# An investigation of a sympathetic innervation of the vertebral artery in primates: is there a neurogenic substrate for vasoconstriction?

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Vasoconstriction of the vertebral artery may be neurogenic in origin. Although the existence of a perivascular sympathetic plexus of the vertebral artery is not in doubt, no method used to date has conclusively demonstrated a direct sympathetic innervation of the vascular smooth muscle cells and, hence, vasomotor function. It was the aim of this study, therefore, to visualise and localise noradrenergic fibres in the wall of the vertebral artery. Intracranial vertebral artery specimens (10 vervet monkeys and 10 baboon vessels) were sectioned (40 μm serial sections) and treated with anti-tyrosine hydroxylase, anti-dopamine β-hydroxylase, and anti-chromogranin-A antibodies. Some evidence of catecholaminergic fibres in the tunica adventitia but not penetrating the external elastic lamina or tunica media of the vertebral artery wall was seen. These findings were confirmed by electron microscopy. It was concluded that although a perivascular sympathetic plexus exists, the vertebral artery of primates was not shown to have a direct sympathetic innervation and a neurogenic vasoconstrictor function is unlikely.

Key words: immunocytochemistry, vasoconstriction, sympathetic innervation, vertebral artery.

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Vasoconstriction of the vertebral artery, a muscular artery, may be neurogenic in origin. This assumption follows from the widely-held belief that the perivascular sympathetic plexus must play a role in the innervation of the artery. There have been several methods used in an attempt to identify sympathetic fibres or the associated neurotransmitter, noradrenaline, in a variety of vessels, over the past decades. For example, many authors have used electrical stimulation of perivascular nerves to demonstrate release of noradrenaline and subsequent vasoconstriction, in several different animal species such as the rat (Hirst et al., 1982; Hart et al., 1988; Buchholz and Pickles, 1990; Garcia-Villalon et al., 1997), and dog (Rorie and Tyce, 1983; Lunn et al., 1990; Kohno et al., 1995).

Using high performance liquid chromatography, Hunter et al. (1988) identified the neurotransmitter in the trachealis muscle of the dog, and Reekie and Burnstock (1992) measured noradrenaline in the trachealis muscle and mucosa of the rat, while Mbaku et al. (2000) found catecholamines in the middle cerebral artery in the sheep. Other researchers, using confocal microscopy, attempted to identify the concentration or oscillations of calcium ions within rat vascular smooth muscle cells in situ, after stimulation of perivascular nerves electrically (Iino et al., 1994; Kasai et al., 1997) or with noradrenaline (Gonzales et al., 2001; Saino et al., 2002). However, none of these studies visualised the innervation of the smooth muscle cells of the artery wall, nor did they include the vertebral artery.

There has been no conclusive evidence, in the studies reported in the current literature involving immunocytochemistry (ICC), of a direct sympathetic innervation of the smooth muscle cells of the vertebral artery, in primates or humans. Few studies have investigated catecholamines in vessels, and none have described findings in the vertebral artery. For example, Scott et al. (1992) studied the carotid artery in the rabbit, and Bleys et al. (1996) report-

ed their findings in human basal cerebral arteries, but neither study included the vertebral artery.

These findings are supported by the few ultrastructural studies reported. Cuevas et al. (1987) studied the middle cerebral artery in humans; Nakakita et al. (1983) examined cerebral veins, and Kadowitz et al. (1981) described electron-dense noradrenergic granules in pulmonary vessels in the dog. Scott et al. (1992) also demonstrated a perivascular network and the typical dense-core noradrenergic granules in the carotid artery in the rabbit. However, in all of these studies, the sympathetic nerve varicosities were shown to lie in the outer layers of the tunica adventitia and in the tunica adventitia-media junctional areas. No penetration of the tunica media or direct innervation of vascular smooth muscle cells were shown. Furthermore, no ultrastructural studies of the innervation of the vertebral artery were found in the current literature.

