

## **Vasa nervorum in rat major pelvic ganglion are innervated by nitrergic nerve fibres**

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## **Abstract:**

**Introduction:** The vasa nervorum comprises a network of small diameter blood vessels that provide blood supply to nerves and ganglia. The cell bodies of autonomic nerves innervating the urogenital organs are housed in the major pelvic ganglia (MPG) in rats. The vasa nervorum of rat MPG have not been characterised previously and it is not known whether these blood vessels are innervated by neuronal nitric oxide synthase (nNOS) containing nitrenergic nerves.

**Aim:** To characterise the blood vessels in and around the rat MPG and to assess their nitrenergic innervation.

**Methods:** Major pelvic ganglia were obtained from healthy Sprague Dawley rats, fixed in paraformaldehyde, frozen and sectioned using a cryostat. The blood vessels and their nitrenergic innervation were assessed with immunohistochemistry using antibodies against alpha-smooth muscle actin (smooth muscle marker), CD31 (endothelial marker), collagen IV (basal membrane marker) and nNOS. The immunofluorescence was imaged using a laser scanning confocal microscope.

**Results:** The neuronal cell bodies were contained within a capsule in the MPG. Blood vessels were observed within the capsule of the MPG as well as outside the capsule. The blood vessels inside the capsule were CD31-positive capillaries with no smooth muscle staining. Outside the capsule both capillaries and arterioles were observed. The extra-capsular arterioles, but not capillaries were innervated by nNOS-positive nerve fibres.

**Conclusions:** This study, to our knowledge, is the first to demonstrate the blood vessel distribution pattern and their nitrenergic innervation in the rat MPG. While similar studies in human pelvic plexus are warranted, these results suggest that the blood flow in the MPG may be regulated by nitrenergic nerve fibres and reveal a reciprocal relationship between nerves and blood vessels.

**Key words:** nitric oxide, autonomic, nNOS, nitrenergic, urogenital, erectile dysfunction, lower urinary tract symptoms

## **Introduction:**

Neurones, relative to other mammalian cell types, are critically dependent on oxygen and have a high metabolic rate which necessitates a constant supply of nutrients [1]. This demand for oxygen and other nutrients is fulfilled by blood vessels often referred to as the vasa nervorum, the blood vessels that supply the nerves [2]. Although the vasa nervorum of large nerve trunks such as sciatic nerves have been studied previously [3], such studies in ganglia, which hold the neuronal cell bodies of autonomic or sensory nerves, have been limited [4]. One such ganglion is the major pelvic ganglion (MPG) in the rat which holds the neuronal cell bodies of autonomic nerves innervating the urogenital organs [5,6]. The MPG has been considered to be the equivalent of the inferior hypogastric plexus or pelvic plexus in humans [5,6]. Although the neuronal cell bodies and blood flow in the rat MPG have been studied previously in healthy and diabetic rats [7,8], there has never been any structural study of the vasa nervorum of rat MPG.

Neuronal cell bodies to have a higher oxygen demand compared to the nerve trunks [9]. Therefore it is not surprising to observe the presence of a dense network of small sized blood vessels around ganglia [10]; arterioles and venules of diameters between 20 and 200  $\mu\text{m}$  and capillaries of diameters less than 20  $\mu\text{m}$ . The tone of these small diameter blood vessels is regulated by factors released from the endothelium lining their lumen as well as the autonomic nerves circumventing their outer wall. These endothelial and autonomic factors include nitric oxide (NO), endothelin, acetylcholine, noradrenaline and several neuropeptides [11]. NO is synthesised by endothelial NO synthase (eNOS) in the endothelial cells of the vasa nervorum [12]. However the expression of neuronal NO synthase (nNOS) in the nerve fibres around the vasa nervorum has yet to be demonstrated.

Nitric oxide released from the vascular endothelium and nitrergic nerve fibres is the most important factor mediating cavernosal and arterial smooth muscle relaxation for penile erections [13]. NO has also been suggested to be involved in micturition, by inhibiting neurotransmission in the urethra [14] as well as regulating the prostate smooth muscle tone and glandular secretory function [15]. NO is delivered to urogenital organs via nitrergic autonomic nerves whose cell bodies are housed in the MPG as mentioned above. These nitrergic neuronal cell bodies in the ganglia and their axons (which partially form the cavernous nerve) have been studied in physiological and pathological conditions such as diabetes [16]. It is also known that nNOS in nitrergic neurons requires relatively high levels of oxygen to generate NO [17] and that the nitrergic neurons are more susceptible to pathological insults such as hypoxia than those nerves which do not express nNOS [18-20]. It is therefore essential to understand how nitrergic neurons are supplied with blood and how this blood flow is regulated.

We aimed to investigate the small diameter blood vessels (less than 200  $\mu\text{m}$  in diameter) in and around the rat MPG and assess the expression of nNOS expression in the nerve fibres around these blood vessels.

