J. Anat. (1994) 184, pp. 111–117, with 2 figures Printed in Great Britain

The effect of age on dendrites in the rat superior cervical ganglion

TIMOTHY J. ANDREWS[1], DAQING LI[2], JAMES HALLIWELL[1] AND TIMOTHY COWEN[1]

[1] *Departments of Anatomy and Physiology, Royal Free Hospital School of Medicine, and* [2] *Norman Sadie Lee Research Centre, Laboratory of Neurobiology, National Institute of Medical Research, London, UK.*

(*Accepted 5 August 1993*)

ABSTRACT

Intracellular injection of a biotinylated probe in fixed superior cervical ganglia followed by confocal microscopy was used to investigate the effects of age on the dendritic arborisation of sympathetic neurons in rats aged 6 wk (young adult), 7 months (fully grown adult) and 24 months (aged). In accordance with other studies considerable dendritic growth was observed during postnatal development. However, in old age dendritic growth did not continue, and significant atrophy was observed. Quantitation of neuronal morphology showed significant reductions in soma size, total dendritic length, number of branch points and total area of dendritic arborisation in old age. Unexpectedly, significant reductions in the numbers of primary dendrites were observed in maturity and in old age. Concomitant with this atrophy there was an increase in age-related morphological abnormalities. The similarities between the atrophy and dendritic abnormalities shown by our aged neurons and those seen in other studies of young adult sympathetic neurons following axotomy or trophic factor deprivation are discussed.

INTRODUCTION

The impairment of thermoregulatory control in aged humans is an important clinical problem. Elderly people show attenuation of sweating and peripheral vascular reflexes with age which may result from impairments of the autonomic nervous system (Collins, 1991; Schmidt, 1991; Abdel-Rahman et al. 1992). In a search for an anatomical determinant of autonomic dysfunction, a number of studies have investigated the effect of age on the morphology of autonomic neurons (for review see Cowen, 1993). They show that old age affects autonomic neurons in a locally and temporally specific manner: cell loss in old age has been reported in enteric (Santer & Baker, 1988; Gabella, 1989) but not sympathetic ganglia (Santer, 1991*a*); atrophy of the autonomic innervation of peripheral target tissues has been described in a number of areas including some supplied by the superior cervical ganglion (SCG) (Cowen et al. 1982; Dhall et al. 1986; Cowen & Thrasivoulou, 1990); abnormal dendritic and axonal processes have been seen in ageing autonomic ganglia although the extent of these lesions varies between and within different ganglia (Schmidt, 1991). However, atrophy is not a general phenomenon (Santer, 1991*b*); indeed in some instances hypertrophy of autonomic fibres has been observed (Mione et al. 1988; Andrews & Cowen, 1991).

It is well established that sympathetic neurons depend on their target tissues for survival and development (Purves, 1988). Recent studies suggest that this relationship continues in old age (Ruit et al. 1990; Gavazzi et al. 1992; Gavazzi & Cowen, 1993*a*). Target tissues regulate their innervating sympathetic neurons by mechanisms including the local production of the protein nerve growth factor (NGF) (Levi-Montalcini, 1987; Hendry & Hill, 1980). Increased target size and resulting increased NGF synthesis is believed to underlie the dendritic growth of sympathetic neurons that occurs predominantly in the postnatal period but continues well into adulthood (Snider, 1986; Voyvodic, 1987; Purves et al. 1988; Ruit & Snider, 1991). In this study we have investi-

Correspondence to Dr Tim Cowen, Department of Anatomy and Developmental Biology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, UK.

