NERVE-TARGET INTERACTIONS
IN THE MATURE AND AGED
PERIPHERAL NERVOUS SYSTEM

by

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This study has centred on neuronal ageing in the autonomic and peripheral nervous system with particular reference to the instructive role that target tissues may have in regulating neuronal phenotype. Previous work in our laboratory has demonstrated a significant decrease in the total population of sympathetic, sensory and parasympathetic nerves innervating cerebral blood vessels (CBV) and eccrine sweat glands using the pan-neuronal marker, protein-gene-product 9.5, in the aged rat. This study investigates the sub-populations of nerves that innervate these targets using novel techniques in an attempt to identify which group/s are affected in old age and then goes on to study the underlying mechanisms of age-related changes. Morphometric assays were developed using confocal and light microscopy and image analysis which could reliably and repeatedly detect changes in nerve density, transmitter expression and other aspects of neuronal phenotype. Critical areas of technique were identified. These techniques demonstrated significant age-related decreases in the innervation of (CBVs) by the sympathetic and sensory populations as demonstrated by tyrosine hydroxylase (TH) and calcitonin gene-related protein (CGRP) or neurofilament (NF-R30) immunostaining, respectively. There was a large reduction (70%) in numbers of trigeminal
sensory neurons projecting to the middle cerebral artery (MCA) in old compared to young rats. Moreover, the number of traced neurones which were CGRP/NF-R_{39} positive were also reduced (80%) in old trigeminal ganglia compared to young, either as a result of age-related changes in retrograde axon transport or by neuronal cell death. *In oculo* transplantation was then used to discover whether target tissues regulate these age-changes in sympathetic and sensory nerve growth and whether they influence changes in neurotransmitter phenotype. Sympathetic nerve density as well as neurotransmitter phenotype was found to be regulated by the target tissue and not by intrinsic differences of the neurons. Nerve growth factor (NGF) induced nerve growth onto aged implants to levels over and above that found in young implants. Target tissues appeared able to influence the extent of their sensory innervation, although the pattern and density of innervation was atypical following *in oculo* transplantation. Following surgical sympathectomy, NF-R_{39} sensory nerve density showed significant increases in young middle cerebral artery (MCA), perhaps as a result of the increased pool of available NGF. Finally, double-immunohistochemistry was used to assess expression of the low affinity NGF receptor (p75) colocalised with sympathetic and sensory nerves. Changes in p75 expression were found to mirror the decline in sympathetic and sensory nerves with age. The component of p75 staining which was not colocalised with sympathetic and sensory nerves was not altered in old age.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$^{125}$I-NGF</td>
<td>radio-iodinated NGF</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetyl cholinesterase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Area%</td>
<td>Percentage area of positive nerve staining</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>CBV</td>
<td>Cerebral blood vessels</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged-coupled device</td>
</tr>
<tr>
<td>CDF</td>
<td>Cholinergic differentiation factor</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related neuropeptide</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>Cyt C</td>
<td>Cytochrome C</td>
</tr>
<tr>
<td>DY</td>
<td>Diamidino yellow</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FB</td>
<td>Fast Blue</td>
</tr>
<tr>
<td>FG</td>
<td>Fluorogold</td>
</tr>
<tr>
<td>FM</td>
<td>Fluorescence microscopy</td>
</tr>
<tr>
<td>GA</td>
<td>Glyoxylic acid</td>
</tr>
<tr>
<td>GV</td>
<td>Grey value (measure of stain intensity)</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>ID/mm</td>
<td>Intercept density of nerves/mm length of tissue</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>kD</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>-LI</td>
<td>-like immunofluorescence</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia-inducing factor</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>MV</td>
<td>Mesenteric vein</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical apperture</td>
</tr>
<tr>
<td>NAd</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-beta</td>
</tr>
<tr>
<td>NF-R39</td>
<td>Neurofilament</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide-Y</td>
</tr>
<tr>
<td>NT3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>NT4/5</td>
<td>Neurotrophin-4/5</td>
</tr>
<tr>
<td>p75</td>
<td>Low affinity NGF receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PGP9.5</td>
<td>Protein-gene product 9.5</td>
</tr>
<tr>
<td>PSB</td>
<td>Pontamine sky blue</td>
</tr>
<tr>
<td>PSF</td>
<td>Point spread function</td>
</tr>
<tr>
<td>RAM</td>
<td>Random access memory</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive index</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SCG</td>
<td>Superior cervical ganglion</td>
</tr>
<tr>
<td>SP</td>
<td>Substance-P</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>Trk A</td>
<td>Tyrosine kinase A (high affinity NGF receptor)</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>VECM</td>
<td>Video enhanced contrast microscopy</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal polypeptide</td>
</tr>
<tr>
<td>Z-section</td>
<td>Optical section thickness</td>
</tr>
</tbody>
</table>
Acknowledgements

This thesis is dedicated to my wife Joy and children, Jason, Zoë, Thomas and Natalie. They have had to endure many months of their father working late through the night and seeing very little of him weekend after weekend. Without the support and understanding of Joy I could never have completed this work and I give my sincere thanks to her for what she has done for me and our children through the good and difficult times. I would also like to thank my parents George and Kay, and Joy's sister Maria for their help with our children throughout the years. I wish to express sincere appreciation to my supervisor Dr Tim Cowen for his friendship during all the years we have worked together and his assistance in the preparation of this manuscript, without his help the writing of this thesis would have been all the more difficult. In addition, I would like to thank Professor John Harris for giving me the opportunity to start this study, Professor Geoffrey Goldspink for his continued support and encouragement and Dr Isabella Gavazzi for her helpful comments and experimental assistance. Finally, I would like to thank my departmental colleagues and friends Mr Tjeu Gysbers, Mr John Norton and Miss Claire Underwood for their support and assistance during the period of these studies.
Chapter 1

General Introduction.

1a) Age-related changes in the CNS

Until recently, ageing of the nervous system was generally thought to coincide with large-scale neuron death. This view may have been generated by studies conducted in the 1950s which demonstrated significant loss of neurons during ageing in certain regions of normal human and rodent brain (Coleman, Flood, 1987; Dayan, 1970a; Dayan, 1970b; Brody, 1955). However, with the development of more accurate procedures for counting neurons, evidence of neuronal loss in aged human cerebral cortex (Brody, 1955) proved to be a result of reduced packing density and not cell loss (Haugh, 1984; Terry et al. 1987) (for recent review see (Morrison, Hof, 1997). The careful application of stereological techniques in several species led to the conclusion that decline in neuron number is not widespread in the CNS during normal ageing. However, human studies have shown selective neuronal loss in areas of the hippocampus (Ball, 1977), the substantia nigra, basal forebrain (Selkoe, 1992), and the locus coeruleus (Vijayshankar, Brody, 1979). Other neuronal populations, such as the hypothalamus (Selkoe, 1992) and the brain stem (Konigsmark, Murphy, 1970), remain unchanged during ageing, although one study has shown loss of dorsal ventral motor neurons in the aged mouse (Sturrock, 1990). In contrast to human studies, a number of animal studies have reported no loss of neurons in rodent cerebral cortex (Diamond et al. 1993) and locus coeruleus (Coleman, Goldman, 1981), whilst others show neuronal loss in the hypothalamus (Duara et al. 1990) (also see (Finch, 1993).
1 a.1) **Age-related changes in the peripheral nervous system.**

Although ageing of the CNS has been investigated extensively, cell loss and fibre atrophy in the peripheral nervous system has not been so widely studied. Neuron cell numbers seem to remain constant in ageing rat superior cervical, coeliac and superior mesenteric ganglia although packing density is decreased (Baker, Santer, 1988). However, neuronal loss in small sub-populations projecting to particular target tissues may not be detected when cell counts in whole ganglia are made. On the other hand, significant reductions of 40% have been observed in the enteric nervous system of *ad-libitum*, but not diet restricted, rats (Gabella, 1979; Wall, Fitzgerald, 1982) (Johnson et al., *J Anat* In press) suggesting that neuronal cell loss is often not normally associated with ageing in peripheral neurons. However, diet does not appear to be involved in the age-related loss of guinea-pig enteric neurons (Gabella, 1989). Therefore, as with the CNS, the peripheral nervous system seems to display spatial variability suggesting selective vulnerability of some neurons to age-related degenerative processes. Nerve fibre atrophy has not been studied in the aged CNS, but where data exists for the PNS, changes range from significant loss of innervation of some targets, to increases in others (for reviews (Cowen, Gavazzi, 1998; Cowen, 1993). For example, projections to the iris from the superior cervical ganglion are largely unaffected by age (Santer, 1991b), whilst projections from the same ganglion to some of the cerebral blood vessels show age related losses (Gavazzi *et al.* 1992; Andrews, Cowen, 1994; Thrasivoulou, Cowen, 1995).

As stated above, neuron counts in autonomic sympathetic and some sensory ganglia, which supply the postganglionic axons to peripheral blood vessels in young and old rats, show decreased packing density (Partanen *et al.* 1982; Baker, Santer, 1988) but little evidence of loss of neurons (Santer, 1991a; Santer, 1991b). Similarly, counts of trigeminal neurons, whose sensory axons project to many of the same targets as those in the above studies, have failed to find any age-related loss of cells in *ad-libitum* and diet restricted rats.
(Biedenbach et al. 1992). However, ageing in the autonomic and peripheral nervous system may exhibit deficits at the target level which do not necessarily involve loss of neurons. Reduced nerve density in aged cerebral blood vessels, demonstrated using the general neuronal marker PGP9.5 (Gavazzi et al. 1992; Cowen, Thrasivoulou, 1990) has been shown in rats. However, because PGP9.5 stains the majority of nerve fibres, these studies were unable to identify which particular populations were affected. Studies using antibodies raised against the rate limiting enzyme of noradrenaline (NAd) synthesis, tyrosine hydroxylase (TH), and catecholamine histochemistry using glyoxylic acid (GA) have shown decrease in the noradrenergic populations (Andrews, Cowen, 1991; Mione et al. 1988). Immunoreactivity for calcitonin gene-related protein (CGRP), usually associated with sensory nerves, increases whilst neuropeptide Y (NPY) associated with parasympathetic and sympathetic nerves remains unchanged in aged cerebral blood vessels (Mione et al. 1988). Studies which use antibodies raised against neurotransmitter or neuropetides have the disadvantage of being unable to distinguish whether the observed changes are due to nerve atrophy or are the results of altered expression of neurotransmitters themselves. For example, levels of the neurotransmitter NAd have been shown to be reduced in sympathetic ganglia with age, whilst levels of TH have been shown to increase (Santer, 1979).

As well as being temporally specific, age changes may also be species specific, for example, sympathetic nerves to the cerebral vasculature decrease in old age in the rat (Andrews, Cowen, 1991; Mione et al. 1988; Santer, 1982) but not in rabbit (Cowen et al. 1982). Data from the many ageing studies done to date are unable to identify a single cause for neurodegeneration of peripheral neurons. What is clear however, is that certain neurons are sensitive to ageing events, whilst others are not impaired suggesting selective vulnerability of sub-populations of neurons.
1 b) Neuronal cell loss in development.

During embryonic and early postnatal life most neuron types in the vertebrate nervous system are subject to a period of cell death, which normally occurs shortly after axons arrive and activate their targets (Hamburger, Oppenheim, 1982). Neuron numbers first of all rise to a maximum as they proliferate and migrate to their final destination, then decline with the onset of naturally occurring cell death, often by apoptosis (Alison, Sarraf, 1992; Edwards, Tolkovsky, 1994; Garcia et al. 1992). The degree of cell loss is not uniform for all neuron types and is somewhat dependent on their stage of differentiation and phenotype. Thus apoptosis may eliminate only a small fraction of the neurons in some sites whilst in others it may eliminate at least half or more of the original neurons. Some populations of neurons may disappear altogether having perhaps served a temporary function (Chun et al. 1987).

The seemingly wasteful process of perinatal neuronal cell death seems to have evolved as an alternative to accurate reprogramming of neuron numbers (Clarke, 1981). However, the nervous system is made up of billions of neurons with $10^{16}$ synapses in cerebral cortex alone (Changeux, 1985) and such perfect precision of neurogenesis seems unlikely. The total quantity of DNA found in cells limits the maximum number of genes it can encode. It has been calculated that the maximum number of genes a fertilized mouse egg, for example, can encode is approximately 2 million (Changeux, 1985). Within this number the majority of genes are not translated into proteins and many genes encoding for a particular protein may have many repeats. Therefore, estimates of the maximum number of structural genes range between 150,000 and 200,000 (Changeux, 1985). Clearly, there is not enough genetic information to generate the varied trophic and tropic agents required for axon guidance of billions of neurons to specific targets. Therefore cell-cell interactions and external, so called 'epigenetic', events have evolved to regulate these processes. Experiments on sensory ganglia and in motor neurons in the ventral
horn of the spinal cord of chick embryos following removal of limb buds before they became innervated, resulted in almost complete loss of neuron groups (for review see (Hamburger, 1980)). Therefore, depriving neurons of their targets had profound effects which led to the hypothesis that targets produce specific agents that are necessary for neuronal survival (Hollyday, Hamburger, 1976).

The same study showed matching of neuron numbers to target size (Hollyday, Hamburger, 1976). Neuronal cell death in sensory ganglia and spinal cord neurons at segmental levels that innervate the limbs is less extensive than at the levels that innervate the trunk regions, where there is much less tissue to be innervated. Moreover, grafting of extra limbs onto early embryos in the limb-innervating regions resulted in less neuronal death than normal (Hollyday, Hamburger, 1976), whilst almost all motor neurons are lost from the lateral motor column of mutant 'limbless' chick (Lanser, Fallon, 1984). Therefore, these results are consistent with the hypothesis that target tissues support the survival of a limited and appropriate number of innervating neurons.

1b.1) Mechanism of developmental nerve cell death.

Neurotrophic hypothesis.

As stated above, observations of cell death in the developing vertebrate nervous system are consistent with the hypothesis that target tissues release trophic factors that are necessary for the survival of their innervating neurons. Failure of neurons to compete successfully for a limited supply of neurotrophic factors, in a system where there is an initial excess of neurons, provides an obvious explanation for the death of some neurons. This hypothesis may also explain why some neurons die if they make contact with inappropriate targets where either an adequate population of neurons is already established, or where the factors available may be unsuitable for the errant neurons.
Evidence for the role of trophic factors in developmental cell death came from the classical studies of Levi-Montalcini (Levi-Montalcini, Hamburger, 1951). An agent, isolated from a mouse sarcoma, was termed nerve growth factor (NGF) following observations of its growth stimulating effects on embryonic chick sympathetic and sensory neurons (Levi-Montalcini, Hamburger, 1951) (also see (Levi-Montalcini, Calissano, 1979)). Snake venom was used as a source of enzymes to find out if the active agent was a protein or nucleic acid. Surprisingly, the venom itself had the same action as the tumour extract which led to the discovery of a range of tissues with similar effects (e.g. male mouse submaxillary and salivary glands, guinea pig prostate gland; (for review see (Thoenen, Barde, 1980)) and showed that the agent was a protein which was subsequently characterised (Cohen, Levi-Montalcini, 1956). Recently, more sensitive techniques have facilitated the detection of NGF protein (Ebendal et al. 1980), and mRNA for NGF (Heumann, 1987; Heumann et al. 1984; Bandtlow et al. 1987) in target tissues of sympathetic and sensory neurons.

The evidence that NGF is essential for the survival of sensory and sympathetic neurons during early development is strong. In vivo experiments where NGF was injected into neonatal rats prevented normal and experimentally-induced neuron death in sympathetic ganglia, and embryonic chick DRGs (Hamburger et al. 1981). In contrast, NGF antiserum injected into neonatal rats resulted in cell death in all sympathetic ganglia (Levi-Montalcini, Booker, 1960), and in embryonic sensory ganglia (Johnson, Jr. et al. 1980). Studies have shown the importance of neuronal activity (Lipton, Kater, 1989; McKee et al. 1989), neurotrophic factors (Thoenen, 1991a; Levi-Montalcini, 1987a), extracellular matrix (Edelman, 1983), glia (Rakic, 1974) and hormones (Devoogd, Nottebohm, 1981) in neural development. The mechanisms behind these target-neuron interactions have been the subject of intense examination spanning over 3 decades. Some of these effects are mediated, at least in part, via release and neuronal uptake of specific neurotrophic molecules. In addition to regulating neuron survival, neurotrophin release from target tissues has been
shown to influence the soma, axonal arborisations and neurotransmitter synthesis of neurons (for review see (Snider, Johnson, Jr. 1989; Purves et al. 1988; Purves, Lichtman, 1985).

1c) Hypothesis of the trophic inter-relationship of targets and their neurons in maturity and ageing.

The continued dependence of mature neurons on their target tissues for trophic support has been suggested by a number of studies (Kandel, O'Dell, 1992; Purves, 1988). Targets may influence several different aspects of neuronal plasticity, including axonal/dendritic growth, collateral sprouting, connectivity, receptor expression and neurotransmitter expression. For example, mature sympathetic neurons grow strongly in response to target associated factors (Andrews, Cowen, 1994a; Gavazzi et al. 1995a; Ebendal et al. 1983a; Uchida, Tomonaga, 1985a) suggesting that targets continue to influence their innervating neurons during maturity.

Experimental manipulation of the target area available to groups of mature neurons has been used to study the influence of target tissues on their innervating neurons. Partial constriction of the small intestine (Gabella, Trigg, 1984; Gabella, 1984; Filogamo, Marchisio, 1970) or bladder (Gabella, Uvelius, 1994; Gabella, Uvelius, 1990; Steers et al. 1990; Steers et al. 1991) results in a ten- to fifteen-fold hypertrophy of the organ wall, with matching growth of the innervating neurons of the enteric plexus (Gabella, 1984; Williams et al. 1993) and bladder (Gabella et al. 1992; Steers et al. 1990). Soma volume increases by 83% in pelvic neurons supplying the hypertrophic bladder (Gabella, 1984), and by 180% in sensory neurons supplying the hypertrophic ileum (Williams et al. 1993). In addition, nerve fibre density over uterine arteries is increased following hypertrophy during pregnancy in the guinea-pig (Mione, Gabella, 1991). These size changes may resemble experimentally induced hypertrophy and hence may involve growth induction between targets and the innervating neurons. Studies have shown that aged
sympathetic neurons supplying targets such as the middle cerebral artery (MCA) and pineal gland undergo an increase in soma size with an increase in TH expression, but a decrease in low affinity NGF receptor (p75) expression (Kuchel et al. 1997). However, other studies have shown that collateral sprouting of sympathetic axons supplying cerebral arteries of rats requires the presence of preganglionic inputs (Handa et al. 1992) suggesting that trans-neuronal regulation of neurotrophic factors may be involved in some aspects of plasticity.

Transplantation and co-culture studies have demonstrated that targets continue to influence the regulation of processes of growth and rearrangement of adult neurons (Chamley et al. 1973; Todd, 1986; Olson, Malmfors, 1970; Malmfors et al. 1971; Burnstock, 1974). These studies show that when sympathetic neurons are exposed to different targets they produce a density of fibre outgrowth over the target tissue that matches the normal, in situ pattern of innervation. Target tissues appear to contain both nerve growth-promoting, as well as inhibitory influences (Kuromi, 1992; Kuromi, 1991). Studies using the anterior eye chamber model have shown that denervated target tissues, taken from mature donor animals and implanted into the anterior eye chamber of young hosts, show a remarkable capacity to induce organotypic patterns of reinnervation, following reinnervation by host iridial nerves (Todd, 1986; Olson, Malmfors, 1970; Burnstock, 1974).

Maintenance of neuronal number in the mature and ageing nervous system may be due to sustained production of trophic factors by their target tissues, or a decoupling of the survival dependence of neurons on their target tissues and associated trophic factors. For example post-natal sympathetic neurons become less sensitive to NGF deprivation with age (Johnson, Jr. et al. 1989) (Oreke et al., In press) whilst, sensory neurons no longer require NGF for survival after birth (Johnson, Jr. et al. 1980).
Nerve-target interactions appear to influence neurotransmitter phenotype throughout life (Landis, 1990; Patterson, 1978). Therefore, growth and other features, such as neurotransmitter expression, may continue to be affected in adulthood (Lindsay et al. 1989; Lindsay, Harmar, 1989; Lindsay et al. 1990). Developing sympathetic neurons cultured in the absence of other cells develop characteristic noradrenergic phenotype, whereas those grown with heart conditioned medium develop cholinergic phenotype (Patterson, Chun, 1974). Sympathetic neurons which are destined to innervate sweat gland targets start off in early development as noradrenergic and later switch to a cholinergic phenotype (Leblanc, Landis, 1986). Functional contact with sweat glands targets initiate the switch from noradrenergic to cholinergic phenotype (Stevens, Landis, 1990). In the latter study, transplants of sweat gland in the anterior eye chamber induce cholinergic transmitter expression in iridial sympathetic neurons which normally do not express this transmitter (Stevens, Landis, 1990). Despite determined efforts by several groups, the soluble, cholinergic differentiation factor (CDF) associated with sweat glands and involved in the instructive switching of transmitter expression in sympathetic neurons remains unidentified, although it is believed to resemble the cytokine LIF (Rao, Landis, 1990).

Regulation of transmitter expression is another facet of neuron-target plasticity affected by extrinsic factors which continues into maturity. Superior cervical ganglion (SCG) neurons respond to nicotinic receptor stimulation by synthesising TH and dopamine β-hydroxylase (Black et al. 1984), suggesting afferent and target influences. Mature sensory neurons innervating the internal carotid artery of the rat show increased CGRP expression with no collateral sprouting following sympathectomy, providing evidence that targets regulate sensory neurotransmitter expression in response to the additional NGF available to sensory nerves (Isaacson et al. 1995). For a review of changes in neurotransmitter expression with age, see (Burnstock, 1990).
1c.1) Hypothesis 1.

The hypothesis on which this thesis is based is that target tissues in particular and possibly other epigenetic factors continue to be important influences on ageing neurons. Therefore, it is proposed that mature and aged neurons retain their dependence on targets for trophic support and that selective age-related breakdown of these mechanisms may underlie neurodegenerative processes.

1d) Neurotrophic factors in the mature nervous system.

NGF has important effects on transmitter expression in sympathetic and sensory neurons, as well as in central, cholinergic neurons of the basal forebrain (for reviews see (Patterson, 1993; Thoenen, 1995). In contrast to the instructive effects of CDF/LIF, NGF appears to have permissive effects on transmitter levels, producing a well-established upregulation of catecholamines and associated enzymes in sympathetic neurons (Thoenen et al. 1971; Miller et al. 1994) and of substance P (SP) and CGRP in NGF-sensitive sensory neurons (Lindsay, Harmar, 1989). These effects are important not only in the induction of the appropriate neurotransmitter phenotype during development, but also in the maintenance of transmitter levels and expression in maturity. Studies of the iris in mature rats (Bjorklund et al. 1985; Kessler, 1985; Kessler et al. 1983) and rabbits (Cole et al. 1983) have shown that NGF availability can exert complex effects on the transmitter expression of different populations of NGF-sensitive neurons. Thus, sympathetic denervation resulted in upregulation of CGRP and SP in sensory nerves, which could be blocked with anti-NGF antibodies (Kessler, 1985; Cole et al. 1983).

The continued dependence of sympathetic neurons on NGF and perhaps NT3 for survival has been studied in vivo in mature and aged mice (Zhou, Rush, 1996; Ruit et al. 1990; Ruit, Snider, 1991; Bjerre et al. 1975) and mature rats
(Goedert et al. 1978; Otten et al. 1979; Gorin, Johnson, 1980) either by prolonged treatment with large, systemic doses of anti-NGF antibodies or by using an experimental autoimmune approach. The role of target derived NGF and neurotrophin 3 (NT3) and other members of the neurotrophin family in neuronal survival has been extensively reviewed (Farinas et al. 1994; Crowley et al. 1994; Birren et al. 1993; Vogel, 1993; Ernfors et al. 1994; Davies, 1996; Thoenen, 1991; Hamburger, 1993; Levi-Montalcini, 1987) (for recent review see (Cowen, Gavazzi, 1998)). Adult sympathetic and sensory neurons become relatively independent of NGF for survival (Johnson, Jr. et al. 1980; Uchida, Tomonaga, 1985). Reduction in SCG neuronal numbers of 35-40% have been observed (Gorin, Johnson, 1980) in adult rats treated with anti NGF, whilst others found only reversible effects on adult sympathetic neurons (Otten et al. 1979). Adult and aged mice systemically treated with large doses of anti-NGF appear to have reduced numbers (~24%) of SCG neurons compared to controls (Ruit, Snider, 1991) but counting methods used in this study are open to question. Therefore, the question of survival dependence of neurons for target derived factors remains unresolved.

1e) Regulation of NGF low affinity receptors.

Neuronal responses to NGF are believed to be mediated by a high-affinity membrane-bound NGF receptor (Green et al. 1986). The principle molecular component of the high-affinity receptor for NGF is TrkA, the product of a tyrosine protooncogene, that is phosphorylated and activates cellular signalling events upon NGF binding (Jing et al. 1992; Cordon-Cardo et al. 1991; Kaplan et al. 1991a; Kaplan et al. 1991b; Klein et al. 1991). In addition, the p75 receptor binds most, if not all, neurotrophins with relatively low affinity (Rodriguez-Tebar et al. 1992; Rodriguez-Tebar et al. 1990), and is a transmembrane glycoprotein that also exists in an extracellular truncated form generated by post-translational cleavage (Barker et al. 1998; Distefano, Johnson, Jr. 1988). Although the functional role of the p75 receptor has been the subject of much recent controversy (for review see (Bothwell, 1995),
recent studies disrupting this gene in transgenic mice show that it plays an important role in both neuronal survival and growth (Lee et al. 1992). In addition, p75 may contribute to high affinity binding of NGF (Hempstead et al. 1991).

Expression of both trk and p75 receptor increases significantly during late embryonic development (Wyatt, Davies, 1995) and continues between postnatal day 15 and 70 in the rat (Ehrhard, Otten, 1994) suggesting that both are likely to be involved in NGF binding and retrograde transport in adult nerves. Studies have confirmed the presence of p75 (Gong et al. 1994) and Trk A (Vega et al. 1994) in many groups of adult neurons, including sympathetic neurons. In the CNS, some neurons, particularly those involved in local circuitry, express only Trk, whilst projection neurons appear to express both receptors (for review, see (Bothwell, 1995)), leading to the suggestion that p75 expression is important in retrograde signalling in neurons with extended axons (Korsching, 1993). p75 is also expressed in Schwann cells where it may have a role in cell migration (Anton et al. 1994; Bothwell, 1995) as well as sequestrating NGF for neurons.