A pilot study by the author using high performance liquid chromatography and electrochemical detection (HPLC), without perivascular nerve stimulation (Mitchell, 2002), found evidence of catecholamines in vertebral artery tissue in the baboon. This was supported by a further study using confocal immunofluorescence miscroscopy to visualise noradrenergic fibres (Mitchell, 2002), which appeared to be in the tunica adventitia of the vertebral artery in some baboon specimens. These investigations indicated that further research was needed to localise the sympathetic fibres in the vessels more precisely.

Immunocytochemistry, using the indirect method, is the most commonly employed and sensitive technique to visualise enzymes such as those involved in the synthesis of catecholamines, for example (Bjorklund and Hokfelt, 1983; Polak and van Noorden, 1986; Clark Brelje et al., 1993). Furthermore, histological counterstaining is often used to differentiate the related tissues, such as elastic laminae, from the catecholaminergic fibres.

The aim of the present study, therefore, was to use ICC and electron microscopy to precisely localise the noradrenergic fibres, indicated by the earlier studies in the baboon by the author, in relation to the smooth muscle cells in the wall of the vertebral artery, in an attempt to provide evidence of a neurogenic basis for vasoconstriction and vasospasm in the vertebral artery.

## Materials and Methods

### *Specimens*

As fresh human material was not available, and the anatomy of primates is comparable to that of humans (Tominaga et al., 1995), the vertebral arteries studied were obtained from baboon and vervet monkeys. Ethical approval for the study was obtained from the Animal Ethics Committee, University of the Witwatersrand, Johannesburg, South Africa.

The animals were euthenased by injections of sodium pentobarbitone (200 mg/mL to effect) (Bayer Animal Health (Pty) Ltd., Johannesburg). The sample for ICC was composed of the right intracranial (fourth) part of the vertebral artery from ten adult, male and female vervet monkeys (*Cercopithecus aethiops*) and ten adult, male and female Chacma baboons (*Papio ursinus*). For the electron microscopic study, the left intracranial (fourth) part of the vertebral artery from nine Chacma baboons was obtained. The arteries were dissected from the base of the brain of each of the animals immediately after death. Pieces of the medulla of a baboon adrenal gland, and the corpus striatum and substantia nigra of a rat brain were obtained to use as the positive control in the ICC study.

### *Tissue preparation for ICC*

Following dissection, each vertebral artery was cut into lengths of approximately 2mm and fixed in 4% paraformaldehyde (2 weeks), and post-fixed in 15% picric acid in 4% paraformaldehyde (2 to 4 days). Following this, the vertebral artery specimens were washed in phosphate buffered saline solution (PBS) and treated at room temperature in a series of solutions containing 5%, 10%, 15% and 20% sucrose in PBS to cryoprotect the tissue. Lastly, each specimen was rapidly frozen in an Eppendorf tube, in isopentane cooled in liquid nitrogen, and mounted onto a cork disc with Tissue-Tek O.C.T. compound (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA). Pieces of the adrenal glands and rat brain of approximately 2 mm by 4mm were processed as above at the same time.

Serial sections of 40 μm were then cut from the blocks of the intracranial vertebral arteries and adrenal glands of the baboon and monkey, and of the brain of the rat, using a Reichert-Jung Cryocut

1800 cryostat at –15°C. These sections were stored in small glass bottles containing antifreeze (1:1 ethylene glycol and glycerol in PBS) at -20°C. Sections were treated with rabbit anti-tyrosine hydroxylase (a'TH) (Eugene Tech International Inc., Ridgefield Park, New Jersey, USA), anti-dopamine β-hydroxylase (a'DBH) (Biogenesis Ltd., Poole, England, UK), and anti-chromogranin-A (a'CGA) (Zymed Laboratories Inc., San Francisco, California, USA) (concentration of 1:1000 each), using the floating method of Totterdell et al. (1992) as described below. The a'TH and a'DBH were used as both are antibodies against known enzymes in the catecholamine synthesis pathway. The a'CGA was used as this is an antibody against the protein chromogranin-A which binds to the noradrenalin formed, as part of the dense-core granule found in the nerve terminals.

