THE EFFECTS OF NERVE GROWTH FACTOR ON ADULT AND AGED DORSAL ROOT GANGLION NEURONS MAINTAINED IN PRIMARY CULTURE

By

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ABSTRACT

Nerve growth factor (NGF) is, a prototype neurotrophic factor, well documented for its biochemical character, distribution and functions in embryonic and neonatal animals. However, only very tentative evidence exists for its effects on mature neurons, and results are controversial. Studying NGF by using mature neurons should be a significant and attractive field, not only for its clinical value in nerve regeneration by adults, but also for understanding the mechanisms of aging. Studies *in vitro* present a comparatively simple environment for analysis of the effective factors. Dorsal root ganglia (DRGs), containing an enormous heterogeneity of sensory neuronal types, are a useful model in understanding the nervous system. Hence, in the present study, mouse DRG neurons were maintained *in vitro* supplemented with NGF *vs* cultures without NGF or with anti-NGF present, to examine any NGF effects on survival, morphological phenotype, neuropeptide expressions and neurite outgrowth (equivalent to regeneration *in vitro*). Comparison of adult (6 months) and aged (2 years) animals was made throughout the present study.

In the survival study, a range of NGF (25-200ng/ml) was added to adult neurons in co-cultures, and neuronal survival monitored for 14 days in vitro (div). 100ng/ml NGF effectively maintained survival, compared with cultures without exogenous NGF. Aged as well as adult neurons were cultured [with non-neuronal cells (NNCs) present] without exogenous NGF or with 100ng/ml NGF added for up to 29div. NGF enhanced the survival of both adult and aged neurons ($P<0.005$ by ANOVA). To exclude possible endogenous NGF from NNCs, or any mediation of an NGF effect in co-culture by NNCs , a neuron-enriched culture system was also used. In these neuronal loss was avoided during cell preparation and well dispersed neurons were obtained. 100ng/ml NGF and/or 1:100 anti-NGF were re-examined in the modified neuron-enriched

cultures for 9div and an enhanced survival of both adult and aged neuron persisted, although this effect was lower than in co-cultures. In general, about 20% of DRG neurons were NGF-dependent for their survival in adult and aged cultures. By size analysis of over 6500 neurons, intermediate-sized neurons (24-33μm in diameter) were predominant for both adult and aged in co-culture in the presence of NGF.

The immunocytochemistry (ICC) study using the avidin-biotin-peroxidase complex (ABC) method revealed that higher proportions of SP-immunoreactive (ir), CGRP-ir and NPY-ir aged, as well as adult, neurons were present in enriched cultures supplemented with 10 or 100ng/ml NGF than in cultures without NGF added ($P<0.05$-0.01 by ANOVA and t-tests), whereas SOM-ir adult and aged neurons showed little difference in cultures without or with NGF added. By counting at the peak day (9div) of SP expression, enhancement of the SP-ir subset in the presence of *vs* absence of NGF was similar for adult or aged neurons; the proportion of the SP-ir subset was not reduced following aging. Hence the SP-ir subset, together with the subsets of CGRP-ir and NPY-ir, were considered as NGF-dependent survival neurons in adult, and even aged mouse DRGs. Enhancement of the SP-ir proportion was more rapid in aged neuronal cultures supplemented with NGF than in cultures without exogenous NGF. Also, the staining intensities for CGRP and NPY were greater in both adult and aged cultures with NGF added than those in cultures without NGF.

In addition to maintaining survival, NGF affected the mature neuronal phenotypes. Scanning electron microscopy demonstrated that in the presence of NGF, abundant microvilli projections were distributed on the neuronal surface; in contrast, neurons were deformed and lost their smooth appearance, if 1:100 anti-NGF was present in enriched cultures.

In the study of neurite geometry in enriched culture, major neurite length (maximal extension of a neurite), entire neurite length (lengths of major neurite plus all its branches), total neurite length (lengths of all neurite outgrowth from each neuron), soma size, neurite number (for each neuron) and branch number (per neurite) were selected as parameters. Tracing was carried out blindly at 1, 3, 6 and 9div by computer image analysis and a digitizing tablet. Neurites and neurons from at least 5 mice for each adult or aged cultures were measured directly from culture or from photomicrograph montages and data were statistically tested by ANOVA. The results demonstrated that NGF enhanced neurite outgrowth ($P<0.0005$) at 6 and 9div for both adult and aged neurons Total neurite lengths of adult and aged neurons were distinct in the presence of NGF: neurite length was predominantly enhanced by adult neurons, whereas increased branch lengths were evident for aged neurons. Neurites predominantly extended from intermediate- and large-sized neurons.

In summary, aged neurons showed comparatively lower abilities for survival and functional peptide expression compared to adult neurons *in vitro*, but continued to be responsive to exogenous NGF. In addition, reconstruction of neurites by aged and adult neurons *in vitro* was demonstrated in the present study.

