivity (Persson et al. 1998). Nitrergic relaxations induced by EFS in the rat urethral smooth muscles are abolished by bilateral cryoganglionectomy of the major pelvic ganglion (Persson et al. 1998), suggesting that nitrergic nerves projecting to the lower urinary tract are predominantly parasympathetic origin. Consistently, some NOS-immunoreactive nerves co-express acetylcholine esterase in the pig lower urinary tract (Persson et al. 1995).

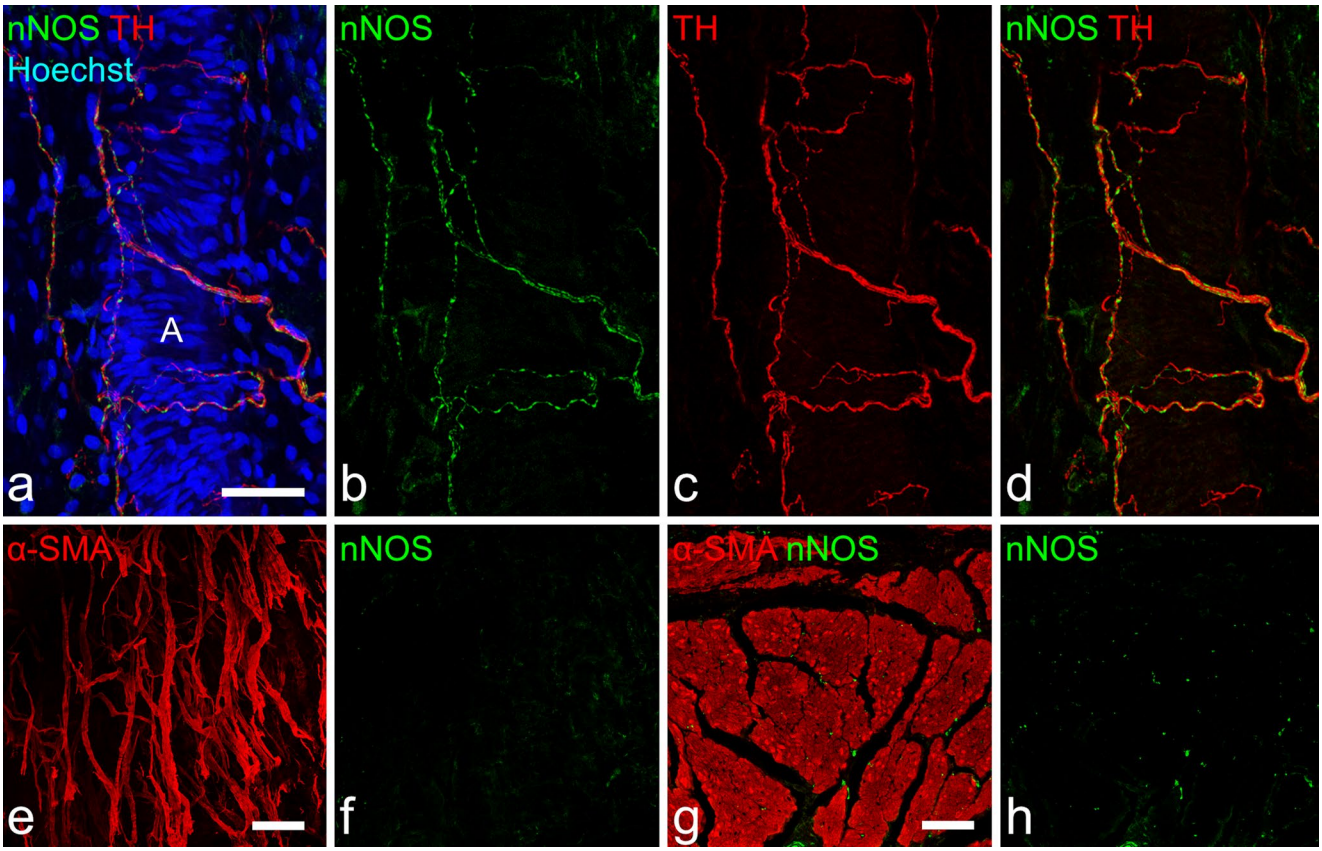

Fig. 7 *Distribution of nitrergic nerve fibres in the pig bladder trigone.* In a mucosal whole mount of pig trigone, *nNOS*-positive nitrergic (*green*) and *TH*-positive sympathetic (*red*) nerves were detected around an arteriole (*A*) (**a**–**d**). In a different whole mount, scattered smooth muscle cells were detected by *α-SMA* immunoreactivity; these cells lacked the innervation of nNOS-immunoreactive fibres (**e**, **f**). Nitrergic nerve fibres were detected in the section of smooth muscle layer of pig trigone (**g**, **h**). The *scale bars* indicate 50 μm (a, g) and 100 μm (e)

