Morphological and neurochemical characterisation of anterogradely labelled spinal sensory and autonomic nerve endings in the mouse bladder

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Highlights

- Immunohistochemistry was combined with anterograde labelling to identify extrinsic nerve endings in the bladder.
- This approach distinguished spinal afferent and autonomic efferent (motor) nerve endings in the bladder.
- Morphology alone could not distinguish simple and branching endings of afferent and autonomic neurons.

Abstract

The bladder is innervated by axons of sympathetic and parasympathetic efferent nerves, and by spinal afferent neurons. The objective was to characterise anatomically and immunohistochemically the terminal endings of sensory and autonomic motor nerve endings in wholemount preparations of the mouse bladder. We used both anterograde labelling of pelvic and hypogastric nerves *ex vivo* and anterograde labelling from lumbosacral dorsal root ganglia (DRG) *in vivo* in male and female mice. These were combined with immunohistochemistry for major markers of sensory, sympathetic and parasympathetic nerves. Selective labelling of spinal afferent endings following dextran biotin-labelling from DRGs *in vivo* showed no co-localisation of VAChT or TH in sensory terminals in the detrusor and suburothelial plexus. Biotinamide was applied *ex vivo* to nerve trunks arising in the pelvic ganglion and running towards the bladder. Among the filled axons, 38% of detrusor fibres and 47% of suburothelial axons were immunoreactive for calcitonin-gene related peptide (CGRP). Vesicular acetylcholine transporter (VAChT) immunoreactivity was present in 26% of both detrusor and suburothelial axons. For tyrosine hydroxylase (TH), the proportions were 15% and 17%, respectively. Three major morphological types of CGRP-immunoreactive nerve endings were distinguished in the bladder wall: simple, branching and complex. VAChT-immunoreactive parasympathetic axons had simple and branching endings; TH immunoreactive axons all had simple morphologies. Our findings revealed that different subtypes of sensory and autonomic nerve endings can be reliably identified by combining anterograde labelling *ex vivo* with specific immunohistochemical markers, although morphologically some of these types of endings were indistinguishable.

Keywords: bladder innervation, sensory nerves, parasympathetic nerves, sympathetic nerves

1. Introduction

The bladder is innervated by sensory and autonomic nerves, each of which has a specific physiological role in controlling function. Spinal afferent neurons are responsible for sensations from the bladder, which range from fullness, urgency to discomfort and pain. Working together with sympathetic and parasympathetic efferent nerves, spinal afferent neurons play a key role in reflex control of urinary storage and micturition. Sensory stimuli include distention and physiological or pathophysiological changes in the chemical environment within detrusor, suburothelium and urothelium. In rodents, cell bodies of spinal afferents are located in dorsal root ganglia (DRG), predominantly at the lumbosacral level (L6-S1) and, to lesser extent, in the thoracolumbar segments (T12-L3) (Keast and de Groat, 1992; Vera and Nadelhaft, 1992; Nandigama et al., 2010). Spinal afferent axons travel alongside efferent autonomic axons in the pelvic and hypogastric nerves (Vera and Nadelhaft, 1992; Brumovsky et al., 2012; Janig et al., 2017).

Retrograde labelling of DRG neurons from the bladder of rodents *in vivo* has been combined with immunohistochemistry. This approach has been instrumental in determining the neurochemical coding of sensory nerve fibers innervating the bladder. Interestingly, some DRG nerve cell bodies contain immunohistochemical markers which are normally associated with autonomic efferent nerve fibers such as choline acetyl transferase (ChAT) (Sann et al., 1995) and tyrosine hydroxylase (TH) (Brumovsky et al., 2012; Usoskin et al., 2015). In DRG nerve cell bodies innervating the mouse bladder, TH is often co-localised with CGRP (Brumovsky et al., 2012), which is a major marker of peptidergic sensory nerves in the bladder in many species (Su et al., 1986; Gabella and Davis, 1998; de Groat et al., 2015). Whether peripheral sensory axons also express TH and vesicular acetylcholine transporter (VAChT) is unresolved. This would raise the interesting possibility that such axons might release noradrenaline and acethylcholine. Most immunohistochemical studies of the mouse bladder have been used thin sections (Lagou et al., 2006; Rahnama'i et al., 2017; Eggermont et al., 2019). They revealed the penetration of nerve fibers into the urothelium layer. However, the downside to this approach is that it obscures the three-dimensional, structural features of sensory and autonomic terminal axon endings innervating the detrusor and suburothelium.

Recently, we distinguished three distinct morphological types of spinal afferent endings in the mouse bladder by selective anterograde tracing by dextran tracer injection into lumbosacral DRG *in vivo*. Interestingly, in the mouse bladder, more than 90% of these bladder sensory endings were immunoreactive for CGRP (Spencer et al., 2018). Electrophysiological recordings of spinal afferents that innervate the bladder have identified several distinct functional classes in cats, rats, guinea-pigs and mice (Habler et al., 1993, Shea et al., 2000, Zagorodnyuk et al., 2007; Xu and Gebhart, 2008; Zagorodnyuk et al., 2009). One of the fundamental unresolved questions, which to date remains unanswered is which morphological type of endings belongs to which functional classes of sensory neurons? We have answered this question for some sensory axons in the gut by combing electrophysiological recordings with subsequent anterograde labelling technique (Tassicker et al., 1999) of the recorded nerve trunk *ex vivo* (Zagorodnyuk and Brookes, 2000; Zagorodnyuk et al., 2001; Song et al., 2009).

The aim of the present study was to apply an *ex vivo* anterograde labelling technique to the mouse bladder in order to determine whether different types of sensory endings in the bladder can be distinguished and differentiated from autonomic efferent axons. For this, we used both *ex vivo* and *in vivo* anterograde labelling, combined it with immunohistochemistry, for major

sensory and autonomic markers. The findings revealed that different types of sensory and autonomic nerve endings can be reliably identified by combining anterograde labelling *ex vivo* with specific immunohistochemical markers, although by morphology alone some of these types were indistinguishable.

2. Materials and methods

2.1 Animals

Male and female C57BL/6 mice (6-8 weeks old, in approximately equal numbers between sexes) were obtained from the Animal Facility at the Flinders University, South Australia. All experiments were approved by the Animal Welfare Committee of Flinders University of South Australia (animal ethics # 861-13 and #945-17) and all protocols were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Health & Medical Research Council (NH&MRC) of Australia. All surgery was always performed under isoflurane anesthesia and pain relief was provided.

2.2 Anterograde labelling ex vivo

C57BL/6 mice were euthanised, then a ventral midline incision was made to remove the bladder with the attached ureters and pelvic ganglia were removed and the preparation was placed in Krebs solution (118 mM NaCl, 4.75 mM KCl, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 11.1 mM D-glucose, 2.5 mM $CaCl_2$, bubbled with 95%O_2/5%CO_2). The ureters and urethra were pinned in a Sylgard (Dow Corning, Midland, MI)-lined Petri dish then the bladder was opened up longitudinally starting from the urethra up to the apex. Superficial fat and connective tissue were removed and the remaining wholemount preparation was pinned serosa side up. Krebs solution was regularly changed during dissection. Nerve trunks, coming out from either left or right pelvic ganglion towards the bladder, were dissected free of connective tissue over a length of at least 5 mm. In most cases, nerve trunks filled with biotinamide entered the bladder wall above the area of the trigone. The end of the nerve trunk was sealed in a small Perspex isolation chamber with a glass coverslip, coated with high vacuum silicon grease (Ajax, Auburn, NSW Australia) before the chamber was filled with paraffin oil. A small drop of 5% biotinamide (N-[2-aminoethyl] biotinamide, hydrobromide; Molecular Probes, Eugene, OR)) dissolved in artificial intracellular solution (150 mM monopotassium, L-glutamic acid, 7 mM $MgCl_2$, 5 mM glucose, 1 mM ethylene glycolbis-[b-aminoethyl ether] N,N,N',N'-tetra-acetic acid [EGTA], 20 mM hydroxyeicosapentaenoic acid [HEPES] buffer, 5 mM disodium adenosine-5-triphosphate [ATP], 0.02% saponin, 1% dimethyl sulphoxide [DMSO], 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamycin sulfate) (Tassicker et al.,1999) was placed on the dissected nerve trunks. The Krebs solution in the Petri dish was replaced with sterile culture medium (Dulbecco's modified Eagle's [DME]/F12, Sigma; supplemented with 10% fetal bovine serum, 1.8mM $CaCl_2$, 100 IU/ml penicillin, 100 μg/ml streptomycin D, 2.5 μg/ml amphotericin B, 20 μg/ml gentamycin, Cytosystems, Castle Hill, NSW, Australia) and placed on an orbital mixer in a humidified incubator at 37ºC and in 5% CO_2 in air. It was not possible to apply the biotinamide tracer to the exact same nerve trunk due to variability between animals. This inevitably results in significant difference in innervation pattern and

density between bladder preparations. Therefore, it was not possible to determine differences between males and females in this study.

