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Nerve Fibres Immunoreactive for Calcitonin Gene-Related Peptide and Substance P in the Rat Superior Cervical Sympathetic Ganglion: Distribution, Incidence and Increase with Synapse Formation Following Preganglionic Denervation

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Abstract: Objective: In rat sympathetic ganglia decentralization by preganglionic denervation leads to new synapse formation. We explored the extent to which these synapses might be derived from peptidergic nerve fibres. **Material and Method**: The distribution of immunoreactivities for calcitonin gene-related peptide and substance P was determined in superior cervical ganglia of normal rats and of rats following preganglionic denervation with prevented reinnervation. **Results:** This study has shown that networks of nerve fibres immunoreactive for calcitonin gene-related peptide alone, for both peptides together and for substance P alone become profuse and widespread in the decentralized ganglion in the longer term, in the absence of reinnervation by the preganglionic nerve fibres. No principal neurones of the ganglia showed immunoreactivity for substance P, but a few were weakly immunoreactive for calcitonin gene-related peptide. These were not seen to contribute fibres to the networks. The newly-formed networks persist over at least 12 to 16 months, and their constituent fibres form axo-dendritic synaptic contacts upon ganglionic neurones. To the extent that these fibres originate extrinsically to the ganglion, activity in their parent neurones should be capable of inducing or modulating activity in the ganglionic neurones. **Conclusion**: It is suggested that activity in such new sensory collateral branches may contribute to the low levels of ganglionic activation observable in the autonomic failure of multiple system atrophy in man. The actions of the released peptides might moreover play an important role in the maintenance of the denervated neurones, pending possible reinnervation by regenerating preganglionic nerve fibres.

Key words: Sensory nerve fibres, nerve collateral sprouting, substance P, calcitonin gene-related peptide, immunoreactivity, superior cervical ganglion.

INTRODUCTION

Decentralization of the superior cervical sympathetic ganglion (SCG) in the rat by the cutting of the cervical sympathetic trunk leads to a rapid loss of over 98% of the intraganglionic synapses. This is followed progressively, in the absence of reinnervation by preganglionic nerve fibres, by the formation of new intraganglionic nerve endings which form synapses, up to approximately 10% of the original incidence per neurone (Ramsay and Matthews, 1985). The nerve terminals which form these new synapses are mostly adrenergic in character, as shown by the presence of small dense-cored vesicles and by a capacity for labelling by uptake of the false adrenergic transmitter, 5-hydroxydopamine (5-OHDA). These adrenergic terminals are likely to arise intrinsically from adrenergic neurones in the same ganglion. A lesser proportion (approximately 30%) of the new synapses is non-adrenergic. These synapses could either be cholinergic, originating from the few intrinsic cholinergic neurones of the ganglion, or might arise extrinsically from other sources, such as sensory nerve fibres (Ramsay and Matthews 1985; Zaidi and Matthews, 1999). The new nerve terminals are found to be capable of function in that those of adrenergic type, and probably also the non-adrenergic terminals, show exocytotic release of material from their vesicles upon appropriate stimulation (Zaidi and Matthews, 1999). Thus the neurones of chronically decentralized ganglia are not necessarily silent, in the sense of being devoid of potential sources of activation. This could be particularly relevant in the case of any new synapses which are extrinsic in origin, since these might elicit potentially useful functional activity of the ganglionic neurones.

Chronic preganglionic denervation appears to be an important underlying cause of the very disabling autonomic failure which may occur in the human neurological disorder, multiple system atrophy (Low *et al.,* 1978, Oppenheimer, 1980). In this condition there may be a normal or near-normal level of plasma noradrenaline in the recumbent subject at rest (Polinsky *et al.,* 1981) but there is a failure of normal reflex

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recruitment of sympathetic activity to protect the arterial blood pressure in the standing position, which leads to orthostatic hypotension (Johnson *et al.,* 1966). These observations suggest that there may be persistence of a low level of ongoing activation of the ganglionic neurones, in the presence of extensive preganglionic denervation. It is therefore of some interest and possible clinical significance to define further the origins and potential extent of such activation.

The prevertebral sympathetic ganglia (coeliac, superior mesenteric, inferior mesenteric) which provide sympathetic postganglionic innervation to the alimentary tract and its vasculature are known to receive inputs from various sources in addition to their major innervation from the intraspinal, preganglionic neurones. These sources include neurones of the enteric nerve plexuses (Lindh *et al.,* 1988), other prevertebral ganglia (Elfvin, 1980), and sensory neurones of dorsal root ganglia. The last of these inputs is in the form of synaptic collateral branches from sensory nerve fibres terminating in the wall of the alimentary tract, which traverse the ganglia on their path between the gut wall and the dorsal root ganglia and central nervous system, depositing synapses *en route* in the prevertebral ganglia in 'axon reflex' fashion. These sensory nerve fibres comprise or include fibres immunoreactive for substance P (Baker, Cuello and Matthews, 1980; Matthews and Cuello, 1982, 1984; Dalsgaard *et al.,* 1982; Matthews, Connaughton and Cuello, 1987).

Substance P(SP) is present in a proportion of the small neurones of sensory ganglia (Hökfelt *et al.,* 1977). Calcitonin gene-related peptide (CGRP) is present in some small and some medium-sized neurones of the ganglia, and co-exists with SP in a subset of the small SP-immunoreactive (SP-IR) neurones (Gibson *et al.,* 1984, Gibbins *et al.,* 1985; Lee *et al.,* 1985a, b; Skofitsch and Jacobowitz, 1985). SP and CGRP are therefore both potentially markers for sensory nerve fibres in sympathetic ganglia. Immunohistochemistry for these peptides might thus be used to explore the incidence of such fibres in sympathetic ganglia in general and to reveal possible changes following preganglionic denervation. Surveys in adult rats (Hökfelt *et al.,* 1977; Heym *et al.,* 1993b) have shown that the normal SCG in vivo contains a few SP-IR nerve fibres but no SP-IR neurones. CGRP-immunoreactive (CGRP-IR) nerve fibres have been demonstrated in both the rat SCG (Yamamoto *et al.,* 1989) and pelvic paracervical ganglion (Inyama *et al.,* 1985). These might include fibres derived from sensory neurones immunoreactive for both SP and CGRP (Heym *et al.,* 1993b). Reservations must however be applied in interpreting findings in the case of each of these peptides. Yamamoto *et al.,* (1989) showed by retrograde neuronal tracing that most of the CGRP-IR fibres in the rat SCG were of preganglionic origin and that only a minor proportion was demonstrably sensory, with cell bodies in cervical dorsal root ganglia. In the guinea-pig stellate ganglion Heym *et al.,* (1993a) made similar observations concerning CGRP, and found co-existence of SP with CGRP in the sensory neurones retrogradely labelled from the ganglion. Immunoreactivity for SP without CGRP was demonstrated in a few nerve fibres, which they considered to be preganglionic (Heym *et al.,* 1993a). In the rat SCG, as in the guinea-pig, Heym *et al.,* (1993b) again found two types of SP-IR nerve fibres, one of which was also CGRP-IR. This second type of SP-IR fibre disappeared following preganglionic axotomy. A further caveat to be observed in denervation experiments is that decentralization and, or, axotomy may change the expression of peptides in sympathetic ganglionic neurones, in some cases dramatically so (Kessler *et al.,* 1981, 1983; Kessler and Black, 1982; Bohn *et al.,* 1984; Heym *et al.,* 1993b; Benarroch, 1994).