In the pig urethra, falls in the urethral pressure during voiding are accompanied by a rise in the mucosal blood flow (Greenland et al. 1996). Considering parasympathetic nerves dominant neural activity in the lower urinary tract during voiding phases, the increased blood flow likely results from arteriolar dilatation that is mediated by NO released from parasympathetic nerves. Neurally released NO would also reduce the arteriolar resistance in the bladder despite the extravascular compression by detrusor contractions, minimising the reduction in the bladder blood flow during voiding (Greenland and Brading 1996).

Functions of nitrergic transmission in suburothelial arterioles of bladder and urethra

Nitrergic nerve–mediated vasodilation responses in peripheral tissues are generally not clearly detectable (Toda and Okamura 2015), although perivascular nitrergic nerve–mediated dilation is evident in the cerebral artery (Toda and Okamura 1990). In suburothelial arterioles of the rat bladder, EFS induces guanethidine-sensitive sympathetic constrictions that are mediated by α-adrenoceptors (Hashitani et al. 2011). In suburothelial venules, sympathetic transmission accelerates spontaneous phasic constrictions via α-adrenoceptors activation, while exerting a β-adrenoceptor-mediated vasodilatory action that appears to involve endothelial NO release (Shimizu et al. 2014). Therefore, it was thought that the contractility of suburothelial arterioles and venules in the bladder are predominantly regulated by sympathetic nerves. However, our recent study demonstrated functional nitrergic transmission in suburothelial arterioles of rat and mouse bladder (Tanaka et al. 2021), highlighting the roles of neurally released NO in modulating vascular contractility.

In the present study, L-NA unmasked or enhanced EFS-induced suburothelial arteriolar constrictions of pig bladder or urethra and also reduced their basal diameter, indicating that NO is continuously released presumably from the endothelium. Time-dependent loss of EFS-induced vasoconstrictions seen in the present study is likely due