Table 1. *Development and ageing of sympathetic neurons from the rat SCG*

Age	No. of cells	Soma size (μm^2)	Total dendritic length (μm)	Primary dendrites	Branch points	Area of dendritic arborisation (μm^2)
6 wk	47	660±34	894±49	7.0±0.38	17.1±1.2	9240±766
8 months	45	1130±58***	1325±69***	5.9±0.29*	22.5±1.4**	18824±1276***
24 months	49	876±56*	836±50***	4.7±0.32*	17.5±1.3*	13126±854***

Values represent means±S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; significantly different from value at preceding age.

gated how ageing affects the dendritic arborisation of sympathetic neurons. Do mature and aged sympathetic neurons continue to grow as they do in development? Do their dendritic arborisations stabilise when growth ceases? Do they atrophy perhaps as a result of lost trophic support in old age? To answer these questions, we have used intracellular injection of a biotinylated probe, Neurobiotin (Li et al. 1990), into fixed sympathetic neurons from the SCG of young, adult and aged rats with confocal microscopy to visualise and quantify the dendritic arborisation of these neurons.

MATERIALS AND METHODS

Male Sprague-Dawley rats were killed at 6 wk (young adult), 7 months (fully grown adult) and 24 months (aged) of age. In order to monitor animal growth we measured the length (nose to anus; anus to end of tail) of the animals at each age stage. We believe that animal length may give a more accurate indication of body size compared with body weight which may be unduly influenced by increased fat deposits. The dendritic morphologies of sympathetic neurons from the superior cervical ganglion (SCG) were studied at each age group. Twelve rats of each age group were killed with an overdose of sodium pentobarbitone (500 mg/kg) and perfused through the heart with Tyrode solution followed by 4% paraformaldehyde in PIPES buffer. A good perfusion was necessary for proper fixation of the tissue. SCG were dissected free, desheathed, postfixed in 4% paraformaldehyde for 2 h and stored in phosphate buffered saline (PBS) containing sodium azide at 4 °C until used.

Ganglia were immersed in a 1:10000 solution of DAPI (Molecular Probes, USA) in PBS for 10 min in order to visualise the nuclei of the neurons prior to being pinned out on a Sylgard-coated culture dish. The dish was placed on the stage of a standard epifluorescence microscope (Leitz Orthoplan, Germany) equipped with a 100 W mercury lamp. The dish was filled with PBS so that fluid just covered the ganglion. It is important that the ganglia are not submerged too deeply as the refractive index of the liquid can impair the impaling procedure and conversely, if liquid does not cover the ganglion it can dry out causing irreversible structural damage. Sympathetic ganglion cells were visualised using a long working distance ×32 objective (Leitz; 4.5 mm, NA = 0.4) and impaled with glass electrodes (Clarke Electromedical, UK) containing a mixture of 4% Neurobiotin (Vector, UK) and 2.5% Lucifer Yellow (Molecular Probes); electrodes had resistances of 30–50 MΩ. Using the ultraviolet filter block (Leitz D2 cube) the neurons (DAPI) and the electrode (Lucifer Yellow) could be seen simultaneously, thus permitting accurate placement of the microelectrode tip with a motorised micromanipulator (M. M., Germany). A satisfactory impalement was characterised by the rapid filling of the cell with Lucifer Yellow following a short burst of hyperpolarising current. If the cell body appeared bright and the primary dendrites were beginning to fill, depolarising current (3 nA) was passed for 30 min to fill the cell with Neurobiotin. Care was taken to impale cells a sufficient distance from each other in order to avoid cross-over of neighbouring dendritic arborisations. The population of cells studied included superficial cells as well as cells situated deep within the ganglion. After cells on both sides of the ganglion had been injected in this way, it was placed in a 1:50 solution of Streptavidin-Texas Red (Amersham, UK) dissolved in PBS containing 0.3% Triton overnight at 4 °C, washed in PBS and mounted in an antifade mountant (Citifluor, UK). Texas Red fluorescence was used in order to negate the effect of the age-accumulated pigment, lipofuscin, in the imaging of neurons. Labelled ganglia were stored at −20 °C until used.