CONTENTS

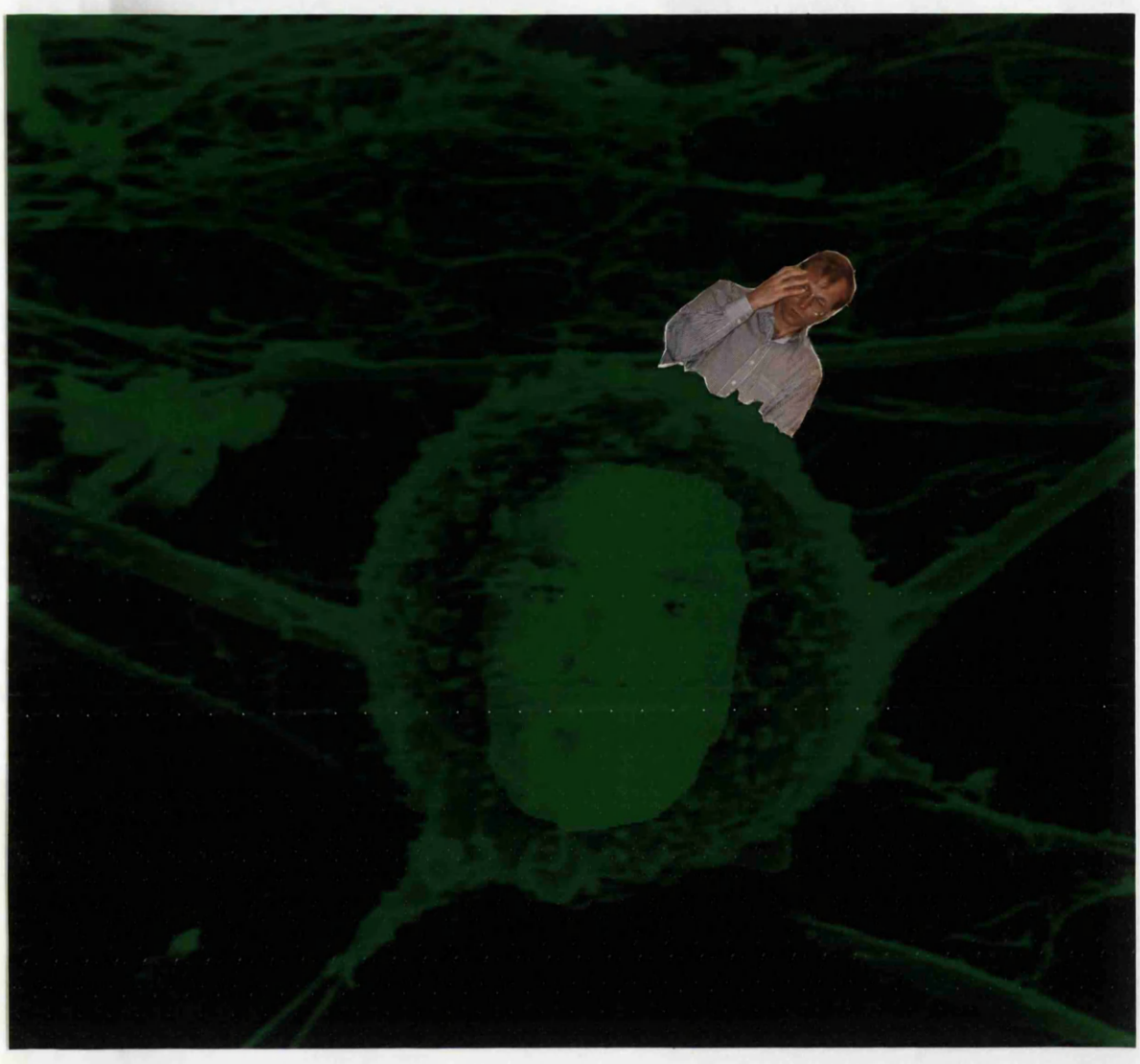
DEDICATION

To my grandmother, grandparents-in-law, parents and sisters, with respect, for their love, previous education and selfless assistance; To my wife and son, with love, for their sacrificing personal interests to support me throughout my study.

DECLARATION

I hereby declare that this thesis is my own composition, except where duly acknowledged, and has not been submitted for consideration of any other degree in this or any other university.

Zhi-Gang Jiang



You have to stay in there!

Have you found any thing of interest yet?

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LIST OF FIGURES AND TABLES

Tables

MAJOR ABBREVIATIONS

Aα, Aβ, Aγ -- Subpopulations of large light DRG neurons in mouse

A1, A2, A3 -- Subpopulations of large light DRG neurons in rat

ABC -- Avidin-biotin-peroxidase complex

Aδ -- A fast-conducting nerve fibre

aFGF -- Acidic fibroblast growth factor

ANOVA -- Analysis of Variance

anti-NGF -- Antiserum Against NGF

Ara-C -- Cytosine arabinoside

Bα, Bβ, Bγ -- Subpopulations of mouse intermediate-sized DRG neurons

B1, B2, B3 -- Subpopulations of rat intermediate-sized DRG neurons

BDNF -- Brain-derived neurotrophic factor

bFGF -- Basic fibroblast growth factor

C -- Small-sized DRG neurons in both mouse and rat

°C -- Centigrade degree

Ca^{2+} -- Calcium

CBA -- Mouse species

cDNA -- Complementary DNA (to mRNA in *reverse* transcription)

C fibre -- A slow-conducting nerve fibre

CG -- Ciliary ganglion

CGRP -- Calcitonin gene-related peptide

ChAT -- Choline acetyltransferase

CNS -- Central nervous system

CNTF -- Ciliary neurotrophic factor

Co-culture -- Neurons in culture with NNCs present

Da -- Dalton (=1.67 x 10^{-24}gram)

DAB -- di-azo-aminobenzene

div -- Days *in vitro*

DMEM -- Dulbecco's Modified Eagle Medium

DNA -- Deoxyribonucleic acid

DRGs -- Dorsal root ganglia

E -- Embryo at a certain day

e.g. -- *exempli gratia* (for example)

EGF -- Epidermal growth factor

et al -- *et alia* (=and others)

FCS -- Fetal calf serum

Fig. -- Figures

FN -- Fibronectin

FRAP -- Fluoride-resistant acid phosphatase

g -- gram

GAP-43 -- A growth-associated phosphoprotein

GFAP -- Glial fibrillary acidic protein

G protein -- GTP-binding regulatory protein

GTP -- Guanosine triphosphate

hrs -- hours

5-HT -- 5-hydroxytryptamine

^{125}I -- Isotopic iodine

ICC -- Immunocytochemistry

i.e. -- *id est* (=that is)

IGF -- Insulin-like growth factor

IL1 -- Interleukin 1

in vitro -- in culture

in vivo -- in body

-ir -- immunoreactive

K -- A thousand

K^+ -- Potassium

KV -- A thousand volts

L -- Large light neurons (or litre in certain contexts)

L3 -- Lumbar-3

LM -- Light microscopy

LNGFR -- Low-affinity NGF receptors (=$p75^{NGFR}$)

M -- Mole or molar, a concentration unit

MAP -- Microtubule-associated protein

mg -- Milligram

min -- Minutes

ml -- Millilitre

mm -- Millimeter

mRNA -- Messenger ribonucleic acid

MW -- Molecular weight

N -- Neuron

n -- Number

Na -- Sodium

NCAM -- Neural cell adhesion molecule

NF -- Neurofilaments

ng -- Nanogram

NGF -- Nerve growth factor

NGS -- Normal goat serum

nM -- Nanomolar

NNCs -- Non-neuronal cells

NPY -- Neuropeptide Y

NRS -- Normal rabbit serum

NT-3, -4, -5 -- Neurotrophin-3, -4 or -5

$p75^{NGFR}$ -- Low-affinity NGF receptor

$p140^{trk}$ -- High-affinity NGF receptor

$p140^{trkB}$ -- High-affinity receptor prdominantly for BDNF

$p140^{trkC}$ -- High-affinity receptor prdominantly for NT-3

PB -- Phosphate buffer

PBS -- Phosphate buffered saline

pH -- Hydrogen ion concentratrion

PNS -- Peripheral nervous system

RT-97 -- A monoclonal antibody against the phosphorylated form of 200 KDa neurofilament

S -- Svedberg unit (=sedimentation coefficient); or small dark neuron

SEM -- Scanning electron microscopy

SOM -- Somatostatin

SP -- Substance P

T2 -- Thoracic-2

tau --A microtule-associated protein

TEM -- Transmission electron microscopy

TGFβ -- Transforming growth factor beta

TH -- Tyrosine hydroxylase

TMN -- Trigeminal mesencephalic nucleus

TMP -- Thiamine monophosphatase

TRG -- Trigeminal ganglion

TRH -- Thyrotropin-releasing hormone

µl -- Microlitre

µm -- Micrometre

VIP -- Vasoactive intestinal peptide

vs -- Versus

NB: The data on graphs and histograms is expressed as mean values and standard errors of the mean in all cases.

Chapter 1

GENERAL INTRODUCTION

Dorsal root ganglia (DRG), situated in the intervertebral fossae, link peripheral nerve fibres with the central nervous system. DRG contain an enormous heterogeneity of primary sensory neuronal types with different neuropeptide phenotypes (Price, 1985), and mediating diverse physiological functions. Therefore investigations of the complexity of DRG neurons are of great importance in further understanding the vertebrate nervous system, including that of human beings. Sensory neurons from DRGs have become, over the years, a useful model for a variety of studies of neuronal properties, including investigations of histogenesis, morphology, electrophysiology, biochemistry, immunocytochemistry, and molecular biology (Scott,1992).

Many early studies concentrated on fetal ganglia (Levi-Montalcini and Angeletti, 1968) whilst in other *in vivo* studies, DRG neurons of mature animals were examined (for review see Lieberman 1976; Johnson and Yip, 1985). Parallel with these whole animals studies, fetal (Baron-van Evercooren *et al.*, 1982; Kim *et al.*, 1984; Yong *et al.*, 1988) and adult DRG neurons were maintained *in vitro* (Scott, 1977, 1982). A refined culture system (Smith and McInnes, 1986a) was introduced for adult DRG maintenance *in vitro* and the effects of substrata upon survival were investigated (Smith and Orr, 1987). Such cultures have also been applied to the evaluation of neurotoxicity (Smith and McInnes, 1986b; Smith, 1991) and also permit the possibility of studying neurotrophic factors in mature neurons in greater detail.

