Histol Histopathol (1996) 11: 303-311

Histology and Histopathology

Ultrastructural localization of substance P-like immunoreactivity in the intermediolateral column of spontaneously hypertensive rats and Wistar-Kyoto rats

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Summary. The distribution of substance P in the intermediolateral column of the upper thoracic spinal cord of spontaneously hypertensive (SHR) rats and Wistar-Kyoto (WKY) rats was studied by combined retrograde tracing of choleragen subunit-B horseradish peroxidase (CB-HRP) and immuno-electronmicroscopy. In the T_1-T_3 segments of the spinal cord, SP-like immunoreactive products were localized in the cell bodies and dendrites of the sympathetic preganglionic neurons as well as in a few pre-axon terminals or axon terminals. In the neuropil of the intermediolateral column (ILN), different synaptic configurations were observed including synaptic contacts between SP-like positive dendrites and negative axon terminals, and between SP-like positive axon terminals and SP-like positive dendrites. Furthermore, a single SP-like positive dendrite was sometimes postsynaptic to several axon terminals, a feature typical of glomerular synapses. The present findings suggest that most of the SP-like immunoreactive elements in the ILN were of intraspinal origin derived mainly from the sympathetic preganglionic neurons in SHR and WKY rats. Since there was no ultrastructural difference in the distribution of SP between the neural elements in the ILN of SHR and WKY rats, the present findings also suggest that SP may not be directly involved in the hyperactivity of the sympathetic nervous system in hypertension.

Key words: Sympathetic preganglionic neurons, Substance P, Intraspinal origin, Hypertensive rats

Introduction

Substance P (SP) in the spinal cord is distributed not only in the dorsal horns (Hökfelt et al., 1975; Pickel et al., 1977; Ljungdahl et al., 1978; Barber et al., 1979; Davis et al., 1984), Lissauer's tract (Barber et al., 1979; Davis et al., 1984) and ventral horns (Hökfelt et al., 1975, 1978; Singer et al., 1979; Helke et al., 1982), but also in the lateral horns (Cuello and Kanazawa, 1978; Ljungdahl et al., 1978; Davis et al., 1982, 1984; Helke et al., 1982; Holets and Elde, 1982; Davis and Cabot, 1984; Oldfield et al., 1985; Krukoff et al., 1985a,b; Bacon and Smith, 1988; Poulat et al., 1992; Yung et al., 1992) and the subependymal region (Hökfelt et al., 1975; Barber et al., 1979; Krukoff et al., 1985a,b; Oldfield et al., 1985). It has been reported that the SP-containing fibers in the dorsal horns are derived from cells in the dorsal root ganglia (Hökfelt et al., 1975; Barber et al., 1979; Nagy and Hunt, 1983) and the local circuit interneurons (Barber et al., 1979) while those in the ventral horn are described to originate from the ventral medulla (Helke et al., 1982). The SP-containing fibers in the intermediolateral column appear to be of multiple origins from the dorsal root ganglia, supraspinal regions and intraspinal neurons (Light and Metz, 1978; Holets and Elde, 1982; Davis et al., 1984; Oldfield et al., 1985; Tang et al., 1985b). Most of the studies on the distribution of SP in the intermediolateral column have been at light microscopic level (Holets and Elde, 1982; Krukoff et al., 1985b; Oldfield et al., 1985; Tang et al., 1995b). Thus, the precise localization of SP in the intermediolateral column, i.e. whether it is localized within the local preganglionic sympathetic neurons and/or their associated axon terminals cannot be determined with certainty. The distribution of SP in the spinal cord appears to show considerable segmental variations with a preferential concentration in the sympathetic subgroup nuclei (Krukoff et al., 1985b; Oldfield et al., 1985; Tang et al., 1995b). But the ultrastructural localization of SP has only been reported in the lower thoracic cord of the rat by Bacon and Smith (1988). Its ultrastructural localization in the upper thoracic segment of the cord has not been reported. The present study seeks to ascertain this by using a combination of retrograde neuronal labelling and immunoelectron microscopy. In addition, the present study will also attempt to ascertain: 1) whether SP is localized in the sympathetic preganglionic neurons that innervate the superior cervical ganglion in both spontaneously hypertensive

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rats (SHR) and Wistar-Kyoto (WKY) rats; and 2) whether there is any difference between the SP reactivity in the intermediolateral column of SHR and WKY rats. Such studies have important functional implications since there have been suggestions that dysfunction of the SP system at the spinal cord level may be the underlying cause of hypertension (Unger et al., 1980; Yashpal et al., 1987).