### *ICC antibody staining procedure*

For all of the above primary antibodies, the immunolabelling procedure was the same (Totterdell et al. 1992). Sections of corpus striatum and substantia nigra of the rat, adrenal gland and vertebral artery of the baboon, and vertebral artery of the monkey were washed in PBS (2 X 10 minutes), treated in 3% hydrogen peroxide and 10% methanol (in PBS) ($H_2O_2$/M) (5 minutes), then washed in a solution of Tris buffered saline (TBS) and the detergent Triton X-100 (TBS/TritonX-100: 1 ml/L) (3 X 10 minutes) (Clark Brelje, 1993). Following this, the sections were incubated in 10% normal goat serum (NGS) (Dako A/S, Denmark) in TBS/Triton X-100 (one hour) (Clark Brelje, 1993; Arroyo-Jimenez et al., 1999), and incubated overnight at room temperature in the primary antibody (eg rabbit a'TH, a'DBH or a'CGA, respectively) (concentration of 1:1000 each). For the negative control, adjacent sections were incubated overnight at room temperature in either normal rabbit serum (NRS) (Dako A/S, Denmark) or in TBS, replacing the primary antibody.

After this, the sections were washed in TBS/Triton X-100 (3 X 10 minutes); treated with biotinylated goat anti-rabbit serum (IgG) (Vector Laboratories, Burlingame, California, USA), for two hours at room temperature; and washed in TBS only (3 X 5 minutes). The ABC solution (avidin-biotin-peroxidase complex) (Vector Laboratories, Burlingame, California, USA) was applied to the sections for two hours at room temperature, followed by rinses in TBS (3 x 5 minutes), and Tris/HCl (1 x 5 minutes).

Bound peroxidase was revealed by incubating the sections at room temperature for three to five minutes in the capture reagent DAB (diaminobenzidine-tetrahydrochloride) (BDH Laboratory Supplies, Poole, England, UK) (1 mg in 2 mL Tris/HCl and 30 µL $H_2O_2$). Following this, the sections were washed in distilled water and PBS (5 minutes each). Lastly, the sections were mounted onto gelatin-coated slides and left to dry overnight. The sections were then rehydrated in distilled water, dehydrated through a graded series of alcohols, cleared in xylene, and coverslips were applied with Eukitt.

Alternate antibody-treated sections of vertebral artery from each of the monkey and baboon specimens were counter-stained with Weigert's resorcin-fuchsin elastic stain (30 seconds). All sections were examined under the light microscope at magnifications of 40X to 1000X for evidence of catecholaminergic fibres. Examples of these fibres were photographed, using a Nikon Optiphot II photomicroscope.

### *Tissue preparation for electron microscopy*

The vertebral arteries were cut into small pieces of approximately 1 mm in length, and routinely processed for ultrastructural examination by fixing in glutaraldehyde and osmium tetroxide, and embedding in epon/araldite (Electron Microscopy Services, Fort Washington, Pennsylvania, USA). Thin gold sections were cut from blocks containing transversally- and longitudinally-orientated specimens, using a Reichert-Jung Stereostar Zoom AO 570 ultramicrotome. The sections were mounted on copper grids and stained with uranyl acetate and lead citrate (Agar Scientific Ltd., Stansted, Essex, England, UK).

The sections were viewed in a JEM-100S Jeol transmission electron microscope (Jeol Ltd., Tokyo, Japan). Particular attention was paid to the junction of the tunica media and tunica adventitia in the vertebral artery wall where autonomic nerve terminals and varicosities would be expected to occur.

## Results

### *ICC*

The labelling patterns with a'TH, a'DBH and a'CGA found in the positive controls (rat corpus striatum and baboon adrenal medulla) are similar to those previously described (Bjorklund and

Hokfelt, 1983; Polak and van Noorden, 1986; Clark Brelje et al., 1993).