Nerve growth factor (NGF) has become a prototype neurotrophic factor, which was firstly identified (Levi-Montalcini and Hamburger, 1951) and then purified (Cohen, 1959). Although vast numbers of other neurotrophic factors have been purified or cloned in following decades (for review see Fallon and Loughin, 1993), NGF remains as the best documented with respect to its structure, its receptors and its mechanism of action. The effects of NGF on

mature sensory neurons continue to represent an interesting research field which is still not fully understood. In discussing the effects of NGF on mature DRG sensory neurons, it is necessary to consider their histogenesis (see section 1.1), the heterogeneous neuronal types present in DRG (see section 1.2), and the requirements of developing sensory neurons for neurotrophic factors (see section 1.3). Further, neurite regeneration/outgrowth (1.4.) and the general effects of neurotrophic factors on adult and aged DRG neurons will be outlined (see section 1.5), together with the methodology (1.6) and the aims of the present study (1.7).

1.1 Histogenesis of DRG Neurons and Fate of Neural Crest Cells

In the early stages of embryonic development, i.e. the neurula (0-3 somite) stage, a dorsal groove appears on the ectodermal surface of the gastrula, followed by a thickening of ectoderm along its margins to form neural folds (Couly and Le Douarin, 1988). The neural tube is created when the neural folds join, and the neural crest arises between the presumptive epidermis and the neural tube. It is from neural crest cells that the spinal and autonomic ganglia, the glial cells of the peripheral nervous system, and a variety of nonneural tissues such as melanocytes, and chromaffin cells of the adrenal medulla and other tissues arise. Epidermal placodes, from five separate thickenings of the ectoderm of the head region in vertebrates, give rise to the sensory ganglia of cranial nerves (Noden, 1978; Le Douarin, 1982; Patterson and Purves, 1982).

Dorsal root ganglia, the superior cervical and jugular ganglia all contain neurons which originate exclusively from the neural crest. The distal portions of both lobes of the trigeminal, geniculate, petrosal and nodose ganglia (associated with cranial nerves V, VII, IX, and X respectively) contain neurons derived from the 4 placodes located on the branchial archs (Le Douarin, 1982).

In forming the DRGs, neural crest cells migrate in both rostral and caudal directions, following a longitudinal pathway of the mediodorsal aspect of neural tube (Teillet *et al.*, 1987). Neural crest cells arising from the region opposite the posterior half of the corresponding somite contribute to about 50% of the ganglion, and localize predominantly in its caudal half. Cells from the posterior half of the preceding somite populate the rostral 20% of the ganglion; and cells arising at the anterior half of the corresponding segment populate the remaining 30% of the ganglion. Even cells derived from rostral and caudal regions relative to the somites, by and large, maintain a topographical segregation within the ganglion, although a few cells of either segmental origin are always found within the territory predominantly colonized by the other.

Sympathetic neurons are also derived from the neural crest and retain nerve growth factor (NGF)-dependent survival in mature animals; two hypotheses have been proposed for autonomic gangliogenesis (Bronner-Fraser and Fraser, 1991). In the first, neural crest cells are thought to be multipotent giving rise to multiple derivatives in a variety of sites, and with the cell's differentiation dependent on instructive cues encountered along their migratory pathway or at their final sites of localization. Alternatively, sensory and autonomic precursor cells arise from a common progenitor during neural crest cell individualization, e.g. neural crest cells are predetermined to give rise to a single cell type. Cell lineage studies *in vitro* have demonstrated that multipotent neuronal crest cells also generate some clonal progeny that form either only neurons or glia; substrate manipulation can alter the fate of these multipotent cells (Stemple and Anderson, 1992).

1.2. Neuronal Heterogeneity in DRG

Originally, DRG neurons were classified into two main subpopulations, called types A and B, or referred to as large light (L) and small dark (S) neurons (Lieberman, 1976; Lawson *et al*, 1984). L and S neurons in mouse and rat DRG can be distinguished within 1 day after birth by their soma size distributions (Lawson, 1979), and by the immunoreactivity of type A neurons with RT97, a monoclonal antibody raised against the phosphorylated form of the 200 KDa neurofilament subunit (Lawson and Waddell, 1991).

L type neurons showed an aggregation of Nissl substance separated by pale areas of cytoplasm, whereas S type neurons had relatively evenly distributed Nissl substance, and were therefore darkly stained. The ultrastructure was confirmed with transmission electron microscopy (TEM) (Lieberman, 1976). L neurons contained clumps of ribosomes and rough endoplasmic reticulum separated into regions by the presence of microtubules and large amounts of neurofilaments (NF). In contrast, S neurons showed a denser distribution of organelles, few neurofilaments and plenty of Golgi bodies (Yamadori, 1970; Duce and Keen, 1977). L neurons in DRGs were labelled by RT97 against a structural protein, but S neurons were not (Sharp *et al.*, 1982; Perry *et al.*, 1991). In contrast, antibodies to peripherin, another intermediate filament, mainly labelled NF- negative neurons in mouse and rat DRG (Troy *et al.*, 1990). Electrophysiologically, L neurons (RT97-positive) have A fibres, whereas most S neurons (RT97-negative) have C fibres (Lawson and Waddell, 1991), Although S neurons also carried some Aδ fibres, approximately 60% of these in rat DRG have a long-duration somatic action potential, similar to S neurons with C fibres (for review see Lawson, 1992). Neurons with an L type ultrastructure were evident over the entire size range of the neuronal population in mouse and rat DRGs, whereas structurally the S neurons were limited to the

lower end of the distribution (Lawson *et al.*, 1984). The overlapping size distributions of two subpopulations also existed in cat and chick DRG neurons (Plenderleith *et al.*, 1988).

More recently, by using a combination of ultrastructural features and cytochemical markers, neuronal phenotypic heterogeneity has been demonstrated in mouse DRGs *in vivo* to consist of seven distinct subpopulations (Sommer *et al*, 1985): The neuronal soma size for A (representing 36% of the whole ganglionic population), B (63%) and C (1%) main neuronal types were 30-50μm, 20-35μm and <20μm respectively. Both A and B type neurons were further subdivided into three subpopulations: Aα, Aβ, Aγ, Bα, Bβ, Bγ (Sommer *et al.*, 1985). A similar classification has been reported for rat DRG neurons: A1 (25-31%), A2 (2-8%), A3 (5-8%); B1 (46-56%), B2 (10%), B3/C (0-1%) (Duce and Keen, 1977; Rambourg, 1983). In these classifications, A type neurons are comprised of the L neurons with respect to the ultrastructural distributions of rough endoplasmic reticulum, but do not fully represent the entire population of L neurons. The ultrastructural differences among subpopulations are not dependent on the neuronal activity status since stimulation did not alter the relative proportions of each group (Duce and Keen, 1977).