2.3 Anterograde labelling in vivo

C57BL/6 mice were anaesthetised by isoflurane inhalation (induced at 4%, maintained at 1.5% in oxygen) and a 1cm long incision was made along the dorsal surface to expose the vertebral segments of the lumbosacral region, ~2 cm rostral to the anus. An incision was made into the skeletal muscle lying superior to the dorsal root ganglia (DRG) and L5-S2 DRGs were exposed. A 1µL volume of biotinylated dextran biotin (10-20%, Cat # D1956, Molecular Probes, Eugene, Oregon, USA), dissolved in saline and then injected into glass micropipettes (inner diameter: 1.5 mm; Cat # TW150-4; World Precision Instruments (WPI) pulled on a Flaming Brown micropipette puller (Model P-87; Sutter Instrument Co. USA) with a tip diameter of approximately 5µm. Micropipettes were advanced into a DRG using a manual micromanipulator (Narashige model #M-4001002; Japan). Approximately 50-100nL of dextran tracer solution was injected by controlled pulses of nitrogen delivered via a solenoid operated valve (1s duration at 0.3Hz; 10-15psi). The fine microelectrode tips did not cause visible damage to the injected DRGs. Following DRG injection with dextran biotin, the skeletal muscle around the spine was sutured with 2.0 suture (Dynek, Australia) and the incision closed with fine sutures. The skin was closed using 7 mm clips (Cat #: 12032-07; Fine Science Tools, Canada) and mice were then given at least 7 days to recover. Buprenorphine (0.1mg/kg, s.c.) was administered twice daily for 3 days post-operatively. All mice were euthanised between 7-9 days after DRG injection by isoflurane inhalation overdose, followed by cervical dislocation. The entire bladder was removed and placed immediately into a Petri dish containing phosphate buffered saline (PBS). An incision was made to open the bladder in half along the rostro-caudal axis, then the full thickness preparation was pinned to the base of a Sylgard-lined petri dish. The preparation was then fixed in 4% paraformaldehyde for 4-6 hours, cleared 3 times for 10 minutes in dimethyl sulfoxide (DMSO), followed by washing in PBS 3 times (each for 10 minutes duration) and then incubated (full thickness) for 2-3 hours in Cy3-conjugated Streptavidin (Cat # 016 160 084, Jackson Immuno Research Laboratories Inc, West Grove, PA, USA). Preparations were then rinsed again 3 times (each time 10 minutes duration) in PBS and mounted in carbonate buffered glycerol on glass slides. After confirming presence of anterograde labeling, preparations were unmounted and washed again in PBS two times for 20 minutes ready for immunohistochemical labelling.

2.4 Immunohistochemistry

Bladder preparations were incubated with antisera to combinations of markers (Table 1) at room temperature for 3 days. Preparations were rinsed three times in PBS and incubated with secondary antisera (Table 2) for 4 hours at room temperature. Biotinamide was labelled with streptavidin conjugated to either Alexa Fluor488 or Cy3 (dilution 1:2000; Molecular Probes). After a final rinse with PBS, preparations were equilibrated with 50%, 70%, and 100% carbonate-buffered glycerol, and mounted in 100% carbonate-buffered glycerol (pH 8.6).

Table 1: Primary antisera used in this study

Primary Antibody	Host/dilution	Source/cat
CGRP	rabbit/1:2000	Peninsula/ IHC 6006
CGRP	goat/1:1000	Biogenesis/1720-9007
TH	sheep/1:200	Abcam/Ab113
VAChT	rabbit/1:1000	Synaptic systems/139103
VAChT	goat/1:200	Promega/G4481
NF200	rabbit/1:1000	Sigma/N0142
SP	rat/1:500	Seralab/SPcc
NOS	sheep/1:1000	Emson/K205

Abbreviations: CGRP, calcitonin gene-related peptide; TH, tyrosine hydroxylase; VAChT, vesicular acetylcholine transporter; SP, substance P; NF200, neurofilament 200; NOS, nitric oxide synthase.

The urothelium together with suburothelium were then sharp dissected off the detrusor muscle as an intact sheet, creating two preparations. These were then immersed in blocking solution for 1 hour (1% bovine serum albumin; BSA, 5% normal donkey serum & 1% triton X100) made up in PBS and then incubated in primary antibody for 72 hours. The tissue was washed again 3 times (x 10 minutes duration) in PBS and then incubated in secondary antibodies at 1:200 dilution for 3 hours. Specimens were mounted in carbonate-buffered glycerol and examined with an Olympus IX71 microscope (Japan) equipped with epifluorescence and highly discriminating filters (Chroma Technology, Battledore, VT). Images were captured using a 20x objective lens by a Roper scientific (Coolsnap) camera at 1392 x 1080 pixels, using AnalySIS Imager5.0 (Olympus-SIS, Münster, Germany) and saved as TIFF files.

Table 2: Secondary antisera used in this study

Secondary antibody	Conjugated fluorophore/dilution	Source/cat
Donkey anti-rabbit IgG	Cy3/1:200	Jackson/711165152
Donkey anti-rabbit IgG	Cy5/1:200	Jackson/711175152
Donkey anti-rabbit IgG	FITC/1:200	Jackson/711095152
Donkey anti-sheep IgG	Cy5/1:200	Jackson/711175147
Donkey anti-sheep IgG	FITC/1:200	Jackson/713095147
Donkey anti-goat IgG	Cy3/1:200	Jackson/705165147
Donkey anti-rat IgG	Cy3/1:200	Jackson/712165153
Donkey anti-rat IgG	Cy5/1:200	Jackson/712175153

Abbreviations: IgG, immunoglobulin; Cy3, indocarbocyanine; Cy5, indodicarbocyanine; FITC, fluorescein isothiocyanate

2.5 Analysis of data

We used a previously validated method to identify the coexistence of biotinamide or dextran biotin with immunohistochemical markers in varicosities (Chen et al., 2015). Briefly, matched micrographs of immunohistochemically labeled axons and their endings were displayed in ImageJ v1.45 (NIH, Bethesda, MD) as a stack. By flipping between the images in the stack, we tested whether biotinamide/dextran-filled varicosities was immunoreactive for any of the other immunohistochemical markers. In preparations where anterograde labelling with dextran-biotin was carried out, labelled sensory axons and terminals were relatively scarce. This made it possible to be confident that we did not repetitively sample terminals arising from the same parent axon. In *ex vivo* anterograde labelling experiments using biotinamide, we captured 10 random sites with filled axons, either in the detrusor or suburothelium. Then, 10 individual biotinamide-labelled endings were analysed within each site. In a few cases it was not possible to find 10 separated endings within a single captured image. In these cases 100 filled widely spaced varicose axons were included in the analysis in order to minimize double counting of individual endings and varicose axons.

Results are expressed throughout as means ± standard deviation, with n referring to the number of animals. Statistical analysis was performed by Student's two-tailed t-test for unpaired data using Prism v.6 software (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered significant if $P<0.05$.