Materials and methods

Pre-embedding immuno-electron microscopy of substance P

For pre-embedding immunoelectronmicroscopic study, 8 males of both SHR and WKY rat (4-5 months old) were used. These rats were obtained from the Animal Resource Center, Murdoch, Western Australia. Following deep anaesthesia with chloral hydrate (0.40 gm/kg), the animals were sacrificed by perfusion which began with an intracardiac injection of 500 units of heparin and 0.25 ml of 1% sodium nitrite immediately after thoracotomy, followed by 50 ml of saline. After that, the animals were perfused with 500 ml of a mixture of 4% paraformaldehyde and 0.4% glutaraldehyde in 0.1M phosphate buffer (PB) (pH 7.4) for 30 minutes. The tissue samples obtained from T_1 to T_3 segments of the spinal cord were postfixed in the same fixative for 2 hours and kept overnight in 10% sucrose in 0.1M PB, pH 7.4. Vibratome sections 40 µm thick, were cut in the transverse plane. The sections were treated in 4% normal goat serum for 2 hours at 4 °C, after which they were washed in 0.1M phosphate-buffered saline (PBS) and placed overnight in primary rabbit anti-SP polyclonal antibody (Incstar, Minnesota) diluted 1:800 in PBS at 4 °C. The next day, the sections were washed in PBS and placed for 1 hour in biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, Calif.) diluted 1:2000 in PBS at 4 °C. After two washes in PBS, the sections were placed in ABC reagent (Vector Laboratories Burlingame, Calif.) in PBS for 1 hour at 4 °C. The sections were then washed in PBS and reacted in a solution of 0.002% H_2O_2 and 0.076% 3,3'-diaminobenzidine (Sigma) in tris buffer for 15 minutes at room temperature. They were then postfixed in 1% OsO_4, stained *en bloc* with 1% uranyl acetate in 70% alcohol, dehydrated through alcohol and flat embedded in Araldite. Ultrathin secitons were stained with lead citrate only.

Combined retrograde tracing and pre-embedding immunohistochemistry of substance P

For this study, 5 males of both SHR and WKY rats (4-5 months old) were used. Following deep anaesthesia with chloral hdyrate (0.40 gm/kg) the right superior cervical ganglion (SCG) of the rats was exposed through a midline incision in the neck. 5 µl of 1% CB-HRP (List biological Laboratories, Inc. USA) were then injected slowly into the ganglion. All injections were made with a glass micropipette. After 72 hours, the animals were sacrificed by perfusion which began with an intracardiac injection of 500 units of heparin and 0.25 ml of 1% sodium nitrite immediately after thoracotomy, followed by 50 ml of saline. After that, the animals were perfused with 500 ml of a mixture of 4% paraformaldehyde and 0.4% glutaraldehyde in 0.1M PB (pH 7.4) for 30 minutes. At the end of the perfusion, the spinal cord was cut into 3 blocks at the following levels: T_1, T_2 and T_3 removed. All the tissue blocks were postfixed in the same fixative for about 2 hours at 4 °C before they were immersed in 10% of sucrose in 0.1M PB overnight at 4 °C. Vibratome sections, 40 µm thick, were cut in the transverse plane, and processed according to the procedure of Weinberg and Van Eyck (1991). Briefly, after pre-incubation in 0.05% paratunsgstate and 0.2% tetramethylbenzidine (TMB) in 0.1M PB (pH 6.0) for 30 min, 10 µl of 30% H_2O_2 were added. Fifteen minutes later, the sections were incubated in a medium containing 0.05% diaminobenzidine (DAB), 0.02% $CoCl_2$ and 0.006% H_2O_2 for 10 min. The sections were then rinsed and processed for immunoreaction as mentioned above. After final immunoreaction, all sections were treated for 1 hour with 1% OsO_4 in phosphate buffer, dehydrated through an ascending series of ethanol and embedded in Araldite mixture. Ultrathin sections were cut on an Ultracut-E ultramicrotome (Reichert-Jung, AO American Optical) and double stained with uranyl acetate and lead citrate. All sections were viewed under a Philips 400T or JEOL 1200 EX electron microscope.