In the present study, in the light of these considerations we have explored the distribution and incidence of SP-IR and CGRP-IR nerve fibres in the rat SCG and have used double-labelling to look for co-existence of CGRP and SP, both in the normal ganglion and in ganglia following long-term preganglionic denervation with prevented reinnervation.

Experimental Procedures:

SCGs of Wistar rats were used in this study. Sites of immunoreactivity for SP and for CGRP were detected and quantified separately by peroxidase immunohistochemistry in cryostat sections from normal SCGs, from SCGs after long term (210d) sympathetic denervation, and from ganglia contralateral to the above. Some sections from this material were processed for immunofluorescence double labelling. Sections from two SCGs chronically denervated for 365d and 450d were processed for electron microscope immunocytochemistry. All rats were obtained from an approved breeder (Harlan, UK) and were maintained and handled under Home Office approved conditions, with unrestricted access to food and water.

Normal Ganglia:

Ganglia for control observations were taken from 7 normal rats received at the same weight and age as the experimental rats and housed with them for 14d (n = 2), 56d (n = 2) and 210d (n = 3) before sacrifice. Both right and left SCGs were removed under surgical anaesthesia induced by intraperitoneal chloral hydrate (3. 5g/kg), and the rats were then immediately killed by an overdose of anaesthetic. The ganglia were fixed in Zamboni's fixative (Stefanini *et al.,* , 1967) at 4°C for 4-6h. These normal ganglia were removed and processed together with experimental ganglia from rats of the same ages, for which they served as controls.

Chronic Decentralization:

In 5 male Wistar rats of weight 150-200g, under surgical anaesthesia induced as above the left cervical sympathetic trunk (CST) was exposed and cut 5mm below the lower pole of the superior cervical ganglion. The proximal end of the CST was sutured into the sternomastoid muscle with 7/0 silk to prevent the regeneration of preganglionic fibres back to the ganglion. The skin was sutured and the rats were allowed to survive for 210d (3 rats), 365d (1 rat) and 450d (1 rat). The success of the operative procedure was confirmed by observing maintained ptosis on the left side, both immediately following recovery from the first operation and again just before the second operation, at which both right and left SCGs were removed under terminal chloral hydrate anaesthesia, and it was confirmed that the left CST had remained embedded in the sternomastoid muscle. The ganglia were fixed in Zamboni's fixative as described above.

Quantitative Immunohistochemical Assessment:

Pairs of consecutive sections incubated for separate detection of SP and CGRP immunoreactivity were scanned at x400 under a Zeiss light microscope fitted with an eyepiece graticule engraved with 100 small squares. The numbers of intersections formed with the graticule by SP-IR and CGRP-IR fibres were counted in successive frames throughout each 10μm section of a 1 in 6 series for each peptide in each ganglion. Intersections formed by immunoreactive fibres with the leading edges of the graticule were excluded during counting to avoid repetition. Counts were expressed as mean numbers of intersections per graticule area per ganglion, ± S. E. M., for comparison.

Counts were also made of sites in each section where SP-IR or CGRP-IR fibres were seen forming basket-like wrappings around neuronal cell bodies (perineuronal baskets), and the mean numbers of baskets per graticule area per ganglion, ± S.E.M., were compared.

In all the immunochemical processing one section from each set was processed without treating with primary antibody for assessment of the extent of background nonspecific labelling.

RESULTS AND DISCUSSIONS

Light Microscopy:

Normal And Contra Lateral Ganglia:

In ganglia contralateral to long-term (210d) denervation, and in ganglia from normal rats of the same age, both CGRP-IR and SP-IR nerve fibres were consistently observed. CGRP-IR fibres were in all cases the more abundant. Immunoreactive fibres of either type were non-varicose and beaded, varicose in appearance. The non-varicose fibres were of two classes.

Infrequent, Relatively Coarse But Non-Myelinated Fibres:

These were not often seen to branch and lay singly or in small bundles, running for considerable distances through the ganglion, usually along its longitudinal axis (Fig. 1 a, b; Fig. 2 b, c, d). These bundles could contain also non-immunoreactive nerve fibres, including occasional myelinated fibres, recognizable by the presence of a refractile sheath. The more substantial bundles tended to be superficially placed. Smaller bundles were often paravascular, accompanying small arteries or arterioles within the ganglion. Bundles were seen to enter or leave the ganglion either independently (Fig. 1 c, d, f, g) or with the blood vessels. These entry or exit points often lay close to the attachments of the three major nerve trunks of the ganglion (the cervical sympathetic trunk (CST) and the internal and external carotid nerves (respectively ICN, ECN)) but were also regularly situated along the border of the ganglion opposite the base of the ECN, in the region of the carotid sinus nerve. The same bundle, identified in consecutive sections, could typically be seen to contain both CGRP-IR and SP-IR nerve fibres, the latter being usually the fewer (Fig. 1 a, b, c, d). These bundles are interpreted as being most likely to contain sensory nerve fibres, since they persisted following preganglionic denervation (Fig. 1 e). Fibres of this class, lying singly in intraganglionic nerve fascicles (Fig. 2 b, c, d), were occasionally seen to give branches which entered the perineuronal neuropil and gave simple encirclements to one or two neurones, with or without a few coarse varicosities.

Fibres of Fine Calibre:

Small numbers of fine-calibre non-varicose fibres were found running singly and unbranched, among and parallel with the nerve fibres of the major ganglionic nerve trunks, listed above. Those fibres within the ECN and ICN were possibly postganglionic. From the region of entry of the CST a few similar fine-calibre non-varicose immunoreactive nerve fibres, both CGRP-IR and SP-IR, were seen running singly along through the ganglion in the longitudinal intraganglionic nerve fascicles, becoming less numerous in the mid- to cranial

