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Nerve growth factor enhances the dendritic arborization of sympathetic ganglion cells undergoing atrophy in aged rats

T. J. ANDREWS and T. COWEN*

Department of Anatomy and Developmental Biology, The Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, UK

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Summary

We have investigated whether dendritic growth can be induced from sympathetic neurons of aged rats by the application of exogenous nerve growth factor to their target tissues. A previous study showed that significant dendritic atrophy (19%) occurs during ageing in the sympathetic neurons innervating the middle cerebral artery and that dendritic atrophy correlated with loss of axon colaterals in the same population of neurons. Using retrograde tracing in conjunction with intracellular injection of fixed tissue and confocal microscopy, we now demonstrate that infusion of nerve growth factor over the peripheral processes of the same neurons from aged rats induces significant dendritic (45%) and cell body (60%) growth. However, not all aspects of the dendritic arborization were affected. Primary dendrites and branch points were not altered by nerve growth factor. In contrast, nerve growth factor induced a significant increase in the number of primary dendrites and branch points (100%) of neurons from young adults. Our results demonstrate that sympathetic neurons undergoing dendritic atrophy during ageing can exhibit significant dendritic growth in response to the *in vivo* infusion of nerve growth factor, although the lack of regenerative response displayed by some parts of the dendritic tree leads us to believe that these neurons also show signs of reduced plasticity.

Introduction

Target tissues have an important role in the establishment of neuronal connectivity during embryogenesis (Levi-Montalcini, 1987). The trophic influence of the target on neurons in the PNS and CNS has been shown to continue through postnatal development and into adulthood (for review see Purves, 1988; Varon *et al.*, 1988). Target tissues exert their effect via the release of neurotrophic factors that are taken up by the axons of innervating neurons and retrogradely transported to the cell soma (Hendry & Hill, 1980; Seiler & Schwab, 1984). Nerve growth factor (NGF) is the best characterized neurotrophic factor (Thoenen, 1991). It has been shown to influence the size (Snider, 1988), axonal arborization (Saffran & Crutcher, 1990), neurotransmitter synthesis (Thoenen *et al.*, 1971) and preganglionic innervation (Nja & Purves, 1977) of sympathetic neurons.

Because most synaptic input to sympathetic neurons is on their dendrites (Forehand, 1985), the regulation of dendritic growth is important in neural organization. The dendritic growth of sympathetic neurons occurs predominantly during postnatal development (Snider, 1986; Voyvodic, 1987) through a continuous process of growth and retraction (Purves *et al.*, 1986). Fluctuating levels of NGF are believed to regulate these processes. Exogenous NGF can induce increased dendritic growth in neonatal and adult sympathetic neurons, primarily through increased dendritic branching (Snider, 1988; Ruit *et al.*, 1990). In contrast, primary dendrites have been described as a stable morphological feature set up early in development (Voyvodic, 1987; Ruit & Snider, 1991). They play an important role in the synaptic organization of neurons and their numbers are proportional to the number of innervating neurons (Purves & Hume, 1981). However, unlike other aspects of the dendritic arborization, NGF has only been shown to induce changes in the number of primary dendrites in neonatal animals (Snider, 1988; Ruit *et al.*, 1990; Ruit & Snider, 1991).

Recent evidence suggests that sympathetic neurons continue to depend upon their targets in old age and a

* To whom correspondence should be addressed.

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breakdown in this relationship may result in neuronal atrophy (Gavazzi *et al.*, 1992; Gavazzi & Cowen, 1993a). The hypothesis that intrinsic changes in the neurone are not the primary cause of age-related atrophy receives further support from studies showing that the plasticity of neurons is not impaired in old age (Buell & Coleman, 1979; Crutcher, 1990; Andrews & Cowen, 1993; Gavazzi & Cowen, 1993b). A decrease in the availability of neurotrophic factors has been associated with age-related changes in the nervous system (Perez-Polo *et al.*, 1990; Cowen, 1993). However, a proven link has yet to be established. In the CNS, NGF can prevent the neuronal atrophy and cell death of cholinergic forebrain neurons that occurs following axotomy in aged rats (Montero & Hefti, 1989) and in the PNS, the application of anti-NGF antibodies to aged sympathetic neurons causes cell death and cell body shrinkage (Ruit *et al.*, 1990). However, to our knowledge no studies have investigated the ability of NGF to enhance dendritic arborization from aged neurons *in vivo*, particularly those undergoing atrophy.