For control immunohistochemical incubation, some sections previously reacted histochemically for the demonstration of CB-HRP were incubated in 1% normal goat serum without anti-substance P antibody.

Results

SHR

Pre-embedding immuno-electron microscopic study

Pre-embedding immuno-electronmicroscopic study revealed that in the T_1-T_3 segments of the spinal cord of SHR, some of the neurons in the intermediolateral column contained amorphous SP-like immunoreactive products which were distributed throughout the cytoplasm (Fig. 1). The immunoreactive products were also localized in the dendrites (Fig. 2) and in axon terminals and preterminals (Figs. 3, 4). In the neuropil of the intermediolateral column, different synaptic configurations were observed. These included synaptic contacts between SP-like positive dendrite and negative axon terminal (Fig. 3), and between SP-like positive axon terminal and SP-like positive dendrite (Fig. 4). An SP-like positive dendrite was sometimes postsynaptic to several axon terminals, a feature typical of glomerular synapses (Fig. 5).

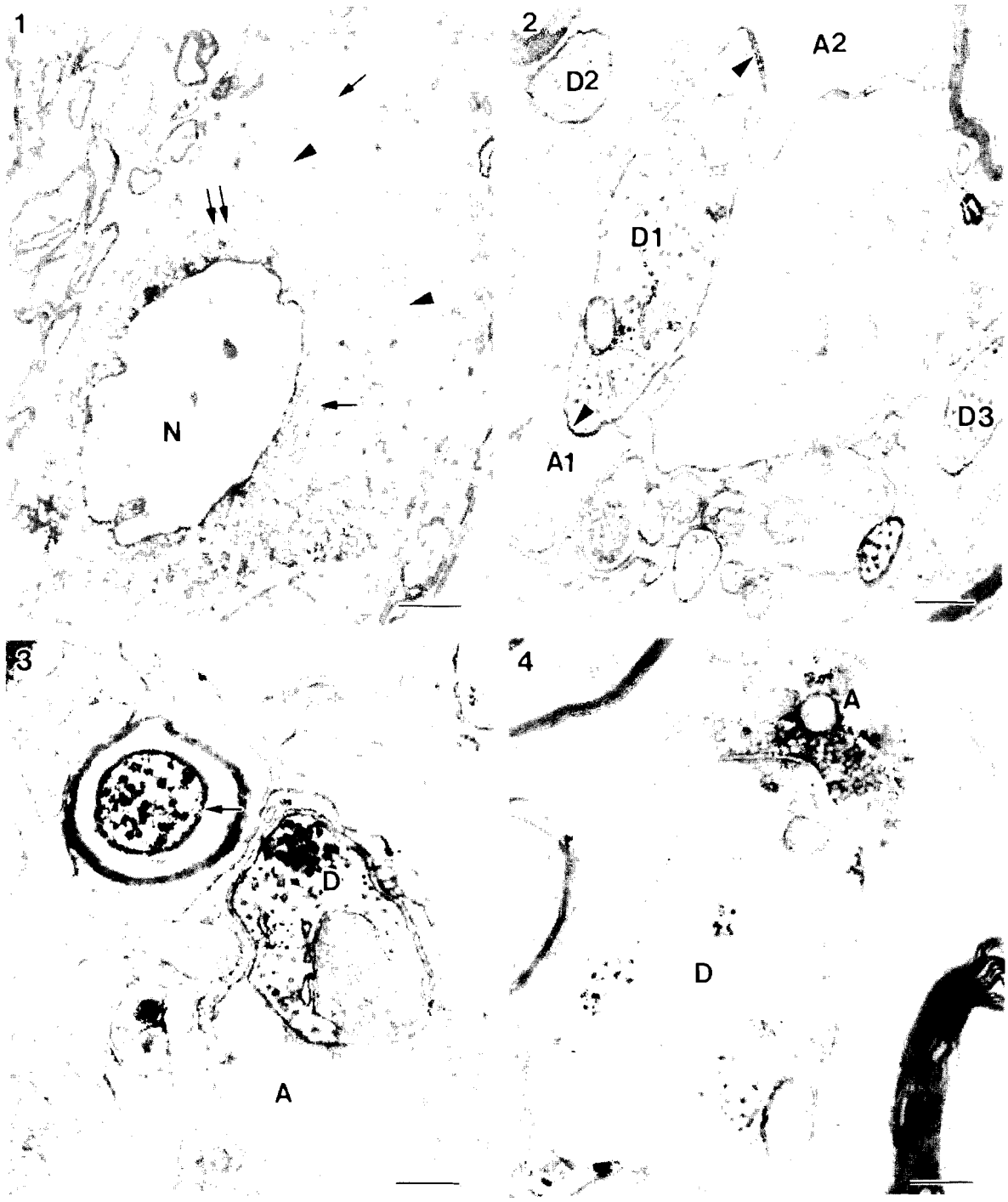


Fig. 1. A substance P (SP)-like immunoreactive neuron in the intermediolateral column of T_1 segment of the spinal cord of spontaneously hypertensive rat (SHR). The immunoreactive product is diffuse throughout the cytoplasm. The nucleus (N) is well delineated by the reaction product. The cisternae of rough endoplasmic reticulum (single arrows), mitochondria (single arrow-heads) and Golgi apparatus (double arrows) are randomly distributed. Scale bar: 1.60 μm.