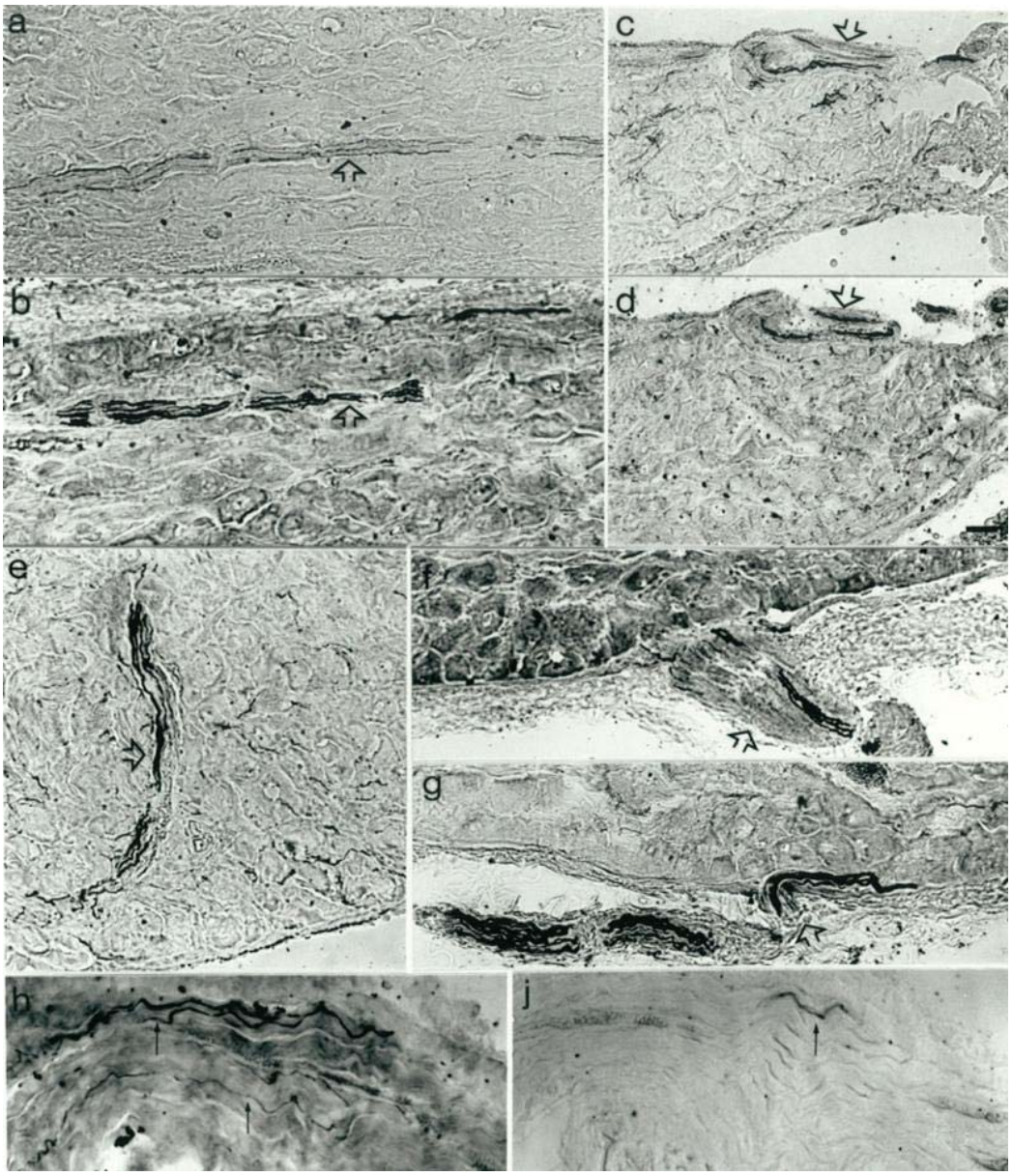

Fig. 1: Light micrographs showing CGRP-IR and SP-IR fibres in normal SCGs in old rats (approximate age 12 months) and in a chronically denervated SCG (survival interval 210d). Scale bar (in j) represents 20μm for a, b, e, f, g, h, j and (in d) 10μm for c and d. a and b are from consecutive sections of a normal SCG showing SP-IR fibres (a) and CGRP-IR fibres (b) in the same intraganglionic fibre bundle (arrows). c and d are from consecutive sections of a normal SCG showing SP-IR fibres (c) and CGRP-IR fibres (d) in the same nerve bundle (arrows) which is either entering or leaving the ganglion near its caudal end. e shows an intraganglionic bundle of SP-IR fibres in a denervated ganglion. f & g are from sections of two different normal SCGs, showing CGRP-IR fibres in nerve bundles entering or leaving the ganglion (arrows). h and j are from consecutive sections of a normal SCG. h shows fine-calibre CGRP-IR fibres in the main part of the cervical sympathetic trunk (e.g. lower arrow) and coarser CGRP-IR fibres in a marginal bundle (upper arrow). j shows a single SP-IR fibre in the same bundle.

regions of the ganglion. These fibres appeared to terminate intraganglionically by forming *varicose nerve networks*. These appeared as patches and trails of richly-branching, profusely and finely varicose immunoreactive networks, lying within the perineuronal and peridendritic neuropil (Fig. 2 a, c, f) and forming occasional densely varicose baskets or caps upon individual neurones (Fig. 2 e, f). They are interpreted as being formed by small populations of preganglionic nerve fibres having the relevant immunoreactivities. The varicose

trails and networks were patchily and unevenly distributed, but they consistently occurred at higher frequency in the wider mid- to cranial regions of the ganglion, apparently involving a greater proportion of the neurones here than in its narrower caudal portion. It was not clear whether the perineuronal baskets were unevenly distributed. Neurones which received baskets in the caudal part of the ganglion tended to stand out conspicuously from immediate neighbours which were not innervated by fibres of the same immunoreactivity (e. g. Fig. 2 e), whereas in the more cranially located patches of varicose networks such isolated baskets were less in evidence but several adjacent neurones in the same cluster might be equally densely innervated by immunoreactive fibres (e. g. Fig. 2 f).

In addition to these varicose networks the small intraganglionic arteries and arterioles, and similar vessels in the periganglionic tissues, were often seen to be innervated by closely-applied perivascular varicose strands or open-meshed networks of fine-calibre immunoreactive nerve fibres, both CGRP-IR and SP-IR. These networks were seen in places to be continuous between intra- and extraganglionic parts of the vessels. They persisted following preganglionic denervation and are interpreted as belonging to sensory nerve fibres, which might here act in both sensory and axon reflex modes. They were not seen to give branches into the neuropil in normal ganglia.

In these normal and contralateral ganglia no neurone was seen to be immunoreactive for SP and only occasional neurones showed more than traces of immunoreactivity for CGRP, despite the small numbers of fine-calibre immunoreactive nerve fibres which were found among the postganglionic nerve fibres in the ICN and ECN (for CGRP, up to 10 per section in the ICN and up to 20 per section in the much larger ECN; for SP the corresponding figures were up to 5 and up to 10).

Chronically Denervated Ganglia:

In the long-term denervated ganglia there was a marked increase of immunoreactive nerve fibres, both SP-IR and CGRP-IR. The fine-calibre, densely branched, richly varicose patchy networks, trails and baskets of the normal and contralateral ganglia were completely absent, but in their place was a new, branching, wider-meshed perineuronal network composed of wavy immunoreactive nerve fibres of moderate calibre, showing occasional coarse varicosities (Fig. 3 a to d). These fibres branched freely in the perineuronal neuropil, crossing, contouring round the neurones and sometimes encircling them (Fig. 3 e, f). Examples of tracings to show the branching of individual fibres, as shown in thick (30μm) slices immunoreacted before re-embedding for electron microscopy, are shown in Figs 4a and 4b. These tracings, taken from a ganglion at 450 days (16 months) after preganglionic denervation, indicate also that the new nerve fibres can persist over long periods. In places single neurones were enveloped in net-like formations (Fig. 3 g, h; Fig. 4 bb). These were coarser and much less richly varicose than the baskets seen in the normal and contralateral ganglia, resembling rather a cage or grille, particularly so in the case of CGRP, but they were found with similar or (for CGRP) slightly greater frequency. Occasionally the intraganglionic networks were locally of perceptibly coarser or finer calibre, and where this occurred it was found in consecutive sections to apply equally to CGRP-IR and SP-IR fibres, which suggested the possibility that both peptides might co-exist in the same fibre.

Strikingly, in these chronically denervated ganglia the other classes (perivascular, etc.) of immunoreactive fibres showed little or no difference in distribution or incidence from the normal and contralateral ganglia. Immunoreactive fibres of coarse or moderate calibre were still seen in small bundles traversing the ganglion, and entering or leaving it, independently or as paravascular bundles, without major alterations in their frequency or composition. Occasional fibres of this class still lay singly, travelling in the intraganglionic nerve fascicles. Small numbers of moderate- to fine-calibre immunoreactive fibres were still occasionally found in the ECN and ICN.