3. Results

3.1 Selective anterograde labelling of spinal afferents in vivo combined with TH and VAChT immunohistochemistry

Unilateral injections of dextran biotin in L5-S2 DRG were performed to investigate possible co-localisation of sensory fibers and their endings with the neurochemical markers TH and VAChT. In both detrusor (283 axons, n=5) and suburothelium (123 axons, n=5), there was no co-localisation with VAChT (Fig. 1A, B). However, VAChT-immunoreactive fibres and endings were often present in close proximity to dextran-labelled spinal afferents. There was also no co-localisation of the dextran-filled axons and endings with TH in the detrusor (292 axons, n=5) and suburothelium (81 axons, n=5) (Fig. 1C, D). Furthermore, dextran-labelled sensory axons and endings, which were also immunoreactive for CGRP, did not co-localise with VAChT (283 axons in muscle and 78 axons in suburothelium, n=5) or TH markers (274 axons in muscle and 119 axons in suburothelium, n=5).

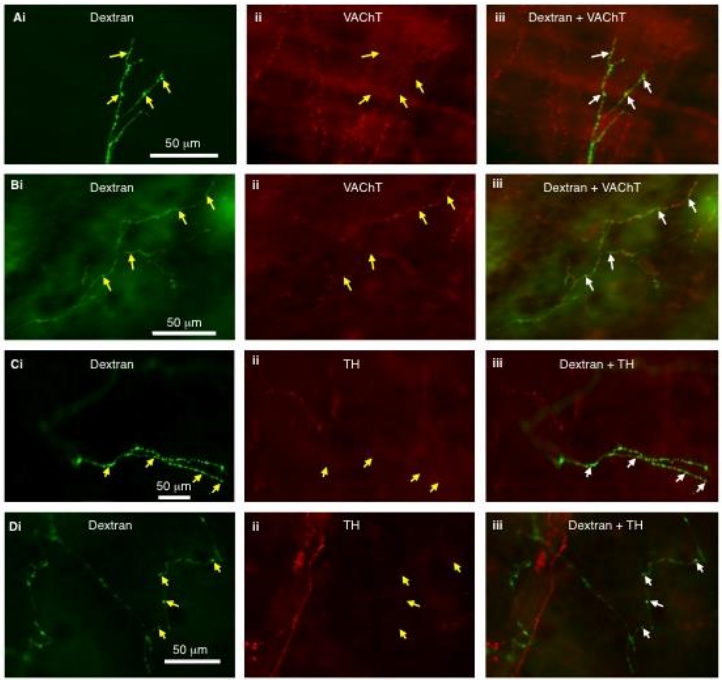

Figure 1: Dextran biotin-filled sensory nerve endings, anterogradely labelled from neurons in L5-S2 DRG *in vivo*. There was no co-localisation of the dextran-filled sensory fibers (Ai and Bi, green) with VAChT marker (Aii and Bii, red) in the muscle layer (Ai-Aiii) or suburothelium of the bladder (Bi-Biii). No co-localisation of the dextran-filled sensory fibers (Ci and Di, green) with TH marker (Cii and Dii, red) in the muscle layer (Ci-Ciii) or suburothelium (Di-Diii).

3.2 Anterograde labelling ex vivo using biotinamide

In bladder preparations anterogradely labelled with biotinamide *ex vivo* (*n*=50), filled nerve fibres from extrinsic nerves entered the muscle layer and formed a mesh that typically ramified over at least half of the bladder preparation (Fig. 2A). Labelled nerve fibres also extended into suburothelium (the lamina propria of the bladder). Biotinamide labelling showed intense staining of nerve fibres (especially their varicosities) after either 4 and/or 16-18 hours of culturing. In this study, the majority of bladder preparations were cultured over a 4-hour period (n=16) as this was associated with less background fluorescence than 16-18 hours incubation (n=10). The nerve trunks labelled with biotinamide include thick axons entering predominantly around upper bladder base area which then projected towards the dome and lateral wall of the bladder.

3.3 Distinct morphological types of endings

Because the majority of biotinamide-labelled nerve fibres terminated in the muscle and the suburothelium, endings in these two layers were investigated in detail. Three types of endings were distinguished using biotinamide anterograde labelling *ex vivo*. “Simple-type” endings terminated in single, straight varicose axons and were present in both detrusor and the suburothelium (Fig. 2B, E). “Branching-type” endings were flattened, varicose axons branching repeatedly from a parent axon, typically running in alignment with smooth muscle (Fig. 2D). “Complex-type” endings terminated in both muscle and suburothelium and were distinguished by projections extending in multiple directions throughout the layers, often not aligned with muscle fires (Fig. 2C, F). Rarely, a single parent axon formed all three types of

these endings in the muscle layer (Fig. 3). These results are similar to previous findings of *in vivo* anterograde tracing of spinal afferents from lumbosacral DRG in mouse bladder (Spencer et al., 2018).

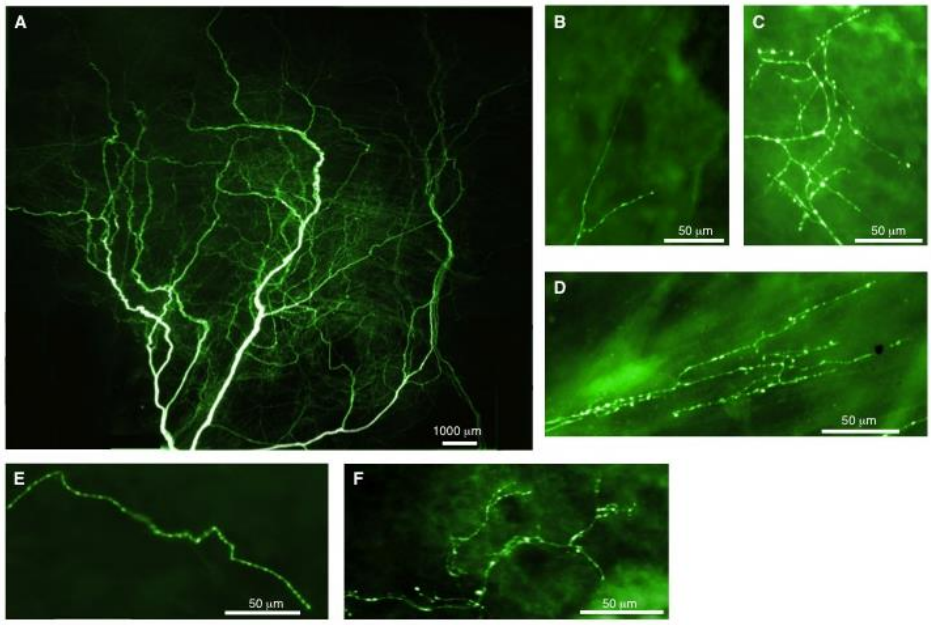

Figure 2: Anterograde labelling *ex vivo* revealed different types of endings in the mouse bladder. A- Montage of biotinamide-filled nerve projecting into the bladder from the vesical plexus, showing high intensity labelling in thick nerve trunks and less intense labelling in thin axons and their endings. Typical example of biotinamide-filled simple (B), complex type (C) and branching type ending (D) in the detrusor muscle layer. Simple type ending (E) and complex type ending (F) are shown from the suburothelium.