Studies by Miller et al., have shown that NGF treatment upregulates p75 synthesis in developing sympathetic neurons both in vivo (Miller et al. 1991), and in culture (Ma et al. 1992), and has no effect on TrkA mRNA levels. It has been proposed that p75 allows sympathetic neurons to respond to NGF over a broader concentration range than predicted by binding to TrkA alone (Chun, Patterson, 1977a; Chun, Patterson, 1977b; Ma et al. 1992b). Miller and her co-workers proposed that p75 might function in an NGF-induced feed-back loop to attenuate the function of TrkA by sequestrating NGF from a high affinity complex, thereby enabling any individual sympathetic neuron to respond to large variations in the amount of territory and/or trophic support (Miller et al. 1994). Supporting evidence for this suggestion comes from studies which show an increased affinity of NGF for TrkA in the presence of p75 receptor (Chao, Hempstead, 1995). NGF treatment of targets combined with retrograde
tracing to the SCG and in situ hybridisation for p75 in identified mature sympathetic neurons revealed that p75 expression was upregulated 2-6 fold, whilst TrkA expression remained unchanged (Miller et al. 1994). The NGF-induced increase in p75 mRNA was accompanied by an increase in α-tubulin and p75 receptor expression on axon terminals. This study is the first to show differential regulation of p75 and TrkA expression in vivo in mature sympathetic neurons and to indicate a possible influence of p75 on growth and transmitter expression of mature sympathetic neurons, although one other study has shown increased p75 mRNA expression in DRG neurons in response to elevated NGF levels (Mearow et al. 1994). It is also suggested that the ratio of p75 to TrkA is important in neuronal responses (Wyatt, Davies, 1995) and that upregulation of p75 expression by NGF forms part of a complex feedback system perhaps reinforcing nerve responses to NGF.

Recent studies have suggested that p75 has signalling pathways independent of TrkA which can lead via the production of ceramide from sphingomyelin to activation of NF-κB (see (Lipton, 1997)). p75 signalling has been reported to induce both apoptosis (Rabizadeh et al. 1993; Frade et al. 1996; Van der Zee et al. 1996; von Bartheld et al. 1994) and cell survival (Davies, 1997; Carter, Lewin, 1997; Taglialetela et al. 1997). The intracellular events of p75-induced apoptosis remain unresolved. However, mechanisms mediated both by ligand binding to the receptors (Frade et al. 1996; von Bartheld et al. 1994), and by unbound receptor (Rabizadeh et al. 1993) have been proposed. The evolutionary advantage of such divergent properties of a receptor is unclear. It is not known whether p75 retains its ability to induce apoptosis in the mature organism. One of the features of NGF signalling which may be important in understanding plasticity in the mature and ageing nervous system is the apparent cell-type specificity of some of these pathways: for example, application of function-blocking p21ras Fab-fragments specifically inhibited survival of NGF-dependent DRG neurons in culture, but not in chick sympathetic neurons (Borasio et al. 1993). Furthermore, the pattern of tyrosine phosphorylation of target protein from TrkA is different in sensory and
sympathetic neurons. Finally, it has also been shown that signalling mechanisms vary with the developmental stage of the particular neurons. Thus, different pathways may be activated by the same signal at different stages in life. How these different intracellular processes interact with the ageing process has not been explored.

In addition to soluble neurotrophic factors, neurons depend on a range of extrinsic cues for their survival, including bound factors such as laminin and other extracellular matrix (ECM) molecules. Experimental studies have implicated ECM molecules in the control of neuronal migration, axonal growth and synaptogenesis (reviewed in (Reichardt, Tomaselli, 1991; Venstrom, Reichardt, 1993; Letourneau et al. 1994). However, evidence linking these factors to neuron survival in the adult nervous system is limited. Recent studies have indicated a role for the ECM in neuronal, as well as glial, differentiation (Bunge et al. 1989; Edelman, 1984). The ECM can have survival-promoting effects: for instance, the survival of early chick sympathetic neurons is independent of NGF, but depends on the presence of a suitable substratum. ECM components can also influence neurons in an indirect, but no less important, way by binding and restricting diffusion, or by modulating the biological activities of soluble growth factors (Flaumenhaft, Rifkin, 1991). Responses of avian sympathetic neurons to NGF, avian sensory neurons to brain derived neurotrophic factor (BDNF) and avian motor neurons to several trophic factors are regulated by ECM components (reviewed in (Reichardt, Tomaselli, 1991)). The ECM molecule laminin is responsible for many of the survival, differentiation and growth promoting effects of the ECM during development (Edgar, 1990; Sanes et al. 1990). Laminin is an ECM component which can bind to a number of cell membrane receptors (reviewed in (Distefano et al. 1992; Reichardt, Tomaselli, 1991)), the best characterized being integrin receptors. Recent studies have shown that laminin immunoreactivity is closely associated with nerve fibres, and is reduced in aged cerebral vessels where a 50% decrease in nerve density had previously been reported (Gavazzi et al. 1995a), suggesting that deficits in bound, as well
as soluble, factors may underlie neurodegeneration. Bound factors such as laminin have also been shown to act synergistically with NGF resulting in increased axon growth and collateral sprouting of mature and aged sympathetic neuron explants in culture (Cowen et al. 1997).

1f) Hypothesis 2

An initial hypothesis that can be drawn from these studies is that failure of targets to synthesise adequate soluble and or bound neurotrophic factors may underlie local atrophy of terminal nerve fibres. Alternatively, neurons may fail to synthesise the appropriate receptors for the ligands being produced by target tissues, rendering them more vulnerable during ageing.

1g) Methodology.

The study of nerve-target interactions in adult and aged tissues requires techniques that can compare and quantify a range of changes of nerve cells and their processes and correlate these changes with altered expression of trophic molecules and receptors. Previous studies have established appropriate methods of specimen preparation and image analysis which can provide this kind of information.

1g.1) Specimen preparation.

Image analysis and quantification of biological specimens requires proprietary methods which are easily repeatable and give consistent results irrespective of the operator. Studies have shown that areal measurements can be reliably quantified (Cowen, Burnstock, 1980; Fuxe et al. 1985; Amenta et al. 1987).
Section 1g.2) Imaging.

Appropriate image techniques are important in quantifying neuronal morphology. All forms of electronic light microscopy involve electronic encoding of an optical image, but they may be divided into two fundamentally distinct types, wide-field microscopy and scanning microscopy. The former includes all those forms of electronic microscopy in which a conventional wide-field two-dimensional optical image of the specimen is formed and focused onto the image plane of an electronic imaging device, such as video camera tube or charged-couple device (CCD), which is then read out to a monitor. Scanning light microscopy involves the physical scanning of the object plane with a diffraction-limited point of light, or in some cases with a one- or two-dimensional array of such points. Most forms of confocal laser scanning microscopy are of this type.

Video enhanced contrast microscopy (VECM) involves the electronic amplification of minute contrast variations produced by the microscope objectives until they become visible. It is often necessary to increase the signal-to-noise ratio by removing unwanted imperfections in the image by subtracting a pre-recorded background image in order to increase contrast. The original developments by Allen, Inoue, and others of VECM produced one of the most powerful techniques now available to biologists for the study of cells (for reviews see, (Weiss et al. 1989; Shotton, 1988; Schnapp, 1986; Inoue, 1986; Inoue, 1988; Allen, 1985).

The second major step in wide-field light microscopy was that of intensified fluorescence microscopy (FM) (Cowen, Thrasivoulou, 1992a; Willingham, Pastan, 1963b; Cowen, Thrasivoulou, 1992b; Reynolds, 1972b). This technique replaces the conventional tube camera with a high-resolution slow-scan CCD array camera, allowing the visualisation of very faint objects which could not be clearly seen by eye nor recorded by direct photomicrography.
Confocal laser scanning microscopy (CLSM) offers superior image quality, particularly in thick preparations containing abundant connective tissues. The ability of (CLSM) to remove out-of-focus blur by optically sectioning the tissue only in the area where nerves are localised and excluding other areas which may generate higher levels of autofluorescence, often associated with connective tissue, results in increased image quality, making quantification of nerves a simpler task.

1g.3) Image analysis.

Computerised image analysis is designed for repetitive measuring tasks and has been used for a number of years to make automated quantitative measurements of field and object data in preparations of nerves from the central and peripheral nervous systems (Cowen, Burnstock, 1982; Cowen, Burnstock, 1980; Johnson, Araujo, 1981; Fasolo et al. 1988; Amenta et al. 1987; Agnati, Fuxe, 1984). These methods have provided sensitive measurements of nerve density from images containing, for example, large numbers of fine nerve bundles. Visual assessment of such images requires the laborious counting of fibre intercepts or a semiquantitative estimation of density on a point-scale. There are several disadvantages of these methods. For example, intercept counts cannot take changes in size of the component nerve bundles into account, whilst visual estimations of nerve fibre density can reliably distinguish at most five or six different degrees of density. Image analysis, on the other hand, has been shown to be capable of quantifying total numbers of nerve bundles as well as providing an estimate of the number of component nerve fibres. Measurements can be made rapidly over large areas of tissue providing sensitive measurements of nerve density when comparing developmental, regional and experimental differences in autonomic perivascular nerves (Cowen, Burnstock, 1986; Cowen, Burnstock, 1982; Cowen, Burnstock, 1980). Fluorescence histochemical and immunohistochemical techniques on whole-mount stretch preparations have been found to give high contrast images of nerve fibres which are suitable for
image analysis and have advantages over transmitted-light images of peroxidase-stained preparations.

Studies leading up to this thesis (Gavazzi, Cowen, 1993b; Gavazzi et al. 1992b; Cowen et al. 1992b; Andrews, Cowen, 1991b) identified the need for a reliable and accurate means of measuring stain intensity as well as nerve morphology. One aim of this thesis was therefore to develop new image analysis procedures which work reliably and reproducibly to measure stain intensity on whole-mount and sections of target tissues and their innervating neurons.

1h) Aims of the thesis.

The main aims of this thesis fall into four areas:

i) Appropriate methodology will be developed and refined in order to quantify age-changes in peripheral nerves.

ii) Considering the varied changes seen in different groups of ageing neurons, the nature of the ageing process will be discussed and quantified in particular subpopulations of nerves in an attempt to characterise the contrasting effects of old age in particular target areas.

iii) Given that neuronal age changes are target-specific, the role of targets in the regulation of ageing events will be investigated using intraocular transplantations.

iv) The molecules which may be involved in the normal neuronal ageing process, *i.e.*, neurotrophic factors and their receptors, will be studied in order to develop a hypothesis about the mechanisms underlying neuronal ageing.
1j) Summary of chapter contents.

Chapter 1 Introduction.

Chapter 2 deals with methods and the establishment and refinement of techniques.

Chapter 3 discusses the results of the methodological refinements.

Chapter 4 describes the sympathetic innervation of young and aged MCA and investigates the role of the target in determining age-changes in sympathetic nerve density using in oculo transplantation. The possibility that NGF treatment can reverse age-related neuronal atrophy is also investigated.

Chapter 5 describes the sensory innervation of young and aged MCA and again investigates the role of the target in determining age-changes in sensory nerve density using in oculo transplantation. Retrograde tracing from MCA and iris is carried out, coupled with immunohistochemistry for sensory markers, in an attempt to correlate nerve fibre atrophy with neuron loss.

Chapter 6 investigates the role of targets in determining age-changes in nerve density and neurotransmitter phenotype in footpad sweat glands of mature and aged animals, using in oculo transplantation and retrograde tracing.

Chapter 7 describes age-changes in the distribution of the low affinity, p75 NGF receptor in sympathetic and sensory nerves projecting to cerebral blood vessels.

Chapter 8 is a general discussion of the principal observations.
Chapter 2

Methodology

2a) Introduction

The central theme of this thesis is the study of nerve target interactions of the peripheral nervous system in mature and aged rats. Since the age changes I have been investigating are primarily morphological involving sometimes subtle changes, it was essential that the qualitative and quantitative techniques employed to study these changes give reliable and reproducible results. Hence, the initial aim of these studies was to standardise the methodological protocols in use in our laboratory and, where appropriate, develop new ones. The main areas that I concentrated on are as follows;

2b) Surgical techniques.

2b.1) Animals.

Young six week old (which are sexually mature) and aged 24 month Sprague Dawley rats where used throughout. All animals where raised in the Royal Free Hospital School of Medicine comparative biology unit. Young rats where kept in groups of four to six per cage and fed ad libitum on a pellet diet. Old animals were kept under the same conditions as the young until the age of six months after which they where kept two to three per cage on a restricted diet of 60% of their ad libitum diet (30g/rat/day). Diet restriction is known to be beneficial to the general health and longevity of the animals (Frol'kis, 1982) and helps to reduce tumour-induced mortality (personal observation). All experimental procedures were licensed by the Home Office.
2b.2) In oculo transplantation.

Transplants of small blood vessels have been extensively used in our laboratory in recent years for studies of nerve target interactions and have been described previously (Gavazzi et al. 1992; Olson, Malmfors, 1970). I have contributed to the development of a similar technique using sweat glands from the rat footpad. A schematic of the in oculo transplantation model can be seen in figure 2.0, showing the sources of sympathetic, parasympathetic and sensory nerve supply to the iris, as well as the size and position of the transplants.

Fig. 2.0
In oculo transplantation model

IRIS
(Young host)

Ciliary ganglion
(Parasympathetic)

Trigeminal ganglion
(Sensory)

Superior cervical
ganglion (Sympathetic)

Young cerebral
artery

Old cerebral
artery

5 mm
2b.3) Cerebral blood vessels

Donor animals of both age groups were sacrificed with an overdose of pentobarbitone sodium and perfused via the left ventricle with Tyrode's solution containing 0.05% pontamine sky blue (PSB) (MERC, UK) to render the vessels visible for dissection and to reduce background autofluorescence (Cowen et al. 1985). Previous experiments have shown that the dye does not affect nerve growth (Gavazzi et al. 1992). Brains were removed and placed in sterile ice-cold Hank's buffered salt solution (HBSS) (Gibco, UK). 5mm lengths of left and right middle cerebral arteries (MCA) were dissected from the brain and cut into a proximal half which was stored in HBSS on ice prior to implantation and a distal half which were fixed and subsequently processed for immunohistochemistry (see below 2c).

2b.4) Sweat glands

Similar perfusions were carried out for the preparations of sweat gland transplants. The presence of PSB in the perfusate allowed sweat glands from the foot pads of the forepaws to be visualised and micro-dissected free of surrounding dermal connective tissue, muscle and epidermis. Glands were cut up into 2mm segments and stored for up to 4 hours in HBSS on ice before transplantation.

Six weeks old host animals were pre-treated with a sub-cutaneous injection of atropine to dilate the iris, then anaesthetized with fluothane and air mixture. Bilateral implants of MCA or sweat gland were inserted in oculo using fine forceps through a small corneal slit made with a microsurgical blade (Rocialle Medical Ltd., Cambridge, UK), then manipulated onto the surface of the iris in the posterior angle of the eye using gentle pressure on the corneal surface with the side of the forceps and, in the case of the MCA's, left for 4 and 8 weeks. Sweat gland transplants were left in place for 4 weeks after preliminary experiments had shown that nerve regrowth was largely complete by this time,
or for 10 weeks in the case of the retrograde tracing experiments (see below 2b.6). Three days before sacrifice, bilateral superior cervical ganglionectomy was carried out on a sub-group of host animals under halothane anaesthesia. Numbers of experimental groups are given in the relevant chapters.

2b.5) Bilateral surgical sympathectomy.

Host rats were anaesthetized with halothane, a mid-line incision was made in the throat and tissue retracted. The sternomastoid muscle was teased away from underlying connective tissue and reflected medially to expose the sternohyoid and omohyoid muscles. These were reflected medially to expose the bifurcation of the common carotid artery. The common carotid was freed from its underlying connective tissue and retracted laterally to expose the superior cervical ganglion (SCG). SCGs were carefully removed making sure to remove all lobes of the ganglion up to the post ganglionic internal and external carotid trunks. The operation site was closed over and the animals were usually left to recover for three days after which they were sacrificed and tissue taken for immunohistochemistry. The level of success of the surgical sympathectomy was assessed by the presence or absence of ptosis of both eyes of the animals and by the absence of noradrenaline staining using the sucrose phosphate glyoxylic acid method (de la Torre, Surgeon, 1976).

2b.6) Retrograde neuronal tracing.

Young and aged rats were anaesthetized with halothane and their heads fixed firmly in a stereotactic head frame. A mid-line incision was made in the scalp and tissue retracted to expose the cranium. The left temporalis muscle was gently teased away at its superior aspect where it is attached to the temporal bone and undermined to expose the left temporal bone beneath. After retracting the temporalis muscle, a small hole was drilled into the left temporal bone, lateral to the sagittal suture with a dental burr. A small cut was made in the dura mater overlying the left MCA and a small piece of foam pre-soaked in
2% fast blue (FB) and 0.2% Diamidino yellow (DY) (Sigma, UK) was placed over the MCA under the dura mater. A small piece of antibiotic gauze was placed over the dura mater, the temporalis muscle was returned to its original position and the operation site sutured. Sometimes in the same animals, the right anterior eye chamber was injected trans-sclerally with a solution of 1% FB and 0.1% DY (1.5 μl in young and 3 μl in aged rats because of the increased volume of the anterior eye chamber in the old animals). Temgesic was administered (intra-muscularly) in order minimise any post-operative pain and animals were left to recover for 4-5 days. Subsequently, under terminal anaesthesia, the rats were perfused with 4% paraformaldehyde in PBS and trigeminal and/or superior cervical ganglia removed. Ganglia were then washed, mounted in agar, vibratome sectioned at 80-100μm and processed as free floating sections for indirect immunohistochemistry using anti-CGRP or anti-NF-R<sub>39</sub>. Since the FB/DY tracer dye fluoresces in the violet spectrum (425nM excitation), the secondary antibody used to visualise the primary antibody was Texas Red conjugated IgG. There was no 'bleed through' of FB/DY into the Texas Red spectrum. Sections were mounted in antifade mountant onto glass slides and viewed on a fluorescence microscope (Olympus Vanox, Olympus Optical Co.(UK) Ltd; London, UK). Cell counts were made on all sections. Only FB stained neurones whose nuclei could be seen to be stained with DY were counted to reduce the risk of over estimating neurone numbers by counting neurons twice from adjacent sections or neurons that may have been FB labelled by diffusion from adjacent cells. Each FB/DY positive neurone was also viewed under green light (565 nM excitation) to check for colocalisation of CGRP or NF-R<sub>39</sub> (see Chapter 6).

2b.7) Nerve growth factor treatment.

MCA transplants of both age groups were treated with 0.5μg (5μl of 100μg/ml solution) mouse 2.5s NGF or Cytochrome C (Cyt C) for 4 weeks. Donor tissue was dipped into a solution of NGF or Cyt C immediately before implanting into the anterior eye chamber as above. Under halothane anaesthesia and
aseptic conditions, trans-scleral injections of the appropriate solution were made into the anterior eye chamber of the host rats once a week for three weeks starting one week after implantation.

2c) Specimen preparation and fluorescence immunohistochemistry.

The initial aim of this study was to test the following key steps in immunohistochemical protocols in order to develop sensitive and reproducible immunohistochemical methods, suitable for quantitative studies. In particular, I wanted to develop methods that could be used to quantify the intensity of immunohistochemical staining as well as morphological parameters such as fibre density.

- Use of buffers with no heavy metal ions.
- Primary and secondary antibodies.
- Antifade mountants.
- Storage of samples prior to image analysis.

In order to satisfy these objectives a model system was developed using 5-hydroxytryptomine (5-HT) uptake into the noradrenergic perivascular nerves of rabbit mesenteric veins. This assay was designed to test whether buffers could significantly enhance the quality of immunostaining and to test the sensitivity of a densitometric assay of immunohistochemical stain intensity (see below 2c.2). HEPES buffer was chosen and tested against PBS; the most commonly used buffer.

Mesenteric veins from 5 young adult New Zealand White rabbits were used. Animals were killed with an overdose of barbiturate (Euthatal, 0.1ml/kg i.v.) and SCG were dissected and removed. The mesenteric vasculature was flushed through with 500ml of warmed Tyrode's solution containing PSB in order to reduce background autofluorescence (Cowen et al. 1985) and to remove non-neuronal sources of 5-HT. Whole-mount preparations of mesenteric veins
were opened, pinned out on silicone rubber strips, cleared of superficial fat and mesenteric membranes and incubated in 10^{-6} M 5-HT for varying periods from 1-60 min. All specimens were fixed in 4% paraformaldehyde in PBS for 1.5hr. SCG were washed overnight in 20% sucrose PBS and sectioned at 10\mu m in the cryostat. Indirect immunohistochemical staining was carried out on all specimens, using a monoclonal antibody against 5-HT (clone YC5/45-HLK, Serotec, UK) and a fluorescein-conjugated, affinity-purified goat anti-rat IgG raised against the Fab2 fragment (Stratech, UK). Some specimens were processed using pH 7.4, 0.1M HEPES buffer (Sigma, UK) instead of PBS at all stages up to, and including, the diluent for the primary antibody.

2c.1) Model system to study antibody-antigen concentration relationships.

In order to study the effects of primary antibodies on immunofluorescence intensity, an adaptation of the technique described by Schipper and Tilders (1983) was used. This technique was ideally suited to test whether use of monoclonal antibodies resulted in a significant increase in image brightness over polyclonal antibodies.

Solutions of 5-HT (10^{-1} to 10^{-8} M) were dissolved in 40% gelatine and allowed to solidify. Small blocks were fixed, vibratome sectioned, at 50\mu m and immunostained for 5-HT. Monoclonal and goat polyclonal (Incstar, UK) antibodies against 5-HT were tested at varying concentrations, using anti-rat and anti-goat FITC-conjugated second layers. An amplified second layer was also used, comprising a biotinylated IgG followed by strepavidin conjugated to FITC (Vector Laboratories). Stain intensity was measured on 6 fields at each concentration of antigen and primary antibody.
2c.2) Effects of storage and ultra-violet radiation on the rate of fading of fluorescence.

The major disadvantage of fluorescence immunohistochemistry is that of photo-bleaching. This occurs when a fluorophore is excited with either ultra-violet radiation or with visible light. Normally when a fluorophore is excited an electron is knocked out of its orbit around the nucleus to the next quantum level. Almost immediately, the electron falls back to its original orbit and in the process releases energy in the form of a photon. It is these photons of light that the observer see when looking at a fluorescent sample. However, because this process involves energy loss, photon emission decreases with time. All fluorophores have a finite life after which they no longer fluoresce. If one excites a fluorophore with a very intense light source or for long periods of time, then photo-bleaching occurs.

In order to investigate the effects of storage and ultra-violet irradiation on the rate of fading of fluorescence, whole-mounts of mesenteric vein and sections of SCG were exposed to UV illumination for 10, 30 or 120 seconds; after which intensity of staining was measured using image analysis. Other sections of SCG were stored frozen for 0, 1, 2, 5 or 12 days after which measurements of fluorescence intensity were made using image analysis.

2c.3) Optimised protocol for indirect-immunohistochemistry.

Tissue was dissected rapidly, pinned to silicon rubber strips and immersion fixed in 4% paraformaldehyde for 1.5 - 2 hours. In some experiments the animals were perfused with 4% paraformaldehyde prior to tissue removal and post fixed in 4% paraformaldehyde for 1 - 2 hours. Tissue was washed in HEPES buffer for 3 x 10 minutes, treated with 0.1% Triton-X 100 in HEPES buffer for 40 minutes followed by a 5% serum wash for 1.5 hours. The serum was specified by the host species of the secondary antibody, which minimises non-specific binding of second layer antibodies and reduces background
fluorescence. Primary antibodies were diluted with HEPES buffer with 1% of the same serum from secondary antibody host species, 0.1% sodium azide, 0.1% Triton-X 100 and 0.1% D-l-lysine. Primary antibodies were applied to the tissue and left in a humidified chamber overnight at room temperature or for 48 hours in the case of the low affinity NGF-receptor (p75) antibody. Tissue was washed 3 x 10 minutes in PBS and the appropriate second layer antibody was applied and left in a humidified chamber for 1.5 hours at room temperature followed by 3 x 10 minutes wash in PBS. Following immunolabelling, specimens were stained for 10 min in 0.025% PSB in PBS and mounted in AF1 antifade mountant (University of Kent, Canterbury, UK) which significantly reduces fluorescence fading (Johnson, Araujo, 1981). Negative controls were carried out on all tissues by omitting the primary antibody and/or using tissues that do not express the antigen. Where possible, positive controls were carried out on tissues which are known to express the antigen in precise locations or cell types.

Nerve staining was visualised using an Olympus Vanox fluorescence microscope and a 200 watt mercury lamp source with stabilised power supply or a Bio Rad MRC 600 CLSM and quantified using a Kontron IPS or Kontron KS400 image analyser (Kontron, GmBH).

2c.4) Sucrose phosphate glyoxylic acid histochemistry.
(de la Tone & Surgeon 1976)

Glyoxylic acid histochemistry was used following surgical sympathectomy to verify the success of sympathectomy, i.e. negative staining indicated that sympathectomy was successful.

Tissue was removed, stretched quickly onto glass slides and air dried. Slides were dipped (3 x 2 seconds) into a solution of 1.5 glyoxylic acid and 7.5% sucrose in phosphate buffer (pH 7.4). Slides were then drained, dried with a hair dryer, placed in an oven at 80° for 5 minutes and mounted in liquid
paraffin. Specimens were viewed on an Olympus Vanox fluorescence microscope and a 200 watt mercury lamp source to confirm the absence or presence of nerve staining. If nerve staining was present then tissue from that animal was not used.

2d) **Fluorescence microscopy**

- Compensation for spatial and temporal illumination of microscope system.

Temporal and spatial unevenness of illumination are commonly observed in microscopical UV illumination systems. A stabilised power supply for the mercury lamp is essential to reduce temporal variation. Evaluation of several leading makes of fluorescence microscope have demonstrated varying degrees of spatial unevenness of illumination caused principally by the mercury lamp and its associated optics. I found the Olympus Vanox fluorescence microscope to provide the least spatial variation of illumination, although the lack of mirror adjustment in this microscope was a disadvantage. A uranyl glass standard (Carl Zeiss, Germany) was used to generate a fluorescence image in the green waveband of uniform intensity. This image was used 1) to adjust lamp intensity to an optimal range for the scanner, 2) to set and maintain a constant lamp intensity for each experiment and 3) to assess the spatial unevenness of illumination generated by microscope and scanner.

2e) **Confocal microscopy.**

Wide-field epifluorescence microscopical images often suffer from out-of-focus blur. The illumination of the entire field of view of the specimen with intense light at the appropriate excitatory wavelength excites fluorescence emissions throughout the whole depth of the specimen, not just at the focal plane of the objective. Much of the emitted light coming from the regions of the specimen above and below the focal plane is collected by the objective
lens and thus contributes out-of-focus blur to the final image of the specimen at the focal plane that results in serious degradation of image contrast and resolution.

While it is possible to remove most of this blur from wide-field images by computational deconvolution (Inoue, 1989) confocal laser scanning microscopy (CLSM) presents an alternative method in which almost all the light causing the blur is prevented, by optical means, from contributing to the original image (Fig. 2.1). This enables one to use CLSM directly for noninvasive serial optical sectioning to give high resolution images essentially free from out-of-focus blur.
Fig. 2.1.