Neurons were observed either with a ×40 (Olympus DPlanApo 40UVPL) or a ×16 (Zeiss Neofluar 16/0.5) lens depending on the extent of their arborisation. Neurons were first located using conventional fluorescence microscopy with filters for Texas Red. Confocal scanning laser microscopy was performed using a Bio-Rad MRC 600 microscope fitted with a krypton–argon laser. The aperture and

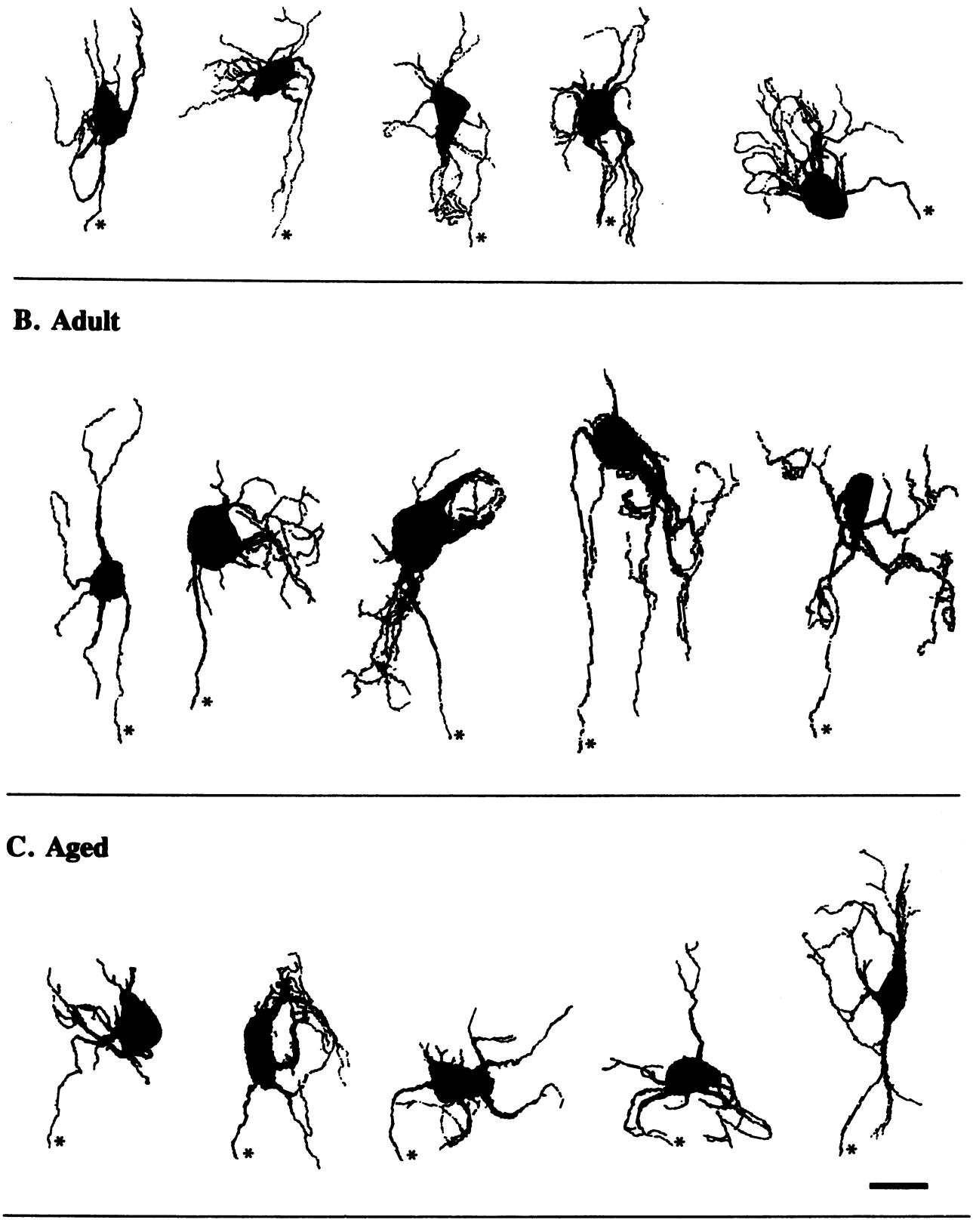

Fig. 1. Neuronal geometry of representative superior cervical ganglion cells at different stages of development and ageing: young (6 wk); adult (7 months) and aged (24 months). Cells were chosen to represent the full range of total dendritic lengths at each time point (see Table). Cells are arranged from left to right in order of increasing total dendritic length. Postganglionic axons are indicated with an asterisk. Images were obtained by binarising 2D confocal projections. Bar, 50 μm for all panels.