Fig. 2. SP-like immunoreactive product in the neuropil of the intermediolateral column of T_1 segment of the spinal cord of SHR is localized primarily in dendrites (D1-3). One of the dendrites (D1) is postsynaptic (arrowheads) to two negative axon terminals (A1, A2). Scale bar: 0.34 μm.

Fig. 3. A pre-axon terminal (single arrow) with a thin layer of myelin sheath is immunoreactive for SP. In its proximity, an SP-like positive dendrite (D) is postsynaptic to a negative axon terminal (A). Scale bar: 0.26 μm.

Fig. 4. An SP-like immunoreactive positive dendritic (D) is postsynaptic to a positive axon terminal (A) containing agranular vesicles. Scale bar: 0.29 μm.

Combined retrograde tracing and pre-embedding immunohistochemical study

The present combined retrograde tracing and pre-embedding immunohistochemical study revealed that the sympathetic preganglionic neurons that innervated the superior cervical ganglion in the intermediolateral column of T1-3 segments of the spinal cord of SHR were SP-like positive (Fig. 6). The sympathetic preganglionic neurons were double-labelled, containing the reaction product of CB-HRP in the form of rod-like crystals embedded in the amorphous SP-like immunoreactive product (Fig. 6). In the neuropil of the intermediolateral column of T1-3 segments, most of the

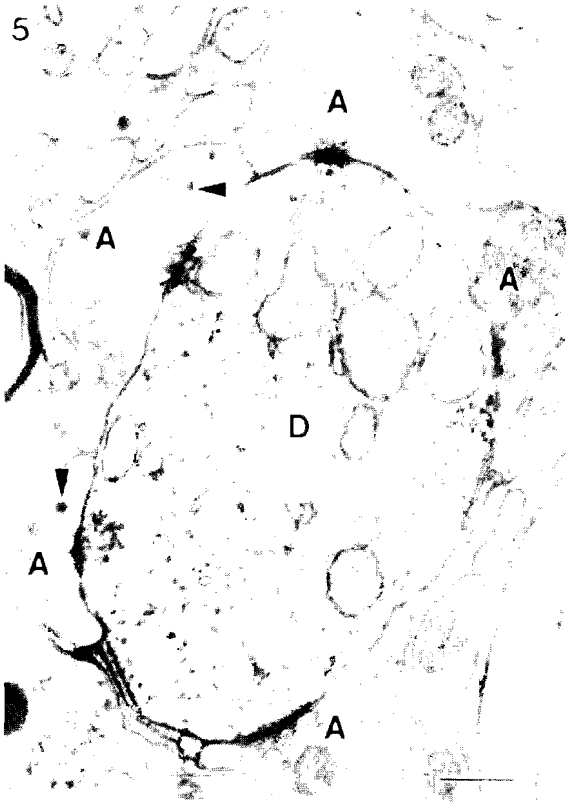


Fig. 5. A centrally-located SP-like positive dendrite (D) in a glomerular synapse is postsynaptic to several SP negative axon terminals (A). The terminals contain agranular synaptic vesicles and a few large dense-cored vesicles (arrowheads). Scale bar: 0.36 µm.