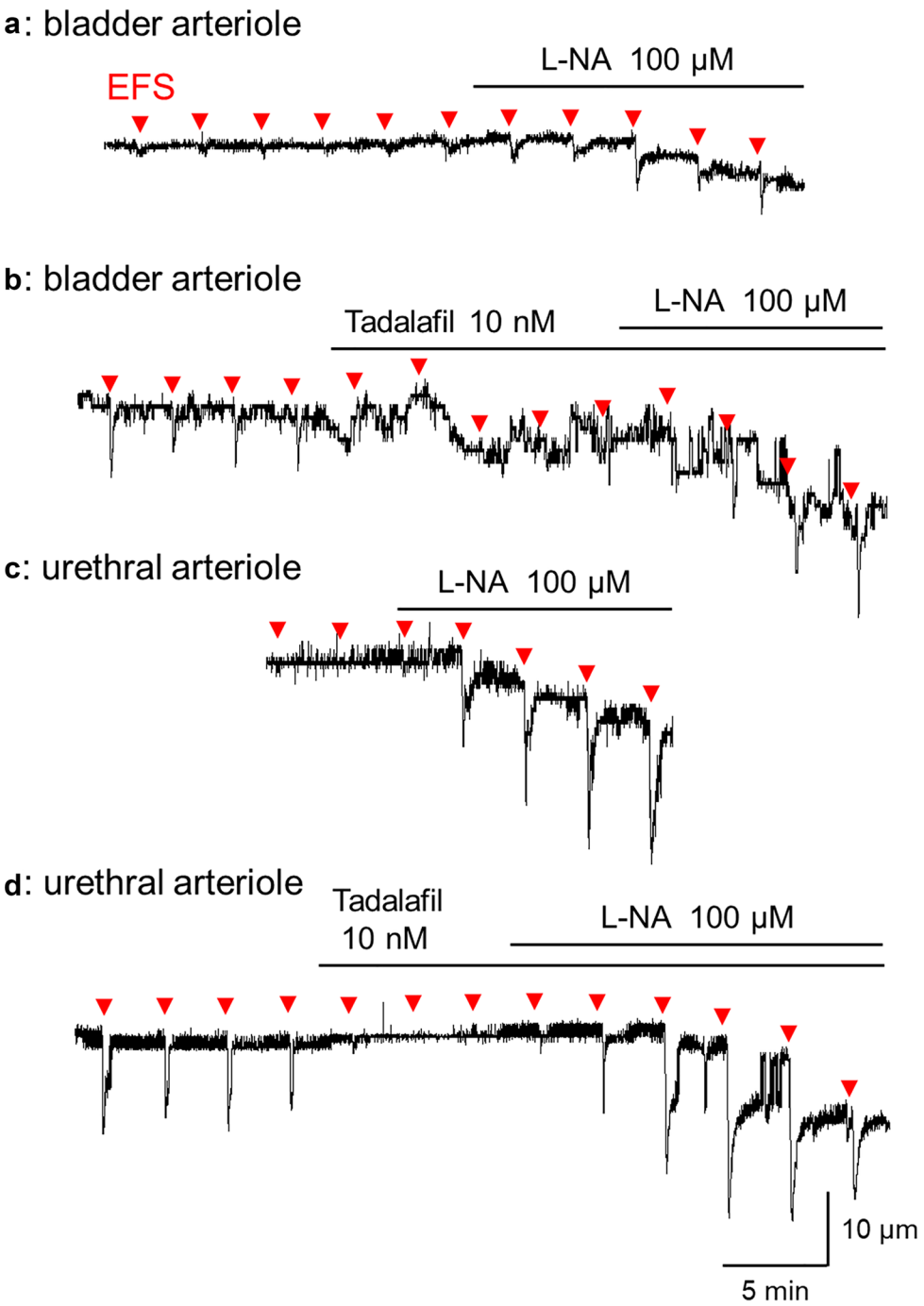

Fig. 8 *Effects of PDE5 inhibitor tadalafil on contractility of mucosal vasculatures in the pig bladder and urethra.* Electrical field stimulation (EFS; 100 μs duration, 20 Hz, 2 s) indicated by *red arrowheads* did not distinctly changed the diameter of arteriole in a pig bladder mucosa preparation, while, in the presence of L-NA, it induced a diameter reduction (i.e., vasoconstriction) (**a**). L-NA reduced the resting diameter of arteriole. In a different bladder mucosa preparation, EFS-induced an arteriolar constriction which was inhibited by tadalafil (**b**). Subsequent L-NA enlarged EFS-induced constriction. In a mucosal preparation of pig urethra, EFS had no effect, while EFS-induced constriction was detected in the presence of L-NA (**c**). In a different urethral preparation, EFS evoked a constriction which was abolished by tadalafil (**d**). EFS-induced constriction was reappeared after subsequent L-NA application. Resting diameters were 35 μm (a), 78 μm (b), 62 μm (c) and 56 μm (d). Scale bars in d apply to all traces

to the increased production of endothelial NO with time under our in vitro experimental conditions. Consistent with the findings in rat bladder arterioles (Tanaka et al. 2021), tadalafil, a PDE5 inhibitor, diminished EFS-induced constrictions of suburothelial arterioles in the pig bladder and urethra without changing their basal diameter, suggesting that the blockade of PDE5 enhances the cGMP-dependent relaxation in arteriolar smooth muscle cells that was triggered by neurally released NO. Unfortunately, L-NPA, an nNOS-specific inhibitor, that enhances EFS-induced constrictions in rodent bladder arterioles without changing the basal diameter (Tanaka et al. 2021) had no effect in the pig vasculature, possibly due to the splicing variant in nNOS amongst different species. In the mucosal blood vessels of rat bladder expressing PDE5 (both in vascular smooth muscle cells and endothelial cells), the PDE5 inhibitor improves bladder hypoxia in spontaneously hypertensive rats (Morelli et al. 2010). Therefore, nitrergic nerve–mediated inhibition of the contractility in bladder microvessels (Tanaka et al. 2021, the present study)