In a previous study we showed that the axon arborization of sympathetic neurons innervating the middle cerebral artery undergoes atrophy in old age. However, following the *in vivo* infusion of exogenous NGF, substantial axonal regrowth was observed (Andrews & Cowen, 1993a). The sympathetic neurons that innervate the middle cerebral artery are found in the superior cervical ganglion (SCG) (Cowen *et al.*, 1986) where they form a small subpopulation, approximately 1%, of the total (Edvinsson *et al.*, 1989). We also showed that the dendritic arborization of sympathetic neurons projecting to the middle cerebral artery of the rat atrophy in old age (Andrews & Cowen, 1993b). In an attempt to reverse this age-related atrophy we have infused exogenous NGF over the axons of the same neurons in freely moving young and aged animals. Previous studies using a similar paradigm have administered NGF or its antibodies via subcutaneous injection (Snider, 1988; Ruit *et al.*, 1990). In an attempt to mimic a target-derived source of neurotrophic factor, we have infused NGF over the axons of the neurons we are studying and have used retrograde tracing and intracellular injection combined with confocal microscopy to quantify any resulting change in dendritic morphology. Our results show that sympathetic neurons from aged animals remain responsive to NGF and that *in vivo* treatment with exogenous NGF can reverse some aspects of age-related neuronal atrophy.

Materials and methods

Male Sprague Dawley rats from a colony maintained at the Royal Free Hospital School of Medicine were used at 6 weeks (young–adult) and 24 months (aged) of age.

Retrograde tracing

Animals were anaesthetized using a mixture of halothane and oxygen and fixed in a stereotaxic head frame. A sagittal scalp incision was made between the eyes. The scalp and the temporal muscle on the left side were retracted laterally. A bone window was drilled below the sagittal suture and between the coronal and lambdoid sutures. Branches of the middle cerebral artery were identified and the dura over the artery incised. A small piece of sponge (Allevyn Cavity Wound Dressing, Smith and Nephew, UK) preabsorbed with 2% Fast Blue (Sigma, UK) and 0.5% Diamidino Yellow (Sigma, UK) was placed underneath the dura and over the artery. A small sheet of antibacterial dressing was placed over the exposed area and the scalp sutured. The analgesic drug, Temgesic (0.06 mg kg^{-1}), was given to animals post-operatively. The specificity of the retrograde tracing to each target was investigated by looking at the sympathetic ganglia contralateral to the labelled tissue. On the contralateral side only occasional neurons, with faintly stained cytoplasm, were observed which did not resemble the more numerous neurons with brightly stained nuclei and cytoplasm on the ipsilateral side.

Nerve growth factor infusion

The middle cerebral artery was exposed as described above. A catheter made of translucent vinyl tubing (ID 0.63 mm and OD 1.4 mm; Portex, UK) connected to a miniosmotic pump (Alzet, USA; pump no 2002) containing 200 µl NGF (100 µg ml^{-1}; Boehringer, UK) or vehicle containing cytochrome C (100 µg ml^{-1}; Calbiochem, UK), was inserted subdurally next to the artery so that it could perfuse over the nerves on its adventitial surface for 14 days. Nerve growth factor and cytochrome C were dissolved in PBS containing 1% rat albumin (Sigma, UK). The pump was sutured subcutaneously between the scapulae, the incision closed, and the rats allowed to recover. Neither old or young animals displayed any infection or other problems following the operation. Animals fed and behaved normally.