Fig 6. A choleragen subunit B horseradish peroxidase conjugate (CB-HRP) labelling sympathetic pre-ganglionic neuron innervating the superior cervical ganglion (single arrows: CB-HRP-TMB paratungstate reaction product) in the inter-mediolateral column of T_1 segment of the spinal cord in SHR is SP-like immunoreactive (asterisk: diffuse reaction product). N: nucleus. Scale bar: 0.95 µm.

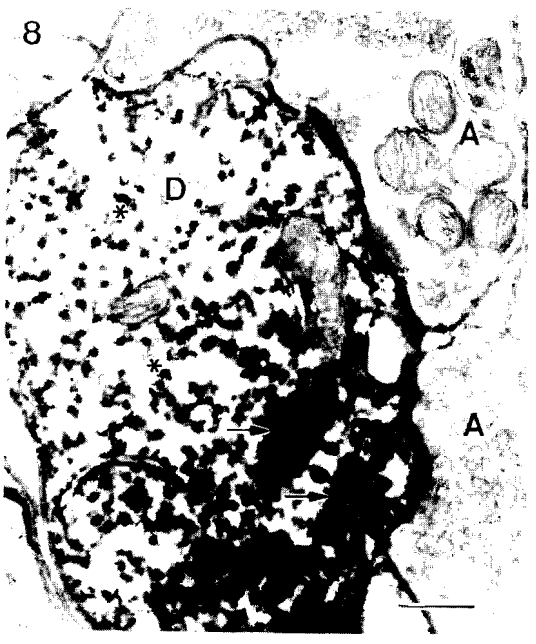


Fig. 7. Several CB-HRP-labelled dendrites (single arrowheads) of the sympathetic pre-ganglionic neurons that innervate the superior cervical ganglion are SP immunoreactive. Single arrows: CB-HRP-TMB-paratungstate reaction product; asterisks: SP immunoreactive product; A: substance P negative axon terminal. Scale bar: 0.95 µm.

Fig. 8. A double-labelled dendrite (D) is post-synaptic to two negative axon terminals (A). Single arrows: CB-HRP-TMB-paratungstate reaction product; asterisks: SP immuno-reactive product. Scale bar: 0.26 µm.

SP-like reaction products were in the dendrites of the CB-HRP-labelled sympathetic preganglionic neurons that innervated the superior cervical ganglion (Fig. 7). Often, they were postsynaptic to SP-like negative axon terminals containing round or pleomorphic agranular vesicles (Fig. 8). A few occasional sympathetic preganglionic neurons double labelled with CB-HRP and SP were in close apposition to the outer walls of blood vessels (Fig. 9).

In the control materials in which the SP antiserum was omitted during incubation, only the CB-HRP-TMB-paratungstate reaction products were shown in the cell body as well as in the dendrites (Fig. 10).

WKY rats

The present results in control normotensive WKY rats paralleled those in SHR, whereby most of the SP-like immunoreactive products were located in the cell bodies (Fig. 11) and dendrites (Fig. 12) of the sympathetic preganglionic neurons that innervated the superior cervical ganglion; occasionally, an SP-like positive axon termial was presynaptic to a positive dendrite (Fig. 13).

Discussion

Methodological advantages - Combined retrograde tracing using a tetramethylbenzidine/tungstate reaction for horseradish peroxidase histochemistry and pre-embedding immunohistochemical procedure for substance P