The sections of the baboon and monkey vertebral artery showed positive immunolabelling with a'TH, a'DBH and a'CGA antibodies (Figure 1). The density of the catecholaminergic fibres and varicosities in the tunica adventitia varied markedly between specimens. Moreover, they did not appear to pass through the blue-green-stained external elastic lamina, nor was there evidence of nerve fibres in the tunica media.

### *Electron microscopy*

All transverse and longitudinal sections (n~250) showed the typical ultrastructure of a normal muscular artery (Fawcett, 1994). There was no evidence of noradrenergic nerve varicosities or terminals intimately related to the smooth muscle cells of the tunica media of the vertebral artery wall.

## Discussion

In this ICC study, the corpus striatum and the adrenal medulla were acceptable as a positive control. In the negative control, some non-specific background staining of the tissues occurred. This result may be expected when using the thick sections (40 μm) recommended for the floating method of Totterdell et al. (1992), in which the non-specific background staining that often occurs (Polak and van Noorden, 1986) would be more obvious than in thinner sections (10 μm). A better result was obtained in this study by incubating the negative control sections in TBS only. Despite these difficulties, however, the negative controls used were considered adequate for the purposes of this study.

The presence of perivascular noradrenergic fibres was demonstrated in this study, using antibodies against the known catecholamine rate-limiting enzyme tyrosine hydroxylase, dopamine β-hydroxylase, and the binding protein chromogranin-A (Cooper et al., 1991; O'Connor et al., 1991; Fawcett, 1994; Ganong, 1997). This is the first ICC investigation of the existence and localisation of perivascular catecholaminergic nerves in the vertebral artery, using the baboon as the primate animal model. It may be inferred, therefore, that similar conditions occur in the vertebral artery of other primates, including humans. The results of the present ICC study support the findings of the HPLC and confocal microscopic pilot studies by the author (Mitchell, 2000), which indicated the presence of noradrenaline, thus sympathetic fibres, in the wall of the vertebral artery. Although the present and this earlier evidence suggest that the fibres were scattered throughout the tunica adventitia, a study by the author using the sucrose-potassium phosphate-glyoxylic acid (SPG) immunofluorescence technique (Mitchell, 2004) did not demonstrate noradrenergic fibres in close proximity to the vertebral artery tunica media of the primate. The present ICC study, however, showed in some sections that the nerve fibres and varicosities lay near the tunica media but external to the external elastic lamina. A possible explanation for this apparent discrepancy in the findings in the present ICC study is that the ICC method is more sensitive and specific for the visualisation of fine nerve fibres than the SPG technique. Furthermore, the apparent paucity of such innervation in the primate vertebral artery makes it difficult to identify and localise the nerves accurately with the less sensitive techniques.

For the noradrenaline-mediated constrictor mechanisms to be activated in the vascular smooth muscle, the noradrenaline would have to be released from varicosities that are closely related to the smooth muscle cells of the tunica media of the vessel (Fawcett, 1994). In the present study, the elastic tissue in the wall of the vessel, stained a blue-green, could be distinguished easily from the dark brown-staining nerve fibres, making it possible to differentiate the latter from the external elastic lamina. These fibres did not appear to traverse the external elastic lamina or penetrate the tunica media in any of the sections of the baboon or monkey vertebral artery. However, at the magnifications used (40X to 400X), it was not possible to visualise precisely how closely related the varicosities were to smooth muscle cells in the region of the discontinuities in the external elastic lamina.

It must be noted that although the thick (40 μm) sections are routinely used with this ICC method (Totterdell et al., 1992), it may not have been possible to visualise and, therefore, detect the presence of very small or fine nerve fibres and varicosities in these sections. Another confounding factor may be the long fixation period used in this study, reducing the sensitivity of the method and resulting in poor visualisation of these fibres.