Biochemically defined neuronal populations have attracted extensive attention following the discovery that a variety of peptides, enzymes and carbohydrates existed in specific DRG subpopulations identified by their size distribution (Prince, 1985; Hunt and Rossi, 1985; Lawson, 1992). By combining studies on the biochemical, cytochemical and morphological properties, S neurons may be divided into three subgroups (for review see Lawson, 1992):

(a) A Fluoride-resistant acid phosphatase (FRAP)/thiamine monophosphatase (TMP) group: FRAP did not overlap with substance P immunoreactive (SP-ir) or somatostatin immunoreactive (SOM-ir) neurons in rat DRGs; TMP localized exclusively within small DRG neurons and had a similar distribution to FRAP (Knyihar-Csillick *et al.*, 1986). This group had a larger mean size than SP ir-neurons and expressed no high-affinity NGF receptor. The FRAP/TMP distributions were restricted to the Bα and Bβ subpopulations of mouse S neurons (Sommer *et al.*, 1985). This group, with pheripheral fibres projecting to skin and skeletal muscle, account for approximately 50% of DRG neurons (Dodd *et al.*, 1983).

(b) A Somatostatin (SOM) group: In this group, SOM-ir was apparently restricted to FRAP-negative neurons, representing 5-15% of rat DRG neurons, and 10-30% of mouse DRG neurons, and co-existing with 2C5 (an antibody against an oligosaccharide epitope, mainly labelling small neurons) (for review see Lawson, 1992). These neurons did not express high-affinity NGF receptor (Verge *et al.*, 1989a, b). They predominantly project pheripheral fibres mainly to skin, and to a lesser extent to muscle (Green and Dockray, 1988), central fibres projecting to laminae II and I of the dorsal horn (Nagy and Hunt, 1982; for review see Hunt *et al.*, 1992). They function in immune responses and heat nociception respectively (O'Dorisio *et al.*, 1985; Kuraishi *et al.*, 1985; Tiseo *et al.*, 1990).

(c) A Substance P (SP) group: These neurons express high-affinity NGF receptor and mRNA for GAP-43, a growth-associated phosphoprotein, involved in axonal regeneration (Meiri and Burdick, 1991). Unlike S neurons in the FRAP/TMP and SOM groups, these neurons have a smaller mean size (Price, 1985; Kawatani *et al.*, 1986; Garry *et al.*, 1989) with C fibres, without neurofilaments, and Aδ fibres with neurofilaments. Their pheripheral fibres

projected mainly to viscera, and to a lesser extent to muscle and skin, whilst central fibres projected to laminae I and II of the dorsal horn. These are involved with neurogenic inflammation (Holzer, 1988), and with sensing mechanical (Kuraishi *et al.*, 1985) and cold noxious stimuli (Tiseo *et al.*, 1990). SP-ir represents 20% of the population in rat DRG and 10-30% in mouse DRG, whereas about 20-30% of the SP ir-proportion in rat DRG were L neurons with Aδ fibers (McCarthy and Lawson, 1989). The 20% SP-ir, 10% SOM-ir and about 50% FRAP neurons roughly represent the total proportion of B and C type neurons (70% in rat, 64% in mouse). L neurons are all neurofilament-rich, and in rat have myelinated fibers as I and II proprioceptive afferents (Szabolcs *et al.*, 1989). Aβ and Aγ neurons form 24% of the mouse DRG neurons, and the A1 forms 25-31% rat neurons and express carbonic anhydrate (CA) (Wong *et al.*, 1983; Robertson and Grant, 1989).

More recently, various classes of DRG neurons were identified by deprivation of neurotrophic factors and by the staining characteristics of central projection; small-sized neurons projecting to laminae I and II of the dorsal horn, and presumably subserving nociception and thermoreception, larger-sized neurons projected to more ventral laminae of spinal cord (DiStefano *et al.*, 1992).

1.3. Neurotrophic Factors

1.3.1. The NGF Family and Their Effects on Developing DRG Neurons: NGF was isolated from adult male mouse submandibular glands (Cohen, 1960), and was further characterised as two distinct forms. 7S NGF (named after its sedimentation coefficient) is a high molecular weight complex containing three different (α, β, and γ) subunits (Varon *et al.*, 1967) and associated as $\alpha_2\beta\gamma_2$ (Varon *et al.*, 1968; Smith *et al.*, 1968). 2.5S NGF (Bocchini and Angeletti,

1969) is indistinguishable from the β subunit alone with respect to its biology and immunology, although proteolytic modification does exist at both termini (for review see Altin and Bradshaw, 1993). The β subunit contains 118 amino acids (Angeletti and Bradshaw, 1971), tightly associated by three interchain disulfide bonds (Angeletti et al., 1971). Conserved residues have been found between different species and considered to contribute directly to receptor interactions or to maintaining the three-dimensional structure (Altin and Bradshaw, 1993). Hence the β subunit is the major polypeptide for NGF bioactivity. NGF mRNA is synthesized from a single-copy gene on chromosome 1 in humans (Francke *et al.*, 1983).

The members of the nerve growth factor gene family consist of NGF (Levi-Montalcini and Hamburger, 1953), brain-derived neurotrophic factor (BDNF) (Barde *et al.*, 1982; Leibrock *et al.*, 1989; Lindsay, 1993), neurotrophin-3 (NT-3) (Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990a, b), neurotrophin-4 (NT-4) (Hallböök *et al.*, 1991) and neurotrophin-5 (NT-5) (Berkemeier *et al.*, 1991). High affinity receptors related to these factors have been determined depending on their gene sequence, including $p140^{trk}$ (Hempstead *et al.*, 1991; Kaplan *et al.*, 1991; Klein *et al.*, 1991), $p140^{trkB}$ (Soppet *et al.*, 1991; Squinto *et al.*, 1991) and $p140^{trkC}$ (Lamballe *et al.*, 1991). The different members exert their function, by binding to the relevant receptors (Meakin and Shooter, 1992). NGF, but not BDNF, binds with $p140^{trk}$ and mainly has effects on the neural crest-derived smaller sized DRG neurons. BDNF, NT-4 and NT-5 bind with $p140^{trkB}$. BDNF affects neural crest-derived DRG neurons of a larger size and placode-derived sensory neurons, but not sympathetic and parasympathetic neurons (for review see Lindsay, 1992). NT-5 affects the neuronal survival and neurite outgrowth of 70% of DRG and 30% of sympathetic neurons, but not parasympathetic neurons (Berkemeier *et al.*, 1991). NT-4 has only been found in Xenopus and snake to date and has a

similar function to BDNF. NT-3 binds with $p140^{trkC}$ (Lamballe *et al.*, 1991), although it was reported to bind also with trk and trkB (Cordon-Cardo *et al.*, 1991), and has a widespread specificity for DRG, sympathetic and nodose ganglionic neurons (Review see Smith and Jiang, 1994). In general, the populations of DRG neurons which express distinct high affinity neurotrophic receptors mediating the relevant factor's function are non-overlapping (Meakin and Shooter, 1992). This also presents the possibility for a further means of classifying DRG neurons. On the other hand, low affinity NGF receptor has been cloned and identified as a 75 KDa protein (Chao *et al.*, 1986), which bound BDNF, NT-3 and NT-4 with similar affinities to NGF (Rodriguez-Tébar *et al.*, 1990, 1992).

Yip and Johnson (1984) suggested that developing sensory neurons received trophic support from both their peripheral and central target fields, although others considered DRG sensory neurons predominantly obtained NGF from their peripheral target (Korsching and Thoenen, 1985). NGF, and a high level of NGF mRNA, is present in developing skin (Harper and Davies, 1990), and in other tissues and organs, such as the heart, ductus deferens and iris (Korsching and Thoenen, 1983b; Shelton and Reichardt, 1984; Heumann *et al.*, 1984; Korsching and Thoenen, 1988). Futhermore, NGF was present in spinal nerves distal to the DRGs, but was not found in spinal nerve roots or in the spinal cord (Korsching and Thoenen, 1985), which suggested it was unlikely that DRG neurons obtained NGF via their central branches. The central nervous system (CNS) is the main site of BDNF mRNA expression, especially in the hippocampus, cerebral cortex and cerebellum, and in their target fields, the spinal cord and hindbrain (Leibrock *et al.*, 1989; Hofer *et al.*, 1990; Maisonpierre *et al.*, 1990a).