the gain settings were set optimally for each neuron to maximise contrast. Kalman filtering was used to remove nonspecific background noise. Successive 2 μm optical sections of the neurons were taken in by the confocal microscope and stored on optical disc, creating files which were then used to generate through-focus images of the entire neuron. Dendrites were traced interactively on screen using Bio-Rad confocal software in order to analyse the neuronal morphology. Total dendritic length was measured automatically from the traces. Branch points were distinguished from crossing dendrites by examining the z-series of images. Soma area was measured interactively by tracing the circumference of the cell soma whilst dendritic area was measured by joining the tips of those dendrites which extended furthest from the soma by straight lines. The enclosed areas were measured using confocal software. Primary dendrites were defined as dendritic processes that arose from the cell body and extended radially for a distance greater than its diameter. One-way ANOVA was used to compare age changes in the different parameters.

RESULTS

Our results show that body length increases by about 50% from 6 wk to 7 months (nose–anus, 18.6–27.25 cm; anus–tail end, 13.9–21.58 cm) but that no significant increase in body length occurs from 7 to 24 months of age (nose–anus, 27.25–28.12 cm; nose–tail end 21.58–23.81 cm). These data support our description of the 7 month rat as fully grown. Measurements of dendrites are summarised in the Table and images of representative ganglion cells at each age stage are shown in Figure 1. The dendritic arborisations were filled to the full extent of their fine processes and the axon (defined as a long unbranched neurite projecting some distance from the cell soma) could often be traced for several hundred micrometres en route towards the postganglionic trunks.

Postnatal development (6 wk to 7 months)

We showed significant growth in the dendritic arborisation of sympathetic neurons from the SCG during the postnatal period. Total dendritic length increased by about 50% ($P < 0.001$), soma size by about 70% ($P < 0.001$) and the number of dendritic branches by about 32% ($P < 0.01$). However, in contrast to the growth of the overall arborisation, we showed that the number of primary dendrites on SCG neurons did not increase in number but a significant decrease of about 1 was detected ($P < 0.05$).

Ageing (7 months to 24 months)

In old age the dendritic arborisation of sympathetic neurons in the SCG showed significant atrophy. The total dendritic length of neurons decreased by about 40% ($P < 0.001$), the number of dendritic branch points by about 20% ($P < 0.05$) and the soma size shrank by about 14% ($P < 0.05$). Abnormal dendritic profiles were seen at all age stages but were increasingly frequent in old neurons. These abnormalities appeared in 2 different forms: distended dendritic processes or cell body swellings (Fig. 2). The number of neurons exhibiting abnormal profiles was 23% at 6 wk, 30% at 7 months and 50% at 24 months.

DISCUSSION

Understanding the cellular mechanisms underlying the impairment of homeostasis in ageing and disease is of major clinical importance. Longitudinal studies have shown a progressive deterioration in the autonomic regulatory capacity of elderly people (Collins, 1991). It has been suggested that this may be due to impairments of the autonomic nervous system (Schmidt, 1991; Abdel-Rahman et al. 1992). In this study we describe changes in the dendritic arborisation of sympathetic neurons of the rat SCG during development and ageing. Previous studies have shown that the dendrites of sympathetic neurons grow considerably throughout postnatal development (Snider, 1986; Voyvodic, 1987; Ruit & Snider, 1991). Our results confirm these findings by showing increases in soma size, total dendritic length, number of branch points and total dendritic area. However, although qualitative observations have been made on the dendritic morphology of aged sympathetic neurons (Schmidt, 1991), there have to our knowledge been no quantitative studies.