To investigate this anatomical relationship further, the electron microscopic examination of the baboon

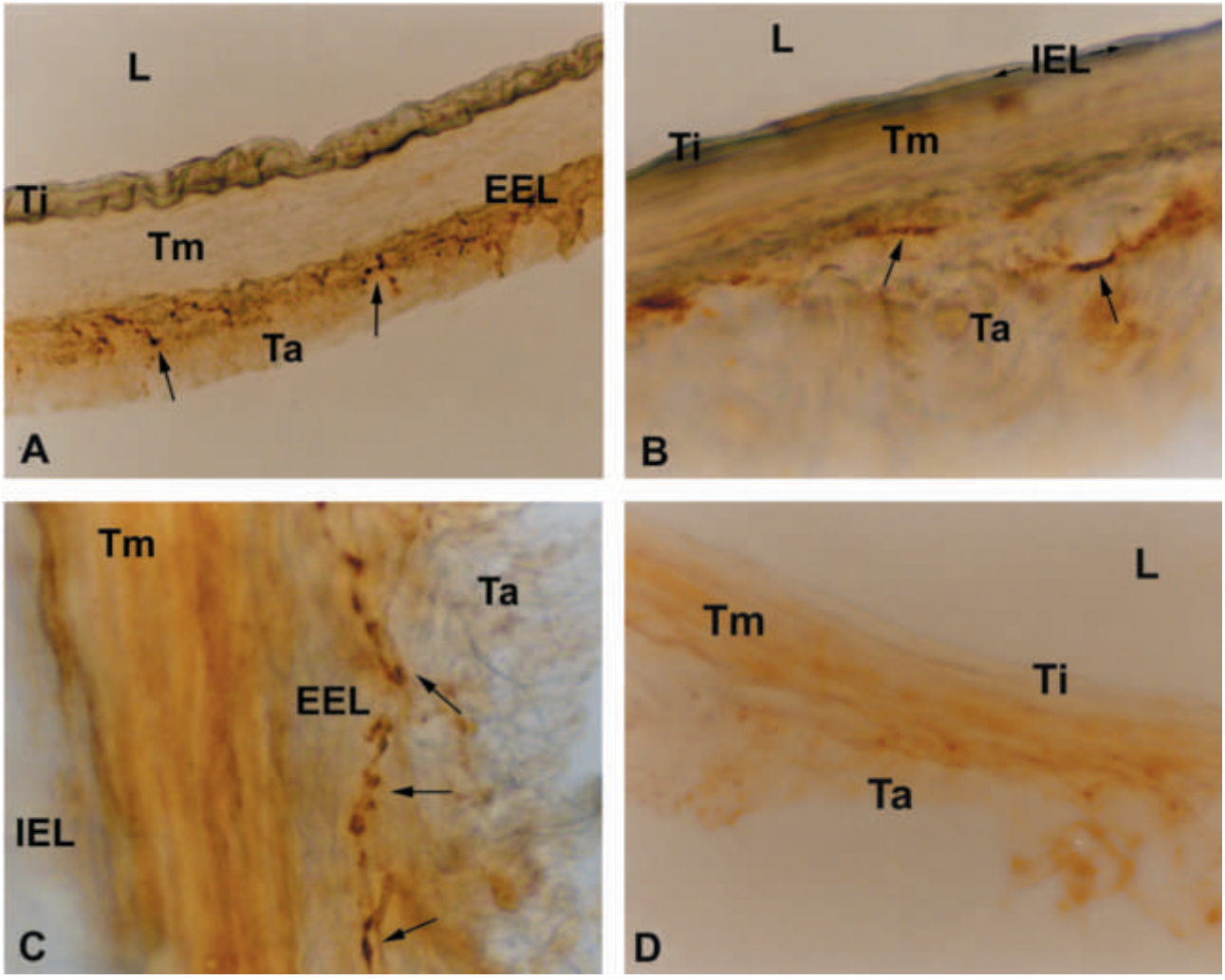


**Figure 1. Visualisation of noradrenergic nerves in the wall of the vertebral artery using immunocytochemistry and a'TH, a'DBH, and a'CGA. A, B, C. Segments of the vertebral artery (transverse sections) incubated with a'TH (baboon), a'DBH (monkey) and a'CGA (baboon), and counterstained with Weigert's resorcin-fuchsin elastic stain. Brown-stained noradrenergic nerve fibres and varicosities (arrows) are demonstrated in the tunica adventitia, near the external elastic lamina. Magnification (microscopic): 100X, 200X, 200X. D. An adjacent section of the vertebral artery (monkey) treated with TBS (negative control). No noradrenergic nerve fibres or varicosities are visualised in this section. Magnification: 200X. EEL: external elastic lamina; IEL: internal elastic lamina; L: lumen; Ta: tunica adventitia; Ti: tunica intima; Tm: tunica media.**

vertebral artery in this study was used. Although normal ultrastructure was evident in the sections, no noradrenergic nerve varicosities or terminals, intimately related to the smooth muscle cells of the *tunica media* of the vertebral artery wall, were demonstrated. This finding at the ultrastructural level was expected because the nerves visualised in the *tunica adventitia* by means of histochemical and immunocytochemical methods suggested a close but not intimate relationship with the smooth muscle cells. For this reason, only the area immediately adjacent to the outermost part of the tunica media, and not the *tunica adventitia*, was studied in the electron microscope.

The only similar reported study is that of Scott et al. (1992), who labelled the perivascular nerves of the rabbit carotid artery with a'TH, and found that the nerve fibres were situated mainly in the tunica adventitia-media junctional area. However, they did not identify a direct innervation of smooth muscle cells within the tunica media. Bleys et al. (1996) reported a similar result in their study of the human basal cerebral arteries, which involved labelling the perivascular nerves with a'TH. However, this study did not include electron microscopy to confirm a close relationship between nerves and smooth muscle cells.