## **Materials and Methods:**

### ***Immunohistochemistry and confocal microscopy:***

A total of thirteen male Sprague Dawley (SD) rats with an average body weight of 400-500g were killed using carbon dioxide. The bilateral MPG were dissected immediately and fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 72 hours at room temperature; were then transferred to 30% sucrose in 0.1M phosphate buffer and stored at 4°C for 24 hours for dehydration. The tissues were then frozen in Optimal Cutting Temperature (OCT) compound (VWR, UK) in individual plastic specimen moulds (10mm x 10mm x 5mm; Thermo scientific) at -80°C. The tissues were then sectioned using a cryostat (Bright Instruments, model OTF; UK) and Feather™ type S35 disposable microtome blades (VWR, UK). The thicknesses of the sections were within a range of 15 to 25 µm. Sections were then transferred on Superfrost Gold Plus glass slides (25mm x 75mm x 1mm; Thermo Scientific, UK) and dried for 2 hours at room temperature.

Slides containing tissue section samples were incubated with 5% donkey serum (Millipore, UK) and 0.1% Triton X-100 (Fisher, UK) in phosphate buffer saline for 1 hour at room temperature. The slides were then incubated with the primary antibodies (Table 1) in PBS overnight at 4°C. The slides were then washed with PBS containing 0.1% Triton X-100 three times, and then incubated with secondary antibodies (Table 1) for 2 hours at room temperature. The slides were then washed again with PBS containing 0.1% Triton X-100 three times and then the sections were covered with coverslips (25 mm x 60 mm; VWR BDH Prolabo, UK) using mounting fluid (Millipore Light Diagnostics, UK). In double staining experiments, the slides were incubated with the two primary antibodies simultaneously overnight at 4°C. The secondary antibodies were applied separately with one hour spacing and PBS wash (3x) between each application.

The immunofluorescence was visualised and the images were captured using a laser scanning confocal microscope (Zeiss, LSM 510 Meta Axiovert 200M, Germany) with LSM 510 software. Excitation and emission wavelengths for fluorescein were 488nm and 520nm respectively. Rhodamine excitation and emission wavelengths were 561nm and 570nm respectively. Digital Z-stacks were obtained to construct 3D projections. Adobe Photoshop™ was utilised to overlay multiple single channel images of identical tissue samples to create a combined image.

### ***Image analysis:***

*Measurement of arteriolar diameters:* The blood vessel diameters were measured in slides stained with smooth muscle actin (SMA) antibody. The circumference of each positively

stained blood vessel in three random fields of view in each slide was measured using the linear measurement tool of the LSM software. As the cross sections of the blood vessels were irregular, direct measurement of the diameter was not possible. The diameter was therefore calculated by dividing the circumference with  $\pi$ ; since the circumference of any circle can be calculated using the following formula:

$$\text{Circumference} = 2 \times \pi \times \text{radius} = \pi \times \text{diameter}$$

*Measuring arteriolar density:* The number of blood vessels with diameters between 20 and 200  $\mu\text{m}$  was counted in slides stained with smooth muscle actin (SMA) antibody. The number of positively stained blood vessels in three random fields of view in each slide was recorded. The field of view was a fixed area with a size of 204 x 204  $\mu\text{m}$  ( $\sim 42,000 \mu\text{m}^2 = 0.042 \text{ mm}^2$ ). The density of the arterioles was then expressed as the number of blood vessels per  $\text{mm}^2$  as described by Vitullo et al in 1993 [21].

*Measuring capillary density:* The area occupied by blood vessels with diameters less than 20  $\mu\text{m}$  was measured in slides stained with CD31 antibody. The total visible area of CD31 positive capillaries was measured in three random fields of view in each slide using the linear measurement tool of the LSM software suite. The field of view was a fixed area with a size of 204 x 204  $\mu\text{m}$  ( $0.042 \text{ mm}^2$ ). The capillary density was then calculated by dividing the measured area of CD31-positive capillaries by the area of the field of view then multiplied by 100 as described by Chantrain et al in 2003 [22].

## Results:

The neuronal cell bodies within the MPG were clustered within a capsule as shown with anti-collagen IV antibody staining (Figure 1). Several small diameter blood vessels were observed outside the capsule (Figure 1).

Immunostaining with anti-smooth muscle actin (SMA) antibody has revealed that most of the blood vessels outside the capsule were SMA-positive. No SMA-positive blood vessels could be observed within the capsule (Figure 2). The density of SMA-positive blood vessels was  $161.4 \pm 28.3$  per  $\text{mm}^2$ . The average diameter of SMA-positive blood vessels was  $44.4 \pm 5.8$   $\mu\text{m}$ .

Using CD31 (endothelial cell surface marker) antibody, we observed several blood vessels with less than 20  $\mu\text{m}$  diameter within the capsule as well as outside the capsule (Figure 3). The total area covered by intra- and extra-capsular CD31-positive blood vessels with less than 20  $\mu\text{m}$  diameter was  $12.4 \pm 3.3\%$ . All of the SMA-positive blood vessels were also positive for CD31 (not shown).

SMA-positive blood vessels that have diameters between 20 and 200  $\mu\text{m}$  had nNOS-positive nerve fibres circumventing their outer wall (Figure 4). No nNOS-positive nerve fibres could be observed around the SMA-negative blood vessels.

## Discussion:

Rat MPG is known to house autonomic neuronal cell bodies in clusters surrounded by a capsule [6]. A similar capsular structure has been reported in intramural ganglia of human male urinary bladder neck [23]. It remains to be established whether human pelvic plexus (human equivalent of MPG) has a similar capsular structure.