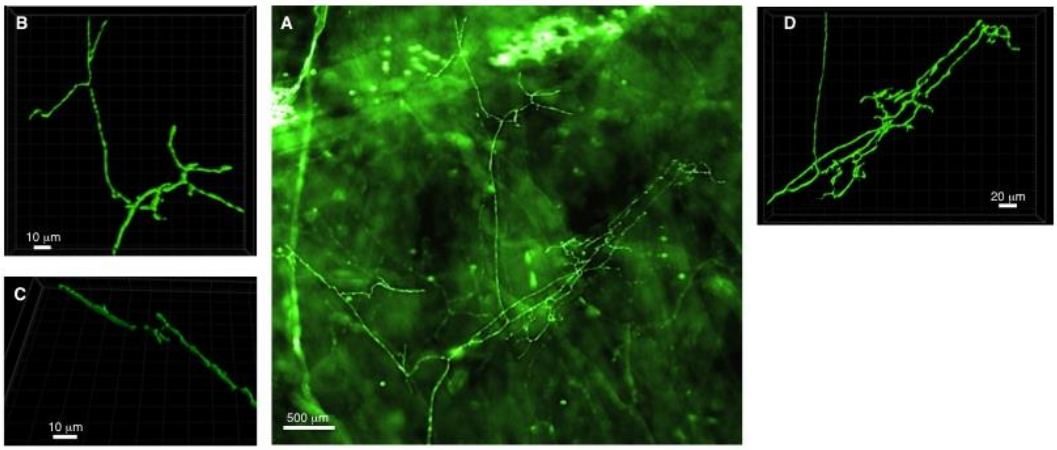

Figure 3: 3-D confocal reconstruction of a single parent axon producing the three distinct types of endings in the muscle layer. A - Fluorescence microscopic image at low magnification, showing the biotinamide-labelled parent axon *ex vivo* and the associated different types of endings in the muscle layer. 3-D reconstruction of z-stacks using laser scanning confocal microscopy of complex type ending (B), simple type ending (C), and branching type ending (D) as shown in A.

3.4 Neurochemical characteristics of sensory and autonomic nerve endings

Based on our selective anterograde labelling of spinal afferents *in vivo* combined with CGRP, TH and VAChT immunohistochemistry (see 3.1 section) and previous studies, biotinamide-filled varicose terminal axons and their endings were determined to be sensory if they were immunoreactive for CGRP (Su et al., 1986; Spencer et al., 2018), parasympathetic efferents if they were immunoreactive to VAChT (Dixon et al., 2000; Rahnama'i et al., 2017) and sympathetic efferents if they were immunoreactive for TH (Nagatsu et al., 1964; Brumovsky et al., 2012). CGRP-containing sensory nerve endings were typically varicose and comprised all three types: simple, branching and complex endings in detrusor (Fig. 4). However, in the suburothelium, all CGRP immunoreactive endings belonged to simple type (Fig. 4).

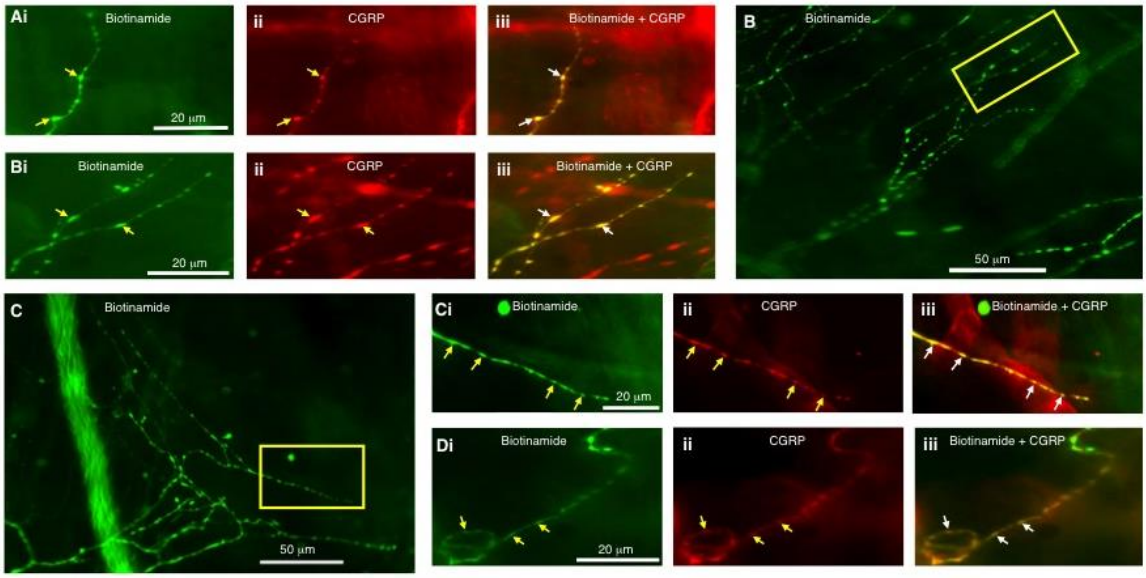

Figure 4: CGRP-immunoreactivity in biotinamide-filled nerve endings innervating the muscle layer and the suburothelium. Simple type biotinamide-filled endings (Ai, green), CGRP-immunoreactive fibers (Aii, red) and their colocalisation (Aiii) in the detrusor. B - biotinamide labelled branching type endings in the muscle layer: fibres in the yellow rectangle are magnified in Bi-Biii and show co-localisation with CGRP. C - biotinamide-labelled complex type ending in the muscle layer: fibres in the yellow rectangle are magnified in Ci-Ciii and show co-existance with CGRP. D - example of simple type sensory endings in the suburothelial layer co-localised with CGRP. Arrowheads show varicosities with co-localisation.

Most VAChT-immunorecative nerve endings in the muscle layer were either simple or branching types. In the suburothelium, all VAChT-positive endings were of the simple type (Fig. 5).

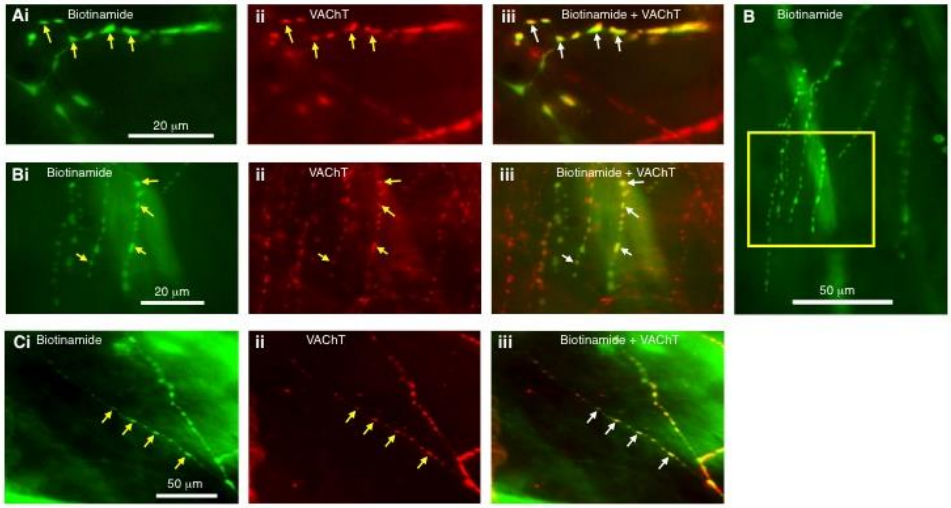

Figure 5: VAChT immunoreactivity in biotinamide-labelled nerve endings innervating the bladder muscle and the suburothelium. A - Biotinamide labelled fibres (green) from nerve trunk projecting into the bladder from the vesical plexus formed simple type ending which is immunoreactive for VAChT (Aii, red). B - biotinamide-labelled branching type endings: fibers in the yellow square are magnified in Bi-Biii and show co-localisation with VAChT in the muscle layer. C- simple type of biotinamide-filled endings immunoreactive to VAChT in the suburothelial layer.

Compared to CGRP and VAChT, TH immunoreactivity was sparser in biotinamide-filled endings in the muscle layer. It was mostly found in nerves around blood vessels, with associated simple type endings in the muscle and the suburothelium (Fig. 6).