Schematic diagram of epifluorescence laser scanning optical microscope. Excitatory laser light from the illuminating aperture passes through an excitation filter, is reflected by the dichroic mirror, and is focused by the objective lens to a diffraction-limited spot at the focal plane within the three dimensional specimen. Fluorescence emissions, excited both within the illuminated in-focus voxel and within the illuminated cones of light above and below it, are collected by the objective lens and pass through the dichroic mirror and the emission filter. However, only those emissions from the in-focus voxel (________) are able to pass unimpeded through the detector aperture to be detected by the photomultiplier. Fluorescence emissions from regions below the focal plane (----------) and from above it (............) have different primary image plane foci and are thus severely attenuated by the detector aperture, contributing essentially nothing to the final confocal image.
Although the confocal principle was invented long ago (Minsky, 1961) and its effectiveness was demonstrated by means of non-electronic direct-vision design (Patran et al. 1968), acceptance was slow until the advent of lasers and electronic frame stores. In recent years, instruments using lasers as a light source have exceeded all previous performance in terms of resolution and sensitivity, particularly in the application of fluorescent specimens (White et al. 1987; Brakenhoff et al. 1985; Brakenhoff et al. 1988; Carlsson et al. 1989; Carlsson, Aslund, 1989).

In confocal microscopy, both axial and lateral confocal resolution are maximized by the use of high numerical aperture (NA) objectives, since this determines the diffraction-limited scanning spot. Individual objectives of the same type differ considerably in their optical characteristics and aberrations, which can be measured by determining their point spread function (PSF). This is done by imaging a plain front-silvered mirror (Sheppard, Cogswell, 1990; Cogswell et al. 1990; Cogswell et al. 1990) making it possible to evaluate objectives and choose the best for confocal work. While in conventional light microscopy (LM) the image formation is determined by the PSF of the imaging objective alone, in a confocal microscope the image formation is determined by the PSF's of both the illuminating and imaging lens. Thus, in the confocal system in-focus PSF is the product of the two lenses. However, in the confocal epi-illumination arrangement these two PSFs are the same, since the objective is used for both illumination and imaging, so that the PSF of the system equals that of the objective lens squared. This improved PSF results in a significant increase in lateral spatial resolution (super-resolution). Since fluorescence emission occurs at longer wavelengths than that of excitation, by a factor determined by the Stokes shift of the particular fluorochrome, the spatial resolution will be determined by the illuminating wave length ($\lambda_1$) and by the emission wavelength ($\lambda_2$), since in the confocal system it is the shorter wavelength, $\lambda_1$, which determines the size of the illuminating spot. Thus in confocal fluorescence microscopy the highest frequency which can be recorded is proportional to $(1/\lambda_1 + 1/\lambda_2)$. This gives the CLSM, when used in
fluorescence mode, an additional resolution advantage over conventional microscopy, in which spatial resolution is determined solely by the longer wavelength, $\lambda_2$, of the fluorescence emission. Since resolution increases with decreasing wavelength, fluorochromes with shorter excitation wavelengths and smaller Stokes shifts will maximize the spatial resolution of the images obtained with CLSM.

Recent advances in technology have made confocal laser scanning microscopes widely available to the biologist. Confocal microscopy has the advantage over conventional bright field or fluorescence light microscopes of increased lateral resolution, of about 7 times, in addition to its optical sectioning capabilities. Since CLSM can achieve optical sections of less than one $\mu$m, image quality is far superior to that produced by conventional microscopes in that it contains none of the artifacts contributed by out-of-focus blur. I have used CLSM for part of this study (Chapter 7) for image acquisition and subsequent image analysis of double-stained immunofluorescence of middle cerebral arteries (MCA).

CLSM was performed using a Bio Rad MRC 600 with a krypton-argon laser and Olympus BH-2 microscope controlled via a 25MHz 486 computer and Bio Rad SOM or COMOS software. Prior to image acquisition, the optical axis of the laser path, confocal aperture, black level, gain amplifier settings, Kalman filtering and zoom settings were optimised to attain good contrast images of about 1-2$\mu$m depth (Z-sections). Where stain intensity measurements were required then all the above settings were standardised for reproducibility. In addition, laser intensity was standardised using a uranyl glass standard (Carl Ziess) and laser power monitored with an ammeter. Image acquisition was performed with Kalman filtering in order to filter out random ‘electronic noise’ generated by the analogue to digital converter (AD converter) and was then kept constant for all subsequent image acquisition. Z-sectioning depth was dependent on the experimental design, lens magnification and numerical aperture of the lens used; but in general 1 or 2$\mu$m optical sections were
collected and saved as 'Z-series' and stored on an optical disk cartridge for future analysis. The number of Z-sections collected was dependent on tissue thickness, whilst image acquisition times were kept to an absolute minimum in order to avoid bleaching of fluorescence. Where densitometric measurements were required one optical Z-section was taken at a predetermined depth in the tissue, in order to minimise bleaching of fluorescence, prior to Z-series collection. Where image analysis of confocal images was required, the individual optical sections of a Z-series were integrated to give a 'maximum brightness projection' where 3D-information from all Z-slices could be visualised on a single 2D-image.

2f) Image analysis.

- Use of solid-state versus cooled-Charged Coupled Device (CCD) cameras.
- Background subtraction routines.
- Micro-densitometric measurements.
- Low-pass filtering.
- Kontron KS400 image analysis system.

2f.1) Scanners for fluorescence imaging.

Fluorescence images are of relatively low intensity, requiring sensitive scanners to transfer images from microscope to image analyser. Whilst image analyzers frequently promise high levels of grey-level (intensity) resolution, in practise, resolution is often limited by the imaging system. The aim was to develop a method of testing the grey-level resolution of low-light video (Panasonic, model WV1900) and cooled digital (PCO Computer Optics GmbH, Germany, Model VL350) scanners. A range of neutral density filters of transmittance ranging from 5-75% were inserted into the microscope light path above an image of the uranyl glass standard and the intensity of the resulting images was measured using image analysis.
2f.2) Image analysis

A Panasonic intensified newvicon camera was used to transfer images from the fluorescence microscope to an IBAS IPS image analyser (Kontron, UK) where they were digitised onto 512 x 512 pixels with 8 bits of grey resolution. Later experiments used cooled CCD scanners after they had been seen to provide significant improvement in grey level discrimination (see below, Chapters 6-7). Lamp intensity was checked at regular intervals during each experiment (see fluorescence microscopy, 2d above). Dark current of the scanner was set at a grey level of 14. Ten images were summed on the analyser and the resulting intensities divided by 10 in order to reduce image noise and short-term temporal variations. Two background images were generated. Uneven illumination caused by the mercury lamp and optics was compensated for by establishing an image (of the pattern of illumination) generated by the uranyl glass standard. This image was reduced to a low level of intensity using neutral density filters, digitised, stored on hard disc and used for multiplicative shade-correction on all subsequent specimen images. In view of the non-linearity of camera response (see fluorescence microscopy, above), I tested whether multiplicative shade correction was preferable to a subtractive method for removal of uneven illumination generated by mercury lamp, optics and scanner.

Low-pass filtering matrices were used to generate a second image representing the uneven background in each specimen resulting from non-specific staining and autofluorescence. This image was subtracted from the specimen image. The fully shade-corrected image was saved for later use. From the shade-corrected image a binary ‘mask’ of the specific staining was generated using standard contour enhancement and segmentation algorithms. The ‘mask’ was superimposed back onto the shade-corrected image using a Boolean operator and the following measurements were made on the staining within the ‘mask’. Fluorescent area provided information regarding the total numbers of stained...
nerve fibres expressed as a percentage of the field area (Area%). Intercept density counted the numbers of intercepts made by the bundles of nerve fibres comprising the terminal plexus in the measuring field (ID/mm). Finally, grey value measured the mean intensity registered by objects within the mask representing images of the terminal nerve plexus (GV).

In the case of measurements made on gelatine sections, assessment of background staining in the specimen and the masking procedure were omitted. Following shade correction, images were segmented and mean grey levels were measured as before. In the tests of grey-level resolution, images were processed with and without the initial shade-correction procedure. Mean grey levels were measured 6 times over a 5-10min period in order to assess residual temporal variations. In order to demonstrate the effects of residual spatial variations, measurements were made over small (400 pixel) measuring frames at the centre and edge of the field, as well as on the whole 512 x 512 pixel measuring frame. Linearisation of camera response was carried out using transformation tables established by linear regression analysis of the camera response curve.

2f.3) Kontron KS400 image analysis system

Part of this thesis used a new generation of image analysis equipment which was acquired midway through my experimental studies. The KS400 software runs on an IBM compatible computer and is therefore easily upgradeable. However, this is not the only advantage. Improvements in microchip technology have made computing extremely fast, so that one can now use very sophisticated algorithms, on full 8-bit grey images, at realistic times periods (seconds, rather than minutes). The speed of algorithm execution is limited only by the clock speed and amount of memory of the computer. In Chapter 7, I describe analysis of confocal images using this system.
Chapter 3

Results of technical studies for the development of an immunohistochemical microscopical assay.

3a) Introduction.

Studies of amines in the terminal plexuses of sympathetic nerves (Violet, Cowen, 1990; Cowen, Thrasivoulou, 1990) have suggested substantial changes in amine uptake in old age. Measurement of the ways in which neurones control their neurotransmitter content has generally been made using biochemical assay, autoradiographic or pharmacological approaches. However, pharmacological studies of similar questions have given contradictory results (Duckles, 1987). Attempts to use HPLC assay with electrochemical detection for these experiments have proved unsuccessful due to the small size of the tissue samples (<2mg) and their heterogeneity (Lincoln J, personal observation). Autoradiographic approaches are likely to be unsuccessful for the same reasons. Peripheral sympathetic nerve plexuses in, for example, blood vessels lie over smooth muscle and endothelial layers and are surrounded by adventitial fibroblasts. All these elements possess amine uptake (Iversen, 1971) and metabolising (Paton, 1976) systems which can compete with neuronal amine uptake in tissue homogenates. The relatively large tissue mass of the non-neuronal elements compared to that of the fine nerve plexus means that they will tend to mask neuronal uptake.

These studies underlined the need for technical developments which would make it possible to quantify the intensity of immunohistochemical staining in a reliable and reproducible way. This chapter represents the results of my attempt to achieve this aim.
3a.1) Confocal microscopy.

Comparisons of LM versus CLSM, combined with image analysis, were made on preparations of PGP9.5 immunostained rat ear arteries to determine which image acquisition method is more sensitive at visualising nerves. I have also contributed to the technical development of other studies in this laboratory which have used CLSM for densitometric quantification of laminin immunofluorescence (Gavazzi et al. 1995a) and for comparison of CLSM versus conventional fluorescence light microscopy for quantitative analysis of the perivascular innervation of human mesenteric and coronary arteries (Buwalda et al. 1997). I have used CLSM for image acquisition and subsequent image analysis (using the KS400 image analysis system) of double stained immunofluorescence of middle cerebral arteries (MCA) (Chapter 7).

3a.2) Image analysis.

Image analysis has proved a valuable tool in quantitative studies of the nervous system (Contestabile et al. 1987; Amenta et al. 1987; Agnati, Fuxe, 1984; Gardette et al. 1981; Cowen, Burnstock, 1985). Field and object measurements have enabled changes to be studied in nerve cell numbers and size, in nerve fibre density and in the localization and colocalization of neurotransmitters. Local (Cowen, Burnstock, 1980; Cowen et al. 1986) and developmental (Vega et al. 1990; Gallen et al. 1982; Cowen et al. 1982; Gale et al. 1989) variations in the pattern and distribution of transmitter-identified populations of autonomic nerve fibres have been made using fluorescence histochemical (Amenta et al. 1987; Todd, 1986) and immunohistochemical (Cowen, 1984) methods of image analysis. During these studies, it has become clear that provision of a clean, high-contrast, uniformly illuminated image is of primary importance for the successful use of automated methods of image analysis. In the present study, the main aim was to develop a 'microscopical assay' using a densitometric application of image analysis in order to study 5-HT uptake in sympathetic nerves within small samples of heterogenous
tissues. To this end, I have investigated problems relating to specimen preparation, immunohistochemical staining, imaging and image analysis (see Chapter 2). The resulting optimised method has been tested by its ability to assess the time-scale of 5-HT uptake into sympathetic perivascular nerves (for methods see Chapter 2).

Recent advances in computer technology, reduction in the price of central processors and computer memory have popularised the use of image analysis in many areas of science. As a result there has been a substantial increase in the range of image processing algorithms available. The majority of the algorithms are not new to the image analyst. Indeed, they were developed many years ago but were not implemented on all but the most expensive systems because the cost of sufficient random access memory (RAM) to enable them to run at reasonable speed was prohibitive. Two key areas have benefited from the increased performance of image analysis systems, namely, full grey morphology algorithms, which results in improved image contrast/edge detection, and thresholding to produce binary masks of regions of interest.

3b) Results

3b.1) Specimen preparation.

Buffers.

The use of HEPES buffer instead of PBS in tissue processing resulted in a significant increase of 40-100% in intensity and sensitivity of staining for 5-HT in perivascular nerves (Fig. 3.1) (p<0.01 @ 10^{-4} M and p<0.001 @ 10^{-6} M 5-HT concentrations) and reduced background, non-specific staining.
Fig 3.1.

Histogram demonstrating the effects of PBS and HEPES buffers on intensity of fluorescence (mean ± s.e.m. grey value). Whole-mounts of rabbit mesenteric vein incubated in $10^{-4}$ and $10^{-6}$M serotonin for 20min. Tissue was subsequently processed using indirect immunohistochemistry. (n=5)

* $p<0.01$

**$p<0.001$
PBS buffer

Hepes buffer

Gray Value

5-HT Concentration (M)

1x10^-4

1x10^-6

* *
3b.2) Fading of fluorescence.

Frozen storage of sections of SCG showed significant decreases of the order of 40% in grey level after 2 days of storage (Fig 3.2a). Storage for up to 2 weeks resulted in no further significant loss of fluorescence intensity. Exposure of the specimens to UV illumination for periods of time up to 2min showed no significant reductions of fluorescence intensity (Fig 3.2b) although 30sec appeared to be a safer upper limit.
Fig 3.2.

Measurements of fading of FITC fluorescence (mean ± s.e.m. grey value) made using image analysis.

a) Effect of frozen storage on sections of rabbit superior cervical ganglion (SCG) stained using a monoclonal antibody to tyrosine hydroxylase followed by indirect labelling with FITC. (n=5) **p<0.01 (Two days compared to Day zero)

b) Effect of exposure to UV light on sections of SCG and whole-mounts of mesenteric vein (MV) from rabbits stained using a monoclonal antibody to serotonin followed by indirect labelling with FITC. (n=5) *p<0.05 (120 seconds compared to time zero)
3b.3) Antibody-antigen concentration relationship

The monoclonal anti-5-HT antibody gave substantially higher stain intensities in gelatine sections impregnated with 5-HT compared to the 'polyclonal' antibody. Antibody concentration had a strong influence on stain intensity: low concentrations resulted in an insensitive response of staining to changes in 5-HT concentration, whilst high concentrations produced the most sensitive staining response. Sensitive changes of stain intensity were observed at concentrations of 5-HT between $5 \times 10^{-4}$ and $10^{-1}$M. No quantitative variation of stain intensity in response to varying concentrations of antigen was seen using amplified second layer antibodies (Table 3.1).

Table 3.1.

Stain intensity (mean ± s.e.m. grey value) in rabbit mesenteric veins ($n=5$) incubated in $10^{-4}$ and $10^{-6}$M serotonin for 20min and processed for normal indirect and avidin-biotin amplified fluorescence immunohistochemistry. **$p<0.01$**

<table>
<thead>
<tr>
<th>5HT (M)</th>
<th>Grey Value (Amplified)</th>
<th>Grey value (Non-amplified)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-4}$</td>
<td>61 ±6.62</td>
<td>57 ± 9.8</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>63 ±5.35</td>
<td>33 ±1.4 **</td>
</tr>
</tbody>
</table>

55
Fig 3.3.

Plot of stain intensity (mean ± s.e.m. grey value) against antigen concentration using mono-(M) and poly-(P) clonal antibodies at dilutions of 1:150, 1:300 and 1:900. Fixed, gelatine, vibratome (50μm) sections containing 10⁻¹⁻¹⁰⁻⁸M concentrations of serotonin were processed for indirect immunohistochemistry. (n=6)
3b.4) Confocal laser scanning microscopy.

3b.4.1) Pilot confocal study

PGP9.5-like immunoreactivity of young rat ear arteries imaged with CLSM showed a significant increase of about 60% in nerve density, (FA%; p<0.01), when compared to images of the same vessels acquired with conventional fluorescence microscopy (Fig. 3.4).

3b.4.2) Summary of results of Buwalda, Colnot, Bleys, Groen, Thrasivoulou & Cowen (1997)

Mesenteric and coronary arteries visualised with CLSM gave qualitative clearer and increased nerve density compared to FM with much reduced background (Fig 3.5).

Statistical analysis (ANOVA) of 45 segments of mesenteric arteries showed no significant differences in nerve density in the parameter FA% between CLSM and FM (Fig. 3.6a). However, ID/mm revealed significant difference in nerve density between CLSM and LM (p<0.0001) (Fig. 3.6b)
Fig 3.4.

PGP9.5 immunoreactive nerve staining of ear arteries of young rats. Comparison of confocal- (CLSM) versus light-microscopical (LM) image analysis. Note the increased sensitivity (~60%) of confocal imaging over light microscopical imaging. (n=6) *p<0.01
Comparison of confocal versus light microscopical image analysis

PGP9.5 like Nerve density of rat ear arteries

![Graph showing nerve density comparison between CLSM and LM methods](image)
Deep nerve plexuses of human mesenteric artery (A-D) and coronary artery (E-H). Note the improved image quality and increased resolution of CLSM (B and F) over LM images (A and E). Binary images were produced of LM (C and G) and CLSM (D and H) during images analysis for quantification of nerve density. (see Fig. 3.6). Scale bar = 50µm
Mean nerve density of 45 segments from human mesenteric arteries imaged with CLSM or LM. (Error bars ± SD)

a) Image analysis revealed no significant difference for area% (p > 0.05)
b) CLSM gave significantly greater intercept density measurements (ID/mm) than LM (*p<0.0001). (n=45)
PGP9.5-LI nerve density of human mesenteric arteries

Comparison of confocal versus light microscopical image analysis

![Graph a](image1)

![Graph b](image2)
3b.5) Image analysis.

3b.5.1) Imaging.

The pattern of UV illumination generated by the low-light video camera and epi-illumination optics of a Leitz Ortholux fluorescence microscope is illustrated by a line profile of grey values obtained using image analysis of an image produced from a uranyl glass standard (Fig 3.7). Note that light intensities can vary from a grey value of about 60 at the periphery of the field to about 90 at the centre.
Fig 3.7.

Line plot showing densitometric measurement of grey value made along a horizontal line across the centre of the field of view to illustrate uneven patterns of fluorescence illumination. Note the marked, central 'hot spot' showing grey values approximately 30% higher than those at the periphery of the image.
The results of grey-level resolution tests on different cameras are illustrated in Fig 3.8. The response curves for 'centre' and 'edge' fields of the low-light video camera illustrate the variation in intensities across the field of view. It should be noted that the spatial variation of intensity indicated by these curves is the sum of variation produced by all components of the imaging system including microscope and scanner. The standard deviations of the 'full' field curve were calculated by summing the effects of temporal and spatial variation (Fig 3.8a). Extrapolation from the standard deviations observed in the 'full' field without background subtraction or linearisation of camera response suggested that under these conditions only about 5 grey levels could be separated between black and white.

The effects of subtractive (Fig. 3.8b) and multiplicative (Fig. 3.8c) shade correction were assessed using the same system. Subtractive shade-correction resulted in a small improvement in grey resolution on the full-field measuring frame to give about 7 separate grey values between black and white. However, multiplicative shade-correction made a substantial improvement, allowing resolution of about 15 grey levels. Camera linearisation provided a small, further improvement (data not shown).

The non-intensified, Peltier-cooled, digital CCD scanner demonstrated a linear response over the full grey scale with low levels of spatial and temporal variation (Fig. 3.8d). The low standard deviation (<1%) suggested that this camera, without correction for non-linearity or uneven illumination, produced a grey-level resolution of a comparable order to that of the image analyzer, i.e. 100-200 grey levels between black and white.
Tests of densitometric resolving power in image analysis.

a) Uncorrected response of a Panasonic (model WV1900) low-light video scanner to a range of image intensities measured over small (400 pixels) measuring frames situated at the centre and edge of the field of view and a larger (200,000 pixels) full measuring frame. Results are the mean of 6 measurements and standard deviations are the sum of spatial and temporal variation. Note the large standard deviation of the data from the full curve.

b) Response of Panasonic video scanner (as (a)) to the same range of image intensities used in (a) after subtractive correction for uneven illumination. Standard deviations are only shown for the full frame.

c) Response of Panasonic video scanner (as (b)) after multiplicative shade correction for uneven illumination. Curves for the 3 measuring frames (see (a)) were virtually indistinguishable from each other. Data from the full measuring frame is shown and standard deviations averaged 7.6%.

d) Uncorrected response of a Peltier-cooled CCD scanner (Model VL350, PCO Optics GmbH, Germany) to the same range of image intensities used in (a). Curves for the 3 measuring frames (see (a)) were indistinguishable from each other. Data from the full measuring frame is shown and standard deviation ranged from 0.2-1%. Note that the field of view of the CCD camera is smaller than that of the video scanner, eliminating some peripheral unevenness generated by the microscope.
3b.5.2) **Time-course of 5-HT accumulation in sympathetic nerves.**

Using a low-light video scanner, the Vanox fluorescence microscope and the correction and camera-linearisation procedures described in **Chapter 2**, I have demonstrated sensitive changes in neuronal accumulation of 5-HT with time as revealed by fluorescence immunohistochemistry. Figure 3.9 shows typical staining patterns of nerve fibres in mesenteric veins following 5 (a) and 30 min (b) incubation in $10^{-6}$M 5-HT. The 'microscopical assay' showed increasing grey values reflecting accumulation of 5-HT up to 30min of incubation followed by a non-significant decrease in 5-HT levels at 60min (Fig 3.10a). The relatively low standard errors resulted in the demonstration of significant differences in the rate of accumulation of 5-HT over time. Field measurements of fluorescent area (FA%) (Fig 3.10b) and intercept density (ID) (Fig 3.10c) showed that FA%, reflecting the apparent size of nerve bundles, is affected by changes in grey value more than ID which measures the density of nerve bundles.
Fig 3.9.

Photomicrographs of rabbit mesenteric vein incubated in $10^{-6}$M serotonin for varying durations and processed for indirect fluorescence immunohistochemistry. Scale bar 50μm. [A] 5min incubation in serotonin, [B] 30min incubation in serotonin.
Fig 3.10.

Histograms demonstrating rate of uptake of serotonin into autonomic nerves on rabbit mesenteric veins, incubated in $10^{-6}$M serotonin for 0, 1, 5, 10, 30 and 60min and subsequently processed for indirect immunohistochemistry. [a] Stain intensity plotted against incubation time, [b] Nerve density (area%) against incubation time and [c] Total intercept density per unit area of vessel wall (Intercept) against incubation time. Data expressed as mean ± s.e.m: (n = 5)
a.

Grey Value

0 10 20 30 40 50 60 70

0 1 5 10 30 60

b.

Area %

0 10 20 30 40

0 1 5 10 30 60

c.

Intercept

0 1000 2000 3000 4000 5000

0 1 5 10 30 60 min
3c) Discussion.

3c.1) Specimen preparation.

Techniques of specimen preparation can influence quantitative studies of the nervous system in a number of different ways. For example, it is well known that fixation and subsequent processing can have a dramatic effect on tissue shrinkage (Cowen, Burnstock, 1980) the extent of which is fixative and tissue type dependent. Fixatives which are solvent based tend to show a more marked effect whereas aqueous fixatives tend to have a lesser effect. Structures which have a relatively large proportion of lipids are more susceptible to shrinkage during fixation and processing. Brain tissue, for example, can shrink by as much as 48% (Mouritzen Dam, 1979) when it is fixed in a range of formaldehyde concentrations. Therefore, it is clearly important that when making quantitative measurement on similar tissues, every care must be taken to control shrinkage effects and, as much as possible, one must compensate for these effects with the use of correction factors when absolute measurements are required or if there is differential shrinkage between experimental groups.

Another key area which can influence fluorescence quantification of the nerves is background auto-fluorescence (Cowen et al. 1985). Connective tissues such as collagen and elastin are highly auto-fluorescent as are certain cell types such as endothelial cells, fibroblasts and hepatocytes. Auto-fluorescence of these structures results in reduced contrast of nerves, particularly in whole mount preparations, and can reach the point where fine nerves are no longer visible. Auto-fluorescence can be reduced by counterstaining with Pontamine sky blue when one is using FITC fluorescence. Connective tissues take up the stain which gives a red emission when excited with blue light at 450-490nM. This can be filtered out by the use of red cut-off filter in the path of emitted light to reveal only the green emission of the FITC localisation (Cowen et al. 1985).
In the present study I have shown that buffers can have an important effect on intensity of staining and on background, non-specific staining. Use of organic buffers resolves these problems, giving substantially enhanced brightness and contrast of specific staining. Inorganic buffers such as PBS contain heavy metal ions which can have electrostatic effects on antigen-antibody binding by cross linking the IgG's of the antibody which may cause steric hindrance resulting in under-saturation of available antigen binding sites. In addition, the presence of positively charged heavy metal ions of inorganic buffers may increase non-specific binding of primary/secondary antibodies by electrostatic bonding, thereby increasing background staining.

It is important to minimise the effects of fading by storing specimens frozen for a maximum of 2 days and by keeping the period during which specimens are exposed to UV light to less than about 30sec. The model system showed that relatively high concentrations of monoclonal antibodies were essential in order to maximise sensitivity of staining. The use of amplifying systems such as avidin-biotin were incompatible with attempts to compare antigen levels with intensity of immunofluorescence staining. Amplifying systems are insensitive to changes in antigen binding sites because they rely on amplifying the signal by building up a 'tree' with multiple avidin 'branches' on the intermediate avidin IgG for the labelled biotin to bind to, therefore relatively small changes can not be detected.

3c.2) Imaging.

CLSM offers superior image quality, particularly in thick preparations containing abundant connective tissues, thereby making quantification of nerves a simpler task. The principle reason for this increased image quality is the ability of CLSM to remove out-of-focus blur by optically sectioning the tissue only in the area where nerves are localised, excluding the deeper areas where connective tissues are localised. A recent study in our laboratory has
compared CLSM versus epi-fluorescence microscopy (FM) combined with image analysis (Buwalda et al. 1997). In this immunohistochemical study, perivascular nerves supplying human coronary and mesenteric arteries, stained for PGP 9.5, were visualised with CLSM and FM followed by quantitative image analysis. The results demonstrated that image acquisition with CLSM was able to reveal significantly (4-25 fold) more fine nerve bundles than FM (Fig. 3.5-3.6). It was evident that images acquired with CLSM resulted in increased contrast of nerves, particularly of the fine terminal nerves localised in the deeper adventitial layers which were scarcely visible with FM.