We have observed that SMA-positive blood vessels were restricted to the extra-capsular space; no SMA-positive blood vessel could be observed within the capsule. Since SMA-positive blood vessels were also CD31 positive and their diameters ranged between 20 and 200  $\mu\text{m}$ , it is safe to assume that they are most likely to be arterioles and venules. The thickness of the smooth muscle layer suggest that most of these blood vessels are likely to be arterioles rather than venules as venules are known to have thinner smooth muscle wall than arterioles. The only blood vessels that could be observed within the capsule were SMA-negative, CD31-positive, with diameters less than 20  $\mu\text{m}$  suggesting that they were capillaries. Thus, the data show that arterioles and venules were restricted to extra-capsular space while only capillaries could be found within the capsule. To our knowledge this is the first demonstration of such a blood vessel distribution in any ganglia. It remains to be established whether a similar vascular distribution exists in human ganglia. The implications of this on oxygen and nutrient delivery to neurons also remain to be investigated.

We have observed nNOS-positive nerve fibres around the SMA-positive blood vessels in the extra-capsular space. No nNOS-positive nerves fibres could be observed around the capillaries either within the capsule or in the extra-capsular space. These results suggest that arterioles supplying the rat MPG are innervated by nitrenergic nerve fibres. To our knowledge this is the first demonstration of nitrenergic innervation of any vasa nervorum. It remains to be established whether a similar nitrenergic innervation can be found in the vasa nervorum of human plexus.

We have observed approximately 160 arterioles within an area of 1  $\text{mm}^2$  around the rat MPG. To our knowledge the density of arterioles in any ganglia has not been reported previously. In rat brain approximately 16 arterioles per  $\text{mm}^2$  have been observed [24]. Although our results may suggest that the MPG may be supplied with a higher density of arterioles than the brain, it should be noted that this striking difference could be due to different measurement techniques. The authors [24] used video imaging techniques in live animals to visualise the arterioles on the cortical surface of the brain. A more detailed immunohistochemical approach like the one we have utilised may have yielded a higher density of arterioles, as not all vessels are necessarily perfused at any one time.

Our results suggest that the dense network of arterioles around the rat MPG has both of the NO sources: eNOS in the endothelium and nNOS in the nerve fibres. These results are



suggestive of a dual NO support for the smooth muscle of the vasa nervorum akin to that observed in other vascular beds such as penile and cavernosal arterioles [13], cerebral blood vessels [25], renal [26] and gastrointestinal [27]. Compared with endothelial NO generation, the nitrergic NO generation is at a distant physical location from NO's principal scavenger red blood cells, which suggest that the amount of NO reaching arteriolar smooth muscle from the nitrergic nerve fibres may be higher than endothelial NO [28]. Nevertheless, dual source of NO may make arterioles more sensitive to phosphodiesterase type 5 (PDE5) inhibitors since the smooth muscle relaxant efficiency of PDE5 inhibitors are known to be dependent on the amount of available endogenous NO [29,30]. This raises the possibility of the use of PDE5 inhibitors to increase blood flow in the vasa nervorum. This is similar to the findings in the vasa nervorum of the sciatic nerve. The function of the sciatic nerve is well known to be highly dependent on its blood flow; in disease states like diabetes the blood flow deterioration precedes neuronal dysfunction [31]. When the blood flow in the vasa nervorum is restored, this is reflected in an improved neuronal function [32]. Indeed, diabetic sciatic nerve dysfunction has been suggested to be ameliorated by PDE5 inhibitors in animal models [33] and patients [34] which has been attributed to improved blood flow. Interestingly we have observed a similar nitrergic innervation around the blood vessels of the rat sciatic nerve (authors' unpublished observations). Although it remains to be established whether the blood flow in the MPG will be affected by PDE5 inhibitors, the recent clinical success of PDE5 inhibitors in ameliorating the lower urinary tract symptoms as well as erectile dysfunction [35] may suggest that increased blood flow to the autonomic nerves and their ganglia by PDE5 inhibitors may be beneficial in improving the autonomic nerve function [36].

Our results suggest that nitrergic nerve fibres are supplying the vasorelaxant NO to the blood vessels which are supplying blood to the nitrergic neurons (Figure 5). To our knowledge such a reciprocal relationship has not been suggested before. Several questions remain to be answered such as where these nitrergic nerve fibres originate from and whether the nitrergic neurons can regulate their own blood flow, a phenomenon that has been observed in the brain [37].

Our previous studies have shown that nitrergic nerve fibres in urogenital, gastrointestinal and cerebrovascular systems undergo a biphasic selective degeneration during diabetes in animal models [7,16,38]. These findings have further been confirmed in samples from patients with diabetes [39,40]. It may be speculated that the current finding of a reciprocal relationship between nerves and blood vessels may suggest that diabetes-induced nerve dysfunction may not necessarily be solely due to impaired blood flow in the nerves but a more complex neuronal component may be involved which obviously necessitates further investigation.