Examples were traced to show the relative abundance and distribution of immunoreactive fibres in representative sections from a chronically denervated ganglion and its contralateral pair (Figs 4c to 4e). The networks in the chronically denervated ganglia were not so patchy as those in the normal and contralateral ganglia but tended, like them, to be more abundant and more extensive cranially than caudally. In the more caudal parts of the denervated ganglia longitudinally-running non-varicose immunoreactive fibres of similar calibre to the networks in the neuropil were relatively more frequent in the intraganglionic nerve fascicles, here tending to predominate over the networks, especially in the case of CGRP. In places the immunoreactive fibres of the networks appeared to arise from these intrafascicular fibres (Fig. 4 aa), and either or both seemed occasionally to arise from the coarser immunoreactive nerve fibres in the small nerve bundles traversing the ganglia.

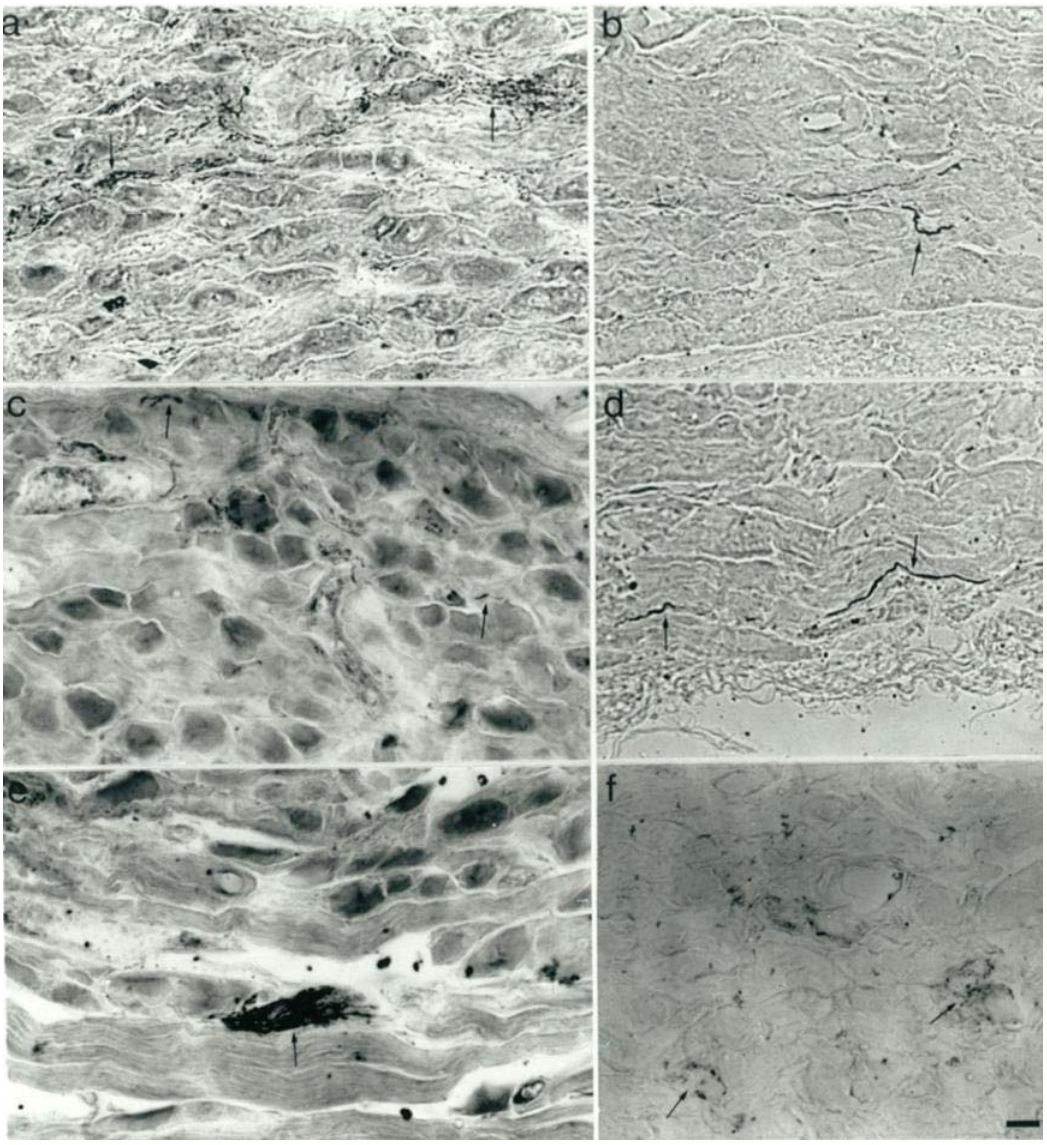

Fig. 2: Light micrographs showing SP- and CGRP-IR in normal SCGs of old rats (approximate age 12 months). Scale represents 20μm. a, c show CGRP-IR fibres (a) running in the neuropil as small bundles of fine, varicose trails and networks along the longitudinal axis of the ganglion and (c) as coarser non-varicose fibres lying singly in intraganglionic fascicles (arrows). In c, finer varicose CGRP-IR networks surround some of the neurones, and the neurones themselves show varying degrees of CGRP-IR. b and d show relatively coarse, nonvaricose SP-IR fibres lying singly, running along the long axis of the ganglion e.g. arrows). e shows CGRP-IR fibres forming a dense perineuronal basket (arrow). A few neurones in this field show moderately intense CGRP-IR. f shows SP-IR fibres forming finely varicose perineuronal baskets (arrows). That on the right involves several neurones in a close group. Other fine-calibre SP-IR perineuronal trails are present, above and to left of the centre.

Counts of Immunoreactive Nerve Fibres:

The major qualitative difference in CGRP-IR and SP-IR fibres between the older normal and contralateral and the chronically denervated ganglia was thus the replacement of fine richly varicose perineuronal networks, patchily distributed, by coarser-meshed, more pervasive perineuronal networks of immunoreactive fibres. This was accompanied by a marked increase in the numbers of intersections per graticule area formed both by SP-IR and by CGRP-IR fibres in the denervated ganglia. Mean counts for the chronically denervated and contralateral ganglia and for ganglia from three normal rats of the same age are given in Table 1. The incidences of