We have used a densitometric application of CLSM to study laminin immunoreactivity in young and aged blood vessels of rats (Gavazzi et al. 1995a). In this study intensity measurements of laminin immunoreactivity were made in aged blood vessels, using specially standardised techniques of specimen preparation, where we had previously shown a decrease in nerve density of approximately 50% when compared to young (Gavazzi et al. 1992). We wished to correlate the decrease in nerve density with laminin immunoreactivity. This morphological approach was necessary because biochemical techniques are unable to distinguish specific, localised changes in laminin at sites accessible to nerves from heterogeneous changes in other areas of the vessel wall, such as the endothelial basal lamina. The results showed that, in aged rats, laminin immunoreactivity was reduced by 50% at the medial-adventitial border in association with the outer layer of smooth muscle cells, where a parallel decrease is observed in innervation density. Axonal terminals were shown to have access to laminin in this region of the blood vessel wall by double staining with laminin and PGP 9.5. Changes in laminin immunoreactivity were region-specific on the same blood vessel, thus excluding the possibility of a generalized decrease in immunoreactivity in old age. Therefore this study supports the hypothesis that laminin may play a role in nerve fibre atrophy in old age.

There have been a number of studies where densitometric measurements have
been made from CLSM images (Liljeborg et al. 1995). A number of standardised procedures are required to make reliable reproducible densitometric measurements. These factors include, a] standardising gain, black-level and confocal aperture settings, 2] monitoring laser output power and anode current during image acquisition sessions and between experiments, 3] standardisation of laser illumination time to reduce and standardise the extent of photobleaching and 4] ensuring that the optical section that is used for GV measurements comes from the same depth from each sample so that observed differences in GV are real and not effects of signal attenuation from layers of different depth within the specimen. With all these pre-requisites satisfied, densitometric measurements are possible and are in some ways preferable to images acquired on FM because measurements can be made from very localised areas in relatively thin optical slices. In other studies it has been shown that if GV measurements are required through the thickness of the specimen it is possible to compensate for the attenuation of the signal from the deeper layers (Liljeborg et al. 1995) which has a linear relationship with depth. However, spherical aberration also increases with specimen thickness depth. In addition, differences in refractive index (RI) of specimen and mounting medium may enhance spherical aberration (White et al. 1996). In the absence of RI data, which in biological systems, is hard to arrive at, RI is normally set to one. This can lead to inaccuracies in measurements. The best solution is therefore to make GV measurements from a predetermined depth within the samples so that no RI or depth correction is necessary.

3c.3) Image analysis.

Densitometric applications of image analysis have been used previously to measure stain intensity (see (Agnati, Fuxe, 1984)) and semi-automated sliver grain counting (Nagata, 1993). However, the technical limits of such measuring systems have not been fully assessed. In the present study, imaging systems and techniques were shown to have significant effects on quantitative measurements of stain intensity. Mercury lamp, microscope optics and video
camera were shown to contribute to significant unevenness of illumination of
the field of view. Linearisation of camera response and a two-stage
background subtraction procedure resulted in substantial improvements in grey
level resolution of video-based imaging systems. The linear response and
uniform spatial imaging characteristics of digital CCD cameras reduced these
problems to give grey level resolutions which were comparable to the
theoretical resolution of an 8-bit image analysis system, thus making these
cameras the instruments of choice for studies of this kind. Furthermore,
because of the uniform image generated by these cameras, background
subtraction is no longer required to compensate for uneven illumination
generated principally by video scanners, although specimen background still
needs to be considered.

Using the microscopical assay system, measurements of 5-HT uptake into
sympathetic nerves were made which reliably separated neuronal from
extraneuronal uptake. Neuronal uptake of 5-HT was relatively uniform over
30 min of incubation in a solution of exogenous 5-HT but became slower over
longer periods, probably due to the increased contribution of passive efflux of
5-HT at high intraneuronal concentrations (Paton, 1976). The standard errors
observed showed that this method provided relatively sensitive measurements
of neuronal uptake. Comparing the grey levels measured in the biological
specimens with those of the model gelatine sections, neuronal concentrations
of 5-HT may be estimated to average about $10^{-3}$ M. These figures compare
with noradrenaline concentrations of similar millimolar values calculated for
sympathetic nerve terminals (Jonsson, 1971) although there is still some
speculation whether 5-HT and noradrenaline use the same uptake mechanism
and storage vesicles. Field measurements showed that the parameter
fluorescent area was more affected by changes in stain intensity than intercept
density. The reason for this is likely to be because FA% is a measure of nerve
bundle size whilst ID/mm is a measure of numbers of nerve fibres. At one-
minute incubation time in $10^{-6}$ M 5-HT all nerve fibres had taken up detectable
amounts of 5-HT. As the incubation period lengthened and more 5-HT was
taken up by the nerves, intraneuronal concentrations increased, immunostaining brightness increased which resulted in much larger nerve bundles with no detectable increase in number of nerve fibres detected.

Recent developments in image analysis have carried these developments a step further. Morphological operators, which work rapidly on 8-bit full grey images (seconds rather than minutes), have made contrast enhancement in images with irregular background staining a more reliable and objective process. The principal algorithm which we have made use of with the KS400 imaging analysis system is the 'white top hat'. This algorithm uses a combination of 'grey open' (erode and dilate) with a user specified shape operator over a number of iterations until the detail that one is interested in starts to lose its definition. This new image is then subtracted from the original grey image to produce an image with very much reduced background staining. Since peripheral axons have very similar diameters one can set the number of iterations at a constant level for each experiment. Another key area in image analysis which suffers from subjectivity is grey level thresholding (segmentation). We have been able to develop semi-automated thresholding procedures which are executed at two stages which reduces the extent of operator subjectivity. The initial thresholding consists of a preliminary grey measurement of the 'white top hat' image where mean grey value and standard deviation (SD)of grey are measured. The mean grey plus 1.7 SD (arrived at empirically) are used to set the lower threshold limit, whilst the upper is set to white (255 GV), to produce a binary image of the bright/large nerve fibres/bundles. The second thresholding step uses a dynamic thresholding procedure where low-pass filtering is used with a matrix size set to match the size of the objects of interest. The low-pass image is then subtracted from the input image. An off-set is then subtracted from the resulting grey image prior to binarisation. The off-set used is calculated from the modal value of the grey level distribution histogram derived from the low-pass subtracted grey image. The rational for setting the off-set at the modal value is that, in our images background constitutes up to 90% of the field, therefore the grey value with
the highest frequency should lie very close to the background, \textit{i.e.}, are least likely to represent specific staining. The resultant binary image which contains just the fine nerve fibres is then added to the first segmented binary image to produce a binary image of the total nerve plexus and can be used as a mask from which to make field, object and grey value measurements.

3d) Conclusions.

The methodology required for densitometry in image analysis is more demanding compared to that for measurement of field and object data. My attempts to deal with the problems associated with specimen preparation, imaging and analysis have indicated several areas of technique which may contribute significantly to improving the accuracy and sensitivity of densitometric measurement:

3d.1) Specimen preparation.

- reduction of background, non-specific staining using buffers free of heavy metal ions combined with serum washes.
- use of monoclonal primary antibodies in preference to polyclonal antibodies.
- avoidance of amplified second layer antibodies for fluorescence immunohistochemistry.

3d.2) Imaging

- the use of standard times of UV exposure and specimen storage to avoid differential fading.
- the use of CLSM results in superior image quality and is well suited for densitometry with the appropriate safe guards.
• the use of cooled CCD scanners for low-light images in preference to video scanners.

3d.3) Image analysis.

• use of background subtraction routines based on analysis of the contributions made to uneven image brightness by the illumination system, microscope optics, scanner and specimen.
• generation of transformation tables to correct for non-linearity of scanner response to image brightness and, where possible.
• attention to the need for objectivity in thresholding procedures.

Many of these techniques, although generated for the measurement of immunostaining in the nervous system, may be found to be of general relevance to quantification of microscopical images using computerised image analysis.
Chapter 4

Regulation of rat sympathetic nerve density by target tissues & nerve growth factor in maturity and old age.

4.a) Introduction.

Alterations in the size of target tissues produce proportional changes in the extent of axonal and dendritic arborization during development and in maturity (Voyvodic, 1989a), whilst NGF-treatment causes expansion of the dendritic arborization in sympathetic neurons of mature mice (Snider, 1988). Target tissues provide neurotrophic factors which ensure the survival of appropriate numbers of neurons during development (Purves, 1988; Oppenheim, 1989), and may also influence the neurotransmitter phenotype of outgrowing nerves. Correlations have been shown between sympathetic innervation of peripheral tissues and expression of NGF mRNA (Shelton, Reichard, 1984; Korsching, Thoenen, 1983). More recently, transgenic mice lacking (Crowley et al. 1994) or over-expressing NGF (Hassankhani et al. 1995) were shown to exhibit hypo- and hyper-innervation of peripheral tissues, respectively. However, other studies have shown high levels of NGF message in non-innervated vascular tissues (Scarisbrick et al. 1993). Sweat glands have been shown to produce a factor or factors which induce a cholinergic phenotype in sympathetic neurones (Landis, 1990), whilst nerve growth factor (NGF), found in some autonomic target tissues, has been shown to influence neuronal morphology during maturity in sympathetic neurones (Ruit et al. 1990), including those that supply cerebral blood vessels (Cowen et al. 1993; Gavazzi, Cowen, 1993) and enhances the expression of catecholaminergic enzymes (Thoenen et al. 1971).

The peripheral nervous system exhibits age changes which involve specific effects on adrenergic, cholinergic and peptidergic nerves, including those
supplying cerebral blood vessels (Mione et al. 1988; Cowen, Thrasivoulou, 1990; Gale et al. 1989; Saba et al. 1984; Dhall et al. 1986; Deckwerth, Johnson, 1993; Lundberg et al. 1976). Mione et al (1988) have shown a decrease in sympathetic nerve density of about 50% in MCA from aged Wistar rats coupled with an increase in density of non-sympathetic nerves. Studies in this laboratory have demonstrated a reduction of approximately 50% in total nerve density in a number of cerebral blood vessels of old Sprague Dawley rats (Cowen, Thrasivoulou, 1990; Gavazzi et al. 1992), using the general neuronal marker PGP 9.5 (Thompson et al. 1983). A decrease of similar proportions with age was also observed in the sympathetic innervation of these vessels in rabbit and rat (Andrews, Cowen, 1994b; Saba et al. 1984b) using catecholamine histochemistry. Recent studies have demonstrated that the sympathetic nerve plexus in cerebral blood vessels declined in density in old animals, whilst non-sympathetic nerves may continue to grow (Mione et al. 1988; Cowen, Thrasivoulou, 1990; Saba et al. 1984).

Investigations in our laboratory have been concerned with the ability of target tissues to influence neuronal phenotype in old age. Specifically, we have used a transplantation model in rats to show that target tissues regulate age-changes in their overall nerve density, demonstrated using the general neuronal marker, PGP 9.5 (Gavazzi et al. 1992). Cerebral arteries which exhibit a reduction of 50% in total plexus density in old age, when transplanted into young host animals, became reinnervated with a nerve density appropriate to the age of the target tissue. Other studies, also using transplantation, have shown that old neurones, whose axons in vivo show age-related atrophy, have an unimpaired capacity for outgrowth (Rosenfield, Kak, 1982; Gavazzi, Cowen, 1993; Stieg et al. 1991).

These studies prompted us to investigate whether target tissues have the capacity to regulate the population-specific changes in nerve plexus density exhibited by ageing cerebrovascular nerves. The aims of the present study were 1) to compare and quantify age changes in the sympathetic innervation of
cerebral blood vessels in rats, using immunohistochemical staining for tyrosine hydroxylase (TH) and light microscopical image analysis, 2) to use in oculo transplantation (Gavazzi et al. 1992; Olson, Malmfors, 1970) to investigate whether target tissues from young and old donors were able to induce appropriate, age-specific density of reinnervation by young host sympathetic nerve fibres, and 3) to study the effects on sympathetic reinnervation of treating in oculo transplants with NGF.

4b) Materials and methods.

Young (6 week) and old (24-26 month), male Sprague-Dawley rats from an inbred colony were used in all experiments.

See Chapter II for methods

4c) Results.

4c.1) Tyrosine Hydroxylase-like immunoreactivity (TH-LI).

16 young and 9 old normal MCA were used to obtain values of TH-LI nerve density for untreated normal vessels, whilst 14 young and 15 old implanted MCA were used for measurements on transplants, of which 7 young and 8 old vessels were implanted for 4 weeks and the remainder for 8 weeks. A further 15 young and 16 old implanted MCA were used for NGF/Cyt C treatment groups, of which 8 young and 6 old were treated with NGF and 7 young and 10 old were treated with Cyto C. Treatment with NGF or Cyt C was carried out for 4 weeks only.

The pattern of TH-LI nerves in the young MCA in vivo was relatively dense and plexiform. TH-LI nerve density was reduced in the old MCA but remained plexiform in character (Fig. 4.1A-B). Image analysis showed significant decreases of 50-60% in nerve density in the old vessels compared to young,
(Area%,, p<0.0001; ID, p<0.0001) (Fig. 4.2A-B). TH-LI stain intensity (GV)
showed a significant increase in old nerves compared to young (p<0.0001)
(Fig. 4.2C). Because of increased background autofluorescence in the aged
blood vessel wall, photography fails to reproduce the increased TH
immunoreactivity shown using image analysis (See Fig 4.1 and 4.3). The
image analysis algorithms used subtract variable background before making
measurements of fluorescence and thus makes reliable comparisons of staining
from different ages (Cowen, Thrasivoulou, 1992b).

In implanted vessels, the pattern of reinnervation by TH-LI nerve fibres was
similar to that seen in non-implanted vessels (Fig. 4.1C-F). At 8 weeks post-
implantation, TH-LI nerve density in both old and young vessels closely
resembled the normal values, thus old implanted MCA had significantly
reduced (50-60%) density of innervation (Area%, p<0.0001; ID, p<0.001)
compared to the young vessels (Fig. 4.2A-B). Grey values resembled those
seen on normal vessels in the young, but were significantly lower (p<0.001) in
old transplanted MCA compared to old in vivo values (Fig. 4.2C).
Reinnervation appeared to occur at a slower rate in the young compared to the
old implanted vessels, thus nerve densities were significantly less than the
normal values (Area%, p<0.0001; ID, p<0.0001; GV, p<0.05) in the young
group at 4 weeks post-implantation (Fig. 4.2A-C). In old implanted vessels,
nerve densities at 4 weeks were higher but not significantly so compared to
values for in vivo or 8 week implanted vessels (Fig. 4.2A-B).

Young and old NGF-treated transplants had a significantly increased density
of innervation (200 and 400%, respectively) (Fig. 4.3A-B), (Area%, p<0.001;
ID, p<0.001) when compared to in vivo and untreated in oculo values (Fig.
4.2A-B). Similar significant increases in stain intensity were also observed in
both young and old NGF treated transplants (GV, p<0.001) when compared to
in vivo and untreated in oculo values (Fig. 4.2C). However, young and old
vehicle-treated transplants also had significantly increased nerve plexus
density (Fig. 4.3C-D) (Area%, p<0.05; ID, p<0.01) and grey value (GV
young: \( p<0.001 \); old: \( p<0.05 \) when compared to \textit{in vivo} and untreated \textit{in oculo} values (Fig. 4.2A-C).

All statistical analysis was performed using ANOVA.
Fig. 4.1.

Tyrosine hydroxylase-like immunoreactivity in nerve fibres in middle cerebral arteries of young (6 week) (A, C and E) and old (24-26 month) (B, D, and F) rats. *in vivo* (A, B), after 4 weeks *in oculo* (C, D) and after 8 weeks *in oculo* (E, F). Scale bar = 50μm

Note the reduced nerve density in aged control tissue (A versus B), and the similar nerve density per age group after four and eight weeks *in oculo* (C - F).
Density (A: Area%; B: ID/mm) and intensity (C: Grey Value) of tyrosine hydroxylase immunoreactive nerve fibres in middle cerebral arteries from young (6 week) and old (24-26 month) rats in vivo (control), after 4 and 8 weeks of in oculo implantation, and following treatment with NGF or vehicle (Cyt C) for 4 weeks in oculo. See text for statistical comparisons.
n=16 young controls
n=9 old controls
n= 14 young in oculo
n= 15 old in oculo
n=15 young in oculo NGF/Cyt C treatment
n=16 old in oculo NGF/Cyt C treatment
* p<0.05; **p<0.01; ***p<0.001 (significant against young control values)
TH-like immunoreactive nerve density of young and aged MCA following in oculo transplantation.

A) Control
- Four weeks in oculo
- Eight week in oculo
- Cyt C treated
- NGF treated

B) Nerve density (%)

C) Stain intensity (GV)

Young vs. Old
Fig. 4.3.

Tyrosine hydroxylase-like immunoreactivity in nerve fibres in middle cerebral arteries of young (6 week) (A, C) and old (24-26 month) (B, D) rats, after 4 weeks in oculo and after weekly treatment with NGF (A, B) or Cyt C (C, D). Scale Bar = 50μm.
4d) Discussion.

Age-changes in the nervous system have been shown to be locally and temporally specific (Cowen, 1993; Haugh, 1984) affecting some neurones, but often leaving other, neighbouring neurones unaffected. Although some age-related decreases in nerves are species and even strain-specific, the present study has demonstrated comparable losses (i.e. about 50%) in the sympathetic terminal nerve plexus surrounding major cerebral arteries of ageing Sprague Dawley rats comparable to those shown in the same population of nerves in Wistar rats (Mione et al. 1988) and rabbits (Saba et al. 1984). The functional implications of this loss of sympathetic nerve fibres are not clear. However, it has been suggested that the sympathetic innervation of cerebral blood vessels protects against stroke (Sadoshima, Heistad, 1982) and it therefore conceivable that loss of nerves with age increases vulnerability to cerebral vascular accidents of this kind.

It has been suggested that a decline in target-derived neurotrophic support underlies the region-specific nature of age-related neurodegeneration in the CNS (Appel, 1981) and autonomic nervous system (Cowen, 1993). However, endogenous changes in neurones, such as reduced responsiveness to neurotrophic factors or a reduction in the rate of retrograde axonal transport of these factors may also contribute. Non-neuronal and neuronal target tissues have been shown to influence the axonal (Oppenheim, 1989) and dendritic (Voyvodic, 1989b) arborization of mature sympathetic neurones, as well as the transmitter phenotype (Landis, 1990).

The present study forms part of a broader investigation of the role of target tissues in regulating neuronal ageing and extends this concept by demonstrating that when MCAs from young and old donors are implanted in oculo, they receive an organotypic and age-specific pattern of sympathetic reinnervation from host iridial nerve terminals. The organotypic nature of nerve ingrowth was not established until 8 weeks in oculo. At 4 weeks there was relatively little difference between the nerve densities on young or old
implants, suggesting non-specific regulation of initial ingrowth, with age-specific pattern and densities established later. Thus the pattern and density of the terminal sympathetic plexus appears to be regulated in the long term by the target tissue, rather than by the host sympathetic neurones. The observed age-related decreases in TH immunoreactive nerve fibres may be due to reduced expression of TH rather than atrophy of nerves. However, this interpretation seems unlikely, since our data are supported by observations of increased TH mRNA (Kedzierski, Porter, 1990) and TH activity (Reis et al. 1977; Yurkewicz et al. 1981). Also, in both in vivo and transplanted MCA from old rats the intensity of TH immunoreactivity was significantly increased, suggesting an upregulation of TH in residual nerve fibres to compensate for the loss of collateral fibres which occurs in old age. Because there is no evidence of loss of neurones from superior cervical ganglia of aged rats (Santer, 1991a; Santer, 1991a), our observations suggests loss of sympathetic axon collaterals with age.

Transplanted target tissues appear to be able to induce an organotypic pattern of sympathetic innervation irrespective of the locality and normal projections of the innervating sympathetic neurones (Gavazzi et al. 1992; Burnstock, 1974; Olson, Malmfors, 1970). The specific neurotrophic influence of target tissues may be mediated by factors which include either the production or availability of NGF (Creedon, Tuttle, 1991). Work in this laboratory has demonstrated a significant reduplication and thickening of basal lamina around smooth muscle cells of cerebral blood vessels in aged rats (Chaldakov et al. 1992), which may physically inhibit the access of sympathetic nerve terminals to NGF and other soluble factors. Alternatively, there may be reduced synthesis of neurotrophic factors by the target tissues. We have preliminary evidence for a lack of change in NGF protein levels in aged peripheral targets, but reduced NGF in aged SCG (Gavazzi et al. 1994). These data suggest that factors other than, or in addition to, NGF synthesis by targets may limit nerve fibre density in maturity.
There have been a number of studies which have demonstrated altered levels of NGF, NGF mRNA, low affinity (p75) NGF receptor and NGF receptor mRNA in various areas of the brain during development and ageing (Crutcher, Weingartner, 1991; Williams, Rylett, 1990; Hellweg et al. 1990; Hefti, Mash, 1989; Gomez-Pinilla et al. 1989; Goedert et al. 1989; Koh, Loy, 1988; Larkfors et al. 1987; Fischer et al. 1987; Goedert et al. 1986). Age-related reductions in the production of NGF and NGF mRNA in the hippocampus and other areas of the CNS have been reported (Pallage et al. 1992; Koliatsos et al. 1990). However, other studies have failed to find changes in target levels of NGF in the same strain (Crutcher, Weingartner, 1991) and in other strains (Alberch et al. 1991; Hellweg, Hartung, 1990). The possibility that reduced neurotrophic support may cause neuronal degeneration has been studied in lesion models in the CNS (Pallage et al. 1992; Koliatsos et al. 1990). However there are to our knowledge no comparable data on the role of neurotrophic factors in the ageing peripheral nervous system.

Sympathetic neurones of the SCG, which innervate the MCA, are responsive to NGF (Hendry, Campbell, 1976). Furthermore, the smooth muscle cells and fibroblasts of the vessel wall have the capacity to produce NGF (Creedon, Tuttle, 1991; Bandtlow et al. 1987). NGF treatment has been shown to increase sympathetic innervation in peripheral targets (Hayashi et al. 1993; Saffran et al. 1989; Bjerre et al. 1975) and we have recently shown that NGF-treatment can induce the reestablishment of a 'young' pattern of innervation on old cerebral blood vessels as demonstrated by PGP 9.5 both in oculo (Gavazzi et al. 1992) and in vivo (Andrews, Cowen, 1994b). However, these studies did not show if the pattern of regrowth induced by NGF was appropriate to the tissue in terms of the sub-populations of nerve fibres which would normally innervate the vessel wall. The present study shows that NGF induces a specific and appropriate regrowth of sympathetic nerve fibres onto the ageing vessel wall, supporting the concept that age-related loss of sympathetic fibres may be the result of neurotrophic deprivation.
Dependence of collateral sprouting on NGF has been demonstrated in vivo (Gloster, Diamond, 1992). However, this group has argued that regeneration of NGF-sensitive sensory and sympathetic nerves occurs independently of NGF. *In oculo* transplantation may provide a model for NGF-dependent collateral sprouting by providing additional target tissue over which uninjured host nerves can grow. It would be interesting to inject anti-NGF antibodies in oculo and study the effect on the reinnervation of implants.

The effects of treatment with vehicle containing Cyt C in increasing sympathetic nerve density and TH levels on old implants were surprising in that some similar studies did not show this response (Gavazzi, Cowen, 1993a; Hayashi et al. 1993a). However, other studies have shown that apparently non-neurotrophic vehicle solutions can induce nerve growth. Our own experiments have demonstrated the capacity of bovine serum albumen in oculo (Gavazzi, Cowen, 1993a) and vehicle infusion in vivo (Andrews, Cowen, 1994a) to induce fibre growth. Other groups have made similar observations in the CNS (Sendtner et al. 1992). One explanation might be an upregulation of NGF synthesis by tissues of the anterior eye chamber caused by the trauma of repeated intraocular injections. NGF has been shown to affect both nerve growth and TH expression in developing sympathetic neurones (Gorin, Johnson, 1979; Gorin, Johnson, Jr. 1980; Thoenen et al. 1971). Indeed our data support their findings in that we found a significant increase in TH expression in young and old MCA implants following treatment with NGF. Shelton and Reichardt (1986) found a 3-4 fold increase in NGF mRNA in irises 6 hours after trans-corneal injections into the anterior eye chamber (Shelton, Reichardt, 1986). The increase in NGF levels caused by trauma might be mediated by an inflammatory response (Weskamp, Otten, 1987) of the iridial tissue, leading to the production of cytokines, which have been shown to induce NGF synthesis in fibroblasts (Lindholm et al. 1988). The increased nerve density and TH immunoreactivity in the young Cyt C-treated group support this hypothesis.
4e) Conclusions.

This study has shown reduced sympathetic (TH-LI) innervation of middle cerebral arteries in old age. Transplantation experiments indicate that target tissues are able to dictate the pattern and density of the sympathetic innervation they receive and that this capacity is retained in old age. Exogenous NGF appears to supplement reduced neurotrophic support by targets in old age allowing regeneration of a 'young' pattern and density of sympathetic nerve fibres. These data suggest that decreases in sympathetic nerve density in old age may be caused by reduced target-derived neurotrophic support. Vehicle-treatment can also have an effect on nerve density in old transplanted MCA, which may be mediated by a trauma-induced inflammatory response.
Chapter 5

Sensory nerves: Role of targets in the regulation of nerve morphology, connectivity and neurotransmitter expression in maturity and old age.

5a) Introduction.

Age changes in the nervous system have been shown to be locally and temporally specific (Todd, Tokito, 1981; Haugh, 1984), affecting some neurons but leaving other, neighbouring neurons unaffected. Some target tissues supplied by the SCG have been shown to have reduced sympathetic innervation in old age (Mione et al. 1988; Cowen, Thrasivoulou, 1990; Gale et al. 1989; Saba et al. 1984). For example, projections of the SCG to MCA show significant decline in old age (Mione et al. 1988; Thrasivoulou, Cowen, 1995). However, projections to the iris remain unchanged (Santer, 1991b) or may even increase in old age (Gavazzi et al. 1996b). The few ageing studies which have investigated the sensory innervation to target tissues supplied by the trigeminal ganglion using immunohistochemistry show that sensory innervation and/or neuropeptide content may increase or decrease, and that these changes are target-specific, like the changes in sympathetic nerves. For example, Gavazzi et al (Gavazzi et al., 96c), have shown a 20% decrease in CGRP-LI in aged Sprague Dawley rat irides, whilst Mione et al 89, showed an increase of about 30% in CGRP-LI in MCA of aged Wistar rats.

The present study follows on from the study of sympathetic nerve-target interactions in Chapter 4 by investigating whether nerve-target trophic interactions are important for the growth, and maintenance of neurotransmitter expression, in sensory neurons projecting to the MCA in young adult and aged rats. Previous studies of peripheral sensory neurons (McMahon, Gibson, 1987; McMahon, Wall, 1989) have supported this possibility but have not
investigated the extension of this idea into old age. Our first aim was to make a thorough descriptive study of the quantitative changes that occur during ageing in sensory nerves, employing a number of different approaches which included:

a) Immunohistochemical localisation and quantification of the sensory marker CGRP in nerves supplying the MCA.

b) Immunohistochemical localisation and quantification of the neurofilament marker NF-R\textsubscript{39} (Dahl et al. 1981) in the same nerves. Because CGRP is only expressed by approximately 25-30\% of sensory neurons, NF-R\textsubscript{39} immunohistochemistry was used which is expressed by the majority of sensory, and very few non-sensory neurons in this target (Seiger et al. 1984).

c) Retrograde tracing from the MCA to the trigeminal ganglion, combined with immunohistochemistry for CGRP and NF-R\textsubscript{39}, in order to estimate changes in the sub-population of sensory neurons projecting to the MCA in mature and aged rats.