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**TABLE 1: Details of the antibodies used in the experiments**

<b>Primary or secondary</b>	<b>Name</b>	<b>Dilution</b>	<b>Species raised in</b>	<b>Source and catalogue number</b>
Primary	Anti-alpha smooth muscle actin (SMA)	1/2500	Mouse	Sigma Aldrich A5228
Primary	Anti-nNOS	1/2000	Sheep	A gift from Prof Garthwaite, UCL (refs)
Primary	Anti-CD31	1/50	Rabbit	Abcam AB28364
Secondary	Anti-mouse IgG (rhodamine conjugated)	1/250	Donkey	Millipore AP192R
Secondary	Anti-mouse IgG (fluorescein conjugated)	1/250	Donkey	Millipore AP192F
Secondary	Anti-sheep IgG (fluorescein conjugated)	1/250	Donkey	Millipore AP184F
Secondary	Anti-rabbit IgG (fluorescein conjugated)	1/250	Donkey	Millipore AP182F

## Figure legends:

**Figure 1:** Collagen IV immunostaining of a rat MPG. The capsule surrounding the neuronal cell bodies of the ganglion is marked with white arrow heads. One of the neuronal cell bodies within the intra-capsular area is circled with a white marker for clarity purposes. Several small diameter blood vessels stained with green can be observed outside the capsule (extra-capsular area). Scale bar is 50  $\mu\text{m}$ .

**Figure 2:** Alpha smooth muscle actin (SMA; green) immunostaining of a rat MPG. The capsule surrounding the neuronal cell bodies of the ganglion is marked with a white line for clarity purposes. Several SMA-positive blood vessels can be observed outside the capsule, blood vessels with more than 100  $\mu\text{m}$  diameters are marked with white arrow heads, while blood vessels with diameters between 20 and 100  $\mu\text{m}$  are marked with white arrows. Two small diameter SMA-positive blood vessels can be observed within the intra-capsular area, but these were not in the same plane with the neuronal cell bodies, they most probably were part of the capsule over that area. Please note that SMA immunostaining was not observed in any blood vessels with less than 20  $\mu\text{m}$  diameter. Scale bar is 50  $\mu\text{m}$ .

**Figure 3:** CD31 (endothelial cell surface marker; green) immunostaining of a rat MPG. The capsule surrounding the neuronal cell bodies of the ganglion is marked with a white line for clarity purposes. Several CD31-positive blood vessels can be observed within and outside the capsule. Blood vessels with diameters more than 20  $\mu\text{m}$  are marked with white arrow heads. Blood vessels with diameters less than 20  $\mu\text{m}$  are marked with white arrows. Please note that all of the CD31-positive blood vessels within the capsule were less than 20  $\mu\text{m}$  diameter. Scale bar: 20  $\mu\text{m}$ .

**Figure 4:** nNOS immunostaining of blood vessels in rat MPG. **A:** nNOS-positive (green) nerve fibres (marked with white arrows) can be observed around the cross section of a blood vessel stained with collagen IV antibody (red). The lumen is marked with a white circle. Scale bar is 50  $\mu\text{m}$ . **B:** nNOS-positive (green) nerve fibres (marked with white arrow heads) can be observed around the longitudinal axis of a blood vessel stained with SMA antibody (red). Several layers of smooth muscle cells (marked with white arrow) can be observed in the wall of the blood vessel. The lumen is marked with a white circle. Scale bar is 20  $\mu\text{m}$ .

**Figure 5:** Schematic representation of the relationship between nitrenergic nerve fibres innervating the blood vessels that supply the nitrenergic neurons in the rat MPG. The neurons in the MPG are contained within a capsule. The individual axons originating from the neurons form nerve bundles such as cavernous nerve which are destined for urogenital organs like penis and urinary bladder. The results of this study suggest that vasa nervorum outside the capsule are innervated by nNOS-positive nitrenergic nerve fibres. It remains to be elucidated where these fibres originate from.