Intracellular injection

At the end of the experiment animals were killed with an overdose of sodium pentobarbitone (500 mg kg^{-1}) and perfused through the heart with Tyrode's solution followed by 4% paraformaldehyde in Pipes buffer. Animals with poor perfusion were rejected. The superior cervical ganglia were dissected free, desheathed, post-fixed in 4% paraformaldehyde for 2 h and stored in PBS containing 0.1% sodium azide at 4° C until used. Ganglia were pinned out on a Sylgard-coated culture dish filled with PBS and placed on the stage of a standard epifluorescence microscope (Leitz Orthoplan, Germany) equipped with a 100 W mercury lamp. Neurons were visualized 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 ultra-violet filter block (Leitz D2 cube) the neuron's cytoplasm (Fast Blue), nucleus (Diamidino Yellow) and the electrode (Lucifer Yellow) could be seen simultaneously, thus permitting accurate placement of the microelectrode tip with a motorized micromanipulator (M.M., Germany). A satisfactory impalement was characterized by the rapid

Table 1. The effect of NGF on the dendritic arbors of aged sympathetic neurons

	No. of animals	*No. of cells*	*Soma size (μm^2)*	*Total dendritic length (μm)*	*Primary dendrites*	*Branch points*	*Area of dendritic arborization (μm^2)*
Untreated	10	33	860 ± 34	670 ± 48	5.0 ± 0.36	15.1 ± 1.4	10 261 ± 832
Cyt C	6	14	932 ± 44	575 ± 51	5.2 ± 0.58	16.9 ± 2.0	11 325 ± 1495
NGF	6	17	1371 ± 164*	978 ± 98‡	5.2 ± 0.49	18.5 ± 1.7	13 186 ± 1669

Errors indicated are ± SEM. Significant differences are given between adjacent groups (* $p < 0.01$, ‡ $p < 0.001$).

Table 2. The effect of NGF on the dendritic arbors of young–adult sympathetic neurons

	No. of animals	*No. of cells*	*Soma size (μm^2)*	*Total dendritic length (μm)*	*Primary dendrites*	*Branch points*	*Area of dendritic arborization (μm^2)*
Untreated	10	32	677 ± 42	823 ± 47	5.7 ± 0.41	17.6 ± 1.4	12 216 ± 874
Cyt C	5	20	717 ± 35	1099 ± 108*	6.9 ± 0.61	22.4 ± 2.6	13 907 ± 1377
NGF	5	17	1035 ± 65§	1227 ± 100	7.6 ± 0.41	34.2 ± 3.7‡	16 318 ± 1730

Errors indicated are ± SEM. Significant differences are given between adjacent groups (* $p < 0.05$, ‡ $p < 0.01$, § $p < 0.001$).

filling of the cell with Lucifer Yellow following a short burst of hyperpolarizing current. If the cell body appeared bright and the primary dendrites were beginning to fill, depolarizing 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 arborizations. 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 minimize the effect on the images of the yellow, autofluorescent age pigment, lipofuscin. Labelled ganglia were stored at −20° C until used.

Neurons were observed with either a × 40 (Olympus DPlanApo 40UVPL) or a × 16 (Zeiss NeoFluar 16/0.5) lens depending on the extent of their arborization. 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 system fitted with a krypton-argon laser. The aperture and the gain settings were set optimally for each neuron to maximise contrast. Kalman filtering was used to remove 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. The area of dendritic arborization was measured by joining those dendrites of maximum extension from the soma with straight lines. Primary dendrites were defined as dendritic processes that arose from the cell body and extended radially for a distance greater than its diameter (Ruit & Snider, 1991). One-way ANOVA was used to compare the effect of age and NGF treatment on the different parameters.

Results

Changes in the dendritic morphology of aged and young adult animals are summarised in Tables 1 and 2 and representative images of SCG neurons projecting to the middle cerebral artery are shown in Figs 1 and 2.

Retrogradely traced neurons projecting to the middle cerebral artery were found predominantly in the rostral half of the SCG. We were able to impale 1–8 traced neurons per ganglion. Because the fluorescent tracers we have used fade relatively quickly following exposure to fluorescent illumination, we could not impale labelled neurons and then assess cell numbers. However, the number of labelled cells was not noticeably different between young and old ganglia. Previous studies have shown that the number of neurons within the rat SCG remains unchanged in old age (Santer, 1991). The number of neurons impaled in this study is less than in investigations of the total population in the SCG (Voyvodic, 1987; Snider, 1988). However, the subpopulation of neurons projecting to the middle cerebral artery is no more than 1% (Edvinsson *et al.*, 1989) of the total population of 26 000 (Purves *et al.*, 1986). Therefore, we believe that our numbers constitute a representative sample.

