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Computer-assisted three-dimensional tracking of sensory innervation in the murine bladder mucosa with two-photon microscopy

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ABSTRACT

A strong association between functional bladder disorders and bladder sensation is well-known, with a relationship between malfunctioning detrusor muscle and abnormal sensation arising from the sub-urothelium and the lamina propria (LP), has been suggested. However, the exact underlying pathophysiology of these bladder disorders is not completely understood. Therefore, it is important to gain knowledge on sensory innervation of the urinary bladder in order to understand the neural network function in healthy and diseased bladder. In the present study we aim at the development of a computer-assisted method for 3D-tracking of sensory innervation in the murine bladder mucosa using two-photon laser scanning microscopy (TPLSM). TPLSM was performed on 10 fixed, stained (CGRP) bladder samples in both the trigone and dome. Nerve tracking was performed in subvolumes ($6.3 \pm 2.9\,10^6\,\mu m^3$; median ± IQR) of 22 stacks with determining total nerve length, nerve segment lengths, curviness, straightness, and locations of branching and ending points in the lamina propria (LP). The results show that the highest concentration of afferent fibres was found at the urothelium-LP interface. Nerve curviness, a presumed indicator of nerve activity, showed an equal value throughout the complete LP. We found a significantly higher median nerve segment length in the LP of the trigone and significantly more curved nerves in the dome of the bladder. This indicates an adaptation to, or an involvement in the detection of, bladder volume changes. Conclusively, we successfully developed a computer-assisted method for 3D tracking of sensory nerve fibres in the LP of the murine bladder wall.

1. Introduction

Impairment of innervation of sensory pathways can be a cause of functional bladder disorders and results in storage or voiding dysfunction, such as the overactive bladder syndrome (OAB) (Natalin et al., 2013) or underactive bladder. A strong association between OAB and bladder sensation is well-known, with a relationship between malfunctioning detrusor muscle and abnormal sensation arising from the sub-urothelium and the lamina propria (LP), having been suggested (Birder, 2013). However, the exact underlying pathophysiology of these bladder disorders is not completely understood. Therefore, it is important to gain knowledge on sensory innervation of the urinary bladder in order to understand the neural network function in healthy and diseased bladder.

It is known that afferent nerve fibres within the lower urinary tract (bladder and urethra) originate from pelvic, pudendal, and hypogastric nerves. They carry sensory information on the filling status of the bladder to the spinal cord, where they subsequently initiate the activation of brain areas involved in regulation of

Abbreviations: CGRP, calcitonin gene-related peptide; CO, connective tissue; KDE, kernel density estimate; LP, lamina propria; OAB, overactive bladder syndrome; ROI, region of interest; 3D, three-dimensional; TPLSM, two-photon laser scanning microscopy; μm, micrometer.

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micturition (Kanai and Andersson, 2010). It is generally accepted that there are two different populations of afferent nerve fibres in the urinary tract: myelinated Aδ and unmyelinated C-fibres (de Groat et al., 2015). The sensitive Aδ fibres are located mainly in the muscle layer of the bladder wall and communicate during bladder filling via mechano-transduction to the central nervous system (Kanai and Andersson, 2010; Andersson, 2011). Under healthy conditions, the unmyelinated, "silent" C-fibres are present but minimally active in the muscle layer, the LP, and sub-urothelium of the bladder wall (Kanai and Andersson, 2010). They can become active in several pathological situations where they respond mostly to noxious stimuli, such as harmful chemicals or inflammation (Cheng et al., 1993; Habler et al., 1990). Xu and Gebhart described four different subtypes of C-fibres in the murine bladder, based on their responses to mechanical stimuli (in vitro study): serosal, muscular, muscular/urothelial, and urothelial afferents (Xu and Gebhart, 2008). Muscular, muscular/urothelial, and serosal afferents were found as lumbar splanchnic nerves, whereas all four types were found as sacral pelvic nerves (Xu and Gebhart, 2008).

To date, studies on sensory innervation of the bladder wall, including quantitative analyses of nerve distribution, are very limited or have not been updated for many years. In 1998, Gabella and Davis examined the afferent nerve distribution in rat bladder samples using classical immunohistochemistry and confocal microscopy (Gabella and Davis, 1998), providing only two-dimensional information. Additional and new insights in nerve pathways by means of three-dimensional (3D) reconstructions could enable the study of e.g. distribution and shape of nerves within the entire organ. As one example, the application of two-photon laser scanning microscopy (TPLSM) to image the transgenic mouse heart revealed the 3D microarchitecture of the sympathetic innervation therein (Freeman et al., 2014). Similarly, the use of TPLSM could potentially yield information on the three-dimensional distribution and orientation of afferent nerve fibres within the bladder wall.