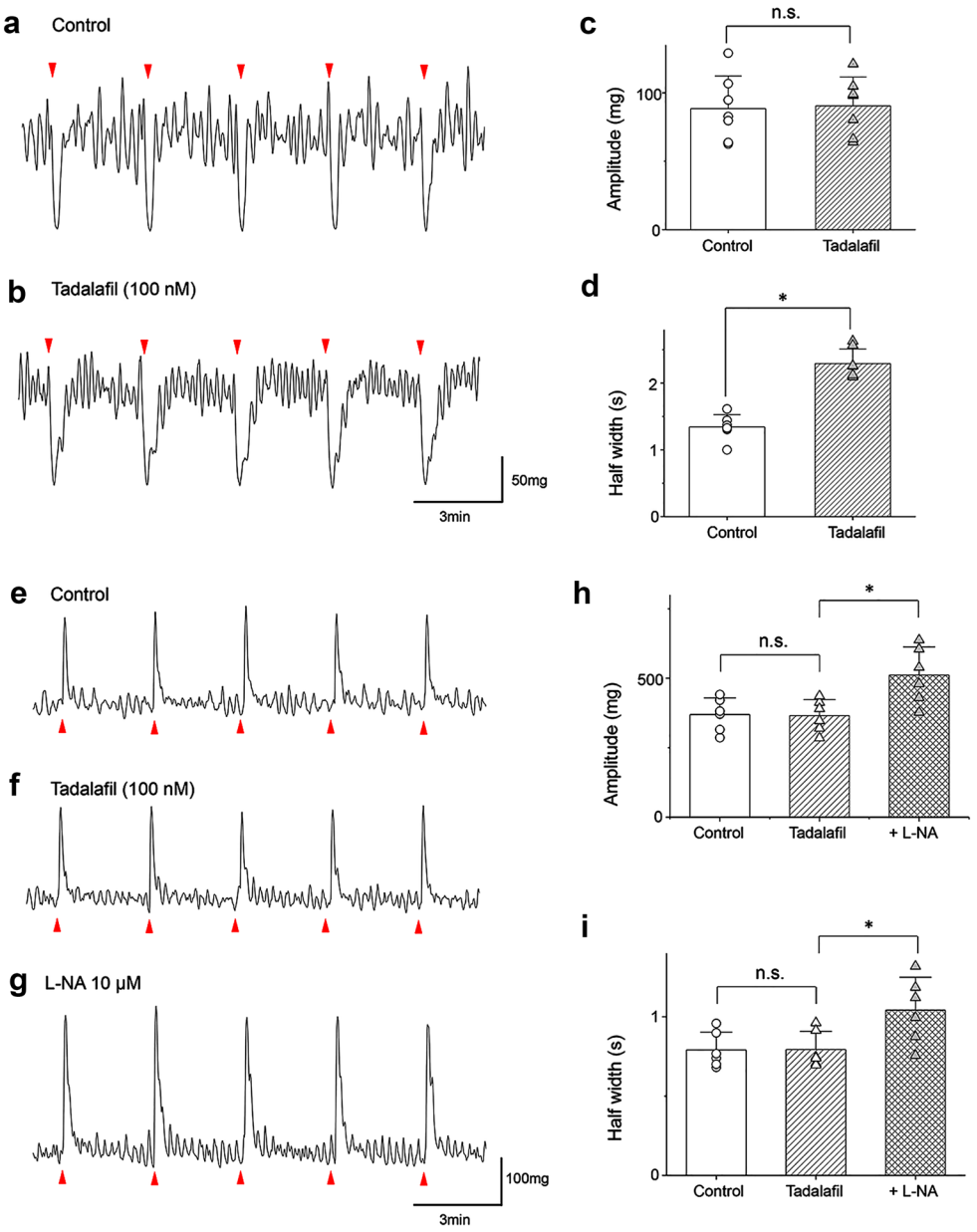

Fig. 9 *Roles of nitrergic innervation in modulating nerve-evoked relaxation/contractions in MM.* **a** In a MM strip that had been pre-constricted with U46619 (100 nM), a thromboxane A2 receptor agonist, and atropine (1 μM), EFS (20 Hz, 1 s every 3 min, *red arrowheads*) evoked transient relaxations. **b** Tadalafil (100 nM) increased the half-width (**c**) of EFS-induced relaxations without changing their amplitude (**d**). Two-tailed paired Student's *t*-test, $*P < 0.05$. **e** In a different MM strip generating spontaneous phasic contractions, EFS evoked phasic contractions. **f** Tadalafil (100 nM) did not affect either the amplitude or half-width of the EFS-induced contractions. **g** Subsequent L-NA (10 μM) increased the amplitude and half-width of the EFS-induced contractions. Effects of tadalafil and subsequent L-NA on the amplitude (**h**) and half-width (**i**) of the EFS-induced contractions were summarised. One-way repeated measures ANOVA with Bonferroni post hoc analysis, $*P < 0.05$

appears to be involved in the bladder protective actions of PDE5 inhibitors. Since the mucosa of lower urinary tract plays a fundamental role in sensing the tissue environment, adequate blood supply to this layer is crucial for maintaining the normal bladder functions. In this regard, a complex and counterbalancing control of arteriolar diameter has physiological relevance.

Arteries rather than arterioles play an important role in regulating blood flow control in the cheek pouch circulation of anaesthetised hamsters (Davis et al. 1986). However, both arterioles and arteries regulate peripheral resistance in the mesenteric circulation of conscious rats (Christensen and Mulvany 1993; Fenger-Gron et al. 1997). The distribution of blood flow within the bladder wall, e.g., between mucosal and muscle layers, is likely to be regulated by the arterioles investigated in the present study, while the upstream arteries may be also involved in the blood flow regulation. This notion appears to be consistent with observation that bladder feeding arteries (vesical arteries) are also regulated by nitrergic nerves (Tanaka et al. 2021).