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FIGURE 1

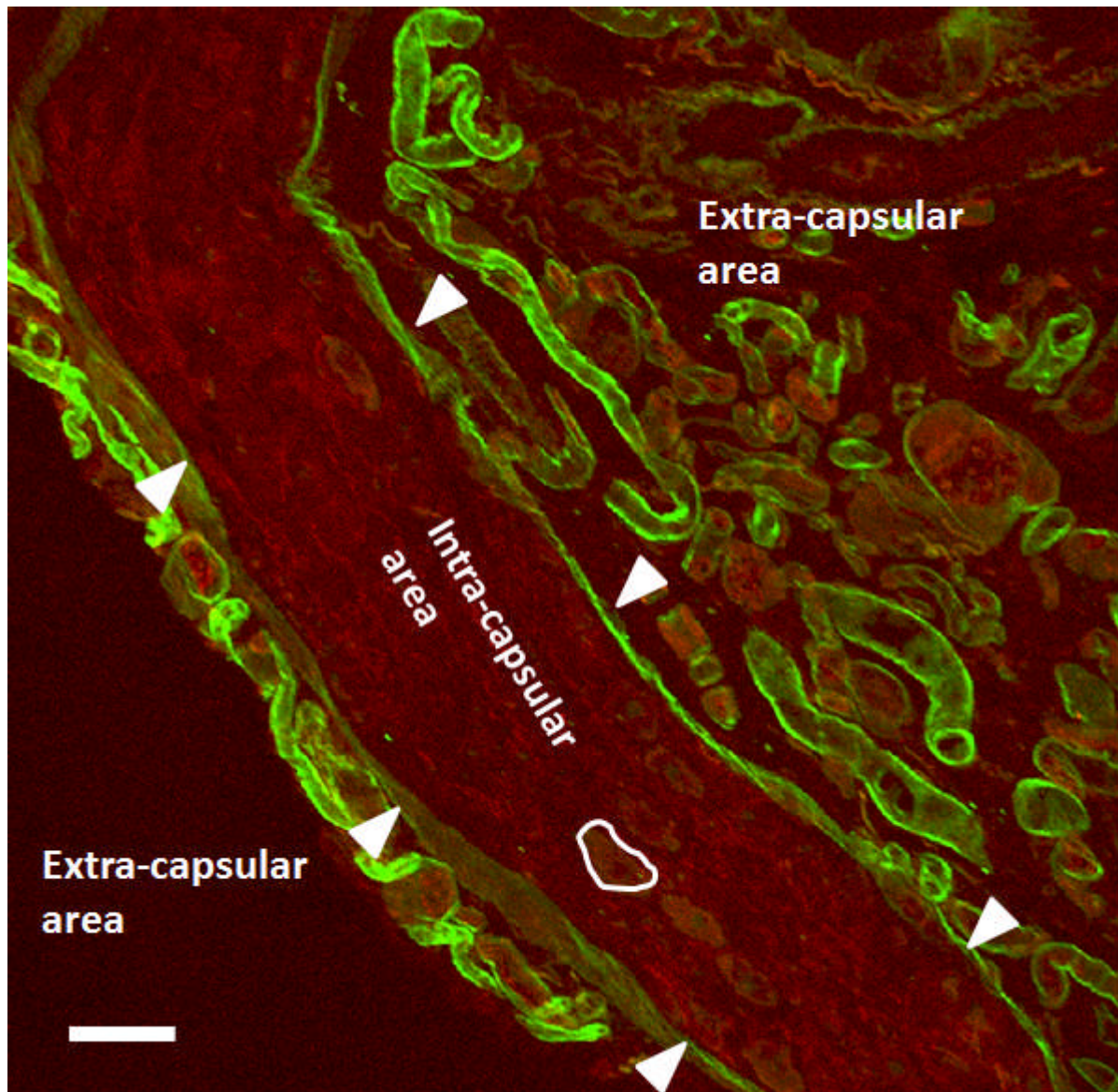




FIGURE 2

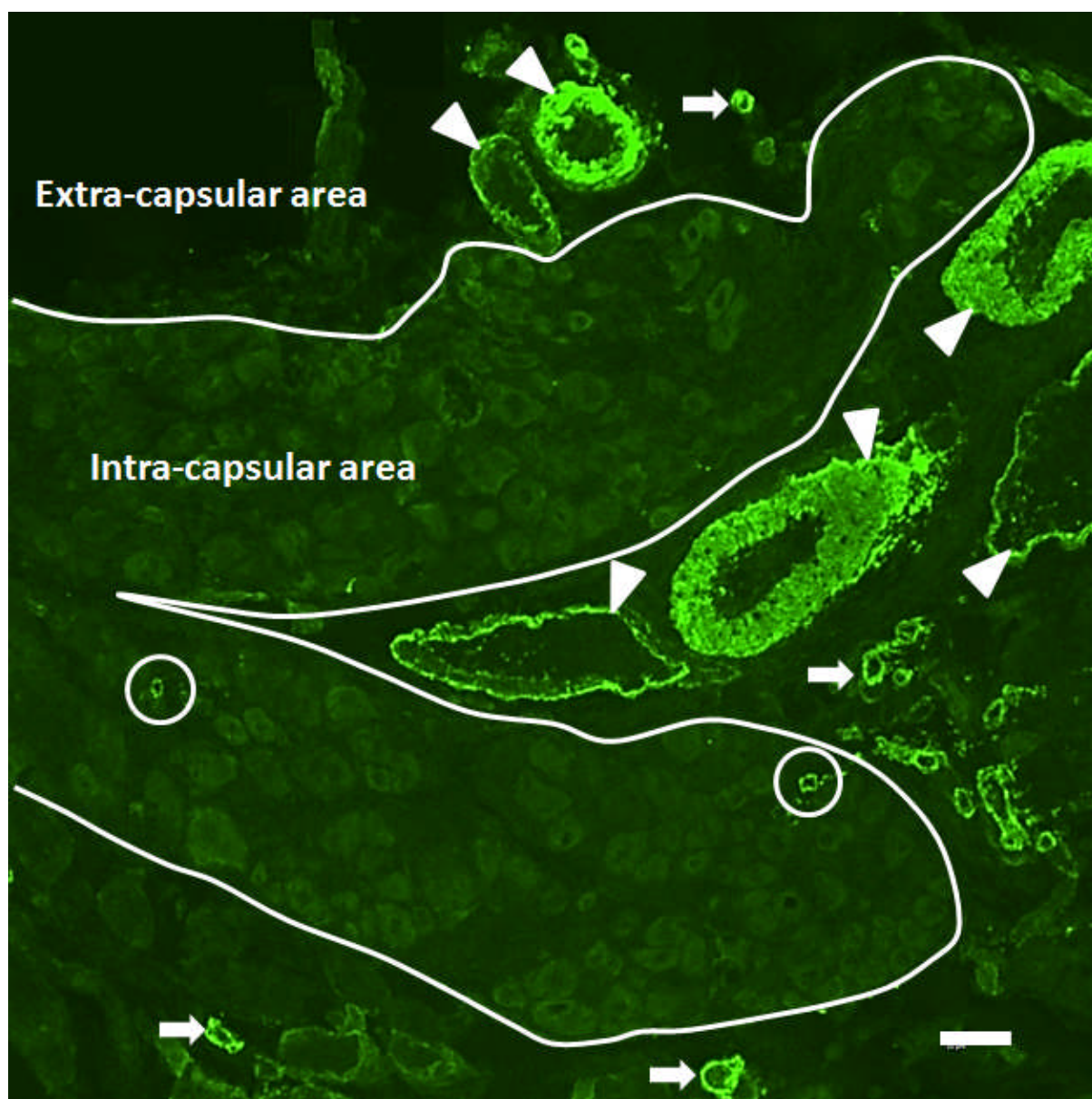