In the present study, we will use TPLSM as a new approach to investigate the 3D structure of afferent nerves within the murine urinary bladder. In a previous TPLSM study, we semi-quantitatively studied the morphological changes in the bladder wall architecture occurring with age, such as the number of nerve fibres and the ratio between collagen and smooth muscle volumes (Schueth et al., 2016). We will continue in line with that research by developing a computer-assisted method for 3D tracking of sensory innervation in the murine bladder wall. Moreover, we aim to quantitatively and objectively analyse distribution, density, length, and shape of afferent nerve fibres as identified by immunohistochemical staining for calcitonin gene-related peptide (CGRP) in the LP of the murine bladder wall. CGRP positive nerves have been shown to form a major part of the afferent nervous circuitry in the bladder of several animal models and even humans. CGRP nerves are thought to play a modulatory role as they are often found in close proximity to motor ganglia and nerves. Therefore these afferent nerves are likely to play a major role in the motor-sensory control of the urinary bladder. To the best of our knowledge, this is the first report of automated TPLSM-3D tracking of sensory nerves in the murine bladder wall.

2. Materials and methods

2.1. Animals

Experimental protocols were approved by the animal experiments committee of Maastricht University, and were carried out according to institutional guidelines and reported in accordance with the ARRIVE guidelines. Ten C57BL/6J wild-type mice (age range between 10 and 25 weeks) were housed in individually-ventilated cages within a temperature-controlled environment with 12-h light/dark cycle, standard chow, and water available ad libitum.

2.2. Tissue preparation and immunohistochemical staining

Nerves were stained with CGRP (Anti-Calcitonin Gene Related Peptide), Rabbit pAB (Merck) with Alexa 594 secondary antibody (Life Technologies™). Fixed bladder tissue was washed 3 times with Tris-Buffered Saline (TBS) (Merck), each for 15 min. Primary antibody (CGRP) was diluted 1:1000 in TBS-Triton (0,3%), applied to the tissue, and incubated overnight at 4 °C. The primary antibody was removed and the sample was washed 3 times, each for 30 min with TBS-Triton (0,3%), TBS and TBS-Triton (0,3%), respectively. Secondary antibody (Alexa 594) was diluted 1:100 in TBS-Triton (0,3%), applied to the sample and incubated in the dark, overnight at 4 °C. The secondary antibody was removed and the sample was washed 3 times, each for 15 min, with TBS-Triton (0,3%), TBS and TBS, respectively

During TPLSM imaging the prepared bladders were placed in a petri dish and submerged under saline solution (Schueth et al., 2014). Images were taken from the urothelial side. In each bladder, both dome and trigone regions were imaged.

2.3. Imaging

For imaging experiments, a two-photon laser scanning microscope (Leica TCS SP5 MP, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany), equipped with a HCX APO L 20×/1.00W water immersion objective was used. Working distance of the objective was 2 mm and the excitation source was a 140 fs-pulsed Ti:sapphire laser (Chameleon Ultra II, Coherent Inc., Santa Clara, CA, USA), mode-locked at 800 nm. To avoid photobleaching and tissue damage, laser power was kept as low as possible (max. 100–125 mW, at the sample surface). Fluorescence emission was detected using photomultiplier tubes (Hamamatsu, R9624, Japan) in three wavelength ranges:

1. 385–489 nm (blue), typically showing collagen fibres as a result of second harmonic generation (Megens et al., 2007),
2. 489–563 nm (green), acquiring the autofluorescence signal of e.g. umbrella cells, and elastic fibres
3. 568–700 nm (red), acquiring the CGRP-Alexa 594 signal of stained afferent fibres.

Images and image stacks were acquired using 12-bit precision with Leica Application Suite Advanced Fluorescence (Leica Microsystems). Image acquisition settings for the experiments are

Table 1
TPLSM image acquisition settings for fixed bladder samples.

mode	scan field resolution	scan field size [μm × μm]	pixel size [μm]	line rate [Hz]	line averaging	frame rate [Hz]
examination	512 × 512	738 × 738	1.4	400	1	0.8
single slice and stack recording	1024 × 1024	738 × 738	1.4	100 or 200	2	0–0.2

Stacks were recorded with a slice spacing ("z-step") of 1 μm.