We show that in old age significant dendritic atrophy occurs in the sympathetic neurons of the rat SCG. Soma size, total dendritic length, primary dendrites, numbers of branch points and dendritic area were all significantly reduced. In addition to, or perhaps as a consequence of, their atrophy aged sympathetic neurons exhibited abnormal dendritic profiles with increased frequency. Sympathetic neurons depend upon the target tissues they innervate for their survival and growth throughout postnatal

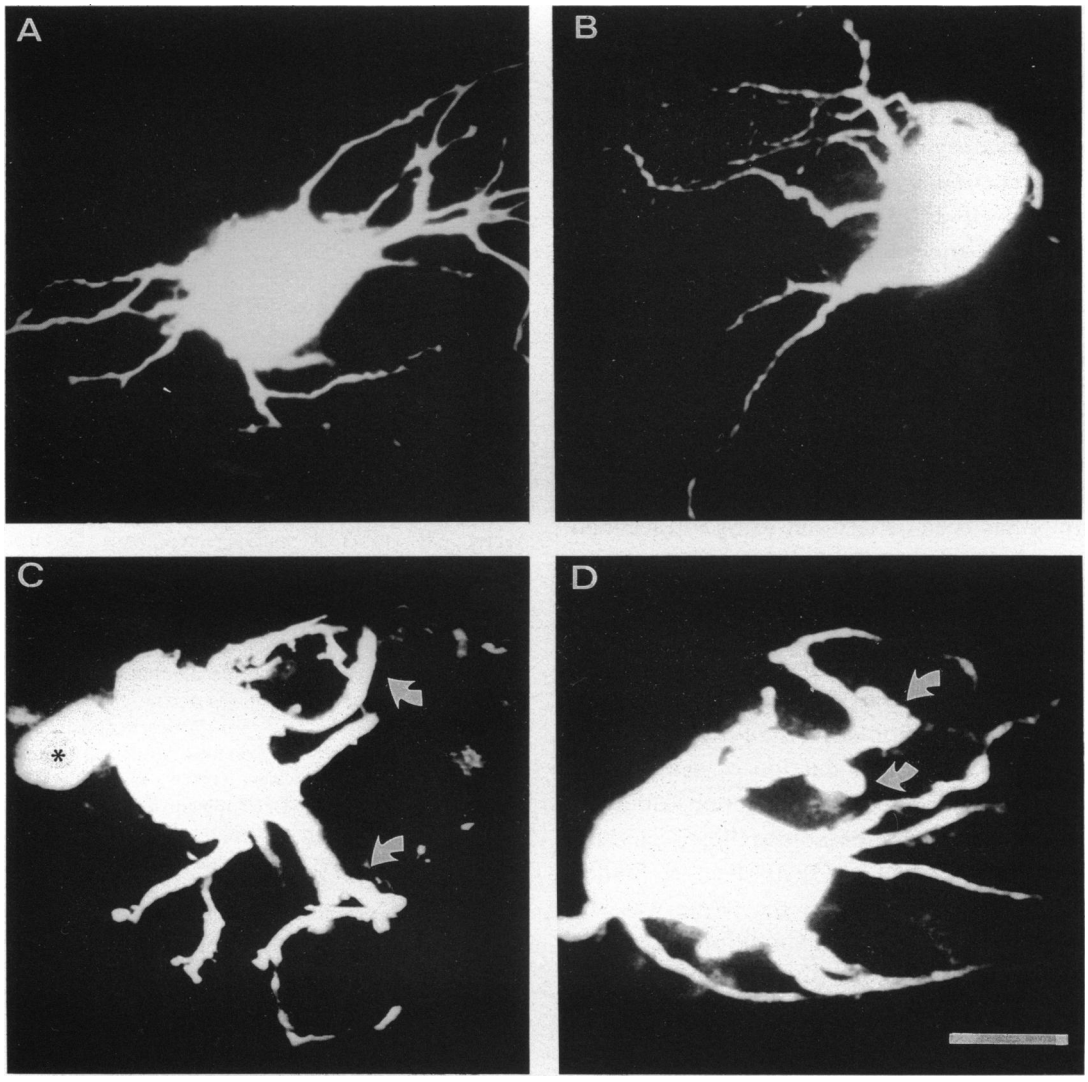

Fig. 2. Photomicrographs of confocal optical sections through superior cervical ganglion cells that have been intracellularly injected with Neurobiotin. Neurons are shown from 2 age groups; young-adult (6 wk) (*A*, *B*) and aged animals (24 months) (*C*, *D*). There was an increasing incidence of dendritic abnormalities with age. Abnormalities could be defined as distended dendrites (arrows) or cell body swellings (asterisk). Bar, 25 μm.

development (Yawo, 1987; Voyvodic, 1989*a*, *b*). Target tissues influence their innervating sympathetic neurons by the local production and release of the neurotrophin, NGF, which is taken up and retrogradely transported by the axon to the cell soma (Hendry & Hill, 1980; Levi-Montalcini, 1987). The abnormal dendritic structures and atrophy we see in aged sympathetic neurons show strong similarities with those seen in studies of young adult sympathetic neurons following axotomy (Yawo, 1987) and the application of anti-NGF antibodies (Ruit et al. 1990). This raises the possibility that the neuronal atrophy and abnormal dendritic profiles we observe may be a result of reduced neurotrophic support from the targets. A decrease in the availability of neurotrophic factors has been postulated as a cause of age-related neuronal atrophy (Appel, 1981; Perez-Polo et al. 1990; Gavazzi et al. 1992; Gavazzi & Cowen, 1993*b*); however, the link has yet to be proven. Further studies on the synthesis and availability of neurotrophic factors in the aged peripheral targets of sympathetic neurons may identify loss of trophic support as the cause of age-related neuronal atrophy.

Dendritic arborisations have an important role in the integration of presynaptic electrical potentials (Miller & Jacobs, 1984) and in determining the number of different inputs that converge on individual cells (Purves & Lichtman, 1985). In vivo imaging of sympathetic neurons has shown that their dendritic arborisations are in a continual process of growth and retraction throughout postnatal development and into adulthood (Purves et al. 1986). Our results show that this process of growth and retraction results in net growth of dendrites in postnatal development and net