The inhibition of sympathetic nerve–mediated vasoconstrictions by neurally released NO is likely due to its inhibitory actions on vascular SMCs. In addition, the proximity of nitrergic and sympathetic nerve fibres in the mucosal vasculatures of pig lower urinary tract may allow NO-induced presynaptic inhibition of sympathetic transmitter release. In the rat mesenteric artery where perivascular nitrergic and sympathetic nerves are in close proximity, an NOS inhibitor

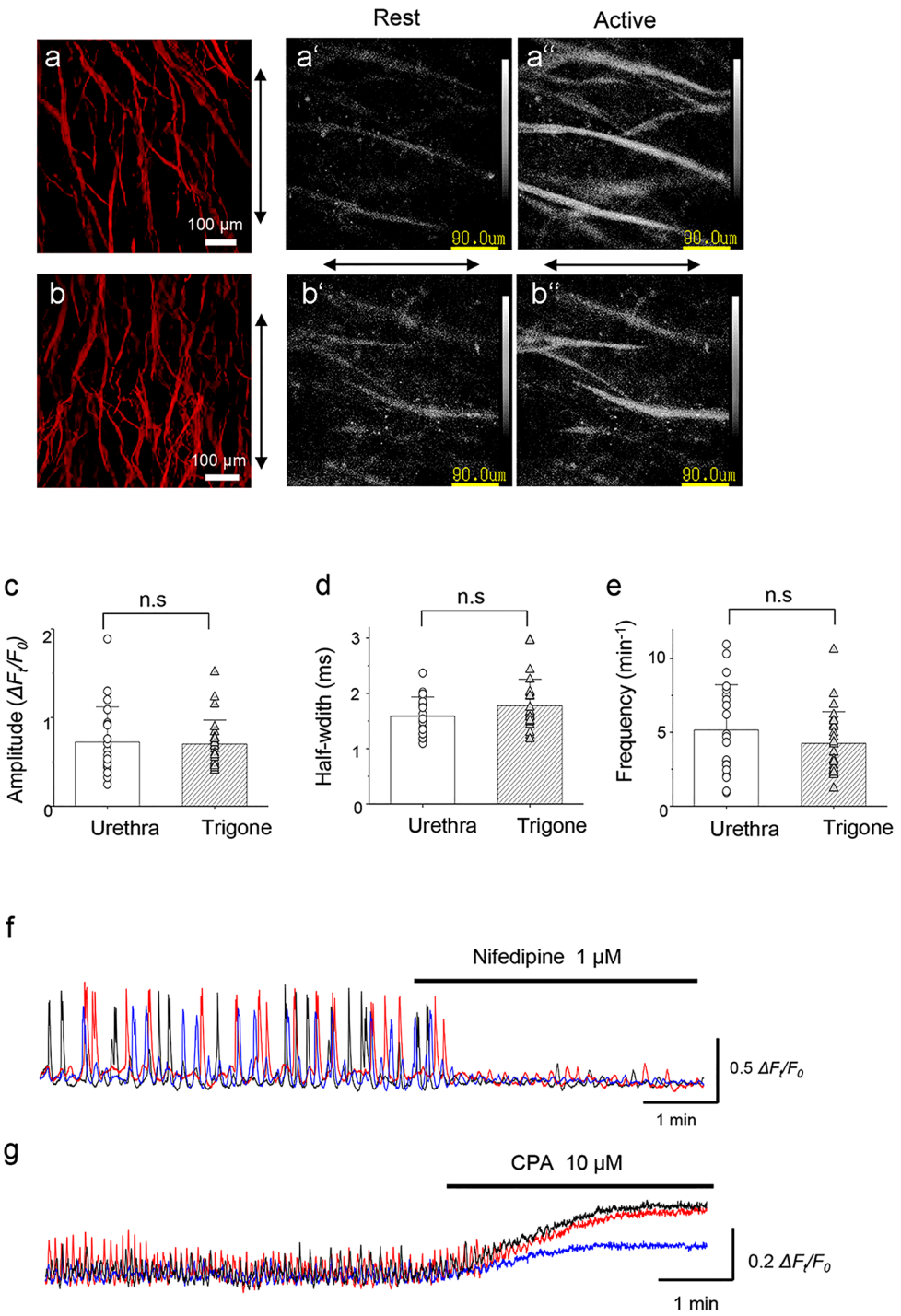

Fig. 10 *Spontaneous Ca^{2+} transients in smooth muscle cells of lamina propria (SMC-LP) in the pig urethra and trigone.* **a** A micrograph of α-SMA-immunoreactive SMC-LP in the pig urethra (*left*) and Ca^{2+} florescence images of urethral SMC-LP (*centre*; basal condition, *right*; during Ca^{2+} transient). **b** Corresponding α-SMA micrograph and Ca^{2+} images of SMC-LP in the pig bladder trigon. Two-way arrows in **a**, **b** indicate the longitudinal axis of the urethra. The amplitude (**c**), half-width (**d**) and frequency (**e**) of spontaneous Ca^{2+} transients in urethral and trigonal SMC-LP are summarised for a comparison. Two-tailed unpaired Student's *t*-test, $^*P < 0.05$. **f** In three urethral SMC-LP developing asynchronous spontaneous Ca^{2+} transients, nifedipine (1 μM) largely suppressed their generation. **g** In three SMC-LP that had been exposed to nifedipine (1 μM), CPA (10 μM) abolished the residual spontaneous Ca^{2+} transients with a rise in the basal Ca^{2+} level

increases EFS-evoked neuronal noradrenaline release (Hatanaka et al. 2006).

Nitrergic innervation to MM in pig bladder

The present study demonstrates that varicose nNOS-immunoreactive nitrergic nerve fibres project to MM in the pig bladder (Fig. 12), indicating that EFS-induced, NOS inhibitor–sensitive relaxations of pig bladder MM (Mitsui et al. 2020) are mediated via NO released from nitrergic nerve fibres. The lack of sympathetic fibres in MM is consistent with the previous finding that the blockade of sympathetic transmission with guanethidine fails to affect MM EFS–induced contractions or relaxations (Mitsui et al. 2020). Considering that nitrergic nerves in the lower urinary tract are predominantly parasympathetic