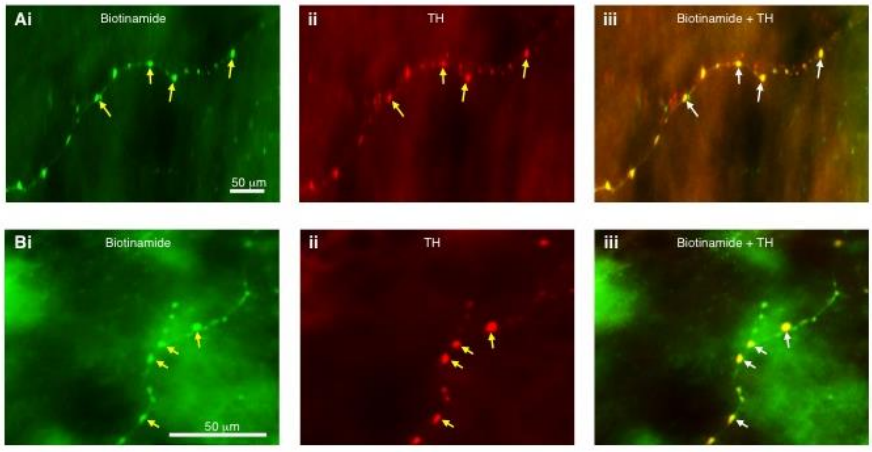

Figure 6: TH immunoreactivity in biotinamide-labelled nerve endings innervating the bladder muscle and the suburothelium. Biotinamide-labelled simple type ending (Ai, green) is immunoreactive to TH (Aii, red) in the muscle layer and in the suburothelial layer (Bi-Biii).

On average, 37.5 ± 6.4% (n=6) of biotinamide filled nerve fibres in the muscle layer were immunoreactive for CGRP and 46.7 ± 9.3% (n=6) in the suburothelial layer (Fig. 7). VAChT-immunoreactive fibres comprised 26.0 ± 5.6% (n=3) in the muscle layer and 26.3 ± 4.9% (n=3) in the suburothelium (Fig. 7). Biotinamide-labelled nerve fibres, immunoreactive for TH, comprised 15 ± 6.1% (n=3) in the muscle layer and 16.7 ± 7.8% (n=3) in the suburothelium (Fig. 7). No CGRP-immunoreactive biotinamide-labelled nerve fibres were

also immunoreactive for TH or VAChT (n=3). From this brief analysis, it is clear that 3 markers (CGRP, VAChT and TH) account for nearly 80-90% of axons in both detrusor muscle and suburothelium.

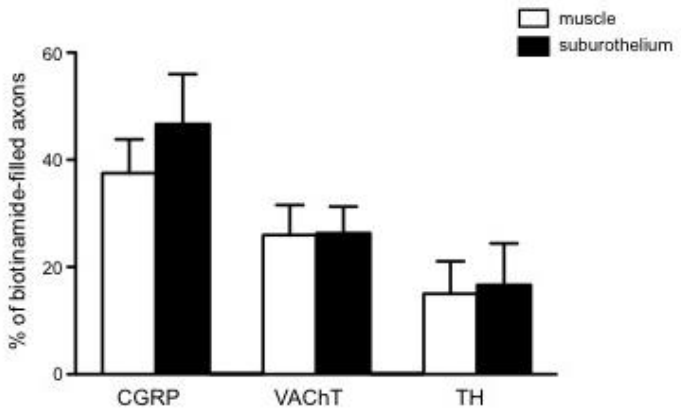

Figure 7: Group data of biotinamide-filled nerve fibres in the muscle and suburothelium layers combined with three immunohistochemical markers to identify different populations. Values are expressed as percentage of biotinamide-filled axons. The graph shows average and their standard deviations values from 6 animals for CGRP and 3 animals for VAChT and TH.

3.5 Further subdivisions of CGRP-immunoreactive sensory neurons

To identify subpopulations of sensory nerve endings, CGRP-immunoreactive nerve endings in the bladder were examined for NF200 immunoreactivity (a marker of myelinated Aδ nerve fibres) (Lawson et al., 1993; Yoshimura et al., 1998; Rahnama'i et al., 2017), substance P (SP) or NOS (Su et al., 1986, Gabella and Davis, 1998; Andersson and Hedlund, 2002; Arms and Vizzard, 2011; de Groat et al., 2015). Most NF200-immunoreactive fibres were non-varicose, long axons running in the nerve trunks that coursed through the detrusor, with single fibres often leaving the main trunks. Few NF200-immunorecative endings were seen, suggesting that this neurofilament in most cases is not expressed in the terminal endings of the axons. Most CGRP immunoreactive fibres in the detrusor lacked NF200: only 2 ± 1% (n=3) of CGRP fibres were co-localised with NF200 axons. In the suburothelium, only few nerve trunks (0.3 ± 0.1%, n=3) were NF200-positive. In triple labelling experiments, the majority of CGRP detrusor fibres colocalised with SP (57 ± 6.7 %, n=3) but lacked NF200-imunoreactivity (34 ± 2% of CGRP fibres lacked SP immunoreactivity). In suburothelium, 87 ± 3.5% (n=3) of CGRP axons were co-localised with SP but all were NF200 negative (13 ± 3.5% of these CGRP fibres lacked SP immunoreactivity). Thus, SP is abundant in CGRP-immunoreactive sensory fibres in the mouse bladder, but is concentrated in C fibres, based on the absence of expression of NF200.

We also investigated co-localisation of NOS with CGRP and VAChT. In the biotinamide labelled nerve fibres in the muscle, NOS-immunoreactive was found in both spinal afferent and parasympathetic motor nerve endings. Thus, 22 ± 5.0% (n=3) of NOS-immunoreactive fibres co-localised with CGRP and 45 ± 4.4% (n=3) of NOS-containing fibres were immunoreactive for VAChT. In the suburothelium, 12.3 ± 3.2% (n=3) NOS-immunoreactive fibers co-localised with CGRP and 21.3 ± 1.9% (n=3) with VAChT. In the detrusor, 19.3 ± 8.6% (n=3) of CGRP fibres were co-localised with NOS and in suburothelium - 9.3 ± 4.0% of CGRP fibres contained NOS marker.

3.6 Effect of organ culture on expression of immunohistochemical markers

In previous studies in the gut, anterograde-labelling of extrinsic spinal nerves with biotinamide *ex vivo* was performed primarily overnight for approximately 16-18 hours (Olsson et al., 2004; Chen et al., 2015). In the mouse bladder, background fluorescence was considerably higher after 16 hours of organotypic culture than in 4 hours. We also noticed that immunohistochemical labelling appeared less after 16 hours (Fig. 8). Therefore, we investigated the effect of culture duration on as CGRP, TH and VAChT immunoreactivities. We found that there was significant reduction in the proportion of biotinamide-filled axons expressing CGRP, VAChT and TH after 16 hours, compared to 4 hours. Only 22 ± 2.7% (n=3) of biotinamide filled fibres were immunoreactive for CGRP, 11.3 ± 5.8% (n=3) for VAChT and 2.7±1.5% (n=3) for TH labelling in preparations cultured 16 hours (n=3) (Fig. 8). Because of this significant loss in immunoreactivity, we restricted immunohistochemical analysis of biotinamide labelled preparations to those which had been cultured for 4 hours.

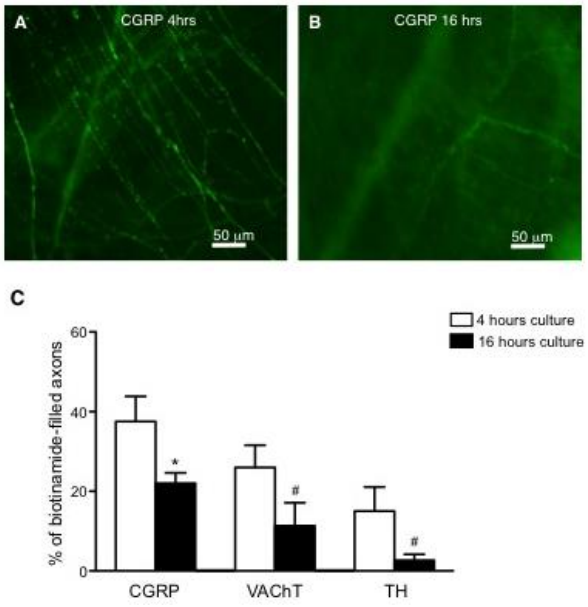

Figure 8: Difference in immunohistochemical labelling of biotinamide-filled axons in the detrusor in 4 and 16 hours of tissues culture. CGRP in the muscle layer in **A-** biotinamide-filled 16 hours cultured compared to **B-** 4-hour cultured bladder preparations. **C** - Group data analysis of co-localisation of biotinamide filled extrinsic nerve fibres in the muscle layer with major immunohistochemical markers with regards to effect of culture duration. The number of nerve fibres co-localised are presented as percentage of co-localisation between biotinamide-filled nerve fibres and the three immunohistochemical markers (*n*=3-6 for each marker). * $P<0.01$, $^{\#}$ $P<0.05$.