BDNF mRNA was detected in heart, lung and muscle at very low levels (Review see Davies, 1992). Embryonic DRG neurons *in vitro* are partially supported by BDNF (Barde *et al.*, 1982). Evidence suggests that DRG neurons may obtain the trophic support of BDNF via a central projection since the highest levels of BDNF are found in the brain, together with appreciable amounts in the spinal cord also (see Davies, 1992). DRG neurons may obtain neurotrophin-3 (NT-3) from both their central and peripheral targets. NT-3 mRNA is present in the spinal cord at much higher levels than BDNF mRNA during the period of innervation by DRG neurons (Maisonpierre *et al.*, 1990b). The level of NT-3 mRNA is also higher than that of NGF mRNA in developing cutaneous tissue, and is expressed in several peripheral tissues innervated by sensory neurons for example skeletal muscle, heart, skin, gut and liver (Davies, 1992), and may promote survival of proprioceptive neurons (Davies, 1986).

DRG sensory neurons demonstrate a variable requirement for neurotrophic factors. Traditonally, NGF was believed to promote the survival of the neural crest-derived neurons in DRG (Levi-Montalcini and Angeletti, 1968; Greene, 1977), in the dorsomedial part of the trigeminal ganglion (Levi-Montalcini, 1962; Ebendal and Hedlund, 1975; Davies and Lumsden, 1983), and in the jugular ganglion (Davies and Lindsay, 1985). NGF did not however support the survival of the placode-derived neurons in the ventrolateral part of the trigeminal ganglion which contains cutaneous sensory neurons of large diameter (Levi-Montalcini, 1962; Ebendal and Hedlund, 1975), or in the nodose (Lindsay and Rohrer, 1985), petrosal, geniculate, or vestibular ganglia (Davies and Lindsay, 1985). Recently, several findings have demonstrated that some proprioceptive sensory neurons derived from the neural crest are NGF-independent, whilst BDNF supports their survival during embryonic stages (Review see Davies, 1992). Embryonic trigeminal mesencephalic nucleus (TMN) neurons appeared not to rely on NGF for survival, but BDNF was

effective in maintaining their survival in culture (Manni *et al.*, 1965; Davies *et al.*, 1987b). In contrast to an almost total loss of sympathetic neurons, 85% DRG neurons degenerated when fetal rats were exposed to maternal anti-NGF antibodies via the placenta (Johnson *et al.*, 1980, 1983). The injection of anti-NGF into fetal rats depleted more than 90% of unmyelinated fibres and 35% of myelinated fibers in the dorsal roots of spinal nerves when examined four months after birth (Goedert *et al.*, 1984); proprioceptive neurons were of large-size and had myelinated nerve fibers. NGF did not prevent the death of proprioceptive neurons caused by nerve crush, but maintained the survival of small-diameter sensory neurons (Miyata *et al.*, 1986). Levels of NGF mRNA and NGF are high in developing cutaneous target fields (Harper and Davies, 1990), whereas they are barely detectable in skeletal muscle (Korsching and Thoenen, 1983a; Heumann *et al.*, 1984; Shelton and Reichardt, 1984). It is evident therefore that the specific neurotrophic factor requirements of sensory neurons are dependent upon the types of sensory structure which they innervate, rather than upon their derivation from neural crest or placode (Davies, 1992); for example, NGF supports small-diameter cutaneous sensory neurons, whereas BDNF supports large-diameter cutaneous sensory neurons and proprioceptive neurons. NGF also regulates the development of some neurons in the central nervous system, such as cholinergic neurons in the basal forebrain (Hefti, 1986; Koliatsos *et al.*, 1990).

Davies and co-workers reported that the survival of sensory neurons from early stage (E4) embryos may be independent of NGF and BDNF in studies of cultured sensory neurons prior to innervation of their targets (Davies and Lumsden, 1984; Davies, 1989; Vogel and Davies, 1991), and also from investigations of progenitor cells from cultured neural crest (Ernsburger and Rohrer, 1988), or placodes (Vogel and Davies, 1991). Embryonic chick DRG neurons only expressed low-affinity NGF receptors at E4 (E4: embryo at 4

days), whereas both low-affinity and high-affinity receptors were expressed by E6 (Sutter et al., 1979). In addition, NGF receptor mRNA was detected in premigratory neural crest cells which suggests that at least some undifferentiated neural crest cells may also express NGF receptors (Heuer *et al.*, 1990). On the other hand, certain target cells of sensory neurons, such as Merkel cells, express low-affinity NGF receptors and receptor mRNA prior to the onset of NGF synthesis in developing skin (Wyatt *et al.*, 1990). The low-affinity NGF receptors are not internalized when bound by NGF (DiStefano and Johnson, 1988). NGF, secreted and bound onto the surface of the target cells by their own low-affinity NGF receptors, is ultimately bound by the high-affinity receptors (since the dissociation contant of the high affinity receptor at $2x10^{-11}M$ is lower than that of the low-affinity NGF receptor at $2x10^{-9}M$) on the axons of NGF-dependent neurons (Review see Davies, 1992). The finding that the NGF concentration is reduced in mouse whisker pads, whereas the total amount of NGF mRNA in the target continues to rise after two days of innervation (Davies *et al.*, 1987a), demonstrates that NGF uptake occurs via the axons of the innervating neurons (Sutter *et al.*, 1979). The receptor-ligand complex is conveyed in the manner of fast axonal transport from the target to neuronal soma, to exert its effects on the neuronal survival and differentiation (Korsching and Thoenen., 1983a; Palmatier *et al.*, 1984).

Although the mechanism of NGF's action at the molecular level is still for from being completely understood, some findings may present valuable clues. High-affinity NGF receptors are present on sensory, sympathetic and central cholinergic neurons (Frazier *et al.*, 1974; Sutter *et al.*, 1979; Seiler and Schwab, 1984), but are absent from non-responsive neurons (Zimmerman and Sutter, 1980; Taniuchi *et al.*, 1986; Stach and Perez-Polo, 1987). The receptor-ligand complex is transported retrogradely to the neuronal soma (Seiler and Schwab, 1984; Johnson *et al.*, 1987) and gene expression triggered (Greenberg

et al., 1985; Kaplan *et al.*, 1991b). Long- and short-term NGF effects are initiated, including the induction of neurotransmitter-related synthetic enzymes (Tagg *et al.*, 1989), the synthesis of structural proteins (Leonard *et al.*, 1988), and the control of membrane properties (Skaper and Varon, 1983).