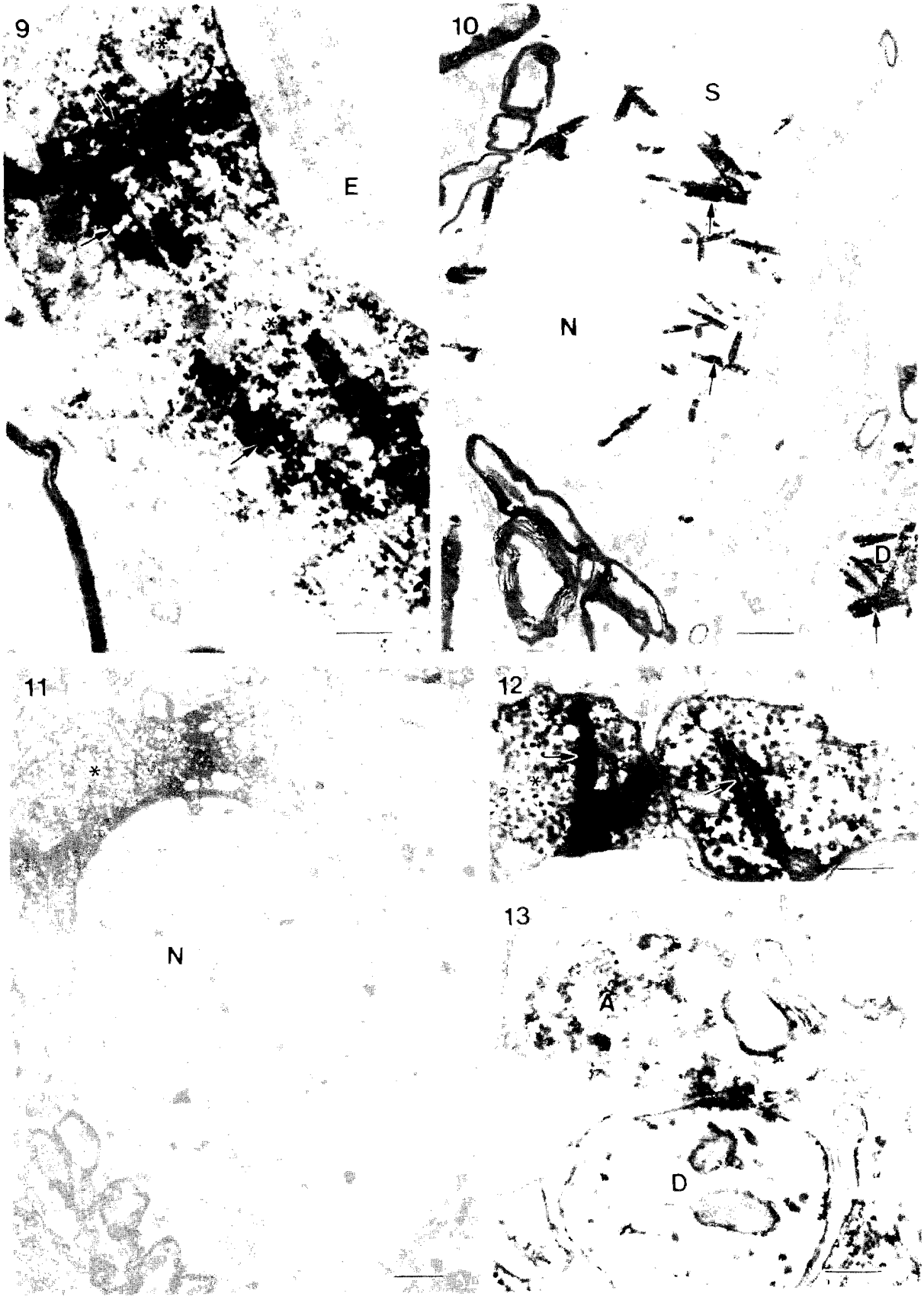
In HRP/TMB reaction, nitroprusside is one of the most sensitive stabilizers, as demonstrated in our previous studies (Tang et al., 1995a-c). However, the crystal-like reaction products dissolved readily in water and ethanol at pH 3.3. The procedure is therefore unsuitable for electron microscopy. Another choice is to use ammonium heptamolybdate for stabilizing the HRP/TMB reaction for electron microscopy, but it is clearly less sensitive. The use of ammonium paratungstate as a stabilizer in HRP/TMB reaction in previous studies (Weinberg and Van Eyck, 1991; Tang et al., 1995c) and in the present one has two major advantages over the two methods mentioned above. Firstly, it produces a more stable reaction product than nitroprusside and, secondly, it has a higher sensitivity than molybdate, as well as better tissue preservation.

The use of combined tetramethylbenzidine/tungstate reaction for horseradish peroxidase histochemistry and pre-embedding immunocytochemistry in the present study, has not only enhanced the sensitivity of HRP/TMB reaction and stability of HRP/TMB reaction products, but has also provided a better contrast between the HRP/TMB reaction products and immunoreaction products than previous studies in retrograde tracing and pre-embedding immunocytochemistry (Bacon and Smith, 1988; Pilowsky et al., 1992). The main advantage being that the CB-HRP/TMB-tungstate reaction product is readily recognized under the electron microscope as rod-like crystals, while the immunoreaction product was diffuse and evenly distributed.