The second aim was to carry out an experimental study of interactions between sensory nerves and their peripheral targets in maturity and old age using \textit{in oculo} transplantation. As previously described, this model allows one to investigate whether the pattern and density of innervation the donor transplant receives from nerves supplying the host iris is controlled primarily by the host iridial neurons or by influences from the target.

5b) Materials and Methods

Materials and methods are as described in \textit{Chapter 2}.
5b.1) In oculo co-transplantation of young and aged MCA.

In order to study the possible inflammatory effects of aged tissue implanted into the anterior eye, young and aged MCA were co-transplanted in oculo for four weeks. Some host rats were treated with a long-lasting, steroidal anti-inflammatory (Depo-Medrone V, 100µl of 40mg Methylpredniolone Acetate and 0.2mg myristyl-gamma-picolinium chloride/ml; UpJohn, UK), injected subcutaneously into the anterior angle of the eyelid immediately after transplantation, whilst others were left untreated. The advantage of using this anti-inflammatory regime, is that a single injection has lasting effects of up to five weeks.

5c) Results.

5c.1) CGRP-Immunoreactivity.

A total of 14 young (six week) and 13 old (twenty four month) rats provided untreated control tissue.

The staining pattern of CGRP-like immunoreactivity (CGRP-LI) from young and old MCA was relatively sparse and comprised a few longitudinally oriented paravascular nerve bundles and some plexiform perivascular nerves (Fig. 5.1a-b). There was a small (20%) but significant reduction of nerve density in old MCA when compared to young, (FA%, p<0.05 ; ID, p<0.001) (Fig. 5.2 a-b). Densitometric measurements of stain intensity (GV) revealed no difference between young and old MCA (Fig. 5.2c).
Fig. 5.1.

Photomicrographs of CGRP-like immunostaining of young six week (a,c,e) and old 24 month (c,d,f) MCA. Control (a-b), after 4 weeks (c-d) or 8 weeks (e-f) in oculo transplantation.

Note the reduced nerve density in aged compared to young MCA (a, b), and the increased in nerve density following four (c,d) or eight weeks (e,f) in oculo transplantation. Aged transplants (d, f) have greater nerve density (ten fold) compared to young (c,e) (six fold) compared to controls.

Scale bar = 50μm
Fig. 5.2.

Quantification of nerve density (a-b) and stain intensity (c) of young six week and old 24 month rat MCA. Control values are normal vessels whilst experimental values are taken from vessels transplanted in oculo for four or eight weeks. Co-transplants of young and old MCA were left in oculo for four weeks and either left untreated or treated with a long lasting anti-inflammatory drug.

Note that in untreated co-transplants reinnervation nerve density was increased in young and decreased in aged MCA but was still greater than that of controls. Treatment with anti-inflammatory had some effect on reinnervation nerve density in young and no effect on aged MCA.

n=14 young controls
n=13 old controls
n=9 young in oculo
n=10 old in oculo
n=8 young and 8 old for in oculo co-transplants, treated and untreated

* p<0.05; ** p<0.01; *** p<0.001
CGRP-LI nerve density of young and aged MCA following in oculo transplantation.

A. Nerve density (A%):

- **Young**
- **Old**

B. Nerve density (ld/mm):

- Control
- 4 wk in oculo
- 8 wk in oculo
- Co-transplants untreated
- Co-transplants treated

C. Stain intensity (GV):

- Control
- 4 weeks in oculo
- 8 weeks in oculo
5c.2) Neurofilament R$_{39}$-immunoreactivity.

A total of 16 young and 25 old animals were used for control tissue, whilst 4 young animals underwent bilateral superior cervical ganglionectomy (operative procedure is described in Chapter 2).

The staining pattern of NF-R$_{39}$ in young and old rat MCA was plexiform in nature and relatively dense (Fig. 5.3). There was a significant reduction (approximately 30%) in nerve density in old MCA compared to young (A%, p<0.002; ID, p<0.0003) (Fig. 5.4). Following sympathectomy (three days), MCA from young rats showed a significant increase (approximately 30%) in NF-R$_{39}$ nerve density compared to non-operated rats (A%, p<0.05; ID, p<0.05) (Fig. 5.4).
Fig. 5.3.

Photomicrograph of neurofilament R$_{39}$ -like immunostaining of young six week (a) and old 24 month (b) rat MCA, from control animals and three days post-sympathectomy (c). scale bar = 50µm

Note the reduced neurofilament staining in aged compared to young MCA (a versus b) and the increase after sympathectomy (c).
Fig. 5.4.

Quantification of NF-R39 in nerve fibres of young six week and old 24 month rat MCA. Area % (a) and ID/mm (b). Note the significant decrease in nerve density between old and young vessels (**p<0.002), and the significant increase in nerve density of young vessels following sympathectomy (* p<0.05).

n=16 young controls
n= 25 old controls
n=4 young sympathectomy
NF-R39 immunoreactive nerve density of young and aged MCA.

A

Nerve density (A%)  

B

Nerve density (ID/mm)

Young Old

*  

*  

Control Sympathectomised
5c.3) Cell counts of retrogradely traced trigeminal ganglion neurons.

A total of 15 (11 for CGRP & 4 for NF-R$_{39}$) young six week and 10 (5 for CGRP & 5 for NF-R$_{39}$) twenty four month rats were used for retrograde tracing from the MCA, using fast blue/diamidino yellow, to the trigeminal ganglion (Fig. 5.5 b). In addition, retrograde tracing was carried out from the right iris using FB/DY in 10 young six week old rats to determine the number and neuropeptide expression of trigeminal ganglion neurons projecting to the iris (Fig. 5.5a).

Approximately 25% of young and aged trigeminal ganglion neurons labelled with FB/DY from the MCA were positive for CGRP or NF-R$_{39}$ (Fig. 5.6). Colocalisation of CGRP and NF was not assessed but it is probable that some MCA projecting neurons are positive for both markers as 75% of trigeminal ganglion neurons are positive for NF-R$_{39}$ (Seiger et al. 1984). Some labelled neurons were not positive for either marker (Fig. 5.7). The number of trigeminal ganglion neurons projecting to young iris (350±40) was twice that projecting to MCA (170±18), and of these iris-projecting neurons approximately 30% expressed CGRP or NF-R$_{39}$ (Fig. 5.8).

There were significant reductions (about 70%) in numbers (57±14) of FB/DY-positive trigeminal ganglion neurons retrogradely traced from the aged MCA compared to young (p<0.001). There were also significant reductions (80% for both markers) in the number (8±2) of retrogradely traced FB/DY neurons with positive staining for either anti-CGRP or NF-R$_{39}$ in aged trigeminal ganglia (p<0.01) (Fig. 5.8).
Fig. 5.5.

Representative sensory trigeminal ganglion neurons retrogradely traced from (a) iris and (b) MCA projecting neurons using fast blue/diamidino yellow. Note that iris projecting neurons are generally larger than MCA projecting neurons.
Fig. 5.6.

Photomicrographs of FB/DY-positive (a,c) and CGRP-positive (b,d) trigeminal neurons traced from MCA in young rats. Arrow heads indicate neurons showing colocalisation of FB/DY and CGRP.

Note the two CGRP-positive neurons (d) which are FB/DY negative and hence are not projecting to the MCA (c).
Fig. 5.7.

Photomicrographs of trigeminal neurons in young rats; (a) FB/DY-positive neuron (arrow head) and (b) the same neuron as in (a) which is CGRP-negative (open circle). Note the presence of other neurons which are CGRP-positive, FB/DY-negative (double arrows).
Fig. 5.8.

Numbers of sensory trigeminal ganglion neurons projecting to MCA and iris of young six week and 24 month rats combined with fluorescent immunofluorescence staining for CGRP and NF R₃₉. Note that approximately 25% of MCA projecting neurons are positive for CGRP/NF R₃₉, whilst ~30% iris projecting neurons are positive for CGRP/NF R₃₉.

n = 15 young traced from MCA (11 for CGRP and 4 for NF-R₃₉).

n = 10 old traced from MCA (5 for CGRP and 5 for NF-R₃₉).

n = 10 young traced from iris.

* = p<0.01

* * = p<0.001
Number of retrogradely traced trigeminal ganglion neurons projecting to the MCA and Iris of young and aged rats

![Graph showing the number of sensory neuron projections to the MCA and Iris in young and old rats.](image)

- **Young**: FB/DY MCA (350), FB/DY Iris (200), CGRP/NF MCA (150), CGRP/NF Iris (50)
- **Old**: FB/DY MCA (300), FB/DY Iris (100), CGRP/NF MCA (80), CGRP/NF Iris (30)

*Significant differences between young and old groups.*
5c.4) In oculo transplantation.

MCA were taken from young and old donor rats and transplanted into the anterior eye chamber of young hosts for either four or eight weeks. A total of eight and six young MCA's, and seven and six old MCA, were used for the four and eight week in oculo experiments, respectively.

Implanted MCA received a very dense innervation of host CGRP-LI nerves at both time periods which was much greater than that seen in young and old control vessels ex vivo (Fig. 5.1 b-c). In addition, in contrast to the reduced sensory innervation of old MCA ex vivo, there was a significantly greater sensory nerve density in old versus young MCA implants (A% & ID, p<0.001) at four and eight weeks (Fig. 5.2 a-b). Both young and old MCA at four and eight week post-implantation time periods showed significantly greater nerve density compared to non-implanted controls (A% & ID, p<0.001) (Fig. 5.2 a-b).

In general, stain intensity tended to be higher in old vessels compared to young, and was significantly so in transplanted MCA at four weeks (p<0.01). Old MCA implants at eight weeks showed slightly elevated GV over normal control values but the difference was not significant. There was a significant lowering of GV in young transplants at four and eight weeks compared to control values (p<0.01) (Fig. 5.2c).

5c.5) In oculo co-transplantation.

During the course of these studies questions were raised as to why transplanted CBV became reinnervated to a greater extent by iridial sensory nerves compared to controls, and why aged MCA were more densely innervated than young. One possibility is that in oculo transplantation per se may cause an inflammatory response in the anterior eye chamber and that aged tissue may do this to a greater extent. Therefore, young and aged tissue were co-transplanted into the anterior eye chamber in order to investigate the effects of
aged MCA on young implants with and without steroidal anti-inflammatory treatment.

A separate group of six young and six aged MCA were co-transplanted in oculo for four weeks. Half were treated with a long-lasting, steroidal anti-inflammatory (Depo-Medrone V, 100μl of 40mg Methylpredniolone Acetate and 0.2mg myristyl-gamma-picolinium chloride/ml; UpJohn, UK), injected subcutaneously into the anterior angle of the eyelid immediately after transplantation, whilst the remainder were left untreated. The advantage of using this anti-inflammatory regime, is that a single injection has lasting effects of up to five weeks.

Untreated, co-transplanted, young MCA became reinnervated by CGRP-positive sensory nerves to a greater density (60%) than its single transplanted counterpart (Fig 5.2 a-b). Co-transplanted aged MCA had a very similar CGRP-positive reinnervation density to its single transplanted counterpart (Fig. 5.2). Therefore, there was a significant increase (p<0.05) in CGRP-LI nerve density in young but not in old co-transplants. This observation may be due to the small n number (three) used in this study.

Anti-inflammatory treated, aged co-implanted MCA, nerve densities were similar to their untreated co-transplanted counterparts (Fig 5.2 a-b). However, there was a significant decrease (p<0.05) in nerve density in young anti-inflammatory treated compared to untreated MCA.

5d) Discussion.

5d.1) Descriptive study.

Data from this study show an approximately 20% loss of CGRP-LI nerve fibres and a 30% loss of NF-R39 expressing nerve fibres in aged compared to young mature MCA in rats. It is not clear at present whether these changes represent the dying back of a significant proportion of peripheral sensory
fibres or age-related changes of neurotransmitter and cytoskeleton within intact axons. For example, CGRP levels decline by as much as 50% in aged rat trigeminal ganglion neurons compared with the highest levels found at 3 weeks of age (Wimalawansa, 1992). Li and Duckles (Li, Duckles, 1993) found reduced CGRP-LI in sensory nerves supplying mesenteric arteries in aged rats, as well as reduced neurogenic vasodilation, indicating that reduced CGRP content may indicate a functional impairment. Axon transport of CGRP in rat sensory nerves declines with age (Fernandez, Hodges-Savola, 1994). The apparent loss of labelled neurons projecting from the aged trigeminal ganglion projecting to the MCA, shown by retrograde tracing in this study, as well as the reduced CGRP immunoreactivity at the periphery, could both be explained by defective axonal transport, and support the possibility of functional impairment of sensory nerves in old age.

The possibility that axon transport is impaired is supported by the decrease in NF-R39 immunoreactivity in aged MCA (this study). Neurofilament protein is an important constituent of myelinated axons and is built up of triplets of polypeptides of approximately 70kD, 150kD and 200kD (Schlaepfer, 1977; Hoffman, Lasek, 1975). NF-R39 is raised against all three polypeptide chains, of which the 200kD triplet has been shown to be related to conduction velocity in CGRP expressing rat primary sensory neurons (Lawson, Waddell, 1991). The observed changes here may therefore reflect impaired transmitter dynamics and/or axon transport in peripheral axons of sensory neurons in old age. Alternatively, the reductions in CGRP-, NF-LI and numbers of retrogradely traced neurons in old age may reflect sensory nerve fibre atrophy, or even neuronal cell death. A previous study has shown that total trigeminal neuron numbers remain stable throughout life in ad libitum feed and diet restricted Fisher 344 rats (Biedenbach et al. 1992), although the possibility remains of an age-related cell loss in a sub-population of neurons. In addition, controversy over neurone counting methods in sensory ganglia (Coggeshall, Lekan, 1996) has bought into question the validity of previous studies. Therefore, it is not possible to deduce whether the present results reflect a
functional impairment of aged sensory neurons, or neurodegeneration, or a combination of both.

Other studies have shown that the period of postnatal cell death in rat trigeminal neurons is complete by P25, with no further cell loss observed at P90 (O'Connor, van der Kooy, 1989). However, a study of trigeminal ganglion neurons projecting to the cerebral vasculature (Horgan et al. 1990) showed that by postnatal day 55 only 15% of the total neuronal pool have died, whilst between postnatal days 55-90, a further 75% of the original pool of neurons die. A proportion of the loss of trigeminal neurons projecting to the MCA in rats between 6 weeks and 24 months of age may therefore occur in early adulthood.

5d.2) Role of axon transport in sensory nerve loss.

To the extent that collateral sprouting of adult sensory neurons depends on target-derived NGF (Diamond et al. 1992), it is possible that defects in axon transport can influence neuronal plasticity, as well as transmitter function. It has been suggested that a decline in target-derived neurotrophins could cause region-specific neurodegeneration in the aged CNS and in the autonomic nervous system (for reviews, see (Gavazzi, Cowen, 1996; Cowen, 1993; Appel, 1981). However, other factors may contribute, such as reduced neuronal responsiveness to neurotrophins (Uchida, Tomonaga, 1985b; Radotra et al. 1994b; Diamond et al. 1992) or the reductions in the rate of retrograde axonal transport discussed above (Fernandez, Hodges-Savola, 1994). Recently, NGF protein levels in MCA have been shown to be unchanged in old age (Cowen et al. 1996a), despite reductions of 50% in sympathetic nerve fibre density, which argues against the possibility that reduced availability of NGF is the cause of the observed age-changes in sympathetic nerves. However, NGF protein is reduced in aged SCG (Cowen et al. 1996a), suggesting that uptake and/or retrograde axonal transport of NGF is impaired. Preliminary data demonstrate a 60% decrease in NGF protein in the external maxillary artery (EMA) of aged rats (Crutcher et al. 1996). In addition, the
major proportion of NGF (60- 80%) is localised within axons in the adventitial connective tissue, rather than in the smooth muscle layer of the vessel wall, suggesting that NGF is rapidly taken up by axons and stored before being retrogradely transported. Furthermore, distal axons of sympathetic neurons treated with $^{125}$I-NGF in compartmentalised cultures bind 70-95% of the available NGF and the half-life of NGF within axons is 3 hours (Ure, Campenot, 1997). Down regulation of CGRP, substance P and neurofilament in sensory neurons has been shown to occur as a direct consequence of decreased availability of target-derived (Fitzgerald et al. 1985) or extrinsic (Verge et al. 1990; Lindsay, Harmar, 1989) NGF. Taken together these studies indicate that alterations in the uptake and retrograde axoplasmic transport of NGF in rat peripheral nerves may underlie reduced neural plasticity, as well as changes in sensory neuropeptides, leading to functional impairments in ageing rats.

5d.3) Role of targets in sensory nerve loss.

Previous studies have shown that sensory neurons are responsive to changes in extra-cellular matrix, as well as to diffusible factors, within their target tissues. Gavazzi et al (Gavazzi et al. 1996a) have performed in oculo transplantation experiments similar to those in this study but where the vessels were repeatedly frozen and thawed before transplantation into the anterior eye chamber. Freeze-thawing kills the cellular components of the tissue, eliminating the source of diffusible neurotrophic factors, but leaves the ECM intact (Gavazzi et al. 1996a). This model enabled the authors to study the capacity of the ECM to influence the pattern and density of reinnervation of young and aged cerebral vessels. After four weeks in oculo, CGRP nerve density on young and aged basilar artery (BA) was similar to ex vivo controls, i.e., nerve density in old transplants was 20% less than in young ones. In contrast, catecholamine-containing sympathetic nerves were very sparse in young and aged freeze-thawed transplants, whereas non-freeze-thawed transplanted vessels in oculo became reinnervated by similar sympathetic nerve densities to those found in vivo (Chapter 4), i.e., young vessels have
relatively dense sympathetic innervation and aged vessels have 50% less nerves than young. These results demonstrate an important difference between sensory and sympathetic nerves: alterations in the aged ECM make the vessels less receptive to reinnervation particularly by sensory nerves, whilst factors produced by cellular elements are important for the growth of sympathetic nerves. In addition, one can deduce that the reduced sensory nerve density observed in aged cerebral vessels is probably due to changes in the ECM and not to changes in soluble cellular neurotrophic factors. Therefore, changes in the ECM are sufficient to explain age changes in sensory nerves, but not changes in sympathetic nerves. It is not yet clear which molecules in the ECM are responsible for the age-related changes in the receptivity of target tissues to innervation. However, a 50% reduction in laminin immunoreactivity has been demonstrated at the medial-adventitial border in aged cerebral vessels (Gavazzi et al. 1996a).

Although sensory and sympathetic neurons are similar in their continued responsiveness to target-derived factors in adulthood, sensory nerve growth is not adversely affected by NGF deprivation and seems to be more dependant on insoluble ECM factors (see above 5d.3, freeze-thaw experiments of Gavazzi et al., 1996a). Initially the predicted outcome of the present study was that the pattern and density of reinnervation of young and old MCA by host sensory nerves would resemble that seen in vivo, suggesting that young vessels would receive a medium to low density of CGRP positive innervation, whilst old vessels would receive a relatively sparse supply of CGRP-positive fibres. Contrary to this prediction, in oculo transplantation resulted in a six-fold increase in CGRP nerve density in young MCA and a ten-fold increase in aged MCA, compared to non-transplanted, age-matched, controls. The density of sensory nerves of old transplants was approximately double that of young transplants after four weeks in oculo. The pattern of reinnervation of the transplant was atypical of CGRP positive, sensory nerves projecting to the MCA i.e., in non-transplanted vessels, the CGRP positive nerves consisted of sparse longitudinally oriented fibres with few circumferentially oriented fibres, whilst in the transplanted vessels, there was a dense plexiform pattern of
innervation typical of vessels and iris. A possible explanation for the hyperinnervation may be an up-regulation of CGRP, mediated by NGF produced by the implants, in axons which are normally undetectable.

Other possible mechanisms to explain the sensory hyperinnervation in young and aged MCA are explored in the general discussion (Chapter 8).
Chapter 6

Role of target tissues in the determination of neurotransmitter phenotype and nerve density in mature and aged sympathetic neurones.

6a) Introduction

The sympathetic innervation of the eccrine sweat gland is unusual in expressing a cholinergic phenotype. In rats, cholinergic characteristics appear during early postnatal development soon after the time when outgrowing sympathetic axons make contact with their targets in the footpad. Experimental studies using in vitro and in vivo techniques have shown that it is the target tissues rather than intrinsic changes in the nerves that are responsible for inducing the switch from the normal catecholaminergic phenotype to a cholinergic one (Stevens, Landis, 1990; Schotzinger, Landis, 1990; Potter et al. 1983; Patterson, Chun, 1974; Landis, 1990). Recent studies have attempted to characterise factor(s) produced by sweat glands which could induce such a switch of phenotype (Rao et al. 1992; Rao, Landis, 1990).

There are numerous examples of the influence of target tissues on neuronal phenotype during development (Purves, 1988). This influence may extend into maturity: for example, reducing the target area of sympathetic neurones causes altered morphology in the mature neurons (Voyvodic, 1989b; Voyvodic, 1989b). We have been investigating the hypothesis that reduced neurotrophic support from target tissues may cause local degeneration of nerve fibres during ageing (Cowen, 1993). Using in oculo transplantation of cerebral blood vessels, we have demonstrated that age-changes in target tissues rather than intrinsic changes in neurons are responsible for regulating the overall pattern and density of innervation of some tissues during maturity and old age (Gavazzi, Cowen, 1993c; Gavazzi et al. 1992c).
Recently, substantial changes have been demonstrated in the innervation of sweat glands in human skin (Abdel-Rahman et al. 1992) and in the footpad of old rats (Abdel-Rahman, Cowen, 1993). Both the overall (PGP-immunoreactive) nerve density (Thompson et al. 1983) and the numbers of nerves exhibiting acetyl cholinesterase staining were markedly reduced. We therefore wished to investigate whether sweat gland targets were capable of regulating both the changes in nerve morphology as well as the reductions in cholinergic features seen in old age. In oculo transplantation was used as before (Gavazzi et al. 1992), combined with surgical sympathectomy and retrograde tracing, to study this question.

6b) Methods

6b.1) In oculo transplantation

Young (6wk) and healthy old (24m) Sprague Dawley rats were used from an outbred colony maintained at RFHSM.

A total of 27 bilateral in oculo transplantations (i.e. 54 transplants; 27 young and 27 old) were carried out on 6wk-old hosts under halothane anaesthesia.

Young and old donors were terminally anaesthetized with i.p. injections of pentobarbital sodium and perfused through the left ventricle with Tyrode's solution containing 0.05% pontamine sky blue. The dye allowed sweat glands from the footpads of the forepaws to be visualized and micro-dissected free of surrounding dermal connective tissue, muscle and epidermis. Previous experiments have shown that the dye does not affect nerve growth (Gavazzi et al. 1992). Glands were stored for up to 4hr in Hank's balanced salt solution (Gibco, UK) on ice before transplantation. A total of 27 bilateral in oculo transplantations (i.e. 54 transplants; 27 young and 27 old) were carried out on 6wk-old hosts under halothane anaesthesia using established techniques.
Transplants were inserted through a small slit in the cornea made with a microsurgical blade (Rocialle Medical Ltd., Cambridge, UK) and manipulated by gentle pressure on the surface of the cornea onto the iris in the posterior angle of the eye. Transplants were left in place for 4wk after preliminary experiments had shown that nerve regrowth was largely complete by this time, or for 10wk in the case of the retrograde tracing experiments (see below). 3 days before sacrifice, bilateral superior cervical ganglionectomy was carried out on 7 host animals (i.e. 14 transplants; 7 young and 7 old) under halothane anaesthesia. Numbers of experimental groups are given in the results.

6b.2) Quantitative immunocytochemistry and histochemistry.

Host animals were terminally anaesthetized with pentobarbitone sodium, transplants were removed with host irides, fixed for 1.5-2hr in 4% paraformaldehyde and washed overnight in phosphate-buffered saline (PBS). 7µm cryosections were cut and mounted on gelatine coated slides and processed for indirect immunohistochemical and histochemical staining. Sections from each transplanted sweat gland were processed for immunocytochemical staining with rabbit antibodies against PGP9.5 (1:800; Ultraclone Ltd, Isle of Wight, UK), tyrosine hydroxylase (TH) (1:100; Eugene Tech, USA) and VIP (1:1000; INCSTAR, UK) and for histochemical staining for acetyl cholinesterase (Abdel-Rahman, Cowen, 1993).

The 'direct-colouring' thiocholine method (Karnovsky, Roots, 1964) was used to demonstrate acetyl cholinesterase, as modified in a previous study (Abdel-Rahman, Cowen, 1993). The sections were rinsed in PBS and pre-incubated for one hour at room temperature in 10⁻⁴M tetraisopropylpyrophosphoramide (iso-OMPA) to abolish non-specific staining or in 10⁻⁵M 1.5 (bisallelydimethylammoniumphenyl) pentan-3-one diiodide (BW284C51, Burroughs Wellcome, UK) which abolished specific staining. The sections were then incubated in fresh acetylthiocholine medium for 30
min. at 37°C, counterstained with 0.5% light green in 0.5% acetic acid, dehydrated through 70-90% absolute alcohols, cleared in xylene and mounted in DPX.

6b.3) Retrograde tracing of sympathetic neurones innervating sweat gland transplants.

The retrograde neuronal tracer, fluorogold, was used in two ways to identify the host neurones innervating sweat gland implants:

1) *pre-loading*: sweat glands were injected using glass microelectrodes attached to a syringe pump with 0.25-0.5µl of a 2% solution in Hanks BSS of fluorogold and washed in Hanks solution before implantation;

2) *post-loading*: trans-scleral injections of 1µl of fluorogold were made bilaterally into the anterior eye chambers of host rats, 4-5 days before sacrifice. Control animals without transplants also received fluorogold injections. All transplants were left *in oculo* for 10 weeks. 6 host rats with 6 young and 6 old sweat gland implants were treated with each of these two methods (i.e. 24 transplants in all). Further SCG were taken from control rats without implants or fluorogold injections.

Host and control animals were anaesthetized as before and fixed by transcardiac perfusion with 4% paraformaldehyde in PBS. Superior cervical ganglia (SCG) were dissected, postfixed, cryosectioned at 20µm and processed as before for immunocytochemistry. Sections were stained for vasoactive intestinal polypeptide (VIP) using a polyclonal antibody raised in rabbits (INCSTAR, UK) (dilution, 1:1000) followed by swine anti-rabbit Texas Red diluted 1:80 as the second layer. In attempts to find alternative cholinergic markers, preliminary experiments showed that acetyl cholinesterase produced non-specific staining of all SCG neurons, whilst choline acetyl transferase antibodies (Boehringer Mannheim, Germany; Chemicon, London, UK) failed
to demonstrate cholinergic sympathetic neurons in the rat stellate ganglion. Control SCG were examined for the presence of VIP-positive neurons with and without prior injection of retrograde tracer. Fluorogold staining was visualised with Violet excitation in a Vanox epifluorescence microscopy equipped with appropriate filters, whilst VIP was viewed with green excitation (560nm). Tests showed that there was no overlap between the excitation spectra of the two labels.