In addition to high-affinity NGF receptors, a low-affinity NGF receptor may be important for neuronal survival (Rabizadeh *et al.*, 1993). The low-affinity NGF receptor contained a GTP-binding regulatory protein (G protein) binding sequence at its carboxy terminus in the cytoplasmic domain (Altin and Bradshaw, 1993). In addition to mediating other intracellular signal systems, G proteins indirectly induce the production of inositol trisphosphate (InsP3), causing a release of Ca^{2+} from an internal calcium-sequestering compartment thus enhancing cytosolic Ca^{2+} concentrations (Alberts *et al.*, 1989). Intracellular Ca^{2+} may be an important index or determinant for altering trophic dependent survival and controlling naturally occurring cell death (for review see Rich, 1992). Ca^{2+} in the cytosol may lead to neuronal death due to unphysiological conditions (Schanne *et al.*, 1979; Choi, 1985; Rothman and Olney, 1986) since physiological concentrations of intracellular Ca^{2+} are required for neuronal survival. Davies and co-workers demonstrated that before nodose sensory neurons become dependent on neurotrophic factors, a reduction in intracellular free Ca^{2+} or depletion of either Ca^{2+}-regulated or InsP3-regulated intracellular Ca^{2+} stores resulted in neuronal death *in vitro*, whereas neuronal survival was enhanced by depolarization-induced Ca^{2+} influx (Larmet *et al.*, 1992). Intracellular Ca^{2+} concentrations of only 100nM are present in embryonic day 15 (E-15) DRG neurons, whereas by postnatal week 2, levels of 225nM are reached and these neurons showed reduced NGF dependence (Rich and Hollowell, 1990). With trophic factor deprivation, high extracellular K^{+} caused enhancement of intracellular Ca^{2+} and prevented neuronal death (Koike *et al.*, 1989).

In addition to NGF inducing the low affinity receptor, $p75^{NGFR}$, Ca^{2+} channels were also induced by NGF treatments (Levi and Alema, 1991). Reduction of $p75^{NGFR}$ and $p140^{trk}$ on adult DRG neurons may reflect NGF-independent survival by establishing a Ca^{2+} regulation system within adult neurons; Up-regulation of $p75^{NGFR}$ by NGF in both developing and adult peripheral neurons (Lindsay *et al.*, 1990; Miller *et al.*, 1991) suggests that NGF still affects Ca^{2+} influx and has a role in neuronal survival. By using embryonic animals, the distribution of $p75^{NGFR}$ was shown to be widespread in tissues or cells, including premigratory neural crest cells, sensory, sympathetic ganglia (Yan and Johnson, 1988; Heuer *et al.*, 1990), placode-derived neurons (all cranial ganglia) (Longo *et al.*, 1993), sciatic nerve (Heumann *et al.*, 1987b) and spinal cord (for review see Longo *et al.*, 1993). $p75^{NGFR}$ was also found in mesenchymal structures, including somites, muscle and tissue around hair follicles (Yan and Johnson, 1988; Heuer *et al.*, 1990). In addition, it was found in thymus (Ernfors *et al.*, 1988) and kidney (Sariola *et al.*, 1991). Motor neurons containing NGF-binding sites (Raivich *et al.*, 1985, 1987) were immunopositive for $p75^{NGFR}$ (Yan and Johnson, 1988) during development and can retrogradely transport ^{125}I-NGF (Wayne and Heaton, 1988; Yan *et al.*, 1988). On the other hand, $p75^{NGFR}$ bound the other members of neurotrophin family, BDNF, NT-3 and NT-4/5 with comparative low affinities (Rodriguez-Tébar *et al.*, 1990, 1992; Hallböök *et al.*, 1991). Depending on the significance of G proteins and Ca^{2+} signal transduction within neurons, the wide distribution of $p75^{NGFR}$ may suggest a general mechanism of cell survival control, which is regulated by various trophic factors depending on location in the body. However, the theory of high-affinity neurotrophin factor receptor-mediated neuronal death cannot explain:

1. How other neurotrophic factors bind to $p75^{NGFR}$;

2. The widespread embryonic distribution of $p75^{NGFR}$, even at very early stages;

3. Why uninervated neurons die in the embryo;

4. How adult neurons obtain levels of NGF independent-survival ability, compared to embryonic neurons;

5. The existence of neurotrophic factors, e.g. BDNF, within neurons themselves.

These questions can only be answered by the developmental balance of the intracellular regulatory systems and support from extracellular trophic factors (or other chemical reagents). Intracellular free Ca^{2+} concentrations and $p75^{NGFR}$ are important candidates to determine neuronal survival or death. Coincidently, intracellular free Ca^{2+} concentration is regulated via $p75^{NGFR}$ (Carge *et al.*, 1989; Albert *et al.*, 1989b). In contrast, $p140^{trk}$ mediates an NGF effect mainly via tyrosine kinase signal conduction, which has a close relationship with cell differentiation. The distribution of $p140^{trk}$ and other high affinity receptors only on neurons gives further evidence for their vital role in mediating neuronal differentiation.

Neurotrophic factors have been shown to be essential for the selective survival of DRG neurons (for review see Smith and Jiang, 1994). In addition to the NGF family, other neurotrophic factors have been discovered and are mainly classified into three other families by their gene sequences (Review see Fallon and Loughlin, 1993). These include fibroblast growth factors (aFGF and bFGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF) families. In addition, ciliary neurotrophic factor (CNTF) (Manthorpe *et al.*, 1982) and transforming growth factor beta (TGFβ) (Manson *et al.*, 1985) have generated interest, although these do not belong to any of the four main families of neurotrophic factors. In addition compounds such as acetyl-L-carnitine (Manfridi *et al.*, 1992), and retinoic acid (Quinn and De Boni, 1991; Chong *et al.*, 1994) have also been seen to have growth factor-like properties.

1.3.2. FGF: Two fibroblast growth factors, aFGF and bFGF have been isolated from embryonic and adult mammalian and chick CNS (Unsicker *et al.*, 1993). Basic FGF has been also found in the peripheral nervous system (PNS), including developing sympathetic neurons (Kalcheim and Neufeld, 1990), adrenal chromaffin cells (Grothe and Unsicker, 1990) and a subpopulation of DRG neurons (Unsicker *et al.*, 1993). A recent study revealed that bFGF was anterogradely transported by retinal ganglion cells (Ferguson *et al.*, 1990). G protein is suggested to be one of the second messengers which is retrogradely transported as the target-derived trigger, rather than FGF itself (Hendry *et al.*, 1993). Basic FGF is synthesized in many other cell types including fibroblasts and endothelial cells (Klagsbrun and Vlodavsky, 1988) and is stored in the extracellular matrix, where heparin-containing molecules may control its release to target cells (Flaumenhaft *et al.*, 1989; Globus *et al.*, 1989; Presta *et al.*, 1989). Various types of aFGF and bFGF receptors have been cloned and characterized (Lee *et al.*, 1989; Ruta *et al.*, 1989). Basic FGF has been found to bind with FGF receptors on cultured fetal hippocampal neurons, internalized and degraded (Walicke *et al.*, 1989). bFGF and aFGF up-regulate neuronal survival, neurotransmitter synthesis and neurite outgrowth *in vitro* by many types of CNS neurons, including those from the neocortex, hippocampus, septum, striatum, thalamus, mesencephalon, cerebellum, and spinal cord (Unisicker *et al.*, 1993). Ciliary ganglion neurons are also affected. It is still uncertain whether the effects of FGFs are direct or glial cell-mediated since the FGF receptor mRNA is lacking in relevant brain regions (Wanaka *et al.*, 1990). Administration of bFGF to the transected adult rat sciatic nerve rescues nearly the same number of DRG neuron as does NGF (Otto *et al.*, 1987), but does not involve specific retrograde transport in the sciatic nerve (Ferguson *et al.*, 1990).

Although fibroblast growth factor (FGF) and ciliary neurotrophic factor (CNTF) have broader effects, their mRNAs are absent in leader sequences for secretion (Abraham *et al.*, 1986; Stockli *et al.*, 1989).