3.7 Anterograde labelling ex vivo using biotinamide applied on peripheral cut of the hypogastric nerve

In the experiments above, biotinamide was applied to the nerve trunks arising from the vesical pelvic ganglion and running towards the bladder. This approach could label axons originating from either hypogastric or pelvic nerves. To investigate selectively innervation of the bladder via lumbar-hypogastric nerves, in this series of experiments biotinamide was applied to the

peripheral cut of hypogastric nerve, before it enters the pelvic ganglia. Biotinamide labelling in the bladder was scarce, revealing only large-diameter axons with faint labelling in the muscle layer (in 6 out of 14 preparations cultured for 16 hours). Only in 1 out of these 6 preparations, branching-type endings were seen in the muscle layer (Fig. 9). CGRP antisera was applied in order to determine whether some of the pelvic ganglia neurons in the mouse could be immunoreactive to CGRP. No CGRP immunoreactivity was detected in the pelvic ganglion neurons (n=4).

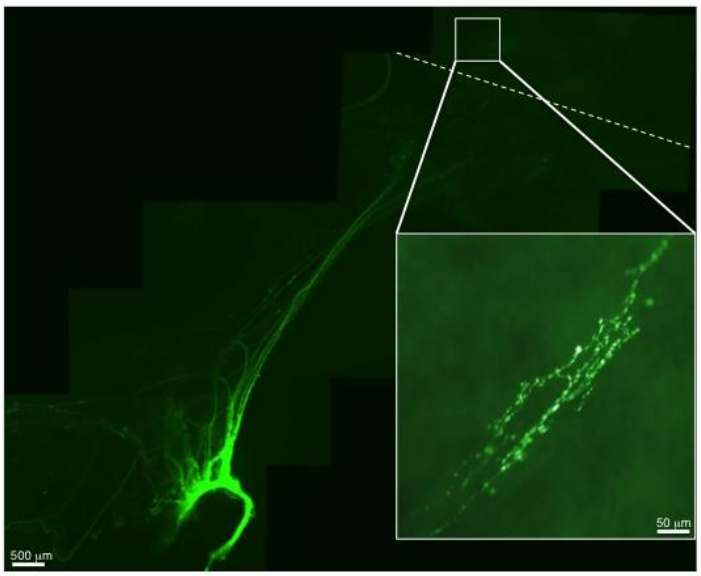

Figure 9: Montage of biotinamide-labelled hypogastric nerve trunk, projecting towards the bladder (edge of the bladder shown by the white dashed line). Expanded region in the white box shows a branching type ending in the muscle layer.

4. Discussion

This study reveals that terminal endings of spinal afferent nerves in the mouse bladder, anterogradly labelled from lumbosacral DRG *in vivo*, do not contain VAChT or TH-immunoreactivities. Using *ex vivo* anterograde labelling, we were able to distinguish terminal endings of sensory, sympathetic or parasympathetic nerves by combining morphological and neurochemical characteristics and analysed their relative proportions in the bladder wall. We also identified subpopulations of CGRP-containing sensory nerve fibres by analysing their co-localisation with either NF200, SP or NOS.

4.1 Are VAChT and TH markers present in the peripheral sensory axons and their terminal endings in the bladder wall?

At the ultrastuctural level, Gosling and Dixon (1974) showed that suburothelial varicose fibres frequently contain large dense core vesicles (most likely containing peptides, in particular they suggested CGRP and SP) as well as small clear vesicles (typical for acetylcholine) (Gosling and Dixon, 1974). However, in later studies, when VAChT was identified as a selective marker for cholinergic neurons, the same authors showed that almost all cholinergic axons contain neuropeptide Y (NPY) in the detrusor and suburothelium (Dixon et al., 2000). Cholinergic nerves containing NPY are likely to belong to motor or secretomotor

axons and have been found in the seminal vesicles, vas deferens and prostate glands (Ottesen and Fahrenkrug, 1995). Early ultrastructural studies and findings regarding distribution of the cholinesterase-positive nerves (Gosling and Dixon, 1974) and of nerve fibers immunoreactive for VAChT (Dixon et al., 2000; Rahnama'i et al., 2017; Eggermont et al., 2019), demonstrated that some projected close to- or into the urothelium. This led to the suggestion that they might be sensory because of the absence of any obvious target sites (Gosling and Dixon, 1974; Rahnama'i et al., 2017; Eggermont et al., 2019). The present study, and previous report (Rahnama'i et al., 2017; Eggermont et al., 2019) did not detect co-localisation of CGRP and VAChT. This suggest that VAChT-immunoreactive fibres in the suburothelium are very unlikely to play a sensory role. Similar to other genitourinary organs, cholinergic innervations of the suburothelium and urothelium is likely to have either a secretomotor function or a neurotrophic action on urothelial cells, regulating their proliferation and repair (Ottesen and Fahrenkrug, 1995; Dixon et al., 2000; Podmolikova et al., 2018; Sellers and Chess-Williams, 2012).

TH is a marker for catecholaminergic neurons, although it is immunohistochemically detectable in the cell bodies of some rodent DRG neurons and intramural neurons of human neonatal bladder (Dixon et al., 1999; Brumovsky et al., 2012). The present study demonstrated that autonomic markers such as TH and VAChT are not immunohistochemically detectable in sensory axons and their peripheral endings in the bladder wall. In contrast, CGRP is present in more than 90% of DRG-labelled sensory endings in the mouse bladder (Spencer et al., 2018). Since pelvic ganglion neurons were not immunoreactive for CGRP in mice, all CGRP-immunoreactive biotinamide-filled axons in the mouse bladder must be sensory in function. This confirms previous data obtained in rats and guinea pigs (Su et al., 1986; Gabella and Davis, 1998; Andersson, 2002).

4.2 Different types of spinal afferent and efferent nerve endings in detrusor muscle and suburothelium

Immunohistochemical studies using antibodies against CGRP have been used to identify spinal afferent endings in the bladder (Gabella and Davis, 1998; Andersson, 2002). In most cases, it was impossible to identify reliably the branching of the individual terminal axons (i.e. receptive fields) in densely innervated areas such as detrusor and suburothelium of the bladder neck. Recently, we have identified unequivocally at least three distinct morphological types of spinal afferent endings in the mouse bladder by selectively labelling spinal afferents by injecting anterogradely transported tracer into lumbosacral DRG (L5-S2) *in vivo* (Spencer et al., 2018). Electrophysiological recordings from spinal afferents that innervate the bladder have identified several distinct functional classes of afferents in cats, rats, guinea-pigs and mice (Habler et al., 1993; Shea et al., 2000; Zagorodnyuk et al., 2007; Xu and Gebhart, 2008; Zagorodnyuk et al., 2009). In the mouse bladder, at least four major classes of sensory neurons were found: (i) low threshold stretch-sensitive muscular and (ii) muscular-mucosal; (iii) stretch-insensitive mucosal and (iv) so-called serosal afferents (Xu and Gebhart, 2008). In order to identify the morphological type of sensory axon terminal endings corresponding to the receptive fields of functionally distinct classes of bladder sensory neurons, a combination of electrophysiological recordings with subsequent anterogradely labelling of recorded nerve *ex vivo* can be used (Zagorodnyuk et al., 2010). It is clear that anterograde tracing of any nerve trunk entering guinea pig bladder labelled a mixture of both sensory and autonomic nerves (Zagorodnyuk et al., 2010). Sensory nerves can release transmitters from their varicose axons via an axon-reflex mechanism (Maggi, 1991). Therefore, the most reliable way to

distinguish sensory endings from autonomic endings after biotinamide tracing *ex vivo* is to use immunohistochemical markers which are selective for particular type of nerves.