6b.4) Image analysis.

Densities of PGP- and AChE-positive nerves were measured using established methods of image analysis (see Chapter 2). A Kontron IPS image-analyser, interfaced to an Olympus Vanox light and fluorescence microscope by a low-light CCD scanner (Model VL350, PCO Optics GmBH, Germany), was used to take measurements from three different sections per specimen for each marker. The perimeter of the sweat gland acini was defined interactively and measured. Because of the presence of autofluorescent lipofuscin (age-pigment) in old sweat glands, the defined glandular areas were masked out of the images prior to measurement of PGP-immunoreactivity. Non-specific background staining was removed using a low-pass filtered image of each specimen, image contrast was enhanced and interactive thresholding was carried out in order to generate a binary image matching the nerve distribution. The area of periacinar PGP- and AChE-positive nerves was measured and expressed as area per μm of acinar perimeter. Mean nerve densities in young, old and denervated transplants were compared using 2-way ANOVA followed by Tukey's HSD test on standard statistical software. Immunostaining for TH and VIP was assessed visually.
6c) Results

6c.1) Organotypic pattern of reinnervation.

The pattern of reinnervation of transplanted sweat glands by host nerves resembled closely that seen in vivo. Dense PGP-immunoreactive nerves were arranged principally around the glandular acini of young transplants (Fig. 6.1), approaching, but not crossing the basement membrane of the glands. Nerves were also seen in the host iris. Dense AChE-positive nerve fibres occupied a similar periacinar position in young transplants (Fig. 6.1). TH-immunoreactive nerve fibres were seen principally in the host irides and only rarely penetrated into the transplants or approached the glandular acini (Fig. 6.1).

6c.2) Age-changes in reinnervation.

Nerves retained their periacinar arrangement in old transplants but there were fewer nerves in old compared to young ones (Fig. 6.1). Image analysis confirmed that the density of reinnervation of old transplants was significantly less than that of young ones (Fig. 6.2). The nerve densities in old and young transplants were closely similar to those observed in vivo (Abdel-Rahman, Cowen, 1993). Thus mean nerve densities in vivo and in oculo were 1.37±0.19 and 1.34±0.12 in young sweat glands and 0.69±0.13 and 0.42±0.04 in old sweat glands, respectively. PGP-immunoreactive nerve area was reduced by about 60% (p<0.01) in old transplants. Acetyl cholinesterase-positive nerve fibres were also significantly (p<0.01) reduced in density in old transplants, by similar proportions (70%) to PGP-stained nerves. PGP-immunoreactive nerve densities tended to be higher than cholinesterase-stained nerves at comparable age-stages, indicating that some nerve fibres supplying the transplants may have been non-cholinergic and non-noradrenergic, although it is difficult to make reliable quantitative comparison between markers which stain with different levels of contrast.
Immunofluorescence and histochemical staining of periacinar nerve fibres *in oculo* transplants of young (a,b,c) and old (d,e,f) sweat glands stained with PGP (a,d), AChE (b,e) and CGRP (c,f). The glandular acini appear as oval groups of cells. The patterns of reinnervation by host nerves closely resemble those seen in the normal, *in vivo* innervation of young and old sweat glands (Abdel-Rahman, 1993). 'i' indicates the position of the host iris, periacinar nerves are indicated by arrows, whilst arrow heads point to autofluorescent lipofuscin granules.

a) Dense periacinar PGP-immunofluorescent nerve fibres in young sweat glands. Note nerve fibres in the host iris.

b) AChE-positive (dark-stained) nerve fibres in young sweat glands. Note the similar pattern of staining compared to (a).

c) TH-immunofluorescent nerve fibres in young sweat glands. Note sparse innervation of the transplant but dense fibres in the host iris.

d) PGP9.5 immunoreactive nerve fibres in old sweat glands. Note the reduced density of reinnervation by host nerves compared to (a) and numerous intra-acinar lipofuscin granules (arrow heads) with high autofluorescence.

e) AChE-positive nerve fibres in old sweat glands. Note the reduced density of staining compared to (b) contrasting with dense nerve fibres in the host iris.

f) TH-immunoreactive nerve fibres in old sweat glands. Note the occasional fibre in periacinar connective tissue (arrows) contrasting with dense nerve fibres in the host iris. Scale bar = 50µm.
Fig. 6.2.

Histogram showing mean density (area of nerves per μm perimeter of sweat gland acini) ± s.e.m. of PGP-immunoreactive and AChE-positive nerve fibres in transplanted young and old sweat glands, with and without prior sympathetic denervation (den). Nerve densities measured using image analysis. Significant differences were assessed by Tukey's HSD test following 2-way ANOVA (**, p<0.01). Note that denervation resulted in the almost total disappearance of AChE nerves from young and old transplants.
Nerve density of transplanted sweat glands.
6c.3) Changes in reinnervation following sympathectomy.

In both young and old transplants, sympathectomy resulted in the virtual disappearance of AChE-stained nerves supplying glandular acini, although cholinergic fibres could still be seen on the host irides (Fig. 6.3). PGP-immunoreactive fibres were also markedly fewer in young and old denervated transplants compared to non-denervated transplants (Fig. 6.3). Image analysis showed that denervation reduced the density of PGP-immunoreactive nerves by 77% (p<0.01) in young transplants, whilst in old transplants the reduction was 60% (p<0.01) (Fig. 6.2). Denervation also caused the disappearance of TH-immunoreactive nerve fibres from host irides and the occasional nerves closer to the transplants (Fig. 6.3).
Immunofluorescence and histochemical staining of periacinar nerve fibres in oculo transplants of young (a-c) and old (d-f) sweat glands which were subjected to sympathetic denervation 3 days before sacrifice. The glandular acini appear as oval groups of cells. The density of nerves is substantially reduced compared to that seen in Figure 1. 'i' indicates the position of the host iris, periacinar nerves are indicated by arrows, whilst arrow heads point to autofluorescent lipofuscin granules.

a) Sparse PGP-immunoreactive nerve fibres in young denervated sweat glands.
b) Sparse AChE-positive nerve fibres in young denervated sweat glands. Note the reduced density of periacinar staining, whilst iridial AChE-positive (presumptive parasympathetic) nerve fibres (insert) are retained.
c) TH-immunostaining of young denervated sweat glands. Fibres are absent from the transplant and from the host iris.
d) Very sparse PGP-immunoreactive nerve fibres in old denervated sweat glands. Note the reduced density of reinnervation by host nerves compared with (a).
e) Very sparse AChE-positive nerve fibres in old denervated sweat glands.
f) TH-immunostaining of old denervated sweat glands. Note the absence of periacinar and iridial fibres and intense autofluorescence (arrow heads).

Scale bar = 50μm.
6c.4) Phenotype of sympathetic neurones reinnervating sweat gland transplants.

Sections of SCG from host rats with transplants of young or old sweat glands and with retrograde tracing showed occasional neurones with double labelling for VIP and fluorogold (Fig. 6.4). These double-stained neurones, presumably projecting to the sweat gland transplants on the host iris, were sparse and varied in numbers. The preloading experiment gave low levels of fluorogold staining, presumably because of the long period between loading and sacrifice (10wk). Double-stained neurons were therefore difficult to identify. However, postloading the implants with fluorogold 5 days before the implants were removed increased the yield to give 1-5 double-labelled neurons per ganglion. No obvious differences were seen between the numbers of double-labelled neurons after contact with young or old sweat glands. The combination of VIP and fluorogold staining indicated that the phenotype of mature host sympathetic neurones could be altered by contact with a novel, sweat gland target. Retrograde tracing with fluorogold demonstrated a substantial number of neurones per ganglion (270±80, C.Thrasivoulou, unpublished observations) projecting to the iris in sections of SCG from host rats. In control experiments on rats without sweat gland implants, which were repeated on separate occasions with different observers, none of these iris-projecting neurons showed VIP immunoreactivity. In sections of SCG from host rats, occasional neurones stained strongly for VIP alone (Fig. 6.4). These neurones were generally at the periphery of the ganglion and were presumably supplying sweat or other glands in the head region. Similar neurones were seen in SCG from control rats which had not received implants or retrograde tracing. VIP staining of sections of transplanted sweat glands revealed a medium density of periacinar nerve fibres in young transplants which was reduced in sections of transplants from old donors (Fig.6.5).
Fig. 6.4.

Retrograde tracing of young and old sweat gland implants with fluorogold. 20μm cryosections of SCG neurons, immunolabelled with VIP and Texas Red, showing VIP immunoreactivity (a,c,e,g) with green excitation at 560nm and, on the same field, fluorogold staining (b,d,f,h) with u.v. excitation. Note colocalization of staining for VIP (a) and fluorogold (b) in neurons projecting to young implants, indicating switched neurotransmitter phenotype. Absence of VIP (c), combined with fluorogold staining (d) indicates SCG neurons projecting to the host iris. Staining for VIP (e) without fluorogold (f) indicates native VIP-positive neurons at the periphery of the ganglion which probably supply glands in the head region. Sections of SCG from hosts with sweat gland implants from old donors also show neurons double-labelled with VIP (g) and fluorogold (h), indicating switched phenotype. Scale bar = 50μm.
Fig. 6.5.

VIP-immunolabelling of 10μm cryostat sections from young (a) and old (b) sweat gland implants. Note the reduced density of periacinar fibres (arrows) in the old sweat gland. Autofluorescence is indicated by arrowheads.
Scale bar = 50μm.
6d) Discussion

Target tissues have been shown to exert a range of influences over their innervating neurones during development and in maturity. Target size is reflected in the extent of axonal and dendritic arborizations, in the diameter and degree of myelination of axons and in soma size (Yawo, 1987; Voyvodic, 1989; Voyvodic, 1989). NGF may mediate some of these growth-related changes (Snider, 1988; Ruit, Snider, 1991; Ruit et al. 1990; Purves, 1988). The sympathetic nerves supplying eccrine sweat glands undergo a switch from catecholaminergic to cholinergic phenotype during early postnatal development (Landis, 1990). This change of phenotype has been shown to be regulated by target-derived factors different from NGF and possibly related to CNTF (Rao et al. 1992; Rao, Landis, 1990) which suggests that target-associated factors can also regulate developmental changes in neurotransmitter phenotype. In oculo co-transplants of rodent sweat glands and sympathetic ganglia have shown that target tissues have the capacity to regulate the switch in phenotype in neonatal sympathetic neurones (Stevens, Landis, 1990) whilst other transplantation experiments showed that sympathetic neurones that do not normally innervate sweat glands can be induced to express cholinergic characters by contact with transplanted neonatal sweat glands (Schotzinger, Landis, 1990). Neither of these studies answered the question of whether mature sympathetic neurones, already in functional contact with their normal targets, can be induced to switch to a cholinergic phenotype by contact with a novel target.

Whilst target-derived factors can exert a range of influences on neuronal phenotype during development, it remains unclear whether these influences extend into maturity. Such a mechanism could be of particular importance in mature autonomic nerves where continuous growth and retraction of terminal fibres (Purves et al. 1987; Purves et al. 1986), as well as plasticity of neurotransmitter phenotype (Adler, Black, 1984), has been demonstrated.
We have investigated the hypothesis that neuronal ageing is the result of changes in target tissues which act retrogradely on innervating nerve fibres to cause neurodegeneration or other age-related changes, perhaps through a reduced availability of neurotrophic factors (Cowen, 1993). *In oculo* transplantation has been used to investigate this possibility because it allows the study of interactions between allotransplants of donor target tissues and host nerves of different ages (Gavazzi, Cowen, 1993c; Gavazzi *et al.* 1992c). Cerebral blood vessels, where reductions of nerve density occur in old age, were transplanted from old donor rats into young hosts. The pattern and density of total (Gavazzi *et al.* 1992) and sympathetic (Thravouvoulou, Cowen, 1995) reinnervation of the transplanted vessels resembled closely that seen in the normal vessels of the appropriate age. Thus, old transplanted vessels became reinnervated by young host nerves with a lower density than young transplants. In further studies, young and old sympathetic ganglia have been transplanted into young and old hosts and have shown that neuronal survival (Stieg *et al.* 1991) and the capacity for neurite outgrowth (Gavazzi, Cowen, 1993c) appear unaffected by the age of the neurones. These results suggest strongly that it is age-changes in targets rather than in neurons that determine nerve fibre loss in old age in some peripheral tissues.

Recently, loss of nerve fibres has also been demonstrated in sweat glands of aged people (Abdel-Rahman *et al.* 1992) and rats (Abdel-Rahman, Cowen, 1993). In addition, marked reductions in nerve fibres staining for AChE were observed in sweat glands from old rats. We therefore wished to use *in oculo* transplantation to study whether changes in ageing sweat glands were responsible for inducing the altered pattern of innervation seen in old age. In the course of the study, a method was developed for microdissecting footpad sweat glands free of connective tissue after rendering them visible by perfusion with pontamine sky blue. *In oculo* transplants of sweat glands treated in this way were readily and reproducibly reinnervated by host nerves.
Transplanted sweat glands have been shown to induce a highly organotypic pattern of reinnervation by nerves growing from the host iris. Fine, dense, varicose nerves were arranged in baskets around the glandular acini of transplants, just as the normal innervation. The contrast between the pattern of reinnervation of transplanted small blood vessels, which typically receive a largely 2-dimensional plexus of host nerves at the adventitial-medial border (Gavazzi et al. 1992), and the 3-dimensional pattern observed here, provided strong visual evidence of the influence of the target tissue on the pattern and density of nerve growth. Furthermore, the neurotransmitter phenotype of the innervating fibres appeared to be typical of the normal innervation of the target: the majority of fibres expressed AChE whilst virtually none expressed TH. Nerve fibres staining for TH-immunoreactivity and glyoxylic acid histochemical staining for noradrenaline (data not shown) were seen in host irides adjacent to the transplants.

Sympathectomy of the transplants showed that a majority of the cholinesterase-positive nerve fibres were of sympathetic origin, but did not rule out the possibility that some fibres were of parasympathetic origin. Occasional cholinesterase-positive fibres, remaining after sympathectomy, may have been sprouts from the native parasympathetic innervation of the iris. Inappropriate matching of cholinergic nerves and targets of this kind has been demonstrated in the re-routing of parasympathetic nerve fibres which occurs in the phenomenon known as gustatory sweating (Drummond et al. 1987).

Retrograde tracing showed that a small number of sympathetic neurons in the SCG, projecting to host irises with transplants *in situ*, were induced to express VIP immunoreactivity. VIP-stained fibres were observed in sections of the transplants, indicating that a small number of sympathetic neurons may have altered their neurotransmitter expression to express VIP as a result of contact with the transplanted sweat glands. In support of this contention, no VIP staining was seen in fluorogold-positive neurones projecting to irides without transplants. VIP is found as a co-transmitter in several groups of glandular
cholinergic nerves including those supplying sweat glands and has been used as a marker for induction of cholinergic phenotype (Stevens, Landis, 1990). The induction of VIP expression, combined with acetyl cholinesterase staining of nerve fibres, may therefore indicate a switch to a cholinergic and VIP-positive phenotype. No obvious differences were observed between old and young sweat gland implants in their ability to induce the switch to VIP expression. We were unable to find a more effective label for the altered character of these neurons. The small numbers of neurons with double labelling was consistent with the small (1-2 mm diam.) size of the transplants.

Contact of mature catecholaminergic neurons with transplanted sweat glands, presumably by growth of axon collaterals, resulted in a switch to a cholinergic phenotype which appeared to affect the cell body and axonal arbor of the innervating neuron. A cholinergic-inducing signal received from a relatively small part of the axonal arbor of a sympathetic neuron may therefore override the 'default' catecholaminergic state existing throughout the rest of the neuron. Plasticity of neurotransmitter expression therefore appears to be retained in maturity in these neurons.

Transplanted sweat glands from donor animals of different ages were able to induce a pattern and density of reinnervation appropriate to the age of the donor tissue. This supports data from similar experiments using different (vascular) target tissues (Gavazzi et al. 1992; Davies, 1994). In the present study, nerve density and neurotransmitter phenotype in transplanted sweat glands closely resembled those seen in normal sweat glands in vivo, with old glands markedly less densely innervated than young ones. Thus, the overall nerve density as indicated by PGP-immunoreactivity was reduced in old transplants, indicating an attenuation in the capacity of the target to support innervating nerves. However, the proportion of nerves expressing AChE on old transplants remained similar to that seen in vivo and the results of sympathectomy on young and old transplants were similar, suggesting that there was no change in the capacity of old targets to induce a cholinergic
phenotype in sympathetic neurones. The decline in PGP- and AChE-stained nerves in old transplants is therefore likely to be the result of reinnervation by fewer fibres rather than an age-change in neurotransmitter phenotype.

In conclusion, further evidence is now provided that targets regulate age-changes in the density of their innervating neurones. We show that the predominant catecholaminergic phenotype of mature sympathetic neurones can be overridden by contact with appropriate target tissues. Contact between a sweat gland implant and a few collaterals of the total axonal arbor of a sympathetic neuron appears to be sufficient to induce a switch to a cholinergic phenotype in the whole neuron. There is no apparent reduction in old age in the ability of sweat glands to induce this switch to a cholinergic phenotype. These results suggest that separate target-derived factors regulate nerve density and neuronal phenotype, the former being affected by ageing whilst the latter is not.
Chapter 7

The low affinity NGF receptor (p75) and its relationship with sympathetic and sensory nerves of the middle cerebral artery in young and aged rats.

7a) Introduction.

Recent studies have provided evidence that aged sympathetic nerves become less able to take up exogenous NGF from their target area. For example, a) aged iridial sympathetic neurones are affected more by anti-NGF treatment than young, whereas, aged sensory nerves are apparently not affected by this treatment (Gavazzi et al. 1996b). b) NGF protein levels remain unchanged in the aged MCA, despite reductions of up to 50% in nerve density (Thrasivoulou, Cowen, 1995), but are decreased in the aged SCG (Cowen et al. 1996a) suggesting that access to or uptake of and/or retrograde transport of NGF is impaired; c) aged tissues transplanted in aged host anterior eye chamber do not become reinnervated to the same degree as when they are transplanted into young hosts, and are less responsive to NGF treatment (Gavazzi, Cowen, 1994); d) aged cultured neurons require higher doses of NGF to elicit the same growth response as young neurons (Cowen et al. 1997); e) aged sympathetic nerves supplying the pineal gland fail to send out collateral sprouts into adjacent areas of denervated pineal gland, following unilateral denervation (Kuchel, 1993); f) in cultured aged sympathetic neurons, NGF does not enhance neurite elongation whereas heart conditioned medium does, indicating reduced responsiveness to NGF (Uchida, Tomonaga, 1985b).

Although the functional role of the p75 NGF receptor remains unclear, recent studies disrupting this gene in transgenic mice indicate that it may play an important physiological role (Lee et al. 1994a; Lee et al. 1994b) including the
regulation of nerve growth in those sympathetic neurons with long projecting pathways. In addition, deleting the p75 gene reduces the sensitivity of the NGF dose response curve in knockout compared to wild type sympathetic neurons (Davies et al. 1995; Lee et al. 1994b), whilst other studies have shown that p75 is important for survival of sympathetic and sensory neurons (Ryden et al. 1997; Wyatt, Davies, 1995). There is also limited evidence that p75 is upregulated in collateral sprouting (Kuchel et al. 1992) and may be reduced in ageing sympathetic neurons (Kuchel et al. 1997). These studies led us to investigate further the role of p75 in the ageing sympathetic and sensory nervous system.

This led to the hypothesis that reduced NGF responsiveness, shown previously, particularly in ageing sympathetic nerves, is the result of reduced p75 expression. A morphological approach was used to locate and quantify the amount of p75 within nerves of the MCA using immunohistochemistry. Other, more quantifiable methods for measuring p75 protein or message, such as two-site ELISA and RNase protection assay, can not identify where and hence in which nerve populations p75 is expressed. Therefore, the main aim of this study was to determine if p75 immunostaining in the aged MCA correlated with the known losses seen in sympathetic and sensory nerves. Therefore the extent of colocalisation of p75 with TH and CGRP was studied in order to distinguish which population of nerves, if any, displayed altered receptor density in aged tissue.

7b) Material and methods.

See Chapter 2 for general description of methods.

7b.1) Tissue preparation and double immunohistochemistry.

All tissues used for this study were taken from animals that had been perfuse-fixed with 4% paraformaldehyde for 10 minutes (approximately 150 ml) via
the left ventricle with descending aorta clamped off. Tissues were removed and then immerse-fixed for a further 1.5-2 hours in the same fixative. Double-labelling immunohistochemistry for TH+p75 or CGRP+p75 was performed on whole mount preparations (see Chapter 2) using cocktails of the two primary antibodies for 48 hours at room temperature, followed by the two secondary antibodies for 90 minutes at room temperature. Mouse monoclonal p75 (Boehringer Mannheim, UK) was used at a dilution of 1:5, rabbit polyclonal TH (Eugene Tech, NY, USA) was used at 1:50 and rabbit polyclonal CGRP (Genosys, UK) was used at 1:600. Secondary antibodies consisted of swine anti-rabbit Texas Red (Jackson, USA) and goat anti-mouse FITC (Serolab, UK), both used at 1:40. Controls for cross reactivity of primary antibodies and non-specific binding of secondary antibodies were carried out and found to give no specific nerve staining.

7b.2) Confocal imaging.

Imaging was carried out on a Bio-Rad MRC 600 confocal microscope with the K1 and K2 filters for simultaneous acquisition of FITC and Texas Red immunofluorescence. Gain settings, black level, Kalman filtering and confocal apertures on both channels were optimised for good signal to noise ratio and contrast and to give a focal depth matching the optical section thickness. For grey value measurements, single optical sections (2μm) from the same depth were acquired from at least three regions of each sample and settings were identical during image acquisition of all samples. Photobleaching was minimised by using antifade mountant, and kept constant for all specimens by capturing images from the same depth within the sample and illuminating for same length of time. For measurements of nerve fibre density where stain intensity was less critical, six to ten optical slices were collected per field covering the full depth of the nerve plexus and 3 to 5 fields were collected per sample.
Images were analysed on the KS400 image analysis system using established methods. (see Chapter 2).

7c) Results.

A total of 16 young (six weeks) and 23 old (24 month) Sprague Dawley rats were used for p75 immunostaining, of which 8 young and 12 old were used for p75+TH double immunostaining and 8 young and 11 old were used for p75+CGRP immunostaining.

7c.1) p75+TH immunoreactivity.

The pattern of TH-LI nerves in young and old MCA was similar to that in the previous LM study (see Chapter 4) although the absolute values for A% in young and old MCA were double that of the previous LM study (Fig. 7.1 b and e, respectively). This can be attributed to the image acquisition method, i.e., CLSM which has been shown to be a more sensitive technique (see Chapter 3). Image analysis showed a 56% decrease in old vessels compared to young (A%, p<0.0001) (Fig. 7.2 a) which is comparable to the decreases observed in the previous LM study, thus despite the increased resolution of CLSM, the proportional changes shown by LM and CLSM were similar. Similar decreases (54%) were also observed in aged MCA when p75+TH-LI colocalisation was compared to young (A%, p<0.0001) (Fig 7.2 a). Colocalisation of TH expressing sympathetic nerves and nerves positive for p75 was not complete and showed a proportion of sympathetic nerve fibres (26-29%) in both old and young that either did not express p75, or expressed the receptor at a level which was undetectable by indirect immunofluorescence (Fig. 7.1 c and f). Thus, within-age-group comparisons of p75+TH revealed reductions in young TH versus young p75+TH of 29% (A%, p<0.05) whilst, old TH versus old p75+TH showed reductions of 26% (A%, p<0.05) (Fig. 7.2 a). No age-related differences in stain intensity for p75 were observed in p75+TH colocalised nerves (Fig. 7.2 b).
Confocal micrographs of p75 fluorescent immunohistochemical staining (green) in young (a,b,c) and old (d,e,f), combined with TH-LI nerve staining (red) in young (b) and old (e) MCA. Merged images (a/b) and (d/e) reveal colocalisation of p75 with young (c) and old (f) rat sympathetic nerves (shown as yellow). Note that the area of p75 staining is far greater than that of TH staining particularly in the young specimens (compare a to b).

Scale bar = 50μm
Fig. 7.2.

Graph showing proportion of TH-immunoreactive nerve fibres colocalised with p75 staining on young and aged rat MCA (a). Stain intensity of TH and p75 staining within nerves supplying young and aged MCA (b).

(a) Note proportional loss of fibre density in all 3 groups with age, contrasting with lack of change of stain intensity (b).

n = 8 young and 12 old
Proportion of TH positive sympathetic nerves colocalised with p75 in young and aged MCA.

A

Nerve density (%)

0 10 20 30

Young

Old

B

Stain intensity (GV)

0 20 40 60 80 100

TH

p75 Co-Localised

p75

*
7c.2) p75+CGRP immunoreactivity.

The pattern of CGRP-LI was very sparse and consisted of a few paravascular nerve bundles running the length of the vessels with some plexiform perivascular nerve fibres (Fig. 7.3 b and e, young and old respectively). Their appearance was similar to that seen at the LM level. There was a significant decrease (30%) in nerve density in aged MCA compared to young (A%, p<0.02) (Fig. 7.4 a). Similarly, there were fewer sensory nerves in aged MCA (23%) colocalised with p75 compared to young (A%, NS). Colocalisation of CGRP-LI sensory nerves and nerves expressing p75 was not complete for both age groups (Fig. 7.3 c and f). Thus, within-age group comparisons revealed about 30% fewer p75+CGRP nerves than CGRP nerves alone in young and aged sensory nerves (Fig. 7.4 a). No age related differences in stain intensity for p75 staining were observed in p75+CGRP colocalised nerves (Fig. 7.4 b).
Fig. 7.3.

Confocal micrographs of p75 fluorescent immunohistochemical staining (green) in young (a,b,c) and old (d,e,f), combined with CGRP-LI nerve staining (red) in young (b) and old (e) MCA. Merged images of (a/b) and (d/e) to reveal colocalisation of p75 with young (c) and old (f) rat sensory nerves (shown as yellow). Note that the area of p75 staining is far greater than that of CGRP staining. (Compare a,b; d,e).

Scale bar = 50µm
Fig. 7.4.

Graph showing proportion of CGRP-immunoreactive nerve fibres colocalised with p75 staining on young and aged rat MCA (a). Stain intensity of CGRP and p75 staining within nerves supplying young and aged MCA (b).

(a) Note that aged MCA have significantly fewer nerves in all three groups (p<0.05 for CGRP and p75+CGRP; p<0.02 for p75 alone) (see Fig 7.3) although the scale of this graph does not illustrate this well (also see Fig. 7.5 for expanded scale). (b) Also note that significant age-related reduction of stain intensity was only seen in the CGRP group and not the other two groups. n = 8 young and 11 old.
Proportion of CGRP positive sensory nerves colocalised with p75 in young and aged MCA.

![Graph showing nerve density and stain intensity]
7c.3) Low affinity NGF receptor (p75) immunoreactivity.