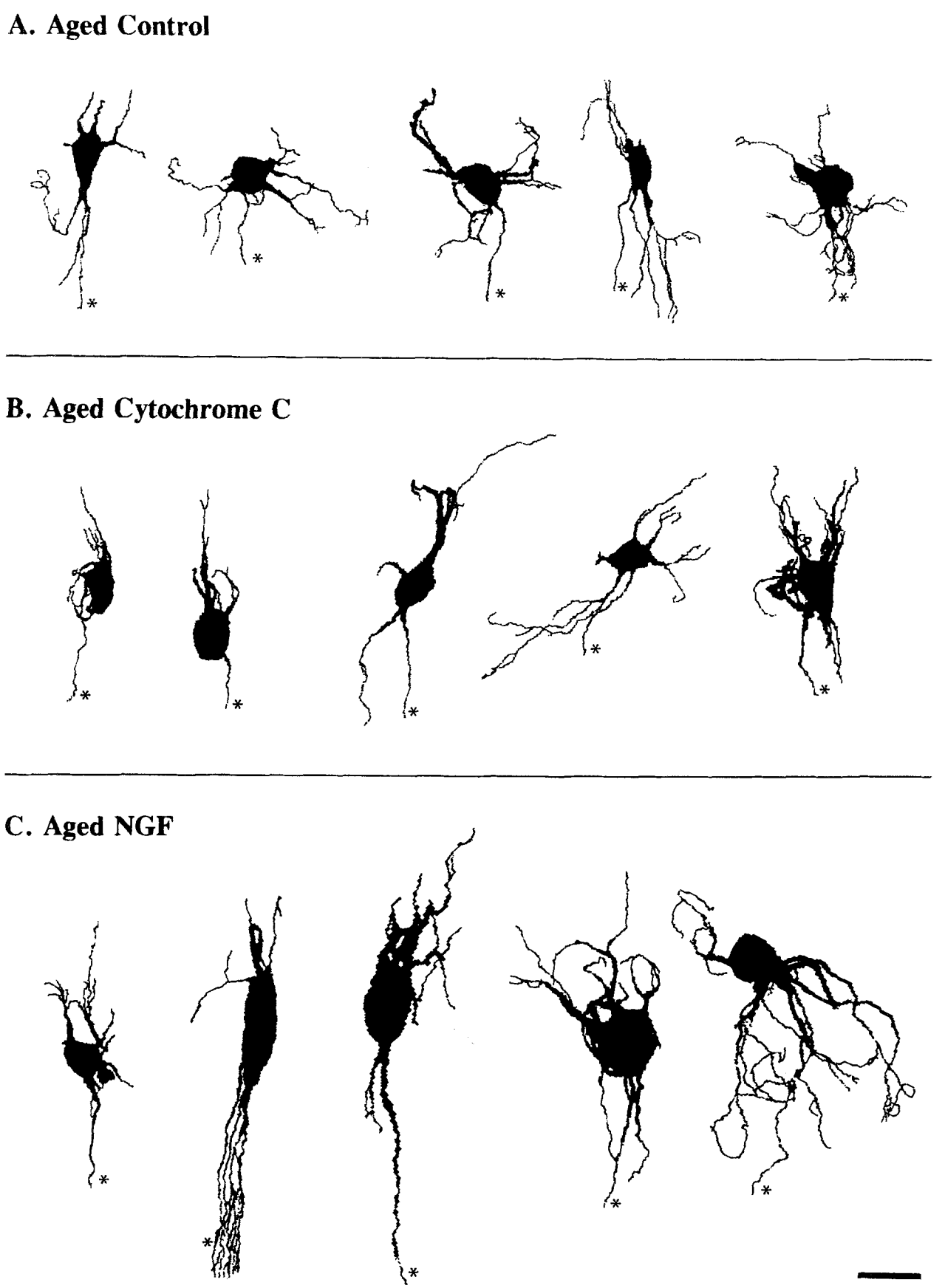

Fig. 1. The effect of 2 weeks' infusion of NGF on aged sympathetic neurons innervating the middle cerebral artery. Cells were chosen to represent the full range of dendritic geometry (see Table 1). Cells are arranged left to right in order of increasing total dendritic length. A striking feature of the aged neurons was the increased appearance of dendritic abnormalities. Examples of this can be seen in (A), neurons 3 and 5, (B), neurons 3 and 5 and (C), neurons 1 and 3. Postganglionic axons are indicated with an asterisk. Images were obtained by binarising 2D confocal projections. Scale bar = 50 μm.