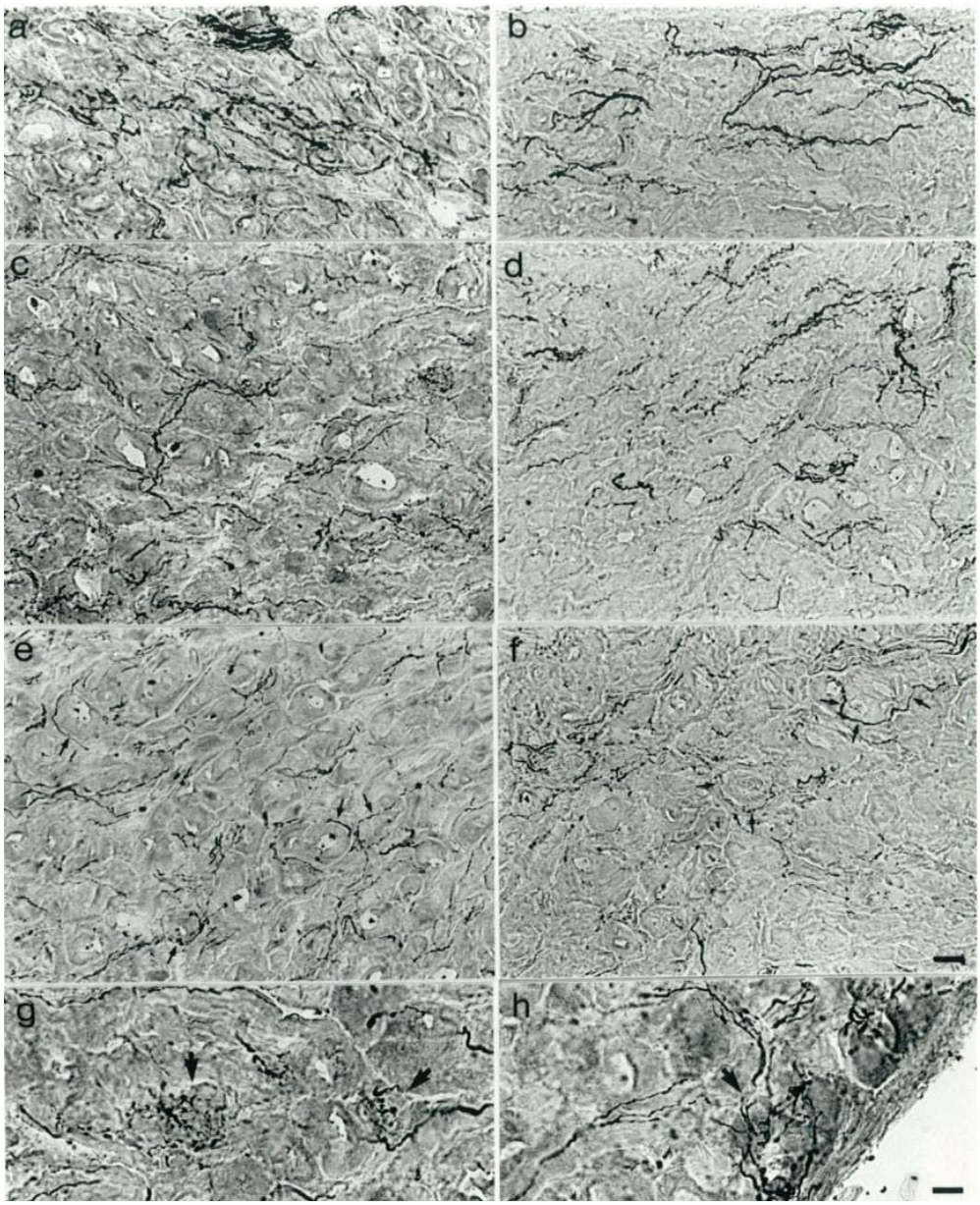

Fig. 3: Light micrographs showing SP-IR and CGRP-IR fibres in chronically denervated SCGs (survival interval 210d). Scales represent 20μm for a to f and 10μm for g and h. a and c show many CGRP-IR fibres and b and d show SP-IR fibres, forming networks throughout the ganglion. The CGRP-IR fibres were more numerous or prevalent than SP-IR fibres. e and f show perineuronal courses (indicated by arrows) of CGRP-IR fibres (e) and SP-IR fibres (f). g and h show perineuronal baskets (indicated by arrows) formed by CGRP-IR fibres.

Table 1:Counts of intersections per graticule area formed by SP-IR and CGRP-IR fibres in 210d preganglionically denervated and contralateral SCGs, and in SCGs of normal rats of the same age. n = number of ganglia.

Intersections per graticule area (mean ± S. E. M.)			
SCGs	n	SP-IR	CSRP-IR
Normal	3	2. 94 ± 0. 62	7. 82 ± 2. 06
Denervated	3	26. 12 ± 6. 33*	48. 55 ± 13. 44*
(210d)	3	2. 08 ± 0. 93	8. 32 ± 3. 62

* P < 0. 05 (Mann-Whitney U test) compared to normal ganglia.

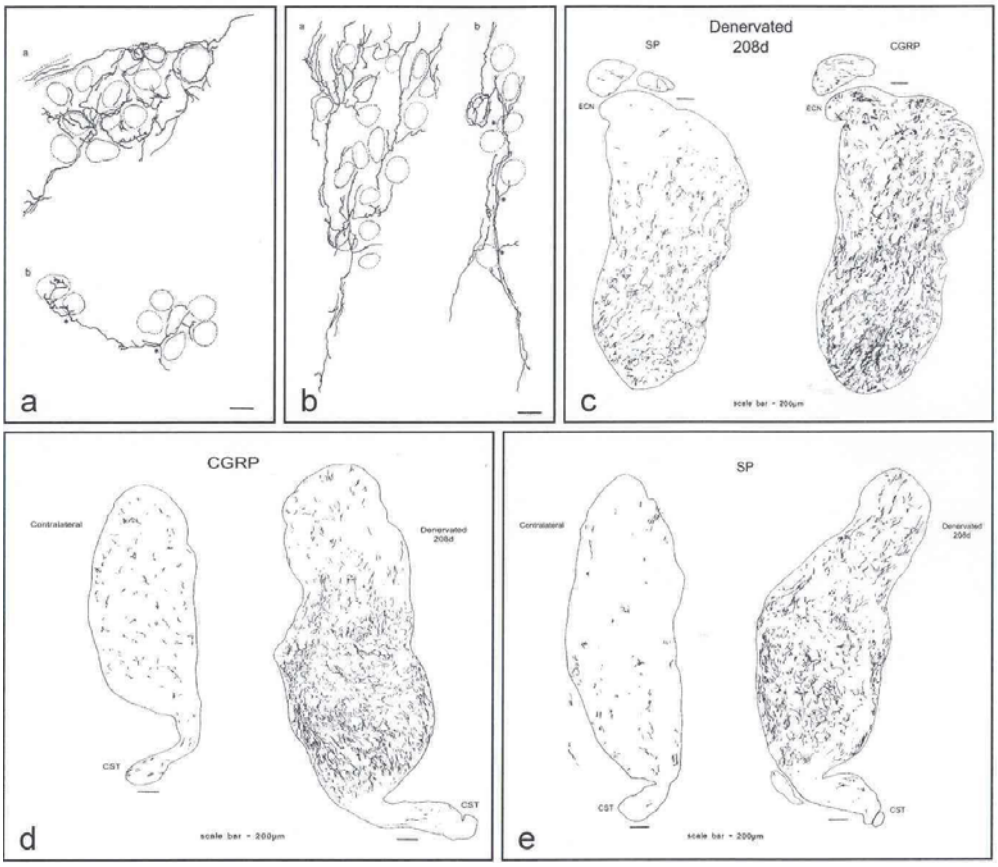

Fig. 4: a. Camera lucida tracings of SP-IR nerve fibres from a 30µm section of a chronically denervated SCG (450d) showing a) inter-neuronal and peri-neuronal courses of a group of fibres and b) a single fibre to show the branching points (some indicated by asterisks) along its course. The dotted profiles indicate neurones and the dotted lines in a represent an intraganglionic nerve fascicle. Scale represents 25µm. Figure 4 b. Camera lucida tracings of CGRP-IR nerve fibres from a 30µm section showing a) inter-neuronal and peri-neuronal courses of several fibres, b) a strand of fibres some of which form a perineuronal basket. Some branching points are indicated by asterisks and the dotted profiles indicate neurones. Scale represents 25µm. Figure 4 c. Camera lucida tracings from 10µm sections showing the relative density and distribution of SP-IR and CGRP-IR nerve fibres in the same chronically denervated SCG (210d). Scale represents 200µm. Figure 4 d. Camera lucida tracings from 10µm sections showing the density and distribution of CGRP-IR fibres in a chronically denervated SCG (210d) and in the contralateral SCG. Scale represents 200µm. Figure 4 e. Camera lucida tracings from 10µm sections showing the density and distribution of SP-IR nerve fibres in a chronically denervated SCG (210d) and in the contralateral SCG. Scale represents 200µm.

intersections did not differ significantly between normal and contralateral ganglia for either SP or CGRP, but in both these groups of ganglia CGRP-IR intersections were approximately 3 to 4 times more numerous than SP-IR intersections. Counts for both peptides in the denervated ganglia were significantly increased above the levels in normal ganglia, by approximately 9-fold for SP and 6-fold for CGRP. CGRP-IR intersections now outnumbered SP-IR intersections by approximately 2:1.