The pattern of p75-LI in young MCA was very dense and plexiform in appearance, resembling the pattern of nerve fibres seen with TH or PGP 9.5 (Fig. 7.1 a, 7.3 a). p75-LI nerves covered approximately 25% of the vessel surface. p75-LI was reduced in old MCA but remained plexiform in character covering about 19% of the vessel wall (Fig. 7.1d, 7.3d). Image analysis revealed a significant decrease of 32% in p75-LI in old vessels compared to young (A%, p<0.01) (Fig. 7.2 a, 7.4 a). However, if the sum of p75 colocalised with TH- and CGRP-positive nerve fibre density values is subtracted from the total p75 nerve density data, then the age related decline in p75 staining was no longer apparent (Fig. 7.5), i.e., the age-related reduction of p75 is accounted for by the portion of p75 staining which is colocalised with nerves. Stain intensity (GV) of p75-LI nerve fibres showed no age-dependent changes (Fig. 7.2 b, 7.4b).

The density of p75-LI nerves was greater that the sum of densities of TH and CGRP-LI nerves, suggesting either a 'hidden' population of p75-LI fibres or non-neuronal distribution of p75-LI in young and aged MCA. Thus a large proportion of p75 staining (33% in young and 57% in old) was not colocalised with TH or CGRP (Fig. 7.2 a, 7.4 a). The non-colocalised p75 staining may be localised largely on glial processes, or in non-CGRP-expressing sensory nerves, non-TH-expressing sympathetic nerves or possibly parasympathetic nerves.
Fig. 7.5.

Summary graph showing nerve density data for TH, p75+TH, CGRP, p75+CGRP and p75 alone with summed values of sympathetic and sensory nerve data subtracted (p75-(TH+CGRP). Note that when the contribution of CGRP positive sensory and TH positive sympathetic nerves are subtracted from the total p75 nerve density data (i.e. "non-neuronal" p75) there is no significant age-related loss of p75 expression.

(See figures 7.2 & 7.4 for significant differences between groups)
p75 staining in MCA from young and old rats

![Chart showing nerve density (A%) for different conditions: TH, p75+TH, CGRP, p75+CGRP, and p75(TH+CGRP). The chart compares young and old rats.](chart.png)
7d) Discussion.

Previous studies have shown a 50% decrease in total and in sympathetic nerve density in aged MCA using PGP9.5 and glyoxylic acid, respectively (Andrews, Cowen, 1994b). I have confirmed a 50% decrease in the sympathetic (Chapter 4) and shown a 22% decrease in sensory (Chapter 5) nerve density. In addition, this study confirms the results of the previous LM studies of sympathetic and sensory nerves (Chapter 4 & 5) in that similar decreases (54-56%) in TH-LI nerves, and (28%) in CGRP-LI nerve density were observed in aged MCA compared to young with CLSM. Confocal and light microscopy therefore demonstrate similar pattern and density of TH-LI nerves in young and aged MCA although using confocal microscopy the absolute values for A% in young and old MCA were approximately double those found by LM. This is attributed to the greater sensitivity of CLSM (see Chapter 3). Despite the increased resolution of CLSM, the proportional changes shown by LM and CLSM were similar.

Early postnatal sympathetic neurones become less dependent on NGF for survival but remain dependent for growth of their axons, dendrites and soma (Ruit, Snider, 1991; Purves et al. 1988) and central connectivity (Yawo, 1987). In vitro studies have shown that sensory neurones become independent of NGF for survival but require it for regulation of neurotransmitter expression (Lindsay et al. 1989). It could be argued therefore that the regulation of growth and survival become to some extent de-coupled in the mature sympathetic nervous system.

An important question therefore becomes to what extent can the neurotrophic hypothesis explain altered growth of adult and ageing sympathetic and sensory nerves? It has been widely hypothesised that neuronal growth in postnatal and adult life is limited by the amount of NGF available at the target (Purves et al. 1988). Studies performed by Miller on neonatal (Miller et al. 1991) and mature sympathetic neurones (Miller et al. 1994) show that target derived
NGF regulates p75, but probably not Trk A expression (also see Chapter 8c.3). An extension of neurotrophic theory may therefore include a positive effect of target-derived NGF on neuronal NGF receptors, reinforcing trophic relations between neuron and target. An initial hypothesis is therefore that growth of adult neurons remain dependent on target derived NGF, and reduced levels of NGF in aged target tissues, combined with reduced expression of p75 receptors, leads to dendritic and axonal loss in the aged MCA.

Regarding NGF availability, recent studies using ELISA assays for NGF protein have shown that NGF levels in the aged MCA (and in other target tissues of SCG neurons) remain largely unaltered (Crutcher et al. 1996) and do not correlate with observed local atrophic changes in sympathetic and sensory nerve density (Thrasivoulou, Cowen, 1995; Cowen et al. 1996). These observations cast doubt on the relevance of the neurotrophic hypothesis to understanding age-related changes in peripheral neurons. However, NGF availability to nerves in the ageing vessel wall may be reduced, despite the maintenance of protein levels. Thickening of basal lamina has been shown around smooth muscle cells in aged MCA (Chaldakov et al. 1992) which may form a diffusion barrier limiting the availability of NGF to axons.

Further studies have shown that, although NGF levels in target tissues are not reduced in old age, NGF protein in the aged SCG is markedly (~50%) lower than in young animals, and is almost unaffected by axotomy (Cowen et al. 1996a). These results suggest that binding, internalization and retrograde transport of NGF, but perhaps not availability, are the limiting factors in the observed age-related decline in nerve density in the MCA and elsewhere. Reduced axon transport has been shown to be reduced in aged peripheral nerves (Fernandez, Hodges-Savola, 1994) which supports the hypothesis that functional deficits may be responsible for these age changes. Further evidence for reduced neuronal responsiveness to NGF in old age has been cited in the introduction to this chapter. The principal result of the present study shows reductions in colocalised p75 staining which parallel the reductions in
sympathetic and sensory nerves. This result appears to be the first indication that p75 receptor expression is reduced in ageing sensory and sympathetic nerves. Approximately 80% of TH-LI sympathetic nerves and CGRP-LI sensory nerves showed colocalisation with p75 at both age stages. Stain intensity (GV) of p75-LI nerve fibres showed no age-dependent changes, suggesting that there is no change in the density of axon membrane-associated receptors with age. Therefore, we can hypothesise that since p75 receptor density parallels the decline in nerve density, then in absolute terms, there is less receptor per neuron and therefore less capacity for NGF uptake in aged sympathetic and sensory neurons. This observation may explain the reduced NGF responsiveness that has been observed in these neurons (see above), and also the reduced capacity for growth of ageing sympathetic and, perhaps, sensory neurons. (For further discussion see 8d.2).

Approximately one-third of the total p75 immunoractivity is not associated with TH and CGRP stained nerve fibres. This non-colocalised staining is most likely to indicate receptors associated with glia (Yasuda et al. 1987) or with non-CGRP expressing sensory nerves. Alternatively, a proportion of the p75 staining may be associated with NGF responsive parasympathetic fibres (Kessler, 1985), although this is controversial (Allsopp et al. 1993; Allsopp et al. 1994). Unlike the nerve-associated p75 staining which declines in old age, the non-nerve associated staining remains unaltered.

It has been proposed that TrkA alone can account for most of the biological effects of NGF and that the p75 receptor is primarily involved in modulating Trk activation. Data from a number of studies suggest that p75 receptors are expressed at higher levels in long projecting neurones (see review, (Bothwell, 1995)) where neurotrophins have to be retrogradely transported to the cell soma over relatively long distances, in contrast with local circuit neurones with short axons which are not so dependent on retrograde transport. Studies on the p75 null mutant mouse display reduced NGF responsiveness of sympathetic and sensory nerves confirming the importance of this receptor
(Lee et al. 1994a). In addition, selective deficits in some sympathetic neurones have been shown (Lee et al. 1994b). Since the affected sympathetic nerves in the latter study have relatively long axonal pathways, the authors deduce that p75 receptors are important for binding, uptake and retrograde transport of NGF (Lee et al. 1994b) indicating that p75 plays a more central role in neuronal signalling than was thought previously. Other knockout studies which render the offspring heterozygotic for an NGF null mutation and homozygotic for the p75 null mutation, show that sympathetic neuron number is unaffected by this condition, but nerve density in the long projecting neurones of the foot pad is very sparse compared to normal mice (Brennan, Landis, 1997), supporting the hypothesis that growth and survival can become de-coupled. In addition, p75 has been shown to be up-regulated during collateral sprouting of adult sympathetic neurones (Kuchel et al. 1992) and down-regulated in atrophic aged sympathetic neurones (Kuchel et al. 1997). Since the axons of the MCA have relatively long distances to travel to their target tissues, this may render them more vulnerable during the ageing process.

Therefore, observations from this study show that reductions of p75 receptor colocalised with TH-LI and CGRP-LI nerves in aged sympathetic and sensory neurons, coupled with a reduction in the amount of receptor per neuron, is consistent with a decrease in TH-LI and CGRP-LI nerve density in vivo (this study, Chapters 4 & 5) and may contribute to age-related neuronal vulnerability.

7e) Conclusions.

Data from this and other studies suggest that p75 plays a crucial role in plasticity of mature neurones and may influence altered NGF responsiveness in adult sympathetic neurones. In addition, the selective atrophic changes in particular nerve populations may be explained by a decoupling of p75 receptor
expression from target derived NGF availability during adult life, or may be
due to intrinsic functional deficits.

The apparent lack of total colocalisation of p75 and TH/CGRP-LI may be
explained by expression of p75 in Schwann cells or by p75 expression in
'hidden' populations of sensory and sympathetic nerves which do not express
CGRP or TH respectively.

It is evident that further studies are necessary in order to answer some of the
problems this study has highlighted. Clearly, in situ hybridisation for p75
mRNA in sympathetic and sensory neurons supplying the MCA is required so
that changes in receptor expression can be quantified in a reliable manner.

In addition, in situ hybridisation or RT-PCR for NGF in target tissues such as
the MCA and iris which show differential changes during ageing will help to
understand whether the neurotrophic hypothesis can be extended to explain
neuronal vulnerability during ageing.
Chapter 8

General Discussion

8a) Methodology.

The study of nerve target interactions in mature and, in particular, ageing autonomic and peripheral nerves requires sound quantitative morphological methods. During the initial phase of these studies it became apparent that in order to make reliable and consistent measurements of mature and ageing neurons on whole-mount and sectioned target tissues, it was imperative to revise existing methodologies in use in the laboratory and, where appropriate, devise new ones. The key methodological areas found to be most important are discussed below.

8a.1) Specimen preparation and fluorescence immunohistochemistry.

8a.1.1) Buffers

These studies have shown that the use of organic buffers results in substantially enhanced brightness and contrast of specific staining by as much as 40-100% (Fig 3.1). The increased brightness of specific immuno-fluorescent staining also facilitated the visualisation of more perivascular nerve fibres which previously may have been below the detection limits of our imaging system when inorganic buffers were used. Trace amounts of heavy metal ions found in inorganic buffers such as PBS have electrostatic effects on antigen-antibody binding by cross linking the IgGs of the antibody and may lead to inappropriate first layer binding. This may cause steric hinderance resulting in under-saturation of available antigen binding sites on the tissue, thereby reducing the total amount of bound fluorescently labelled secondary antibody.
8a.1.2) Storage of samples

Specimen storage and UV illumination of fluorescently stained samples can lead to significant fading of fluorescence. These studies have shown that, where densitometric quantification is required, it is especially important to minimise the effects of fading by storing specimens frozen for a maximum of 2 days prior to imaging, and to keep the period during which specimens are exposed to UV light to less than about 30 seconds per microscopic field. In addition, the model system used to study the effects of fading (Chapter 3) revealed that relatively high concentrations of monoclonal antibodies were essential in order to maximise sensitivity of staining. The use of amplifying systems such as avidin-biotin result in a non-linear relationship between antigen concentration and intensity of immunofluorescence staining.

8a.2) Imaging

8a2.1) Conventional fluorescence microscopy

One of the major developments in wide-field light microscopy is intensified fluorescence microscopy (FM) (Cowen, Thrasivoulou, 1992a; Willingham, Pastan, 1963b; Cowen, Thrasivoulou, 1992b; Reynolds, 1972b). This technique replaced the conventional tube camera with a high-resolution slow-scan CCD array camera. FM allows the visualisation of very faint objects which can not be clearly seen by eye nor recorded by direct photomicrography. In these studies FM has been used for image acquisition prior to image analysis and has revealed a number of advantages over solid-state tube cameras. Firstly, CCDs produce very low levels of background noise (black-level) which can be reduced to negligible levels if the CCD is cooled to sub-zero temperatures. Secondly, all pixels on the image forming area of the chip have the same sensitivity and have a linear response to brightness. Thirdly, since the 'black-level' is so low images can be integrated over several frames to visualise very low-light level samples without degrading contrast.
These properties make CCDs ideal for imaging biological specimens, especially where densitometric quantification is required (see below, 8a.3)

8a.2.2) Confocal laser scanning microscopy

Confocal laser scanning microscopy offers superior image quality, particularly in thick preparations containing abundant connective tissues. The optical sectioning capabilities of CLSM have proved to have significant advantages over conventional FM when imaging thick whole mount preparations such as blood vessels. In these tissues, peripheral nerves are located mostly at the medial-adventitial boarder with some nerve fibres running through the adventitia. When viewed with conventional FM one sees all the layers beneath the nerves, including the highly autofluorescent endothelium and internal elastic lamina, resulting in very high background and reduced contrast, sometimes to the point where no nerves are visible. Therefore, with CLSM one can optically section only in the layers where nerves are localised and exclude the deeper areas. This results in enhanced image quality and makes quantification of nerves a simpler task. A recent study in our laboratory of post-mortem human coronary vessels has demonstrated for the first time that image acquisition with CLSM significantly increased discrimination of fine nerve bundles (4-25 fold) in human vessels compared to conventional FM (Buwalda et al. 1997). It was evident that images acquired with CLSM resulted in increased contrast of nerves, particularly of the fine terminal nerves localised in the deeper adventitial layers which were scarcely visible with FM.

8a.3) Image analysis.

Densitometric applications of image analysis have been used previously to measure fluorescent stain intensity (Agnati, Fuxe, 1984; Cowen, Thrasivoulou, 1990), semi-automated autoradiography (Ramm, Kulick, 1985) and silver grain counting (Nagata, 1993). However, these studies did not fully assess the technical limits of microdensitometry. Therefore, one focus of this part of the
study was to assess the problems associated with quantitative fluorescence densitometry. The results show that a number of components of imaging systems have significant effects on quantitative measurements of stain intensity (see (Cowen, Thrasivoulou, 1992b)). Mercury lamp, microscope optics and video camera were shown to contribute to significant unevenness of illumination of the field of view. Linearisation of camera response and a two-stage background subtraction procedure resulted in substantial improvements in grey level resolution of video-based imaging systems. The linear response and uniform spatial imaging characteristics of digital CCD cameras reduced these problems to give grey level resolutions which were comparable to the theoretical resolution of an 8-bit image analysis system, thus making these cameras the instruments of choice for studies of this kind. Furthermore, because of the uniform image generated by these cameras, background subtraction was no longer required to compensate for uneven illumination generated principally by video scanners, although specimen background still needed to be considered.

Densitometric quantification of confocal images is possible with the appropriate safeguards. A number of standardised procedures are required to make reliable reproducible densitometric measurements (for reviews see (Laurent et al. 1994; Shotton, 1995). These factors include, 1] standardising gain, black-level and confocal aperture settings, 2] monitoring laser output power and anode current during image acquisition sessions and between experiments, 3] standardisation of laser illumination time to reduce and standardise the extent of photobleaching and 4] ensuring that the optical section that is used for GV measurements comes from the same depth from each sample so that observed differences in GV are real and not effects of signal attenuation from layers at different depth within the specimen. With all these pre-requisites satisfied, densitometric measurements are possible and in this context, CLSM is preferable to FM because measurements can be made from very localised areas in relatively thin optical slices. An example of the value of this approach, a recent study in our laboratory used a densitometric
application of CLSM to study laminin immunoreactivity in young and aged blood vessels of rats (Gavazzi et al. 1995a). Laminin has been shown to provide trophic support and facilitate nerve growth (Adler, 1990). In the above study, intensity measurements of laminin immunoreactivity were made in aged blood vessels, where an age-dependent decrease in nerve density of approximately 50% had previously been shown using the standardised procedures outlined above (Gavazzi et al. 1992). The results showed that, in aged rats, laminin immunoreactivity at the medial adventitial border was reduced by 50%, matching the decrease in innervation density. Changes in laminin immunoreactivity were specific to the adventitial-medial border and were not found in the intima. Axon terminals are closely apposed to laminin of the basal laminin of smooth muscle cells in this region of the blood vessel wall supporting the hypothesis that laminin may play a role in nerve fibre atrophy in old age.

Advances in technology and the expansion of the computer industry have substantially reduced the cost of computer hardware. Image analysis systems of the past were constrained by their processing speed, and by the cost of RAM. Typically image analysis systems would have around 2-4 MB of RAM and most algorithms would be 'hard wired' processor arrays, making the systems rather inflexible. The reduction in price of high speed central processors, and much cheaper RAM has enabled programmers to implement complex algorithms into PC which can process 8-bit full grey images in seconds rather than minutes. The principal algorithm that I have made use of with the KS400 imaging analysis system is the 'white top hat'. This algorithm applies a combination of 'grey open' (sequence of erosion and dilations) with a user-specified shape-operator over several iterations to generate an image of local levels of background which excludes the objects of interest (nerve fibres). This image is subtracted from the original grey image to produce an image with very much reduced background staining thus increasing the contrast in the areas of interest. In effect, 'white top hat' amounts to a localised subtraction of background. Since peripheral axons have very similar
diameters, one can set the number of iterations to a constant level for each experiment.

Subjectivity in grey level thresholding (segmentation), to produce a binary image from which subsequent measurements are made, has always been a major problem in image analysis (see, (Rosenfield, Kak, 1982; Spencer et al. 1990)). In this study I have developed a semi-automated thresholding procedure that reduces the contribution of operator subjectivity. This is achieved by a 2 stage semi-automated thresholding procedure. A more detailed description of the thresholding procedures are described in Chapter 3. Image thresholding in this way limits operator subjectivity and results in data that are more robust and reproducible.

8b) Regulation of growth, neurotransmitter expression and survival in adult and ageing sympathetic neurons

In order to study the interactions of target-tissues and their innervating neurons in the mature and ageing autonomic and peripheral nervous system it was essential to identify a target tissue where age-changes occur in one or more sub-populations of neurons supplying that target. Previously we had shown, using the general neuronal marker PGP 9.5 (Thompson et al. 1983), that total nerve density of the MCA was reduced in old age (Gavazzi et al. 1992). The main aims of this study was to identify which population/s of nerve fibres were reduced in the MCA, and to develop model systems to investigate the role of target tissues, trophic factors and their receptors in neuronal survival, growth and neurotransmitter plasticity.

8b.1) Descriptive studies

Decreases of approximately 50% were shown in the sympathetic innervation of aged cerebral blood vessels compared to young (Chapter 4). Mione et al (1988) have shown a decrease in sympathetic nerve density of about 50% in
MCA from aged Wistar rats (Mione et al. 1988). A decrease of similar proportions with age was also observed in the sympathetic innervation of these vessels in rabbit (Saba et al. 1984) and rat (Andrews, Cowen, 1994a) using catecholamine histochemistry and TH-immunohistochemistry. Immunohistochemical localisation of sub-populations of nerves generally relies on the detection of neurotransmitters/neuropeptides, their precursor, or by-products of these substances. Therefore, one can not be certain if the changes one sees are the result of neuronal cell loss, and/or the dying back of a significant proportion of nerve fibres in sympathetic neurons, or age-related changes of neurotransmitter within intact axons. However, a recent morphological study in our laboratory using orthograde tracing from the SCG to the pineal gland showed good correlation with immunohistochemical staining for TH (unpublished observation), thus providing evidence that atrophy of axons and not reduced neurotransmitter expression, is responsible for age-related changes in staining patterns of sympathetic nerves in these targets.

8b.2) Target regulation of sympathetic nerve morphology.

Neuron-target interactions have been shown to influence many features in developing peripheral neurons, including survival, neurite outgrowth, synaptic modulation, and neurotransmitter phenotype (Landis, 1990; Kaneda et al. 1990; Vogel, 1993; Raffioni et al. 1993; Deckwerth, Johnson, 1993; Snider, Johnson, Jr. 1989; Barde, 1989; Levi-Montalcini, Angeletti, 1968; Levi-Montalcini, 1987). The neurotrophic hypothesis (Purves, 1988) proposes that, during development peripheral target organs influence neuronal survival, nerve fibre density and other aspects of neuronal morphology via the production of limiting amounts of neurotrophic factors. Axons growing onto the target from different neuronal pools compete for the neurotrophic factors and the unsuccessful neurons undergo apoptosis. The initial hypothesis of these studies was an extension of the neurotrophic hypothesis which stated that competition for limiting amounts of these factors (probably NGF in
sympathetic and sensory neurons) continues in mature and ageing neurons. During old age, access to, or availability of neurotrophins (either cellular and/or ECM derived) may be disrupted in some aged target tissues leading to a dying back of nerves and possible cell death. The initial observations from the descriptive studies of sympathetic nerves therefore seemed to support the hypothesis of selective changes in nerves resulting from altered trophic support by particular targets.

The role of the target in determining its neuronal morphology and phenotype (see below, 8b.5), has been investigated in this study using an in oculo transplantation model (Olson, Malmfors, 1970). Isogenic in oculo transplantation has been used extensively to study the effects of target-neuron interactions in the past (see Chapter 1 section 1b.2.1, (for review, see (Cowen, 1993)), and provides a good model for NGF-dependent collateral sprouting by providing additional target tissue over which uninjured host nerves can grow. MCA from young and old donor rats transplanted in oculo for four weeks, show that nerve density of total and sympathetic reinnervation by young host iridial nerves closely matches the pattern and density seen in situ, i.e. total and sympathetic nerve densities are reduced by about 50% on old, compared to young, transplanted vessels (Chapter 5; also see (Gavazzi et al. 1992)). These data show clearly that target tissues have a strong influence on the density of sympathetic innervation they receive, and suggest that changes in the aged MCA are responsible for the reduced nerve density of the aged transplant.

8b.3) Role of cellular factors.

Sympathetic neurones of the SCG, which innervate the MCA, are responsive to NGF (Hendry, Campbell, 1976). Furthermore, the smooth muscle cells and fibroblasts of the vessel wall have the capacity to produce NGF (Creedon, Tuttle, 1991; Bandlow et al. 1987). NGF treatment has been shown to increase sympathetic innervation in peripheral targets (Isaacson et al. 1990;
Saffran et al. 1989; Bjerre et al. 1975). Recent studies have shown that NGF-treatment can induce the reestablishment of a 'young' pattern of innervation on old cerebral blood vessels as demonstrated by PGP 9.5 both *in oculo* (Gavazzi et al. 1992) and direct application over cerebral blood vessels *in vivo* (Andrews, Cowen, 1994a). However, these studies did not show whether the pattern of regrowth induced by NGF was appropriate to the tissue in terms of the sub-populations of nerve fibres which would normally innervate the vessel wall. **Chapter 4** shows that NGF induces a specific and appropriate regrowth of sympathetic nerve fibres onto the ageing vessel wall to levels greater than those found in young vessels, supporting the concept that age-related loss of sympathetic fibres may be the result of neurotrophic deprivation.

The effects of treatment with vehicle containing Cyt C in increasing sympathetic nerve density and TH levels on old implants were surprising in that similar studies did not show this response (Gavazzi, Cowen, 1993b; Isaacson et al. 1990b). However, studies have shown that apparently other non-neurotrophic vehicle solutions can induce nerve growth. Experiments from this laboratory have demonstrated the capacity of bovine serum albumen *in oculo* (Gavazzi, Cowen, 1993b) and vehicle infusion *in vivo* (Andrews, Cowen, 1994a) to induce fibre growth. Other groups have made similar observations in the CNS (Sendtner et al. 1992). One explanation might be an upregulation of NGF synthesis by tissues of the anterior eye chamber caused by the repeated intraocular injections of foreign substances. Shelton and Reichardt (1986) found a 3-4 fold increase in NGF mRNA in irises 6 hours after trans-corneal injections into the anterior eye chamber (Shelton, Reichardt, 1986). The increase in NGF levels caused by trauma might be mediated by an inflammatory response (Weskamp, Otten, 1987) of the iridial tissue, leading to the production of cytokines, which have been shown to induce NGF synthesis in fibroblasts (Lindholm et al. 1988). The increased nerve density and TH immunoreactivity in the young Cyt C-treated group support this hypothesis. In conclusion, the unexpected effect of Cyt C may be caused by local release of NGF from fibroblasts and glia of the iris or an
upregulation of NGF in the smooth muscle cells of the implanted donor tissue itself in response to injection _per se_ or injection of foreign substances.

NGF has been shown to affect both nerve growth and TH expression in developing sympathetic neurones (Gorin, Johnson, 1980; Thoenen _et al._ 1971). Indeed the data from this study (Chapter 4) support their findings in that we found a significant increase in TH expression and nerve fibre density in young and old MCA implants following treatment with NGF. Therefore, NGF is able to induce hyperinnervation of aged implants compared to young control MCA illustrating that old target tissues have the capacity to support _de novo_ nerve growth.

### 8b.4) Neuronal responsiveness to NGF.

NGF is produced by vascular smooth muscle cells (Creedon, Tuttle, 1991). These cells undergo atrophy in aged cerebral arteries (Chaldakov _et al._ 1992; Kojimahara _et al._ 1973) suggesting that neurotrophic factor synthesis may be reduced in this vascular target tissues. However, recent studies using ELISA assays for NGF protein have shown that NGF levels in the aged MCA are similar to levels of young tissue (Crutcher _et al._ 1996) and do not correlate with observed local atrophic changes in sympathetic nerves (Chapter IV, also see (Gavazzi _et al._ 1994). These observations cast doubt on the capacity of the neurotrophic hypothesis to explain age-related changes in peripheral neurons. However, NGF availability to nerves in the ageing vessel wall may be reduced, despite the maintenance of protein levels. Thickening of basal lamina has been shown around smooth muscle cells in aged MCA (Chaldakov _et al._ 1992) which may form a diffusion barrier limiting the availability of NGF to axons. The insoluble ECM protein laminin has been shown to be reduced by about 50% in aged cerebral blood vessels (see 8e) and may also be involved in the loss of sympathetic and sensory (see 8c.3) neurons.
Although NGF levels in target tissues are not reduced in old age, NGF protein in the aged SCG is markedly (~50%) lower than in young animals, and is almost unaffected by axotomy (Cowen et al. 1996a). These results suggest that binding, internalization and retrograde transport of NGF, but perhaps not availability, are the limiting factors in the observed age-related decline in nerve density in the MCA and elsewhere. Reduced axon transport has been shown in aged peripheral nerves (Fernandez, Hodges-Savola, 1994) which supports the hypothesis that functional deficits may be responsible for these age changes.