FIGURE 3

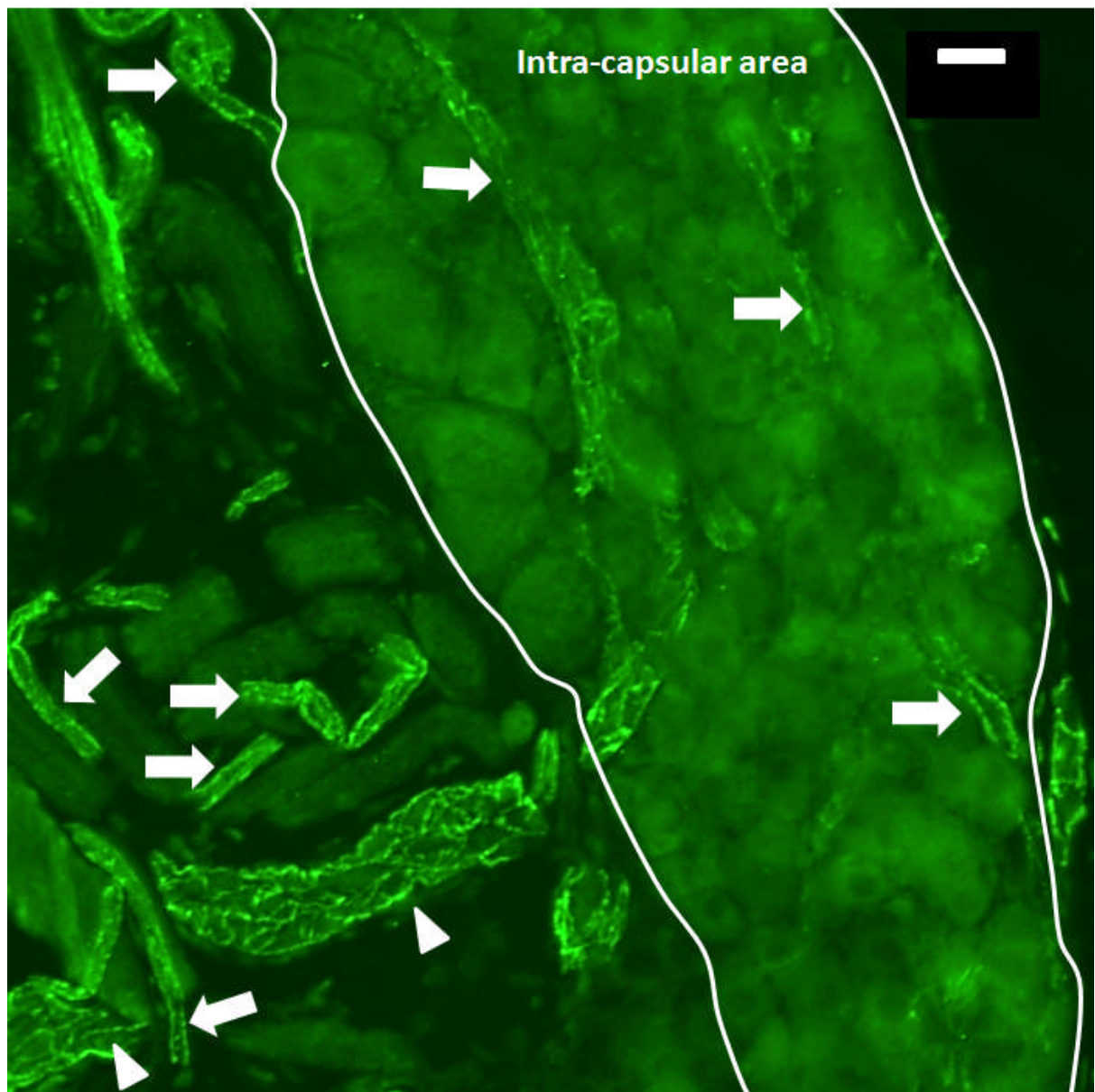


FIGURE 4A

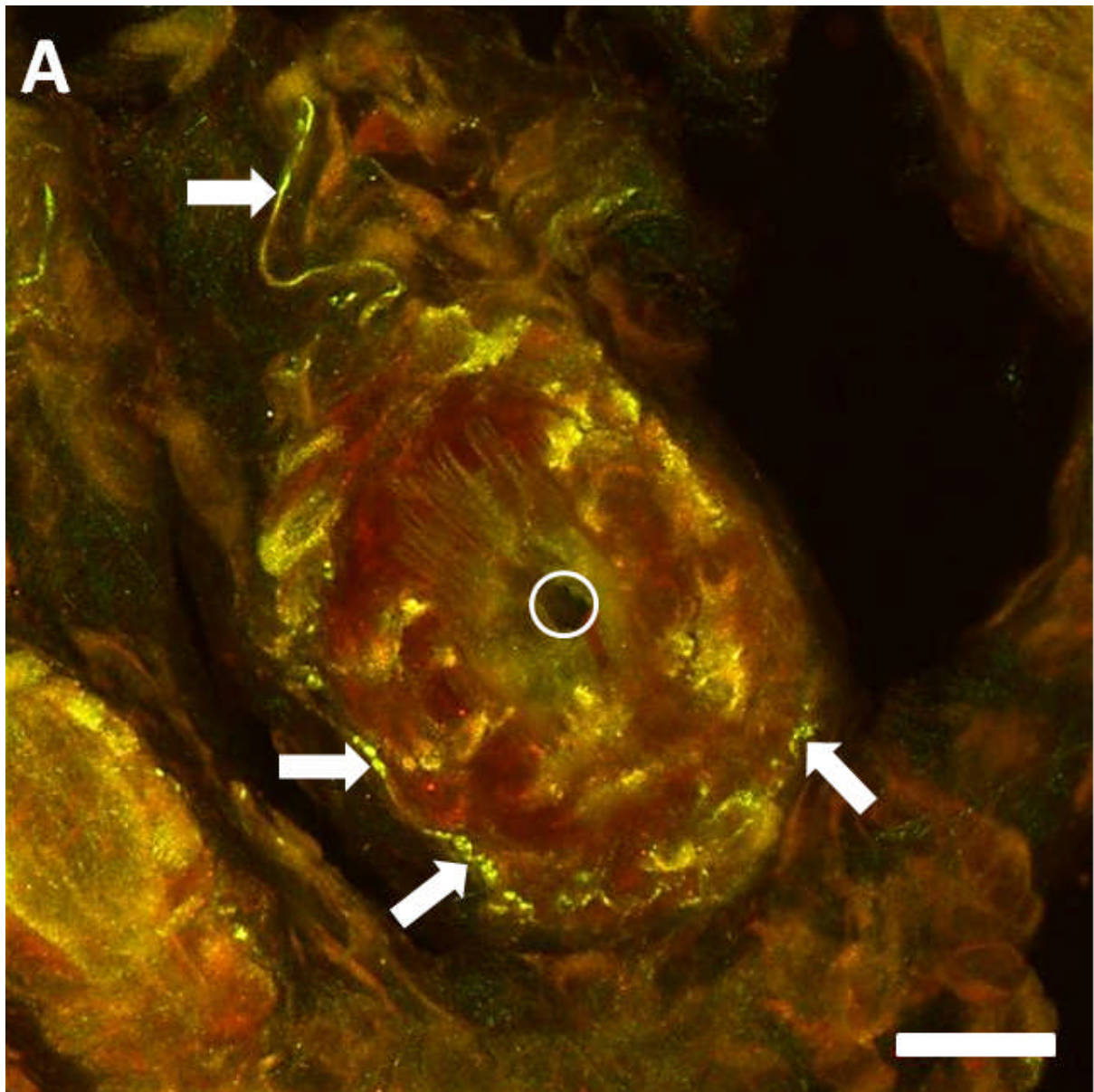


FIGURE 4B