The ICC and ultrastructural findings in the present study may be interpreted as there being an

extremely low density of sympathetic fibres in the vertebral artery wall. This view is supported by Koboyashi et al. (1981) who stated that the posterior part of the cerebral circulation (of which the vertebral artery is a component) is less richly innervated than the anterior part. Saltzman et al. (1992) also stated that there were marked variations in the density of the arteriolar nerve plexuses they studied. However, the presence of nerve fibres in the tunica adventitia does not confirm innervation of the cells in the tunica media. In the present study, even a sparse functional innervation of the artery wall could not be demonstrated. Thus, these results indicate that the vascular smooth muscle in the baboon vertebral artery may not be innervated directly by the noradrenergic fibres that lie in the tunica adventitia, and, as a result, do not appear to have a vasocontrictor function.

Although a vasomotor function was attributed to the smooth muscle cells in the vessels studied by the authors reviewed (Cuevas et al., 1987; Scott et al., 1992; Bleys et al., 1996), only the reports of Kadowitz et al. (1981) described the nerves penetrating the tunica media of the larger arteries. The only investigation of an intracranial vessel was that of the middle cerebral artery (Cuevas et al., 1987). Here again, no direct innervation of the vascular smooth muscle cells was demonstrated. Therefore, it seems likely that the perivascular plexus in these arteries, and in the vertebral artery of the baboon and the monkey, serves as a means by which the nerves reach the intracerebral arterioles that are sympathetically innervated (Jones, 1982; Bevan et al., 1991) and have a vasomotor function (Purves, 1972; Ganong, 1997). Moreover, it seems unlikely that the primate intracranial vertebral arteries, which supply the entire hindbrain with essential oxygen and glucose, would need to vasoconstrict. Thus, given the evidence presented here, it can be concluded that vasospasm in the vertebral artery does not appear to have a neurogenic substrate in primates. Further research to investigate the presence of noradrenergic receptors in vertebral artery smooth muscle cells may provide more and unequivocal evidence for this assertion.

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