retraction in old age. Because most of the synapses in sympathetic ganglia are made onto the dendrites (Forehand, 1985), this change in morphology must go hand in hand with a change in synaptic connectivity. The implied loss of synaptic input in the aged neurons receives experimental support from studies that show the appearance of dystrophic presynaptic axons in aged rat sympathetic ganglia (Schmidt, 1991). The number of synapses that impinge on sympathetic neurons is dramatically diminished following postganglionic axotomy (Matthews & Nelson, 1975) or the application of antiserum to NGF (Nja & Purves, 1977). These studies lend support to the hypothesis that age changes in sympathetic neurons may result from reduced neurotrophic support.

This study has been carried out using intracellular injection of fixed tissue and confocal microscopy. Although the dendritic arborisations we obtained appeared to be filled to the full extent of their fine processes, they were about 40% smaller than those obtained in a comparable study (Voyvodic, 1987) using intracellular injection of HRP of living neurons. Previous studies using light fixation of sympathetic neurons prior to intracellular injection obtained dendritic arborisations comparable to those seen using in vivo injection (Ruit et al. 1990; Ruit & Snider, 1991) although in these studies the tissue was still maintained under physiological conditions in case of insufficient fixation. However, although the dendritic lengths were larger the relative changes we obtained compared well with those of Voyvodic's study. We show a 50% increase in dendritic length during the postnatal development compared with a 55% increase in his study. We offer 2 possible explanations for the different size of dendritic arborisations obtained in the present study and those from comparable studies: (1) the perfusion fixation we have used may cause substantial shrinkage relative to the immersion fixation of other studies (Voyvodic, 1987; Ruit et al. 1990); (2) the fixation we have used may restrict the access of molecules to the fine processes of the dendritic tree. Therefore, although the heavy fixation we used gave smaller dendritic arborisations than those seen in similar studies the relative changes during postnatal development using this technique compared favourably. The advantages of this technique are its flexibility and simplicity.