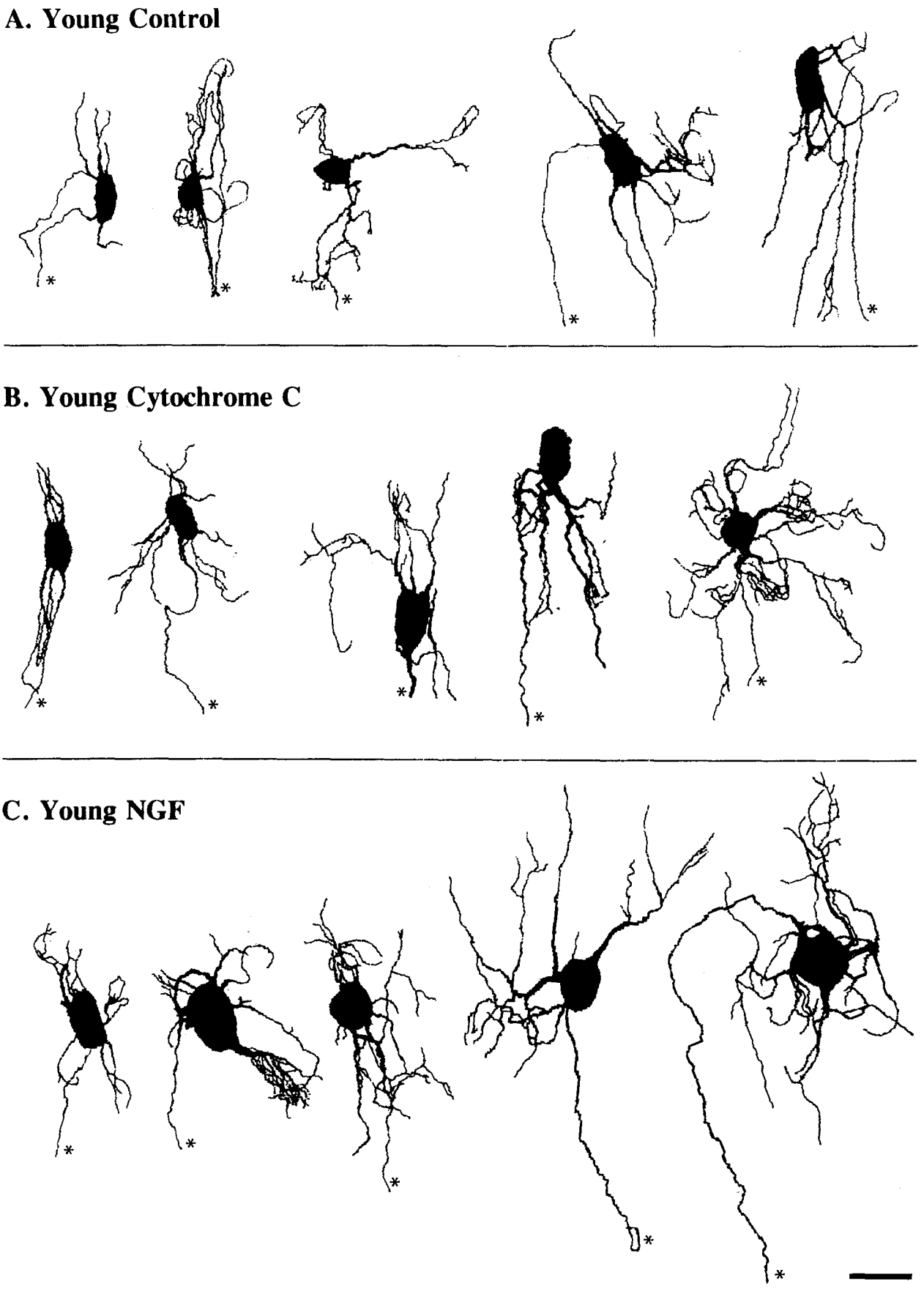

Fig. 2. The effect of 2 weeks' infusion of NGF on young-adult sympathetic neurons innervating the middle cerebral artery. Cells were chosen to represent the full range of dendritic geometry (see Table 2). Cells are arranged from left to right in order of increasing total dendritic length. Postganglionic axons are indicated with an asterisk. Images were obtained by binarising 2D confocal projections. Scale bar = 50 μm.

Changes in neuronal morphology of aged sympathetic neurones following NGF treatment

Here and in a related study (Andrews & Cowen, 1993b) we show that sympathetic neurons projecting to the middle cerebral artery show dendritic atrophy in old age. Total dendritic length was decreased by about 20% ($p < 0.05$). However, NGF treatment increased the total dendritic length of aged sympathetic neurones by about 45% compared to untreated neurones ($p < 0.01$) and by about 70% compared to vehicle-treated neurones ($p < 0.001$). The changes induced by vehicle treatment were not statistically significant. Nerve growth factor also increased the mean cell body size of neurones from aged rats by 60% compared to untreated neurons ($p < 0.001$) and by about 50% compared to vehicle-treated neurons ($p < 0.01$). However, not all aspects of the dendritic arborization were affected by NGF infusion. The number of primary dendrites, branch points and the area of dendritic arborization were unchanged. Aged sympathetic neurons exhibited an increased number of dendritic abnormalities, including swellings on dendrites, primary dendrites and cell bodies (Fig. 1). Nerve growth factor treatment did not have any obvious effect on the number or appearance of these abnormalities. Our measurements of dendritic length may have been affected by altered diffusion of tracer in the dendrites of aged neurons. However, prolongation of iontophoresis beyond 30 min did not demonstrate larger dendritic arbors (data not shown).