The present study revealed that different subtypes of sensory and autonomic nerve endings can be reliably identified by combining anterograde labelling *ex vivo* with specific immunohistochemical markers. However, based on morphology alone, some of these types of endings were indistinguishable. Three major types of nerve endings (simple, branching and complex), were found for peptidergic sensory (CGRP-immunoreactive) nerves, simple and branching for cholinergic parasympathetic (VAChT immunoreactive) nerves and only simple endings for sympathetic (TH immunoreactive) nerves. All three types of CGRP-containing sensory nerve endings (simple, branching and complex endings) were found by anterogradely labelled *ex vivo* in the muscle layer, while only simple endings were seen in the suburothelium. This data is in agreement with our previous data obtained using *in vivo* filling (Spencer et al., 2018). A previous study, using in vivo dye filling showed that 75-89% of complex, simple and branching endings in the detrusor were CGRP-immunoreactive, as were 100% of simple endings in the suburothelium (Spencer et al., 2018). Here, we found that CGRP, VAChT and TH account for 80-90% of biotinamide-filled axons in both detrusor muscle and suburothelium. It is possible that remaining 10-20% of nerve fibres are non-peptidergic sensory fibers. Selective, reliable immunohistochemical markers for non-peptidergic sensory nerves, which include Aδ fibers and some C fibres, are still lacking. However, we cannot exclude the possibility that immunohistochemical staining is underestimated in cultured tissue *ex vivo*, or that errors accumulating from multiple counts reduced the total.

4.3 Further subdivision of peptidergic sensory nerve subtypes with NF200, SP and NOS marker.

In functional studies, Aδ and C fibres play different roles in bladder function in many species (de Groat et al., 2015). In rats, many bladder DRG sensory neurons are lightly myelinated and express NF200 (Hayashi et al., 2009; Russo et al., 2013). However, the majority of pelvic ganglion neurons projecting to the rat bladder were also stained with antisera against NF200 and these are likely to be autonomic efferents in function (Forrest et al., 2014). Present experiments indicated that in mouse bladder only a small proportion (2-3%) of individual sensory CGRP-immunoreactive fibers and their endings contained a NF200 marker. Thus, it is likely that CGRP axons in the detrusor and suburothelial plexus of the mouse bladder are predominantly C fibres. In addition, the present study showed that majority of them contain both CGRP and SP. Co-localisation of CGRP with SP has been documented previously in the bladder of rats and guinea pigs (Su et al., 1986; Gabella and Davis, 1998; Arms and Vizzard, 2011). In contrast, murine cutaneous afferents are mostly CGRP positive SP-negative and most likely Aδ polymodal mechanoreceptors (Kestell et al., 2015). The present study clearly demonstrated two sources of NOS in the bladder nerves - autonomic cholinergic (VAChT positive) and sensory axons (CGRP positive), confirming previous observations in the bladder of many species (Dixon et al., 2000; Andersson and Hedlund, 2002; de Groat et al., 2015). The functional role of NO in CGRP-immunoreactive sensory fibres still needs to be determined.

4.4 Splanchnic-pelvic versus lumbar-hypogastric bladder innervation

In rats, cell bodies of bladder sensory nerves are predominantly located in DRG at lumbosacral (L6-S1) and lumbar (L1-L3) segments (Keast and de Groat, 1992; Vera and Nadelhaft, 1992). Mouse bladder is innervated by thoraco-lumbar (T12-L2) and lumbosacral (L5-S2) DRG neurons projecting to the bladder via lumbar-hypogastric and splanchnic-pelvic nerves, respectively (Nandigama et al., 2010). Previous anatomical studies in the cat and rat suggested that different regions of the bladder received sensory innervation from thoraco-lumbar and lumbar-sacral pathways. Lumbar pathways were concentrated in the bladder trigone while lumbar-sacral axons spread over the entire bladder (Uemura et al., 1973; Vera and Nadelhaft, 1992). In the mouse bladder, functional studies indicated that both pathways contained different populations of mechanosensitive afferents and had distinct location of receptive fields in the bladder similar to those described anatomically (Xu and Gebhart, 2008). We attempted to label thoraco-lumbar afferent endings by applying biotinamide selectively to the peripheral end of hypogastric nerve *ex vivo*. This was largely unsuccessful probably due to long distance to terminal nerve endings in the detrusor and suburothelium. Further studies are warranted to selective label of thoraco-lumbar afferent endings in the mouse bladder from T12-L2 DRG *in vivo*, similar to those reported for lumbosacral innervation (Spencer et al., 2018).

5. Conclusions

Our findings reveal that different types of sensory and autonomic nerve endings (simple, branching and complex) can be reliably identified by combining anterograde labelling *ex vivo* with immunohistochemical markers such as CGRP, VAChT and TH. However, morphology alone could not distinguish the origins of these endings unequivocally. By selectively labelling of sensory axons from lumbosacral DRG *in vivo*, we did not detect any sensory axons or terminals in the bladder detrusor or suburothelium immunoreactive for either TH or VAChT. The next major challenge is to determine how these distinct morphological types of afferent axon terminal endings are related to functional properties of spinal sensory neurons.

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REFERENCES

Andersson, K.-E. 2002. Bladder activation: afferent mechanisms. Urology 59, 43-50.

Andersson, K.E., Hedlund, P. 2002. Pharmacologic perspective on the physiology of the lower urinary tract. Urology 60, 13-22.

Arms, L., Vizzard, M.A. 2011. Neuropeptides in lower urinary tract function. Handb. Exp. Pharmacol., 395-423.

Brumovsky, P.R., La, J.H., McCarthy, C.J., Hokfelt, T., Gebhart, G.F. 2012. Dorsal root ganglion neurons innervating pelvic organs in the mouse express tyrosine hydroxylase. Neuroscience 223, 77-91.

Chen, B.N., Sharrad, D.F., Hibberd, T.J., Zagorodnyuk, V.P., Costa, M., Brookes, S.J. 2015. Neurochemical characterization of extrinsic nerves in myenteric ganglia of the guinea pig distal colon. J. Comp. Neurol. 523, 742-756.

de Groat, W.C., Griffiths, D., Yoshimura, N. 2015. Neural control of the lower urinary tract. Compreh. Physiol. 5, 327-396.

Dixon, J.S., Jen, P.Y., Gosling, J.A. 1999. Tyrosine hydroxylase and vesicular acetylcholine transporter are coexpressed in a high proportion of intramural neurons of the human neonatal and child urinary bladder. Neurosci. Lett. 277, 157-160.

Dixon, J.S., Jen, P.Y., Gosling, J.A. 2000. The distribution of vesicular acetylcholine transporter in the human male genitourinary organs and its co-localization with neuropeptide Y and nitric oxide synthase. Neurourol. Urodyn. 19, 185-194.

Eggermont, M., De Wachter, S., Eastham, J., Gillespie, J. 2019. Innervation of the epithelium and lamina propria of the urethra of the female rat. Anat. Rec. 302, 201-214.

Forrest, S.L., Osborne, P.B., Keast, J.R. 2014. Characterization of axons expressing the artemin receptor in the female rat urinary bladder: a comparison with other major neuronal populations. J. Comp. Neurol. 522, 3900-3927.

Gabella, G., Davis, C. 1998. Distribution of afferent axons in the bladder of rats. J. Neurocytol. 27, 141-155.

Gosling, J.A., Dixon, J.S. 1974. Sensory nerves in the mammalian urinary tract. An evaluation using light and electron microscopy. J. Anat. 117, 133-144.

Habler, H.J., Janig, W., Koltzenburg, M. 1993. Receptive properties of myelinated primary afferents innervating the inflamed urinary bladder of the cat. J. Neurophysiol. 69, 395-405.