Corresponding counts for perineuronal baskets in the normal and contralateral ganglia and for basket-like formations in the denervated ganglia are given in Table 2. Here the counts do not differ significantly between normal and contralateral ganglia, or between SP and CGRP. CGRP-IR baskets are rather more numerous in the denervated ganglia, significantly more so than in their contralateral ganglia but not significantly more than in the normal ganglia.

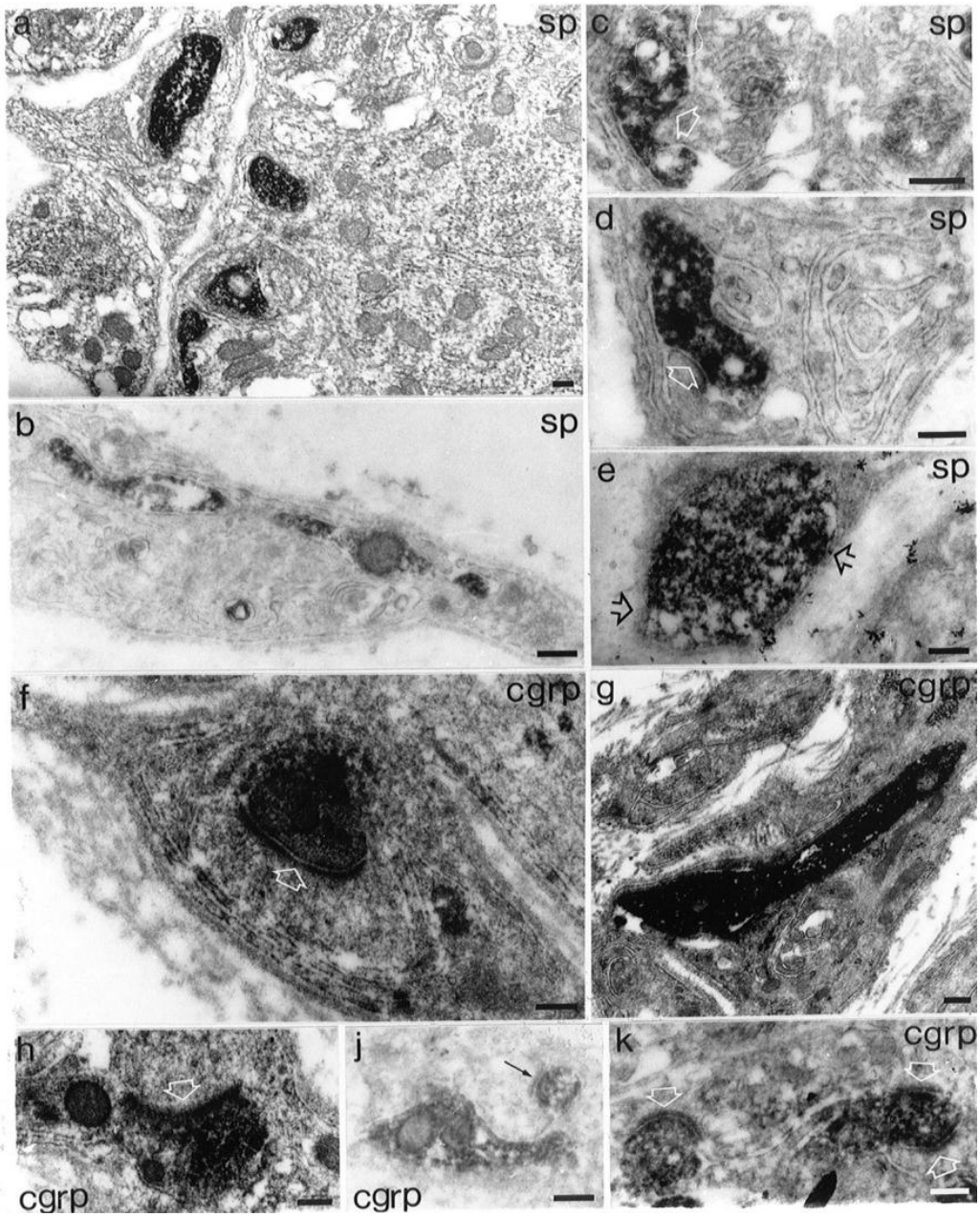

Fig. 5: Electron micrographs showing SP-IR and CGRP-IR in chronically denervated ganglia. Scales represent 0.2µm. a shows SP-IR fibres in the vicinity of a neuronal soma. All the profiles are completely surrounded by satellite cells, and none is directly apposed to the soma membrane. b shows a single SP-IR fibre enclosed completely within a covering of satellite cell cytoplasm. c, an SP-IR profile showing a synaptic attachment to an unlabelled dendritic profile (synapse indicated by arrow). Two other unlabelled vesicle-containing profiles, indicated by white asterisks, can be seen in the vicinity. d, an SP-IR profile forming a synapse (arrow) with a small dendritic profile which shows a distinct postsynaptic density. e, an SP-IR profile which is only partly enclosed by a satellite cell. A region separated only by basal lamina from the endoganglionic tissue space is indicated between arrows. g, a long section of a CGRP-IR fibre which is here enclosed completely within a satellite cell covering. f, h, j and k show (arrows) intraganglionic axodendritic synapses formed by CGRP-IR fibres.

Table 2: Perineuronal baskets formed by SP-IR and CGRP-IR fibres in 210d preganglionically denervated and contralateral SCGs, and in SCGs of normal rats of the same age. n = number of ganglia.

Perineuronal baskets per graticule area (mean ± S. E. M.)			
SCGs	n	SP-IR	CDRP-IR
Normal	3	0. 076 ± 0. 032	0. 088 ± 0. 014
Denervated (210d)	3	0. 075 ± 0. 014	0. 139 ± 0. 019
Contralateral	3	0. 051 ± 0. 034	0. 067 ± 0. 009

Electron Microscopy:

Long-term denervated SCGs (12 and 16 months) still showed numerous SP-IR and CGRP-IR nerve fibres, identifiable by electron microscopy. Most of the immunoreactive fibres which were seen were completely enclosed within Schwann or satellite cell coverings (Fig. 5a, b, g). A few sites were seen at which an immunoreactive fibre lay partly apposed to basal lamina (Fig. 5 e). Figure 5 a shows many SP-IR fibres corresponding to part of a perineuronal basket observed by preliminary light microscopy. In the sections studied, no direct appositions or synapses were found between any immunoreactive fibres and the somata of the ganglionic neurones. However, numerous examples were found both of CGRP-IR fibres and of SP-IR fibres which formed synapses with non-immunoreactive dendrites (Fig. 5 c, d, f, h, j, k). The proportions of immunoreactive profiles seen to form synapses were, for CGRP-IR fibres, 12. 3%, and for SP-IR fibres 6. 25%, of total profiles found in 8 grid squares of side 100µm in two ganglia.