ACKNOWLEDGEMENTS

We would like to thank Professor G. Raisman for his help in establishing the intracellular technique, Chris Thrasivoulou for his expert technical assistance and Isabella Gavazzi for helpful criticism of the manuscript. This work was supported by a grant from the Wellcome Trust. Tim Andrews was supported by a Junior Research Fellowship from the Royal Free Hospital School of Medicine. The confocal microscope was purchased with a grant from the Wellcome trust.

REFERENCES

ABDEL-RAHMAN TA, COLLINS KJ, COWEN T, RUSTIN M (1992) Immunohistochemical, morphological and functional changes in the peripheral sudomotor neuro-effector system in elderly people. *Journal of the Autonomic Nervous System* **37**, 187–198.

ANDREWS T, COWEN T (1991) Target-specific changes in autonomic nerve fibres in old age. *European Journal of Neuroscience*, Supplement 4, 1987.

APPEL SH (1981) A unifying hypothesis for the cause of amyotrophic lateral sclerosis, parkinsonism, and Alzheimer disease. *Annals of Neurology* **10**, 499–505.

COLLINS KJ (1991) The autonomic nervous system in old age. *Reviews in Clinical Gerontology* **1**, 337–345.

COWEN T (1993) Regulation of the autonomic innervation of blood vessels during development and ageing. In *Vascular Innervation and Receptor Mechanisms: New Perspectives* (ed. L. Edvinnson & R. Uddman), pp. 25–40. London: Academic Press.

COWEN T, HAVEN AJ, WEN QIN C, GALLEN DD, FRANC F, BURNSTOCK G (1982) Development and ageing of perivascular adrenergic nerves in the rabbit. A quantitative fluorescence histochemical study using image analysis. *Journal of the Autonomic Nervous System* **5**, 317–336.

COWEN T, THRASIVOULOU C (1990) Cerebrovascular nerves in old rats show reduced accumulation of 5-hydroxytryptamine and loss of nerve fibres. *Brain Research* **513**, 237–243.

DHALL U, COWEN T, HAVEN AJ, BURNSTOCK G (1986) Perivascular noradrenergic and peptide-containing nerves show different patterns of change during development and ageing in the guinea-pig. *Journal of the Autonomic Nervous System* **16**, 109–126.

FOREHAND CJ (1985) Density of somatic innervation on mammalian autonomic ganglion cells is inversely related to dendritic complexity and preganglionic convergence. *Journal of Neuroscience* **5**, 3403–3408.

GABELLA G (1989) Fall in the number of myenteric neurons in aging guinea pigs. *Gastroenterology* **96**, 1487–1493.

GAVAZZI I, ANDREWS TJ, THRASIVOULOU C, COWEN T (1992) Influence of target tissues on their innervation in old age: a transplantation study. *Neuroreport* **3**, 717–720.

GAVAZZI I, COWEN T (1993*a*) Axonal regeneration from rat sympathetic ganglia transplanted in oculo is not impaired by age. *Experimental Neurology*, in press.

GAVAZZI I, COWEN T (1993*b*) NGF can induce a 'young' pattern of innervation in transplanted old cerebral blood vessels. *Journal of Comparative Neurology*, in press.

HENDRY IA, HILL CE (1980) Retrograde axonal transport of target tissue-derived macromolecules. *Nature* **287**, 647–649.

LEVI-MONTALCINI R (1987) The nerve growth factor: 35 years later. *Science* **237**, 1154–1162.