Hayashi, T., Kondo, T., Ishimatsu, M., Yamada, S., Nakamura, K., Matsuoka, K., Akasu, T. 2009. Expression of the TRPM8-immunoreactivity in dorsal root ganglion neurons innervating the rat urinary bladder. Neurosci. Res. 65, 245-251.

Janig, W., Keast, J.R., McLachlan, E.M., Neuhuber, W.L., Southard-Smith, M. 2017. Renaming all spinal autonomic outflows as sympathetic is a mistake. Auton. Neurosci. 206, 60-62.

Keast, J.R., de Groat, W.C. 1992. Segmental distribution and peptide content of primary afferent neurons innervating the urogenital organs and colon of male rats in. J. Comp. Neurol. 319, 615-623.

Kestell, G.R., Anderson, R.L., Clarke, J.N., Haberberger, R.V., Gibbins, I.L. 2015. Primary afferent neurons containing calcitonin gene-related peptide but not substance P in forepaw skin, dorsal root ganglia, and spinal cord of mice. J. Comp. Neurol. 523, 2555-2569.

Lagou, M., De Vente, J., Kirkwood, T.B., Hedlund, P., Andersson, K.E., Gillespie, J.I., Drake, M.J. 2006. Location of interstitial cells and neurotransmitters in the mouse bladder. BJU Int. 97, 1332-1337.

Lawson, S.N., Perry, M.J., Prabhakar, E., McCarthy, P.W. 1993. Primary sensory neurones: neurofilament, neuropeptides, and conduction velocity. Brain Res. Bull. 30, 239-243.

Maggi, C.A. 1991. The role of peptides in the regulation of the micturition reflex: an update. Gen. Pharmacol. 22, 1-24.

Nagatsu, T., Levitt, M., Udenfriend, S. 1964. Tyrosine hydroxylase. The intitial step in noradrenaline biosynthesis. J. Biol. Chem. 239, 2910-2917.

Nandigama, R., Bonitz, M., Papadakis, T., Schwantes, U., Bschleipfer, T., Kummer, W. 2010. Muscarinic acetylcholine receptor subtypes expressed by mouse bladder afferent neurons. Neuroscience 168, 842-850.

Olsson, C., Costa, M., Brookes, S.J. 2004. Neurochemical characterization of extrinsic innervation of the guinea pig rectum. J. Comp. Neurol. 470, 357-371.

Ottesen, B., Fahrenkrug, J. 1995. Vasoactive intestinal polypeptide and other preprovasoactive intestinal polypeptide-derived peptides in the female and male genital tract: localization, biosynthesis, and functional and clinical significance. Am. J. Obstet. Gynecol. 172, 1615-1631.

Podmolikova, L., Mukanyangezi, M.F., Nieto-Marin, P., Giglio, D. 2018. Cholinergic regulation of proliferation of the urothelium in response to E. coli lipopolysaccharide exposition. Int. Immunopharmacol. 56, 222-229.

Rahnama'i, M.S., Biallosterski, B.T., Van Kerrebroeck, P.E., van Koeveringe, G.A., Gillespie, J.I., de Wachter, S.G. 2017. Distribution and sub-types of afferent fibre in the mouse urinary bladder. J. Chem. Neuroanat. 79, 1-11.

Russo, D., Clavenzani, P., Sorteni, C., Bo Minelli, L., Botti, M., Gazza, F., Panu, R., Ragionieri, L., Chiocchetti, R. 2013. Neurochemical features of boar lumbosacral dorsal root ganglion neurons and characterization of sensory neurons innervating the urinary bladder trigone. J. Comp. Neurol. 521, 342-366.

Sann, H., McCarthy, P.W., Mader, M., Schemann, M. 1995. Choline acetyltransferase-like immunoreactivity in small diameter neurons of the rat dorsal ganglion. Neurosci. Lett. 198, 17-20.

Sellers, D.J., Chess-Williams, R. 2012. Muscarinic agonists and antagonists: effects on the urinary bladder. Hand. Exp. Pharmacol., 375-400.

Shea, V.K., Cai, R., Crepps, B., Mason, J.L., Perl, E.R. 2000. Sensory fibers of the pelvic nerve innervating the Rat's urinary bladder. J. Neurophysiol. 84, 1924-1933.

Song, X., Chen, B.N., Zagorodnyuk, V.P., Lynn, P.A., Blackshaw, L.A., Grundy, D., Brunsden, A.M., Costa, M., Brookes, S.J. 2009. Identification of medium/high-threshold extrinsic mechanosensitive afferent nerves to the gastrointestinal tract. Gastroenterology 137, 274-284.

Spencer, N.J., Greenheigh, S., Kyloh, M., Hibberd, T.J., Sharma, H., Grundy, L., Brierley, S.M., Harrington, A.M., Beckett, E.A., Brookes, S.J., Zagorodnyuk, V.P. 2018. Identifying unique subtypes of spinal afferent nerve endings within the urinary bladder of mice. J. Comp. Neurol. 526, 707-720.

Su, H.C., Wharton, J., Polak, J.M., Mulderry, P.K., Ghatei, M.A., Gibson, S.J., Terenghi, G., Morrison, J.F., Ballesta, J., Bloom, S.R. 1986. Calcitonin gene-related peptide immunoreactivity in afferent neurons supplying the urinary tract: combined retrograde tracing and immunohistochemistry. Neuroscience 18, 727-747.

Tassicker, B.C., Hennig, G.W., Costa, M., Brookes, S.J. 1999. Rapid anterograde and retrograde tracing from mesenteric nerve trunks to the guinea-pig small intestine in vitro. Cell Tissue Res. 295, 437-452.

Uemura, E., Fletcher, T.F., Dirks, V.A., Bradley, W.E. 1973. Distribution of sacral afferent axons in cat urinary bladder. A. J. Anat. 136, 77, 305-313.

Usoskin, D., Furlan, A., Islam, S., Abdo, H., Lonnerberg, P., Lou, D., Hjerling-Leffler, J., Haeggstrom, J., Kharchenko, O., Kharchenko, P.V., Linnarsson, S., Ernfors, P. 2015. Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nat. Neurosci. 18, 145-153.

Vera, P.L., Nadelhaft, I. 1992. Afferent and sympathetic innervation of the dome and the base of the urinary bladder of the female rat. Brain Res. Bull. 29, 651-658.

Xu, L., Gebhart, G.F. 2008. Characterization of mouse lumbar splanchnic and pelvic nerve urinary bladder mechanosensory afferents. J. Neurophysiol. 99, 244-253.

Yoshimura, N., Erdman, S.L., Snider, M.W., de Groat, W.C. 1998. Effects of spinal cord injury on neurofilament immunoreactivity and capsaicin sensitivity in rat dorsal root ganglion neurons innervating the urinary bladder. Neuroscience 83, 633-643.

Zagorodnyuk, V.P., Brookes, S.J. 2000. Transduction sites of vagal mechanoreceptors in the guinea pig esophagus. J. Neurosci. 20, 6249-6255.

Zagorodnyuk, V.P., Brookes, S.J., Spencer, N.J. 2010. Structure-function relationship of sensory endings in the gut and bladder. Aut. Neurosci. 153, 3-11.

Zagorodnyuk, V.P., Brookes, S.J.H., Spencer, N.J., Gregory, S. 2009. Mechanotransduction and chemosensitivity of two major classes of bladder afferents with endings in the vicinity to the urothelium. J. Physiol. 587, 3523-3538.

Zagorodnyuk, V.P., Chen, B.N., Brookes, S.J. 2001. Intraganglionic laminar endings are mechano-transduction sites of vagal tension receptors in the guinea-pig stomach. J. Physiol. 534, 255-268.

Zagorodnyuk, V.P., Gibbins, I.L., Costa, M., Brookes, S.J., Gregory, S.J. 2007. Properties of the major classes of mechanoreceptors in the guinea pig bladder. J. Physiol. 585, 147-163.