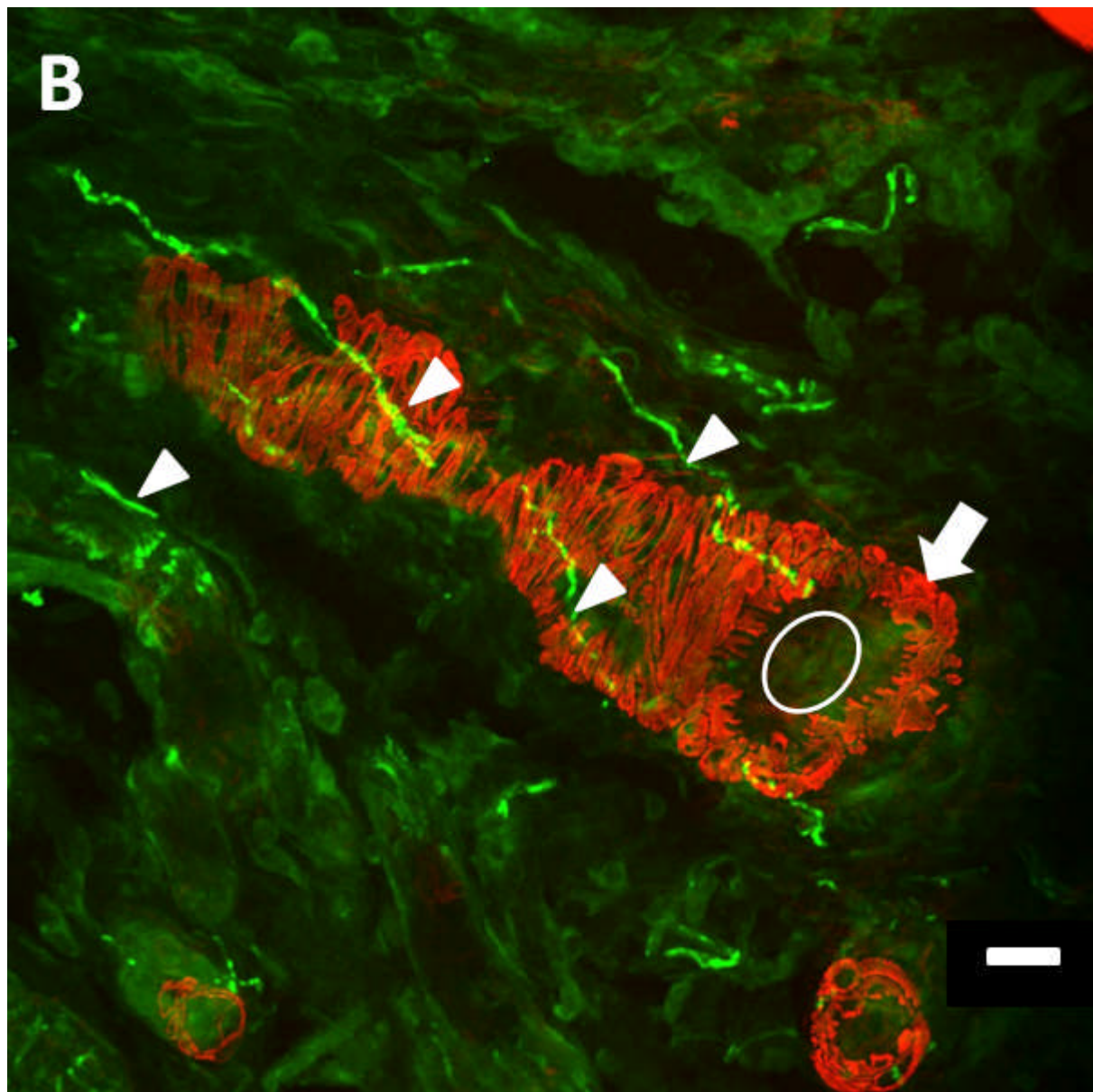
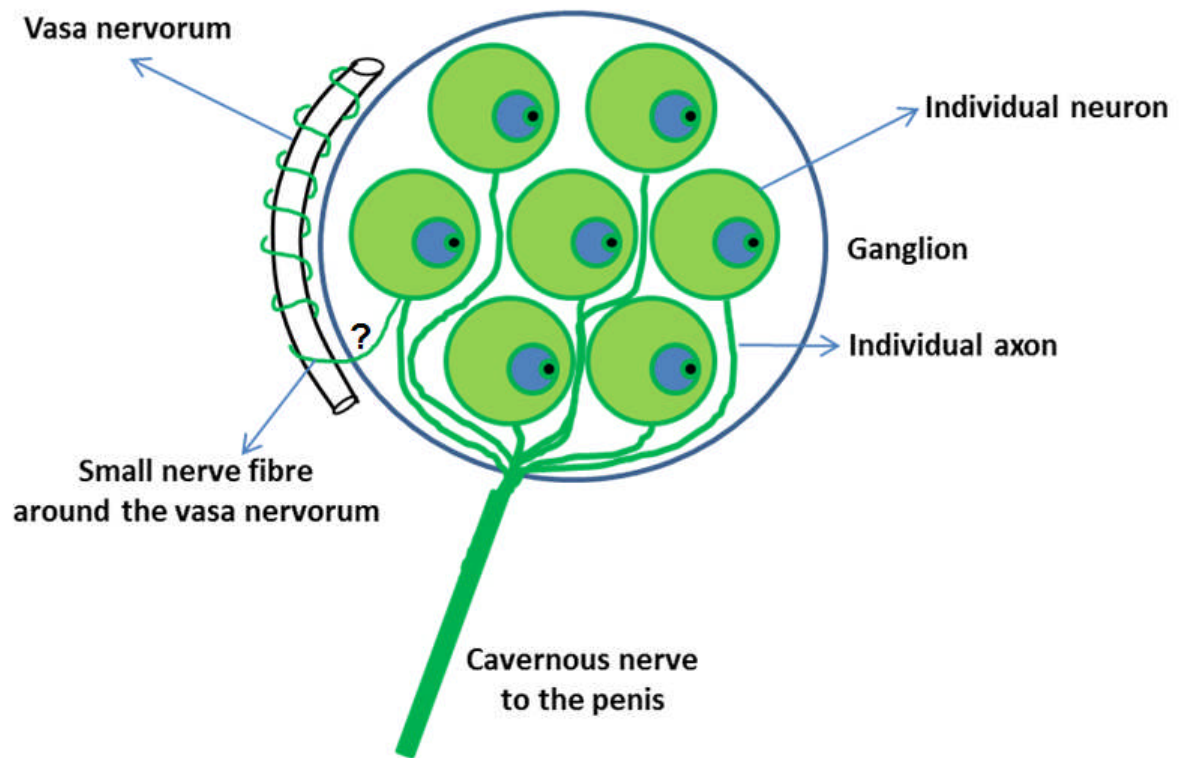


FIGURE 5



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# Vasa Nervorum in rat major pelvic ganglion are innervated by nitrenergic nerve fibers

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