Double Immunofluorescence Labelling for SP and CGRP in Normal and Chronically Denervated SCGs:

Suggestively close correspondence was noted in some instances between individual CGRP-IR and SP-IR nerve fibres in consecutive sections of immunoperoxidase-labelled ganglia. Double immunofluorescence labelling revealed some degree of coexistence of SP-IR and CGRP-IR in the same nerve fibres in normal ganglia and in 7-months-denervated ganglia and their contralateral ganglia. The fibres could be differentiated into three categories: 1) a larger group of fibres which contained only CGRP-immunoreactivity (Fig. 6 a, c); 2) relatively fewer fibres which showed immunoreactivity both for SP and for CGRP (Fig. 6 a, b; c, d) and 3) a very few fibres immunoreactive for SP only. Both varicose and non-varicose fibres were seen in all the three categories. In the chronically denervated ganglia examples were found of perineuronal basket-like formations. These could be CGRP-IR (Fig. 6 g), or CGRP-/SP-IR (Fig. 6 e, f). Baskets formed by SP-IR fibres alone were not found. A few neurones in these ganglia showed immunoreactivity for CGRP in various degrees (e. g. Fig. 6 g, j). Double immunofluorescence labelling revealed no neurones immunoreactive for both SP and CGRP in any of the normal or denervated ganglia (Fig. 10 h, j), and no principal neurone was seen to be immunoreactive for SP, but one cell of SIF cell type was observed which was immunoreactive for both peptides (Fig. 6 l, m). SIF cells are small, catecholamine-rich cells ('small, intensely fluorescent' cells) which occur in small groups and clusters in ganglia and in the rat SCG are interneurone-like (Matthews and Raisman, 1969; Case and Matthews, 1985; Matthews, 1989). They may contain various neuroactive peptides.

Discussion:

This study has shown that, following a preganglionic denervation of the rat SCG, with prevented regeneration of the preganglionic nerve fibres, extensive new networks of nerve fibres immunoreactive for CGRP and for SP appear in the ganglion and there form synapses upon the ganglionic neurones, persisting for up to at least 16 months. The new fibres show immunoreactivity for CGRP alone (the majority), for CGRP and SP together (a lesser group), or for SP alone (the least numerous). No neurone was seen to be immunoreactive for SP nor, with the exception of one SIF cell, for both SP and CGRP, but some neurones were moderately or weakly CGRP-IR. This indicates that not all the SP- and CGRP-IR fibres in the denervated ganglia necessarily have similar origins. Some of the solely CGRP-IR fibres are evidently sensory in origin, but others may be intrinsic, from ganglionic neurones. The fibres which showed immunoreactivity for both SP and CGRP are apparently purely sensory in origin, as are also the few fibres immunoreactive for SP alone, since no neurones were found to show SP-IR. Very low levels of SP-IR such as might be present in neurone somata might however not have been detected by the antibody system used, but might possibly be revealed by further exploration using colchicine to block axonal transport away from the soma.

Immunoreactive fibres accompanying or innervating the blood vessels of supply to the ganglion may also contribute sprouts. Since the immunoreactive fibres concerned survive following preganglionic denervation, we presume them to be sensory. They are relatively small and non-myelinated and are thus not somatic motor fibres. As was noted above in the Introduction, some sensory neurones in the rat, including some which may be labelled retrogradely from the SCG, are immunoreactive for either CGRP or SP, or for both peptides.

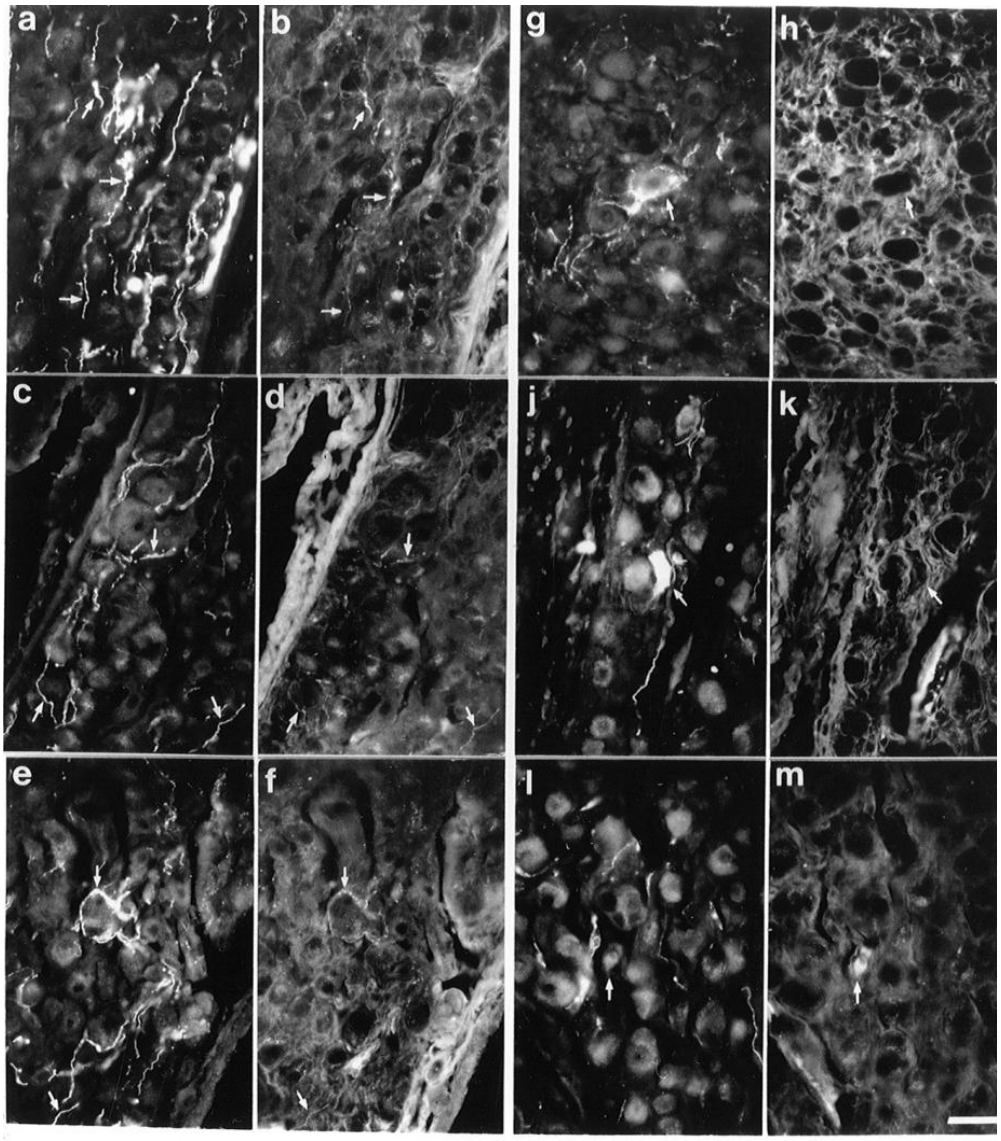

Fig. 6: Micrographs showing double immunofluorescence labelling for SP and CGRP in chronically denervated SCGs. Scale represents 50μm. a and b, c and d, paired micrographs of the same areas showing nerve fibres immunoreactive both for CGRP (a, c, arrows) and SP (b, d, arrows). Many fibres in a and c which show immunoreactivity for CGRP are not seen in b and d, i.e. are not immunoreactive for SP. e, f are paired micrographs showing a perineuronal basket formed by fibres immunoreactive for both SP and CGRP. g and h, j and k are paired micrographs showing CGRP-IR neurones (g, j, arrows) which did not show SP-IR (h, k, arrows). l, m are paired micrographs showing a small cell immunoreactive both for CGRP (l) and for SP (m).