Sympathetic preganglionic neurons: an intraspinal origin of substance P immunoreactive fibers in the intermediolateral column of SHR

The possible origins of SP-containing fibers in the intermediolateral column are the dorsal root ganglia, supraspinal regions or intraspinal neurons (Light and Metz, 1978; Holets and Elde, 1982; Davis et al., 1984; Oldfield et al., 1985). Light and Metz (1978) reported that in the lumbar spinal cord of the cat, primary afferent fibers collateralized into the parasympathetic nucleus and the lateral horn. This had not been substantiated by studies of other authors (Takahashi and Otsuka, 1975; Hökfelt et al., 1975; Barber et al., 1979; Tessler et al., 1981), who found a reduction of up to 90% SP content of the dorsal horn but no alteration in its distribution in the intermediolateral column or ventral horn after dorsal rhizotomies. Holets and Elde (1982) also reported that after spinal cord transection, they were unable to identify any SP immunoreactive fibers in the intermediolateral column below the level of the transection. They therefore suggested that the SP fibers in the intermediolateral column were derived from the supraspinal regions instead of the dorsal root ganglia. This is supported by both anatomical and physiological studies (Loewy and McKellar, 1981; Helke et al., 1982; Gilbey et al., 1983; Davis et al., 1984), which show that following unilateral lesions of the ventrolateral medulla the SP content of the bilateral intermediolateral column is reduced by 40% (Helke et al., 1982), and, furthermore, that iontophoretically applied SP causes an excitation of identified sympathetic preganglionic neurons (Gilbey et al., 1983). However, both the studies by Helke et al. (1982) and Davis et al. (1984) suggested the supraspinal and intraspinal origins of substance P-containing fibers in the intermediolateral column. Davis et al. (1984) even reported that after hemisection at low cervical and/or mid-thoracic levels, the intermediolateral column below the hemisection levels contained normal or elevated amounts of SP, but they did not know the exact site of the intraspinal origin. Krukoff et al. (1985a) and Oldfield et al. (1985) were able to localize some SP-containing cell bodies in the thoracolumbar sympathetic nuclei at light microscopic level, white Hökfelt et al., (1977) and Kuramoto et al. (1985) had found some SP positive fibers, presumably derived from the sympathetic preganglionic neurons, in the superior cervical ganglion and adrenal medulla. These results taken together with our previous light (Tang et al., 1995b) and present electron microscopic findings suggest that the sympathetic preganglionic neurons may be the main source of SP in the intermediolateral column of the upper thoracic cord in SHR and WKY rats.

There is considerable variation in the segmental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distribution of SP-containing fibers in the intermediolateral column of thoracolumbar spinal cord (Krukoff et al., 1985a,b; Oldfield et al., 1985; Tang et al., 1995b). Oldfield et al. (1985) reported that the greatest accumulation of SP fibers was in the T_3-T_5 and L_2-L_4 segments of the spinal cord of rabbits, cats and monkeys, whereas in the cat, Krukoff et al. (1985b) found that the greatest concentration of SP fibers was in the C_8-T_2 and T_{11}-L_1 segments. Our previous study in the upper thoracic cord of SHR and WKY rats showed that the greatest accumulation of SP-like fibers was in the T_1-T_3 segments (Tang et al., 1995b). The precise localization of SP-like immunoreactive products as well as to what extent they were derived from supraspinal area or intraspinal region could not be ascertained because all the above studies were at light microscopic level. These studies could not confirm whether the SP-immunoreactive products were contained in the axon terminals or dendrites. Since the distribution of SP is known to show considerable variation and since the subgroups of the sympathetic preganglionic neurons are described to have a preferential innervation of SP (Krukoff et al., 1985b; Oldfield et al., 1985; Tang et al., 1995b), it is not unreasonable to suggest that the distribution and origin of SP also vary in different segments of the spinal cord.