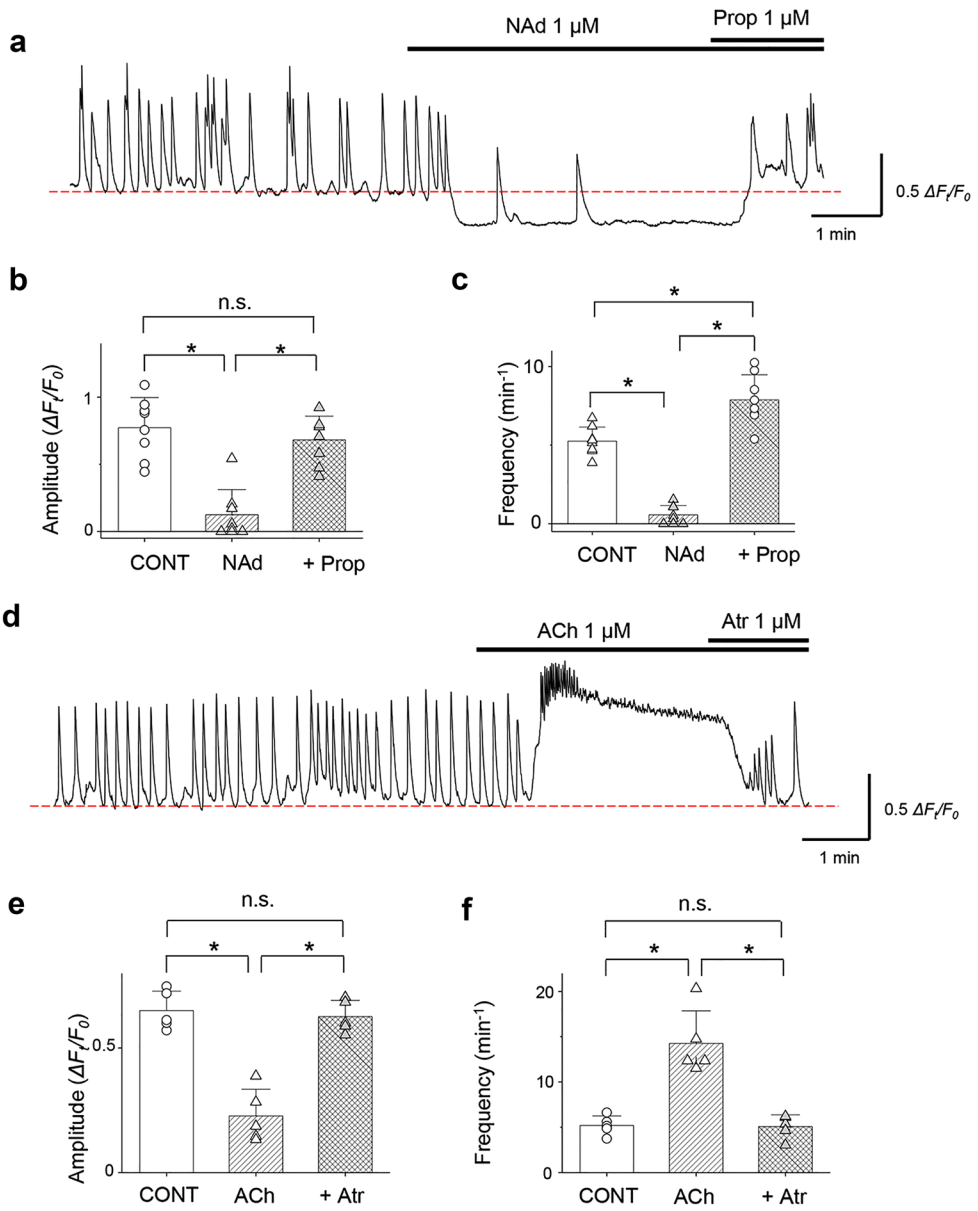

Fig. 11 *Pharmacological profile of spontaneous Ca^{2+} transients in SMC-LP of the pig urethra.* **a** In a SMC-LP of pig urethra exhibiting spontaneous Ca^{2+} transients, noradrenaline (*NAd*, 1 μM) slowed and diminished Ca^{2+} transients with a reduction in the basal Ca^{2+} level in a manner sensitive to propranolol (*Prop*, 1 μM) (**a**). Effects of NAd and subsequent Prop on the amplitude (**b**) and frequency (**c**) of spontaneous Ca^{2+} transients in SMC-LP were summarised. One-way repeated measures ANOVA with Bonferroni post hoc analysis, $^*P < 0.05$. **d** In a different spontaneously active SMC-LP, acetylcholine (*ACh*, 1 μM) transiently accelerated spontaneous Ca^{2+} transients and then suppressed their generation with a rise in the basal Ca^{2+} level in a manner sensitive to atropine (*Atr*, 1 μM). Effects of ACh and subsequent Atr on the amplitude (**e**) and frequency (**f**) of spontaneous Ca^{2+} transients were summarised. One-way repeated measures ANOVA with Bonferroni post hoc analysis, $^*P < 0.05$