CGRP and SP in ganglia are however not exclusively confined to these classes of presumed sensory nerve fibres in the rat. This study has also confirmed that in the rat SCG some of the preganglionic nerve fibres are CGRP-IR, as earlier found by Yamamoto *et al.,* (1989), and has further shown that as reported by Heym *et al.,* (1993b) some are SP-IR, a few in each case, with SP-IR fibres being the fewer. The tendency for SP-IR and CGRP-IR networks in the denervated ganglia to show the same distribution during their establishment as the preganglionic fibre terminations in the normal and contralateral ganglia suggests that the different populations of denervated ganglionic neurones may retain selective receptivity for innervation by fibres having a specific peptidergic phenotype.

The small compact bundles containing coarser non-myelinated CGRP-IR and SP-IR nerve fibres which survive a preganglionic denervation and are therefore presumed to be sensory are consistently found traversing

the ganglion, entering or leaving it close to or along its major associated nerve trunks, or opposite the broad base of the ECN in the neighbourhood of the carotid sinus nerve. The number and size of these nerve bundles in denervated ganglia did not differ appreciably from those seen in normal or contralateral ganglia. Their contribution to the observed increase in intraganglionic SP-IR and CGRP-IR fibres appears to occur via collateral sprouting of the fibres already entering or passing through the ganglion. Various sources of sensory nerve fibres are closely associated with the SCG. The carotid sinus nerve, a branch of the glossopharyngeal nerve, innervates the immediately adjacent carotid sinus and carotid body; the vagus nerve, carrying many sensory nerve fibres from thoracic and abdominal viscera, lies postero-medial to the ganglion, which is partly encircled by the superior laryngeal branch of the vagus and regularly has connecting strands linking its cranial end with the vagus nerve or directly with the nodose ganglion (personal observations); and the inferior end of the ganglion communicates by one or more consistent slips with nerves of the cervical plexus (Case and Matthews, 1986).

The single moderately coarse immunoreactive nerve fibres which run for varying distances along intraganglionic nerve fascicles are apparently derived from fibres in the presumed sensory nerve bundles. Fibres of this class in normal and contralateral ganglia, and also in acutely denervated ganglia, have been seen to enter or branch into the perineuronal neuropil, there encircling individual neurones with fibres of moderate calibre showing occasional coarse varicosities. There are thus in the normal rat SCG indications that sensory nerve fibres form small numbers of collateral terminations upon the ganglionic neurones. A comparable situation is seen in normal prevertebral sympathetic ganglia of the guinea-pig, where SP-IR sensory nerve fibres have been found to form collateral terminations upon the ganglionic neurones (Baker *et al.,* 1980; Matthews and Cuello, 1982, 1984; Matthews *et al.,* 1987).

Fibres of the new class of perineuronal immunoreactive networks which appears in the ganglion after a preganglionic denervation resemble the occasional moderately coarse fibres seen forming encirclements round neurones in normal ganglia. They are of similar calibre and branching pattern to the SP-immunoreactive nerve fibres seen in the prevertebral ganglia of the guinea-pig, and to the immunoreactive nerve fibres observable in the carotid body of the same rats, which are likewise of sensory origin. CGRP-IR and SP-IR nerve fibres in the rat carotid body are derived from the carotid sinus nerve and, through it, from neurones in the petrosal ganglion (Ichikawa, 2002). In the longer term the new intraganglionic networks reach a high density of distribution, extending to all parts of the ganglion, and they persist over long periods, for 16 months or longer, in the absence of reinnervation by preganglionic nerve fibres. Although unlike the preganglionic fibres they show relatively infrequent varicosities, both CGRP-IR and SP-IR fibres of this class form synapses upon dendrites of the ganglionic neurones and can form basket-like configurations round the neurones. Such 'baskets' may eventually become as frequent as in normal ganglia, but are of a coarser, less richly varicose character. This suggests that neurones which normally receive basket-like encirclements from CGRP-IR or SP-IR preganglionic fibres remain receptive to, and accept, comparable encirclements from the new immunoreactive fibres which arise post-denervation. This study leaves open the question whether other types of fibres from the presumptive sensory nerve bundles also sprout into the ganglion and form additional terminations there after a preganglionic denervation.

Since the new nerve fibres ramify so extensively in the neuropil and form synapses upon the ganglionic neurones it is relevant to consider in what ways they may influence the neurones. Non-synaptic as well as synaptic release might contribute, in the case of these peptides which are slow-acting and slowly inactivated, being capable of spreading by diffusion after release, thus most or all of the neurones might come under their influence, to the extent that they possess receptors capable of responding to these peptides. SP has been shown to produce a late, slow excitatory wave of depolarization (late slow EPSP) in sympathetic neurones (Dun and Karczmar, 1979; Dun and Jiang, 1982; Konishi *et al.,* 1983). Both substance P and CGRP are potent vasodilators (Gamse and Saria, 1985; Girgis *et al.,* 1985), and might thus enhance the ganglionic blood flow. CGRP is found also to augment the effect of other neurotransmitter substances with which it coexists, such as acetylcholine in somatic motoneurones or sympathetic preganglionic neurones and possibly fast-acting excitatory amino acids such as glutamate or aspartate in some sensory neurones. Short N-terminal peptide derivatives of CGRP can potentiate acetylcholine actions at neuronal nicotinic receptors of rat chromaffin cells (Di Angelantonio *et al.,* 2002). CGRP might regulate the release or the action of SP; for example CGRP has been shown to potentiate the release of SP from the dorsal horn of the spinal cord in rats (Oku *et al.,* 1987). CGRP may also prolong or extend the action of SP by inhibiting its breakdown by endopeptidases (Le Greves *et al.,* 1985; Schaible *et al.,* 1992). To the extent that the new fibres are of extrinsic, probably sensory origin, activity in their parent nerve fibres may induce activity in ganglionic neurones which would otherwise be silent.

Such an effect might account for the normal levels of circulating noradrenaline which may be found in the type of autonomic failure occurring in man in the clinical condition of multiple system atrophy (Polinsky *et al.,* 1981), in which there has been demonstrated an extensive loss of sympathetic preganglionic neurones (Low *et al.,* 1978; Oppenheimer 1980). In this condition the systemic arterial blood pressure and the level of circulating noradrenaline may be within normal limits while the subject is recumbent but the blood pressure falls to a low level, leading to collapse of the subject, when the erect posture is assumed (orthostatic hypotension). The plasma noradrenaline fails to rise, indicating failure of reflex recruitment of sympathetic vasoconstrictor tone (Polinsky *et al.,* 1981). The presence of circulating noradrenaline during recumbency suggests some ongoing activation of sympathetic neurones (acutely denervated sympathetic ganglia are silent), and its failure to rise in the erect posture indicates that this is not accessible to the normal reflex pathways. Sprouting of sensory nerve fibres into extensively denervated sympathetic ganglia might contribute to this paradoxical situation, by maintaining some sporadic activation of the neurones. This would constitute an expansion of what seem to be normally low levels of innervation of ganglia by collateral branches of sensory nerve fibres. It is unclear whether by any means such a situation might be beneficially exploitable in clinical terms.

Conclusion:

It is concluded that extrinsic, presumptively sensory neurones contribute to the degree of substitutive re-innervation which occurs in chronically decentralized sympathetic ganglia and that activity in these neurones could account at least in part for the moderate level of excitation of ganglionic neurones which may occur in such ganglia. The synaptic effect of such activation is not necessarily completely random, since there is evidence to suggest some selectivity in the establishment of the new connexions, but there may in addition be a wider influence resulting from non-synaptic release. The actions of the released peptides might moreover play an important role in the maintenance of the denervated neurones, pending possible reinnervation by regenerating preganglionic nerve fibres.

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