LI D, SEELEY PJ, BLISS TVP, RAISMAN G (1990) Intracellular injection of biocytin into fixed tissue and its detection with avidin-HRP. *Neuroscience Letters* Supplement 38, 81.

MATTHEWS MR, NELSON VH (1975) Detachment of structurally intact nerve endings from chromatolytic neurones of the rat superior cervical ganglion during the depression of synaptic transmission induced by postganglionic axotomy. *Journal of Physiology* **245**, 91–135.

MILLER JP, JACOBS GA (1984) Relationships between neuronal structure and function. *Journal of Experimental Biology* **112**, 129–145.

MIONE MC, DHITAL KK, AMENTA F, BURNSTOCK G (1988) An increase in the expression of neuropeptidergic vasodilator, but not vasoconstrictor, cerebrovascular nerves in aging rats. *Brain Research* **460**, 103–113.

NJA A, PURVES D (1977) The effects of nerve growth factor and its antiserum on synapses in the superior cervical ganglion of the guinea-pig. *Journal of Physiology* **277**, 53–75.

PEREZ-POLO JR, WERRBACH-PEREZ K, MARCHETTI D, MORGAN B, TAGLIALTELA G, RAMACCI MT, et al. (1990) Nerve growth factor activity and aging in CNS. *International Journal of Clinical Pharmacology Research* **10**, 15.

PURVES D (1988) *Body and Brain, A Trophic Theory of Neural Connections*. Cambridge, Massachusetts: Harvard University Press.

PURVES D, LICHTMAN J (1985) Geometrical differences among homologous neurons in mammals. *Science* **228**, 298–302.

PURVES D, HADLEY RD, VOYVODIC JT (1986) Dynamic changes in the dendritic geometry of individual neurons visualized over periods of up to three months in the superior cervical ganglion of living mice. *Journal of Neuroscience* **6**, 1051–1060.

PURVES D, SNIDER WD, VOYVODIC JT (1988) Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. *Nature* **336**, 123–128.

RUIT KG, OSBORNE PA, SCHMIDT RE, JOHNSON EM, SNIDER WD (1990) Nerve growth factor regulates sympathetic ganglion cell morphology and survival in the adult mouse. *Journal of Neuroscience* **10**, 2412–2419.

RUIT KG, SNIDER WD (1991) Administration or deprivation of nerve growth factor during development permanently alters neuronal geometry. *Journal of Comparative Neurology* **314**, 106–113.

SANTER RM (1991*a*) Sympathetic neurone numbers in ganglia of young and aged rats. *Journal of the Autonomic Nervous System* **33**, 221–222.

SANTER RM (1991*b*) Morphological evidence for the maintenance of the cervical sympathetic system in aged rats. *Neuroscience Letters* **130**, 248–250.

SANTER RM, BAKER DM (1988) Enteric neuron numbers and sizes in Auerbach's plexus in the small and large intestine of young adult and aged rats. *Journal of the Autonomic Nervous System* **25**, 59–67.

SCHMIDT RE (1991) Pathology of the sympathetic nervous system. In *The Pathology of the Ageing Human Nervous System* (ed. S. D. Duckett), pp. 431–442. London: Lea & Febiger.

SNIDER WE (1986) Rostro-caudal differences in dendritic growth and synaptogenesis in rat sympathetic chain ganglia. *Journal of Comparative Neurology* **244**, 245–253.

VOYVODIC JT (1987) Development and regulation of dendrites in the rat superior cervical ganglion. *Journal of Neuroscience* **7**, 904–912.

VOYVODIC JT (1989*a*) Target size regulates calibre and myelination of sympathetic axons. *Nature* **342**, 430.

VOYVODIC JT (1989*b*) Peripheral target regulation of dendritic geometry in the rat superior cervical ganglion. *Journal of Neuroscience* **9**, 1997–2010.

YAWO H (1987) Changes in the dendritic geometry of mouse superior cervical ganglion cells following postganglionic axotomy. *Journal of Neuroscience* **7**, 3703–3711.