origin, NO could be co-released with acetylcholine from parasympathetic nerves. In the present study, the blockade of NOS with L-NA enlarged EFS-induced contractions of MM that are predominantly mediated by neurally released acetylcholine (Mitsui et al. 2020), and thus, neuronal NO may function as a self-limiting factor in parasympathetic nerve–mediated regulation of MM contractility. In contrast to MM, detrusor smooth muscles vigorously contract upon the excitation of parasympathetic nerves as they do not respond to NO.

Since non-voiding phasic contractions of detrusor smooth muscles stimulate mechanosensitive primary afferent nerves (Heppner et al. 2016), increased contractility of detrusor smooth muscles would trigger urinary urgency. In species that have MM in the bladder including human, spontaneous contractions in MM could also be a source of mechano-induced afferent stimulation (Heppner et al. 2011; Isogai et al. 2016; Mitsui et al. 2020). In the present study, CGRP-containing primary afferent nerves projected to suburothelial vasculature but not MM. Nevertheless, the close proximity of MM with the blood vessels would allow MM to mechanically stimulate perivascular afferents. Since tadalafil prolonged nitrergic relaxations of MM, MM could be a therapeutic target of PDE5 inhibitors for the treatment of urinary urgency seen in patients with overactive bladder (Gacci et al. 2016).