8b.5) Regulation of neurotransmitter phenotype.

Altered expression of neurotransmitters, whether qualitative or quantitative, provides a further dimension of neuronal plasticity, enabling a relatively rapid and precise adjustment of neuronal function to altered demands. During development, transmitter expression becomes matched to the target tissue much like the adaptation of neuronal morphology to targets. Selective expression of neuropeptides and neurotransmitters become matched to the particular physiology of the end organ. During ageing and following nerve damage, neurotransmitter expression may again be altered, although the functional implications of these changes are not so obvious.

Increased intensity of TH-immunostaining (20%) was observed in terminal sympathetic nerve plexus around cerebral arteries of aged rats (Chapter 4) (Thrasivoulou, Cowen, 1995), in parallel with nerve fibre atrophy (Andrews, Cowen, 1994b), as revealed by PGP 9.5. Contrasting changes in noradrenaline staining and nerve plexus density have also been demonstrated in the sympathetic nerve supply to the aged rat iris (Gavazzi et al. 1996b), suggesting increased neurotransmitter expression. This may be due to an increase in neurotransmitter expression or it may simply reflect normal levels of transmitter being condensed into a smaller axonal volume, giving a false impression of increased expression. However, increases in TH enzyme
expression in aged neurons is consistent with other studies (Kuchel et al. 1996; Reis et al. 1977). For example, Kuchel et al (1996) showed that in intermediate sized sympathetic neurons, which normally project to the pineal gland and cerebral vasculature, there was a 74% increase in TH activity and mRNA levels in aged SCG. Possible mechanisms are discussed below. Whatever the explanation, these observations suggest that nerve growth and neurotransmitter expression may be regulated separately in mature and ageing sympathetic nerves.

It is well established that retrograde signalling can have profound effects on neurones including their neurotransmitter phenotype (Rao, Landis, 1990; Landis, 1990). Using in oculo transplantation, it has been found that sweat glands retain the capacity to induce a 'switch' to a cholinergic phenotype in neonatal (Stevens, Landis, 1990; Schotzinger, Landis, 1990) and, more recently, in mature sympathetic neurons (Chapter 6) (Cowen et al. 1996b) with an established catecholaminergic phenotype, although the 'switched' neurons retain some noradrenergic characteristics (Potter et al. 1983). Thus, developing and mature neurons appear to depend on contacts with particular target tissues for specification of a precisely appropriate pattern of neurotransmitter expression, which can include the regulation of more than one transmitter substance in a single neuron. For example, as well as neuropeptides, cholinergic enzymes, such as acetylcholinesterase, are upregulated in sympathetic neurons following contact with sweat glands (Chapter 4) (Schotzinger, Landis, 1990).

Thermoregulation provides a clinically important and sensitive index of ageing in humans (for review see (Collins, Cowen, 1997)). Sweat gland function is impaired in elderly subjects over the age of 70 years (Abdel-Rahman et al. 1992) and is correlated with a substantial loss of cholinergic sudomotor nerve fibres, which has also been observed in ageing rats (Abdel-Rahman, Cowen, 1993). Transplantation experiments (Chapter 6) show that aged sweat glands are as potent as younger ones in inducing the switch to a cholinergic
phenotype in adult sympathetic neurons. However, they become innervated by significantly fewer nerve fibres, indicating again that fibre growth and transmitter characteristics are differentially regulated in adult sympathetic neurons.

8b.4) Cell death, functional deficits and selective vulnerability.

In those sympathetic ganglia which have been investigated systematically, the numbers of neurons appear to be maintained in old age (Santer, 1991a; Santer, 1991b; Baker, Santer, 1988a; Schmidt et al. 1995; Schmidt et al. 1990b; Warburton, Santer, 1993a). However these studies are not able to distinguish changes in small sub-populations of neurons within large ganglia which send out axons to many targets. Counts of SCG neurons projecting to the MCA in aged rats show reduced numbers (58%) (165 in young versus 70 in aged SCG) of traced neurons compared to young (personal observation, data not shown), although only a few ganglia were used at both age stages to obtain this data. However, reductions in the number of traced neurons in aged sympathetic ganglia may be explained by reduced axonal transport of retrograde tracer and not neuronal atrophy per se, and may suggest functional impairment in these axons (see section 8d.3, below). The increase in TH-LI stain intensity in aged sympathetic nerves of the MCA would seem at first sight not to support functional impairment, but one can not rule out the possibility that compensatory mechanisms may be at work which counteract the loss of axons arborisations. Alternatively, the increased TH content of these nerves may be as a result of increased storage resulting in concentration of the enzyme, although NAd levels do not normally increase in old age (Hervonen et al. 1978), at least not in the human sympathetic ganglia.
8c) Regulation of growth, neurotransmitter expression and survival in adult and ageing sensory neurons

8c.1) Descriptive studies

The sensory innervation of young and aged cerebral blood vessels was found to be very sparse as demonstrated by CGRP-IHC. Approximately 30% of sensory neurons projecting to the MCA from the trigeminal ganglion express CGRP (see Chapter 5, (Seiger et al. 1984)), whilst 10-12% express substance P (Lee et al. 1985a; Lee et al. 1985b). However, aged MCA showed reduced (20%) nerve density compared to young (Chapter 5). In addition, there was a 30% loss in the cytoskeletal protein NF-R39, usually only expressed in sensory axons of this target (Seiger et al. 1984), in aged compared to young vessels. It is not known whether intrinsic neuronal changes or changes in the MCA target are responsible for the age changes but, as with sympathetic neurons, functional deficits are likely to be implicated (see 8c.6).

8c.2) Target regulation of sensory nerve morphology.

Reinnervation of MCA transplants by host iridial sensory nerves has been investigated in order to answer what role target tissues may have in sensory nerve regulation. After four weeks in oculo CGRP-LI nerve density did not match that seen in vivo, and showed a marked hyperinnervation, which is greater in aged donor tissue than young (six to ten fold in old compared to six fold in young). Clearly, the target is having a profound effect on the density of innervation it receives, although the mechanism/s involved in this response are unclear. Sensory hyperinnervation of MCA implants taken together with data of unaltered NGF protein levels in aged MCA (Gavazzi et al. 1994) throws serious doubt on the relevance of the neurotrophic hypothesis to understanding the regulation of growth in adult sensory neurons. Therefore, an alternative hypothesis must be postulated to explain these results (see 8e).
8c.3) Role of cellular & ECM factors in sensory hyper-reinnervation of targets

Sensory nerves are sensitive to target-derived trophic factors, including NGF (Johnson et al. 1986) and BDNF (Kalcheim et al. 1987; Davies et al. 1986; Barde, 1989). There is evidence during early development that the density of peripheral sensory fibres is related to the availability of trophic factors in the target area (Harper, Davies, 1990). Also, skin becomes hyperinnervated by sensory nerves in transgenic mice which over-express NGF (Albers et al. 1994). Freeze-thawed MCA transplants become reinnervated to levels similar to those in control tissues (Gavazzi et al. 1996a) (see Chapter 5). This suggests that the sensory hyperinnervation observed following transplantation is due to cellular factors, since the cellular components are effectively removed after freeze-thawing and therefore, presumably, only insoluble factors have an influence on the innervating neurons. It is hypothesised that increased expression of NGF, and/or other trophic factors for sensory neurons, by the donor implants may explain the sensory hyperinnervation.

In vivo denervation of the rat iris has shown increases in NGF using both bioassay and radioimmunoassay (Ebendal et al. 1983; Ebendal et al. 1980). Co-culturing rat iris and sympathetic ganglia results in neurite outgrowth towards the iris which is considerably enhanced if the sensory innervation is ablated ten days before removal onto culture (Ebendal et al. 1980). Sympathetically denervated irides also potentiate neurite outgrowth to a greater extent than normal irides in the same model, but to a lesser extent than irides which have been subjected to sensory denervation (Ebendal et al. 1980). Combined sensory and sympathetic denervation resulted in a greater increase in neurite outgrowth than sensory denervation alone and the outgrowth was abolished by anti-NGF treatment. Using two site enzyme-linked immunoabsorbant assay, it has been shown that the NGF content of cultured iris increases approximately 200-fold (Barth et al. 1984), but only two-fold in irises which have been chemically sympathectomized with 6-hydroxydopamine.
(Korsching, Thoenen, 1985). There are several mechanisms which might contribute to the increase in NGF content observed after explantation or denervation. The increased NGF content of a tissue may reflect decreases in degradation or neuronal uptake of NGF. Thus, the absence of NGF-utilising nerves may be responsible for the increase in NGF content seen after surgical (Ebendal et al. 1983) and chemical (Korsching, Thoenen, 1985) sympathectomy.

Alternatively, treatments may cause an increase in gene expression and synthesis of NGF, leading to higher levels of the protein in the tissues. No change in NGF mRNA levels were found following sympathetic or sensory denervation in vivo, although a total denervation, performed by retrobulbar severing of the optic nerve, led to a fivefold increase in NGF mRNA levels (Shelton, Reichardt, 1986). Irises cultured in vitro, however, do show a rapid, large increase in NGF mRNA levels (Shelton, Reichardt, 1986), suggesting strongly that an increase in gene expression is an important contributor to the much more dramatic increases in NGF content observed under these conditions (Barth et al. 1984; Ebendal et al. 1983; Ebendal et al. 1980). Evidence suggests that the rise of NGF mRNA in culture is due to the absence of some activity which is normally present in aqueous humour. When irides are cultured in the presence of aqueous humour there is no induction of NGF mRNA (Shelton, Reichardt, 1986). This is in agreement with the results obtained with sensory or sympathetic denervation in vivo.

This raises the possibility that the MCA responds to denervation and transplantation with a rise of NGF mRNA, in the same way as explants of iris in culture medium, which in turn leads to an increase of NGF in the implants. The increased NGF content of the implants may then promote hyperinnervation by collateral sprouting of iridial sensory nerves. However, the increase in NGF in the transplanted tissue would be available to all nerve populations, raising the question of why sensory, but not sympathetic, hyperinnervation of the transplant is observed. Developmental studies have
shown that sympathetic nerves innervating the cerebral vasculature are relatively dense by E18-19 compared to CGRP positive sensory fibres at this stage (Tsai et al. 1992), indicating that sympathetic nerves arrive in the target before sensory nerves. However, Hiebert and Smith (Hiebert, Smith, 1994) have shown that sensory fibres reinnervate implants of adult tarsal muscle in oculo much more rapidly than sympathetic nerves, suggesting important differences between collateral sprouting of sympathetic and sensory nerves in mature tissues compared to axon growth during development. Other data from this study (Chapter 5) show that at three days post-bilateral superior cervical ganglionectomy, there is 30% increase in NF-R39 immunostaining in the iris and MCA. This suggests that sensory nerves are able to take advantage of the increased availability of NGF and either expand their terminal axons by collateral sprouting, or upregulate NF, thereby enabling the visualisation of a larger proportion of sensory axons. In addition, it is possible that sensory neurons utilise NGF more rapidly than sympathetic nerves, since NGF levels increase to a much greater extent following sensory denervation than after sympathetic denervation (Ebendal et al. 1980). If the increase in NGF after denervation is due to accumulation and not upregulation in NGF synthesis (Shelton, Reichardt, 1986) then Ebendal's results strengthen the argument that sensory nerves utilise more NGF than sympathetic nerves, as a result of either a faster retrograde transport rate or a more efficient uptake mechanism. Treatment of transplants with antiserum to NGF would go some way towards providing an answer as to the involvement of NGF in the observed sensory hyperinnervation.

8c.4) Responsiveness of sensory nerves to NGF.

Recent studies in this lab suggest that sensory iridial nerves are more responsive to NGF than sympathetic nerves, in that sensory nerves display a larger increase in fibre density than sympathetic nerves in response to different concentrations of exogenous NGF (Gavazzi et al. 1996b). The hypothesis is therefore put forward that collateral sensory axons from the iris innervate the
implant before the sympathetic axons and grow more rapidly, and in addition have a stronger affinity for the NGF. As a result, they, and not the sympathetic nerves, are able to take advantage of any increased availability of target-derived factors, leading to the unexpected outcome of sensory hyperinnervation combined with organotypic sympathetic reinnervation (also see 8d.4).

A further question that remains to be answered is why the aged transplanted vessels become more densely innervated by sensory nerve fibres than the young ones? Aged cerebral blood vessels have approximately twice the diameter of young vessels (Andrews, Cowen, 1994a) and consequently have a greater volume of smooth muscle cells, assuming they are not replaced by other cells during ageing. If, as is likely, smooth muscle cells are the principal cells responsible for neurotrophic factor synthesis (Creedon, Tuttle, 1991; Korsching, Thoenen, 1983) then, following transplantation, the capacity to upregulate NGF mRNA may be greater in aged compared to young MCA, explaining the greater extent of hyperinnervation in aged transplants.

The increased CGRP immunoreactivity that we have observed, including elevated grey values in vessels from aged donors, is likely to be due to elevated NGF levels. This may result in the visualization of CGRP immunoreactive nerve fibres in transplanted tissues which under normal circumstances did not express CGRP or were previously below the limits of detection, accentuating the appearance of hyperinnervation. Alternatively, the increased CGRP-LI is axon growth in response to increased NGF. It is clear that, in the absence of a pan-sensory marker, more sophisticated techniques are required to answer this question. Orthograde tracing from the trigeminal ganglion may be one possible solution to investigate growth responses. We are currently developing orthograde tracing techniques in order to visualise the morphology of the peripheral projections of single neurons in the hope of resolving the dilemma posed by the use of immunohistochemical markers,
especially since only 25-30% of trigeminal ganglion neurons projecting to the iris and MCA express CGRP (Seiger et al. 1984) and Chapter 5).

8c.5) Regulation of neurotransmitter expression in sensory nerves.

Although sensory neurons become independent of NGF for survival they still require it for neurotransmitter expression (Lindsay et al. 1989; Lindsay, Harmar, 1989; Lindsay, 1988). Data from the sensory study (Chapter 5) suggests that target tissues have an effect on CGRP expression. Stain intensity (GV) is reduced in aged MCA compared to young, suggesting that availability of NGF is reduced in the aged MCA. Therefore, targets may well regulate neurotransmitter expression of sensory neurons via trophic factors.

8c.6) Cell death, functional deficits and selective vulnerability.

Counts of sensory neurons retrogradely traced from the MCA to the trigeminal ganglion show reduced numbers (70%) in aged animals compared to young (Chapter 5). However, trigeminal neuron numbers have been shown to remain stable throughout life in Fischer 344 rats (Biedenbach et al. 1992). As with sympathetic neurons, the possibility remains of an age-related cell loss in a sub-population of neurons which counts of neurons in whole ganglia may not be sensitive enough to detect. Alternatively, reductions in the number of traced neurons in aged sensory ganglia may be explained by reduced axonal transport of retrograde tracer and not neuronal loss per se. This implies some functional impairment in these axons.

NGF protein levels in aged trigeminal ganglion increase (~40%) when calculated as amount of NGF per ganglion, but show no age difference when results were expressed as NGF/mg wet weight (Cowen et al. 1996a). The increased NGF per ganglion may be due to the increased weight of the ganglion in old age and/or that the additional NGF may be localised in glia. Gliosis has been shown to occur in old age (Johnson et al. 1991). However,
ELISA assay is not able to separate the neuronal versus glial contribution to NGF levels in the ganglion. CGRP levels decline by as much as 50% in aged rat trigeminal ganglion neurons compared with the highest levels found at 3 weeks of age (Wimalawansa, 1992). Other studies show reduced CGRP-LI in sensory nerves supplying mesenteric arteries in aged rats, as well as reduced neurogenic vasodilation (Li, Duckles, 1993), indicating that reduced CGRP is associated with functional impairment. In addition, it has been shown that axon transport of CGRP in rat sensory nerves declines with age (Fernandez, Hodges-Savola, 1994). Therefore, the apparent loss of labelled neurons projecting from the aged trigeminal ganglion projecting to the MCA, shown by retrograde tracing in Chapter 4, as well as the reduced CGRP immunoreactivity at the periphery, may be explained by defective axonal transport, reinforcing the possibility of functional impairment of sensory nerves in old age.

8d) Distribution and regulation of p75 NGF receptor in sympathetic and sensory neurons.

8d.1) Descriptive studies

The confocal study in Chapter 7 has confirmed a 50% decrease in the sympathetic and a 30% decrease in sensory nerve density in aged MCA. Therefore, confocal and light microscopy demonstrate similar patterns and densities of TH-LI and CGRP-LI nerves in young and aged MCA, although using confocal microscopy the absolute values for A% in young and old MCA were approximately double those found by LM. The amount of p75 receptor colocalised with sympathetic and sensory nerves is reduced in ageing MCA and parallels the decreases seen in TH-LI and CGRP-LI nerves, i.e. decreases of 54% and 23% were also observed in aged MCA when p75+TH-LI or p75+CGRP-LI colocalisation was compared to young values, respectively. These data suggest that p75 receptor density per unit area of nerve fibre is unaltered in ageing sympathetic and sensory neurons. However, numbers of
p75 receptors per neuron is likely to be reduced because total axon length is decreased during ageing. Age-related decreases in total p75 (32%) were also observed. However, the non-colocalised proportion of p75 staining showed no age-related decline (see Chapter 7). The non-colocalised proportion of p75 positive immunostaining probably consists largely of glial receptors (Yasuda et al. 1987) and therefore indicate absence of age changes in glia. Alternatively, 'hidden' groups of sensory and/or sympathetic nerves which do not express CGRR or TH, respectively, or express neurotransmitter at levels which are undetectable, may contribute to non-colocalised p75 in vivo.

8d.2) Target regulation of p75 receptor expression.

It is not clear at present what role targets play, and the effects they have on NGF receptor expression in adult peripheral neurons. Studies on early developing sympathetic neurons show that they can not survive for longer than 48 hours in culture without NGF in the medium (Martin et al. 1988; Chun, Patterson, 1977). Later on in early postnatal life sympathetic neurones become less dependent on NGF for survival but remain dependent on it for growth of their axons, dendrites and soma (Ruit, Snider, 1991; Purves et al. 1988; Purves et al. 1987) and central connectivity (Yawo, 1987). Sensory neurones have been show to become independent of NGF for survival but require it for regulation of neurotransmitter expression (Lindsay, Harmar, 1989; Lindsay et al. 1989). Therefore, growth and survival seem to be de-coupled in the mature sympathetic and sensory nervous system. Insoluble factors of the ECM such as laminin have also been shown to have effects on neurite outgrowth in postnatal neurons and have synergistic effects with NGF (Cowen et al. 1997; Grabham, Goldberg, 1997).
What is the role of the NGF receptors in these altered patterns of NGF dependency?

NGF binds to two classes of receptor, the low-affinity receptor, p75 and Trk A tyrosine kinase receptors (For review see, (Cowen, Gavazzi, 1998; Bothwell, 1995)). All neurotrophins bind to their receptors, are internalised and are retrogradely transported to the cell body. Miller et al., 1994, showed that NGF regulates the expression of p75 but not TrkA in mature sympathetic neurones, leading to an increased ratio of p75 to TrkA receptor upon exogenous application of NGF to distal axons (Miller et al. 1994). Her data suggest that extrinsic regulation of p75 receptor synthesis is mediated by an NGF-induced stimulatory feedback loop. Trk expression appears not to be extrinsically regulated. Therefore, p75 receptor may have a specialised role when it is presented with high levels of NGF, i.e., p75 sequestrates NGF from the high affinity TrkA receptor complex, thereby attenuating the function of TrkA and allowing sympathetic neurones to respond to NGF over a broad concentration range (also see Chapter 7).

Miller's et al., data (Miller et al. 1994) would predict that if NGF levels were reduced in old age then p75 receptor expression may also be reduced. However, NGF protein levels are unaltered in aged MCA (Crutcher et al. 1996) but are reduced in SCG of old rats (Cowen et al. 1996a) suggesting impairments in retrograde transport, and, perhaps, p75 expression independent of target levels of NGF. In addition, axon transport has been shown to reduced in aged peripheral nerves (Fernandez, Hodges-Savola, 1994) which supports the hypothesis that functional deficits may be responsible for these age changes. Reduced laminin expression in aged targets (Gavazzi et al. 1995a) may affect NGF responsiveness of neurons and result in reduced uptake and binding of NGF and lead to axon atrophy. Alternatively, NGF availability may be reduced, resulting in reduced amounts of NGF being taken up by nerves. Basal lamina around smooth muscle cells have been shown to be reduplicated in aged MCA (Chaldakov et al. 1992) which may form a diffusion barrier.
limiting the availability of NGF to axons, supporting the hypothesis of reduced NGF availability.

8d.4) Intrinsic neuronal changes may be involved in p75 receptor regulation.

The reduction of receptor expression in sympathetic neurons may be regulated via intraneuronal changes, rather than by NGF levels, and may help to explain the reduced responsiveness and atrophy of these neurons. These intrinsic changes may be a result of free radical damage or other neurodegenerative processes.

Sensory neurons on the other hand seem to retain their NGF responsiveness, since data from the transplantation study (Chapter 5), shows that MCA transplants are hyperinnervated in oculo and are unaffected by the age of the implant but not in the way that was expected (aged implants had increased rather than decreased reinnervation nerve density compared to young). It is postulated that removal of the MCA from the donor results in denervation of the implant which leads to a localised increase of NGF production, which is preferentially utilised by sensory nerves (see 8c.4). However, this does not explain why sensory nerve density and total p75 receptors per neuron are reduced in old age. This may be because of an intrinsic down regulation of p75 receptor which leads to axon atrophy via reduced NGF uptake.

8d.5) Regulation of neurotransmitter expression.

There is some evidence that p75 is involved in neurotransmitter expression of sympathetic and sensory neurons. Sympathetic and sensory neurons regulate neurotransmitter expression via an NGF mediated pathway (Lindsay, Harmar, 1989; Thoenen et al. 1971). It is possible therefore that a reduction of p75 receptor may lead to decreased NGF uptake which results in decreased neurotransmitter expression. Grey value data from the sensory study showed a
small decrease in CGRP-LI stain intensity in aged MCA which is consistent with a decrease in p75 receptor expression, but is inconsistent with the increased TH-LI stain intensity in aged targets.

8d.6) Summary of conclusions.

The principal results of this study show reductions in colocalised p75 staining which parallel the reductions in sympathetic and sensory nerves. This appears to be the first indication that p75 receptor expression is reduced in ageing sensory and sympathetic nerves. The data also suggest that p75 may contribute to the apparent loss of sensory and sympathetic nerves in aged MCA in two ways; 1) by reduced uptake and retrograde transport of NGF as result of reduced receptor expression, or 2) by killing neurons.

8e) Concluding hypothesis.

Sympathetic and sensory neurons are both affected adversely by old age, although not all neurons are affected to the same degree. Neurons projecting to some targets appear more vulnerable than others.

Target tissues have a profound influence on neuronal morphology and phenotype during development which continues into adulthood and even old age. In the case of sympathetic neurons, targets continue to influence their density and transmitter phenotype throughout adult life. However, the original hypothesis that altered NGF levels in target tissues are responsible for age-related neuronal atrophy in sympathetic neurons is not proven. Altered local levels of NGF per se do not appear to be the initiating event in neuronal atrophy and/or cell death. Intrinsically regulated changes in neuronal p75 receptor expression may make ageing sympathetic neurons less able to utilise available NGF.
In the case of adult sensory neurons there appears to be a similar combination of neural and target associated factors involved in plasticity. Age-changes in targets induce differential responses by young host nerves. For example, changes in ECM molecules appear to be important in regulating nerve growth. However, the increased sensory hyperinnervation of targets in oculo may be explained by a denervation-induced upregulation of target-derived NGF i.e., neurons may affect the synthesis of NGF, although this has not been tested. Other neuronal influences, including changes in p75 expression may help to explain the age-related atrophy of sensory, as well as sympathetic, neurons.

The final conclusion of this thesis is that age-related nerve atrophy cannot simply be explained by changes in targets or NGF protein levels, on the principle of the neurotrophic hypothesis. Clearly, intrinsic neuronal changes such as altered receptor expression and effects of neurons on targets are also important.


ALLSOPP TE, ROBINSON M, WYATT S, DAVIES AM (1994) TrkA mediates an NGF survival response in NGF-independent sensory neurons but not in parasympathetic neurons. *Gene Therapy*, 1 Suppl 1, S59


ANDREWS TJ, COWEN T (1994b) In vivo infusion of NGF induces the organotypic regrowth of perivascular nerves following their atrophy in aged rats. *J Neurosci*, 14, 3048-3058.


BJERRE B, WIKLUND L, EDWARDS DC (1975b) A study of the de- and regenerative changes in the sympathetic nervous system of the adult mouse after treatment with the antiserum to nerve growth factor. *Brain Res, 92*, 257-278.


COWEN T, GAVAZZI I, ANDREWS TJ, THRASIVOULOU C (1993) Age-related neurodegeneration is caused by changes in targets and target-derived growth factors, not by intrinsic changes in neurones. *J Auton Nerv Syst*, **43** (Suppl.), 69


CRUTCHER KA, WEINGARTNER JA, GAVAZZI I, COWEN T (1996) NGF in the rat external carotid artery is primarily associated with nerve fibres and shows age-related declines. Society for Neuroscience Abstracts,


GAVAZZI I, COWEN T (1993c) Axonal regeneration from transplanted sympathetic ganglia is not impaired by age. Exp Neurol, 122, 57-64.


GONG QZ, BAILEY MS, PIXLEY SK, ENNIS M, LIU W, SHIPLEY MT (1994) Localization and regulation of low affinity nerve growth factor receptor


HORGAN K, O'CONNOR TP, VAN DER KOOY D (1990) Prenatal specification and target induction underlie the enrichment of calcitonin gene-related peptide in the trigeminal ganglion neurons projecting to the cerebral vasculature. Journal of Neuroscience, 10, 2485-2492.


ISAACSON LG, ONDRIS D, CRUTCHER KA (1995) Plasticity of mature sensory cerebrovascular axons following intracranial infusion of nerve growth


KORSCHING S, THOENEN H (1985) Nerve growth factor supply for sensory neurons: site of origin and competition with the sympathetic nervous


LEE KF, DAVIES AM, JAENISCH R (1994b) p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development*, 120, 1027-1033.


MINSKY M (1961) Microscopy apparatus (Filed Nov 7, 1957). (patent)


O'CONNOR TP, VAN DER KOOT Y (1989) Cooperation and competition during development: Neonatal lesioning of the superior cervical ganglion induces cell death of trigeminal neurons innervating the cerebral blood vessels but prevents the loss of axon collaterals from the neurons that survive. *J Neurosci*, 9, 1490


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. J Neurosci, 6, 1051-1060.

PURVES D, VOYVODIC JT, MAGRASSI L, YAWO H (1987) Nerve terminal remodeling visualized in living mice by repeated examination of the


REYNOLDS GT (1972) Image intensification applied to biological problems. [Review] [20 refs]. Quarterly Reviews of Biophysics, 5, 295-347.


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- 210 -


WALL PD, FITZGERALD M (1982) If substance P fails to fulfil the criteria as a neurotransmitter in somatosensory afferents, what might be its function?. [Review] [32 refs]. *CIBA Foundation Symposium*, 249-266.