1.3.3. EGF: Epidermal growth factor (EGF) and EGF mRNA has been found in various CNS regions, including the hypothalamus, olfactory bulb, cortex, striatum and brain stem, especially cerebellum (Schaudies *et al.*, 1989; Lazar and Blum, 1992). High levels of mRNA of transforming growth factor alpha (TGFα), another member of the EGF family, have been located in all brain regions examined (Review see Morrison, 1993). EGF receptors were found on neurons in the basal forebrain, caudate putamen, lateral septal nucleus, olfactory tubercle, neocortex and hippocampal formation (Loy *et al.*, 1987). EGF and bFGF have similar neurotrophic effects on CNS neurons although, in contrast to bFGF, EGF maintains cerebellar neuronal survival in neonatal rat (Morrison *et al.*, 1988). Even EGF at a concentration of 500ng/ml does not maintain trigeminal, nodose and DRG neurons *in vitro*, whereas TGFα was found to support the survival of cultured DRG neurons from neonatal rat, in a dose-dependent manner (Morrison, 1993). EGF receptor and the EGF receptor related *neu* oncogene product have been detected on DRG neurons (Kokai *et al.*, 1987; Werner *et al.*, 1988). In addition, TGFα is produced by keratinocytes which may present the required factor for both developing and mature DRG neurons (Coffey *et al.*, 1987).

1.3.4. IGF: Insulin-like growth factors I and II (IGF-I, IGF-II) are present predominantly in CNS being most abundant during development (Rotwein *et al.*, 1988). Functionally, IGFs may be involved in dendritic maturation, synaptogenesis or myelinization. IGF-I mRNA is found in peripheral targets of trigeminal and sympathetic nerves during the innervation stage (Bondy *et al.*, 1990; Bondy and Chin, 1991). IGFs support survival and cause an up-

regulation of neurite growth by sensory, sympathetic (Recio-Pinto *et al.*, 1986), cortical (Aizenman and de Vellis, 1987) and motor neurons (Caroni and Grandes, 1990). They induce oligodendrocyte differentiation and myelin synthesis (McMorris and Dubois-Dalq, 1988; Saneto *et al.*, 1988). A recent investigation demonstrated IGF-I also promoted the regeneration of adult sensory neurons in culture (Fernyhough *et al.*, 1993).

1.3.5. CNTF: Ciliary neuronotrophic factor (CNTF) was initially shown to support the survival and growth of chick ciliary ganglion (CG) neurons (for reviews see Manthorpe *et al.*, 1993; Smith and Jiang, 1994). The dependence of CG neurons on CNTF is maximal during development and then declines (Manthorpe *et al.*, 1981). DRG, sympathetic neurons and spinal cord motor neurons are also supported during defined periods (Manthorpe *et al.*, 1993). CNTF and CNTF mRNA have been shown to be present in the optic nerve and olfactory bulb of adult CNS, and were detected in the hippocampus (Stockli *et al.*, 1991). The largest amount of CNTF mRNA occurred in sciatic nerve, mainly produced in Schwann cells (Muir *et al.*, 1989; Rende *et al.*, 1992). It was not detected in adult skin and muscle (Stockli *et al.*, 1989). In addition, CNTF up-regulates the synthesis of neurofilament protein of rat hippocampal (Ip *et al.*, 1991) and ventral spinal cord neurons (Wong *et al.*, 1990) *in vitro*. It also increases ChAT (choline acetyltransferase) content and activity (Saadat *et al.*, 1989; Rao *et al.*, 1990), and induces the expression of LNGFR (low-affinity NGF receptor) in many CNS regions (Magal *et al.*, 1991a, b).

1.3.6. TGFβ: Transforming growth factor beta (TGFβ) 1-2 have been isolated from natural sources (Assoian *et al.*, 1983; Cheifetz *et al.*, 1987), whilst TGFβ 3-5 have been isolated from cDNA libraries (Puolakkainen and Twardzik, 1993). The distribution of TGFβs in the CNS and PNS, and their effects on neurons have been well documented (see Smith and Jiang, 1994). TGFβ2 and 3

have been found in adult DRG neurons *in vivo* and *in vitro*, where TGFβ3 expression can be induced by exogenous NGF (Unsicker *et al.*, 1991). In peripheral nerve injury, neurons synthesised and secreted TGFβ1 (Rogister *et al.*, 1993), which has not been reported in intact mammalian nervous tissue (Millan *et al.*, 1991).

1.4. Neurite Construction and Regeneration

The axonal structural framework is composed of neuronal microtubules which also have a role in vesicle transportation (Bunge, 1973). Tubulin and actin were shown to be present in the growth cones which mediate elongation of the regenerating and developing neurites (Fine and Bray, 1971). A microtubule-associated protein (MAP), *tau* protein, was found to induce microtubule assembly (Cleveland *et al.*, 1977) and to prevent microtubule depolymerization (Drubin and Kirschner, 1986). In the mature rat DRG, a *tau* isoform of 110KDa was discovered (Oblinger *et al.*, 1991), of considerably greater size than the predominant *tau* isoforms (50 to 70KDa) (Cleveland *et al.*, 1977). Neurofilaments (NFs) maintain the integrity and continuity of axons extended from mature neurons, but have very limited roles in actual neurite elongation (Hoffman *et al.*, 1985 and 1987). In adult rat DRGs, three NF subunits and their relevant mRNAs have been identified (Goldstein *et al.*, 1988). Peripherin, another intermediate filament protein, was seen to be up-regulated only in large neurons following axonal injury (Oblinger *et al.*, 1989; Wong and Oblinger, 1990b).

Growth associated protein-43 (GAP-43), a growth cone membrane protein (Goslin *et al.*, 1988), has been functionally implicated in the onset of axonogenesis (Meiri and Burdick, 1991), and also in the modulation of calcium channel conductance (Norden *et al.*, 1991). A virtually complete correlation

was seen between high levels of GAP-43 mRNA and high-affinity NGF receptors, and also between low GAP-43 mRNA and SOM immunoreactivity (Verge *et al.*, 1990). Ca^{2+} ions may regulate the stability of actin (Lankford and Letourneau, 1989) and growth cone behavior, with optimal Ca^{2+} concentrations permitting elongation, but higher levels arresting neurite outgrowth or causing growth cone collapse (Kater and Mills, 1991). In addition, neural cell adhesion molecule L1 (NCAM L-1), present in Schwann cells from cultured or denervated peripheral nerve, specifically supports neurite outgrowth (Bixby *et al.*, 1988; Seiheimer & Schachner, 1988). In contrast, the neurite inhibitors, IN-35 and IN-250, were shown to arrest neurite growth of postnatal DRG neurons (Brandtlow *et al.*, 1990).

In peripheral nerve transection of adult animals, mRNA levels, protein synthesis and the axonal transport of tubulin and actin were shown to be enhanced (Neumann *et al.*, 1983; McQuarrie, 1983; Hoffman *et al.*, 1985; Oblinger and Lasek, 1988; Tetzlaff and Bisby, 1990). Nearly all DRG neurons expressed GAP-43 mRNA (for review see Lawson, 1992), with higher levels of expression in small neurons (Woolf *et al.*, 1990). In contrast, neurofilament proteins (Hoffman *et al.*, 1985; Goldstein *et al.*, 1988) and mRNA for neurofilament subunits were down-regulated until reinnervation occurred (Hoffman *et al.*, 1987; Goldstein *et al.*, 1988). Protein and mRNA levels of rat *tau* protein also decreased following axotomy (Oblinger *et al.*, 1991). The production of tubulin, actin, peripherin and GAP-43 therefore represent essential elements for neurite regeneration; the partial disintegration of the axonal skeleton by down-regulation of neurofilaments and *tau* may be advantageous in permitting elongation and ramification of neurites and their terminals. Identical with these molecular studies, Richardson and Verge (1987) demonstrated that the regeneration rate for central projections of adult L-5 DRG was approximately tripled by ipsilateral sciatic nerve transection at 6 days, with

new thinly myelinated fibres 5-10 times more abundant ipsilateral to the transection 8 days after injury. The peripheral projection following injury was also regenerated in adult DRG neurons (for review see Johnson and Munson, 1992).