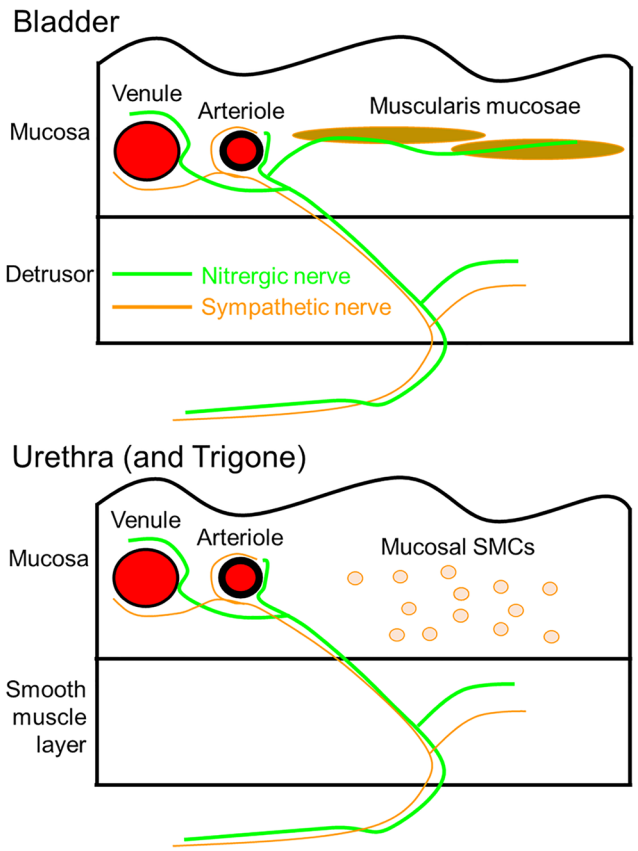

Fig. 12 *Comparison of nitrergic nerve distributions in the pig bladder and urethra.* In the pig bladder mucosa, nitrergic nerve fibres (*green*) project to arterioles and venules and muscularis mucosae. The perivascular nitrergic nerve fibres are intermingled with sympathetic nerve fibres (*orange*), while the muscularis mucosae lacks sympathetic innervation. The activation of perivascular nitrergic fibres counteracts sympathetic constriction of vasculatures in the presence of PDE5 inhibitor, while nitrergic nerves relax the muscularis mucosae even in the absence of PDE5 inhibitor. Note that whether a single nitrergic neuron projects to both mucosal vasculatures and MM remains to be determined. Nitrergic fibres are present in the detrusor layer; their main targets may be interstitial cells rather than detrusor smooth muscle cells (SMCs). In the pig urethra and trigone, nitrergic and sympathetic nerves project to the mucosal vasculatures but not mucosal SMCs. Their smooth muscle layers have inhibitory nitrergic innervation (see Introduction)

Properties of SMC-LP in pig urethra and trigone

SMC-LP scatteredly distributed in lamina propria of the urethra and trigone develop spontaneous Ca^{2+} transients predominantly arising from the opining of L-type voltage-dependent Ca^{2+} channels, suggesting that they fire action potentials as do MM in the bladder (Lee et al. 2018). Nevertheless, SMC-LP also generated nifedipine-resistant, residual Ca^{2+} transients that were blocked by the blockade of sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) with CPA, and thus, spontaneous activity in SMC-LP may primarily depend on SR Ca^{2+} handling. Unlike MM that receive both nitrergic and cholinergic innervations, SMC-LP did not respond to EFS, suggesting their lack of a functional innervation. Nevertheless, bath-applied ACh raised Ca^{2+} level in SMC-LP via the activation of muscarinic receptors, while bath-applied noradrenaline caused β-adrenoceptor-mediated inhibition of spontaneous Ca^{2+} transients. These pharmacological characteristics of SMC-LP are similar to those of bladder MM (Moro et al. 2011, 2013). However, in contrast to MM in the bladder, SMC-LP did not have a nitrergic innervation (Fig. 12) and failed to respond to even bath-applied NO donor. A previous study demonstrated that rabbit urethral lamina propria is contacted upon the activation of α-adrenoceptors with EFS or bath-applied noradrenaline (Mattiasson et al. 1985). Moreover, pre-contracted rabbit urethral lamina propria is relaxed by neurally released NO or bath-applied ACh that trigger NO release (Zygmunt et al. 1993). Although it was not determined if the contractility of rabbit urethral lamina propria ascribed to vascular or non-vascular smooth muscle, SMC-LP in the pig urethra appears to have different properties from rabbit urethral lamina propria.

Conclusion

In the pig bladder and urethra, varicose nitrergic fibres were projected to suburothelial arterioles and venules, and neurally released NO exerts vasorelaxant effects that can be enhanced by clinically relevant concentration (10 nM) of tadalafil (Forgue et al. 2006). Thus, mucosal vasculatures in the lower urinary tract could be the site of action of PDE5 inhibitors for LUTS treatment. The varicose nitrergic nerve fibres also projected to MM of pig bladder where nitrergic relaxation has been reported (Mitsui et al. 2020). SMC-LP in the urethra developed spontaneous Ca^{2+} transients but did not receive a functional nitrergic innervation. Although SMC-LP responded to exogenously applied ACh or noradrenaline, their roles in regulating the function of urethral mucosa remain to be explored.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00441-021-03521-9.

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Declarations

Ethical approval The experimental protocols used in the present study were approved by the animal experimentation ethics committee at

Nagoya City University Graduate School of Medical Sciences (No. H-30 M-44).

Conflict of interest The authors declare no conflict of interest.

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