Changes in the dendritic morphology of young adult sympathetic neurons following NGF treatment

Nerve growth factor increased the total dendritic length of young adult SCG neurons by about 50% relative to untreated ($p < 0.001$) but not to vehicle-treated neurons. Moreover, vehicle increased the total dendritic length of neurons relative to untreated controls by about 30% ($p < 0.05$). In young neurons, the increase in total dendritic length caused by NGF relative to untreated controls was primarily due to an increase in dendritic branching. Nerve growth factor increased the number of branch points by about 100% relative to untreated ($p < 0.001$) and about 55% relative to vehicle-treated ($p < 0.01$) controls. Nerve growth factor also increased the cell body size of neurons from young adult animals by about 50% relative to untreated controls ($p < 0.001$) and by about 45% compared to vehicle-treated controls ($p < 0.001$). The area of the dendritic arborization was also increased significantly ($p < 0.05$) by NGF treatment relative to untreated but not vehicle-treated controls. In contrast to the aged neurons, NGF induced an increase in the number of primary dendrites on young-adult sympathetic neurons by an average of 2 ($p < 0.01$) relative to untreated but not vehicle-treated controls. We believe that this is the first demonstration of plasticity in primary dendrite numbers of young-adult neurons. The use of confocal microscopy with its ability to resolve the origins of primary dendrites above and below the soma may explain the difference between this and previous studies (Voyvodic, 1987; Ruit & Snider, 1991).