The present study showed that in the neuropil of the intermediolateral column of the T_1-T_3 segments of the spinal cord, most of the SP-like positive products were localized in the dendrites of the sympathetic preganglionic neurons, and occasionally in the pre-axons and axon terminals. In the latter, it is speculated that the SP-like immunoreactive axon terminals may arise from the paraventricular nucleus of the hyperthalamus (Sawchenko and Swanson, 1982), the nucleus tractus solitarius, the raphe nucleus (Holets and Elde, 1982) and the ventral medulla (Helke et al., 1982) which are known to project towards the intermediolateral column. It is also not impossible that they represent the collaterals of the sympathetic preganglionic neurons (Bogan and Cabot, 1991; Cabot and Bogan, 1987; Forehand, 1990). The present study has also confirmed the presence of glomerular synapses which had been described by Tan and Wong (1975). In such glomeruli, the central SP-like positive dendrite was contacted synaptically by several axon terminals. These may form the structural basis for convergence of nervous impulses to the sympathetic preganglionic neurons (Nemecek, 1972). Since the majority of the SP-like positive dendrites in the intermediolateral column were double labelled, it can be confidently concluded that in the T_1-T_3 segments, most of the SP-like positive products were from the sympathetic preganglionic neurons, with probably a minor contribution from the supraspinal regions.

The fortuitous finding of some SP-like immunoreactive sympathetic preganglionic neurons in close apposition to the outerwall of blood vessels may have functional implications. This is because our previous light microscopic study (Tang et al., 1995b) had demonstrated the occurrence of some SP-like positive neurons in contact with the cerebrospinal fluid. This is consistent with the notion of the possible neurosecretory nature of the SP system in the spinal cord (Barber et al., 1979). It is tempting to speculate that the sympathetic preganglionic neurons may release their SP contents into the circulation through their close spatial relation with the vascular wall.

Qualitative comparison of distribution patterns of SP-like immunoreactivity in the intermediolateral column of the spinal cord of SHR and WKY rats

It has been proposed that dysfunction of SP systems at the spinal level may be the underlying cause of some models of hypertension and examples of essential hypertension in man (Unger et al, 1980; Yashpal et al., 1987). Our previous light microscopic comparison of the distribution pattern of SP-like immunoreactive products between SHR and WKY showed that the ladder-like configuration of the medially-directed SP fibers in WKY rats was less conspicuous in SHR, some of them forming a dome-like or triangular configuration (Tang et al., 1995b), but that the contents of SP-like immunoreactive products were similar between those of SHR and WKY rats. Previous light (Tang et al., 1995b) and electron microscopic studies (Tang et al., 1995c) also suggested that the suppressive effect on the activity of the sympathetic preganglionic neurons may be attenuated in SHR. It was speculated that this would result in an

Fig. 9. A double-labelled sympathetic preganglionic neuron abuts against the outerwall of a blood vessel. Single arrows: CB-HRP-TMB-paratungstate reaction product; asterisks: SP immunoreactive product; E: endothelial cell. Scale bar: 0.34 μm.

Fig. 10. A CB-HRP-labelled neuronal soma (S) and dendrite (D) of the sympathetic preganglionic neuron that innervate the superior cervical ganglion in the intermediolateral column of T_1 segment. Control material incubated in 1% normal goat serum without anti-substance P antibody. N: nucleus; single arrows: CB-HRP-TMB-paratungstate reaction product. Scale bar: 2.04 μm.

Fig. 11. A substance P (SP)-like immunoreactive neuron in the intermediolateral column of T_1 segment of the spinal cord of Wistar-Kyoto (WKY) rat. The immunoreactive product is indicated by an asterisk. N: nucleus. Scale bar: 1.60 μm.

Fig. 12. Two double-labelled dendrites in the intermediolateral column of the spinal cord of WKY rats. Single arrows: CB-HRP-TMB-paratungstate reaction product; asterisks: SP immunoreactive product. Scale bar: 0.34 μm.

Fig. 13. An SP-like immunoreactive positive dendiritc (D) is postsynaptic to a positive axon terminal (A) containing agranular vesicles. Scale bar: 0.26 μm.

unbalanced activity of some of neurotransmitters regulating the sympathetic preganglionic neurons, thereby leading to the onset of hypertension. However, the results in the present EM study of the ILN of the spinal cord show that there is no difference in the distribution of SP-like immunoreactivity among the neural elements in either SHR and WKY rats. Since SP is present in both SHR and WKY rats and it is not possible to compare them quantitatively, and if the postulated rat SP may be implicated in hypertension, it can only be speculated that there may be a quantal difference in SP levels in both SHR and WKY rats. Alternatively, the present findings may also suggest that perhaps SP is not directly involved in hypertension.

Acknowledgements. This work is supported by a research grant (GR 5173) from the National University of Singapore. The technical assistance of Mr Tajuddin bin M. Ali and the technical staff in the Electron Microscope Unit is gratefully acknowledged.

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Accepted October 6, 1995