Discussion

In previous studies we demonstrated atrophy in the terminal plexus of nerves supplying the middle cerebral artery of aged rats and showed that the application of exogenous NGF can induce neurite outgrowth over this target (Andrews & Cowen, 1993a). In this and a previous study (Andrews & Cowen, 1993b) we demonstrate that the dendritic arborizations of this same subpopulation of sympathetic neurons atrophy in old age. We now show that these dendrites can be induced to regrow following the application of NGF to their peripheral axons. We suggest that an age-related reduction in neurotrophic support may explain the atrophy and dendritic abnormalities seen in aged sympathetic neurons. This hypothesis receives support from studies of adult sympathetic neurons following axotomy (Yawo, 1987) or NGF deprivation (Ruit *et al.*, 1990) that show neuronal atrophy associated with similar abnormal dendritic profiles to those we have observed.

Nerve growth factor treatment induced increases in soma size (60%) and total dendritic length (45%) in neurons from aged animals. However, not all aspects of morphology were affected by NGF. There was no increase in either the number of primary dendrites or branch points, contrasting with the effect of NGF on similar young–adult neurons which showed an increased number of branch points and primary dendrites. Previous studies have shown that primary dendrites are a stable morphological feature set up within the first 2 weeks of postnatal development (Voyvodic, 1987; Ruit & Snider, 1991; Ruit *et al.*, 1990). The increase in primary dendrites that occurs in young-adult neurons is much less than in neurons exposed to NGF in their first weeks of postnatal development (Snider, 1988). We suggest that the decreased ability of adult and the inability of aged neurones to increase the number of primary dendrites in response to NGF infusion may reflect increased age-related morphological restraints on aspects of dendritic growth. In contrast to primary dendrites, the number of dendritic branch points can be modified in adult animals by increasing or decreasing NGF availability to sympathetic neurons (Snider, 1988; Ruit *et al.*, 1990). We confirm these results by showing a dramatic increase in the number of dendritic branch points following NGF treatment in sympathetic neurons from young adult animals. *In vivo* imaging of

sympathetic neurons in adult mice has shown that their dendritic arborizations are in a continual state of growth and retraction (Purves *et al.*, 1986a), with *de novo* dendritic branches appearing after 14 days. However, our results show that 14 days of exposure to NGF fails to induce any increase in the dendritic branching of neurons from aged animals, albeit in a different species. Consequently, it would appear that the ability of neurons to remodel primary dendrites and branch points may be impaired in old age. The dendritic arborization of a neuron has an important role in determining the extent of its presynaptic innervation (Purves & Hume, 1981; Forehand, 1985). The dendritic atrophy and NGF-induced regrowth that we have demonstrated may therefore have implications for synaptic connectivity.

Cytochrome C has been used in studies of NGF as a control with no neurogenic effects. However, in this study, cytochrome C-vehicle infusion induced dendritic growth from young-adult sympathetic neurons projecting to the middle cerebral artery. Nerve growth factor induced no significant additional increase in total dendritic length of young neurons, perhaps indicating physical restraints on the maximum length of these dendrites. Evidence from a number of recent studies leads us to believe that an immune or inflammatory reaction possibly potentiated by the invasive surgery could directly or indirectly induce nerve growth (Sendtner *et al.*, 1992; Andrews & Cowen, 1993; Gavazzi & Cowen, 1993a). Possible mechanisms could include the upregulation of NGF synthesis in fibroblasts by cytokines produced by invading cells of the immune system (Yoshida & Gage, 1992).

In summary, our results support the concept of a continued role for NGF in the maintenance of dendritic morphology of sympathetic neurones in old age. Whilst we do not prove that NGF is the primary causative factor in age-related neuronal atrophy, our results demonstrate, in accordance with other studies (Buell & Coleman, 1979; Gavazzi & Cowen, 1993b), that neurons retain plasticity in old age. However, some aspects of the NGF-induced dendritic growth in old neurones suggest that this plasticity may be limited. A decrease in the availability of NGF has been linked with age-related changes that take place in human neurodegenerative diseases (Appel, 1981; Bartus *et al.*, 1982) and it has been suggested that NGF could be used as a therapeutic agent in these diseases (Tuszynski *et al.*, 1991). This study shows the potential of exogenous NGF to reverse some aspects of age-related changes in neuronal morphology.

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