1.5. Neurotrophic Factors and Mature Neurons

1.5.1. Effects of NGF on Adult Neurons: The distribution, function and effects of NGF on developing neurons have been well studied and compared with other neurotrophic factors (see section 1.3.1.).

In recent years, NGF has also been shown to be an important neurotrophic factor for the differentiation of mature sensory neurons including the expression of neuropeptide phenotype (Johnson *et al.*, 1986; for review see Lindsay, 1992). In addition to NGF synthesis in the tissues and organs of adult animals, high affinity NGF receptors were detected on about 50-60% of postnatal DRG neurons (Verge *et al.*, 1989b; Lindsay, 1988). Adult neurons maintain the ability to transport retrogradely the NGF-receptors complex from the periphery to the cell body (Stoeckel *et al.*, 1975). The pattern of retrograde axonal transport of the target-derived neurotrophic factors of the NGF family has also shown that NGF may still be a significant factor from peripheral targets in adult animals (DiStefano *et al.*, 1992). When NGF, BDNF and NT-3 were injected into the sciatic nerve, fiber tract labeling was evident with NGF retrograde transport, reflecting a greater capacity of DRG neurons for transporting NGF (DiStefano et al., 1992). Retrograde transport of endogenous NGF has been reported by others (Korsching and Thoenen, 1983a; Palmatier *et al.*, 1984), which demonstrates that adult neurons normally transport NGF under physiological conditions. Transport specificity was demonstrated by an effective block with excess unlabeled homologous factor (DiStefano *et al.*,

1992). Once the NGF-receptor complex reaches the neuronal soma, NGF may exert very diverse effects on adult neurons: regulation of gene expression and content of substance P (SP), calcitonin gene-related peptide (CGRP) and somatostatin (SOM) (Lindsay and Harmar, 1989; Schwartz *et al.*, 1982; Delree *et al.*, 1992), modulation of the response of DRG neurons to capsaicin (Winter *et al.*, 1988), the calibre of axons, the content of neurofilaments and the position of the nucleus within the perikarya (Gold *et al.*, 1991). NGF also influenced the size of neuronal soma, neurite arborization and total neurite length (Yasuda *et al.*, 1990; Bedi *et al.*, 1992).

The different characteristics of NGF, BDNF and NT-3 have been shown in comparative studies of their retrograde transports, although their functions on postnatal DRG neurons have not been fully documented (DiStefano *et al.*, 1992). In dorsal root ganglia, NGF prodominantly labels smaller and intermediate populations, whereas BDNF and NT-3 label large neurons. Furthermore, unlike the distribution of NGF in spinal cord, BDNF and NT-3 are retrogradely transported to the ventral spinal cord, which may suggest they have a function as a regulator of adult motor neurons

1.5.2. Effects of NGF on Aged Neurons: Only very tentative evidence is available for the effects of neurotrophic factors on the nervous system of aged animals. In the CNS, the levels of NGF and NGF mRNA were found to be reduced in aged rat brain (Larkfors *et al.*, 1987). A decrease in NGF receptors has also been found in aged humans as well as in rat basal forebrain (Koh *et al.*, 1989; Hefti and Mash, 1989). However, neuronal populations may retain a normal plasticity with age (McNeill, 1983). A limited number of studies on NGF effects on PNS neurons from aged animals have recently been reported *in vitro*, including an up-regulation of neurite regeneration for aged mouse DRG (Horie *et al.*, 1991), enhanced survival of a subset of aged shrew trigeminal

sensory neurons (Fukuda *et al.*, 1991), and an increased survival for DRG neurons from aged rats (Manfridi, 1992) and mice (Jiang and Smith, 1992; 1993a).

1.6. Experimental Models for Studying the Effects of NGF

The distribution of neurotrophic factors, and localization of their receptors may be studied *in vivo* by retrograde transport methods (Stoeckel *et al.*, 1975; Korsching and Thoenen, 1983a), by immunohistochemistry (Loy *et al.*, 1987; Rende *et al.*, 1992), by autoradiography (DiStefano *et al.*, 1992) and by in situ hybridization techniques (Heumann *et al.*, 1984; Leibrock *et al.*, 1989; Hohn *et al.*, 1990). These parameters may be monitored and compared under both physiological and injury condition. However, it is often difficult to investigate the precise effects of the trophic factor due to the complexity of the *in vivo* environment. When neurotrophic factors are administrated into an animal, their diffusion from the site of introduction into injured sciatic nerves, their concentration in the blood, and any antibody production must be considered, and these can alter the effects of trophic factors or anti-trophic factors. Even if these problems are minimised, non-neuronal cell (NNC) and extracellular matrix mediation and the neuro-hormone axis regulation can not be fully determined.

Compared to the *in vivo* environment, neurons in culture are maintained in a simpler state, more suitable for examining the effects of neutrophic factor. However, neuronal loss during cell dissociation has often been a problem in enriched neuronal culture (Lindsay, 1988; Yasuda *et al.*, 1990). The sedimentation coefficient (during centrifugation) and anchorage area (during pre-implantation steps) of different sized neurons can result in neuronal loss. If a subpopulation of NGF-dependent neurons is not retained during culture

initiation, the data obtained from such an experimental model can be seriously limited for the evaluation of NGF's function or role. Neurons in culture often reaggregate, i.e. they mimic neuronal characteristics of normal growth and development *in vivo*. Cell growth in populations is an essential requirement for cell survival and differentiation (Alberts *et al.*, 1989). However, cell reaggregation presents a disadvantage to neuronal monitoring *in vitro*. Although single-cell culture can avoid such problems, it can not satisfy this essential growth requirement of normal cells and as such neurons often only survive for a one week maximum in such cultures (Yasuda *et al.*, 1990). The functional evaluation for a single trophic factor is therefore likely to be impossible by such model systems.

In view of these considerations, two modified cultures were introduced in the present studies which avoided significant neuron loss during the dispersal procedure:- co-culture with NNC's and also in NNC-limited neuron-enriched culture. In the first case with NNCs present (Smith and McInnes, 1986a), the environment is similar to that *in vivo*. In the second method, relatively pure neuronal cultures were obtained by administration of cytosine arabinoside (Smith and Orr, 1987) and enriched dispersed neuronal cultures were achieved, which minimised any NNC-mediated effect of NGF on the neurons.

In the study of neuropeptide-defined subpopulations *in vivo* experiments were also included for comparison with the *in vitro* study.

1.7. The Aim of Present Study

Many questions remain unanswered, or certainly are ambiguous, with respect to the effects of NGF on adult sensory neurons. There is, as stated, a paucity of data concerning the behaviour of neurons from aged animals and

their response to NGF. In view of this the present study set out to investigate further the response of primary cultured DRG neurons prepared from aged (24 months old) as well as adult (6 months old) animals. Three specific questions were addressed:-

(1) The effects of NGF on the survival of aged as well as adult cultured sensory neurons;

(2) The effect of NGF on the phenotype of cultured aged and adult neurons with respect to their content of substance P, somatostatin, calcitonin gene-related peptide, and neuropeptide Y;

(3) The effect of NGF on neurite regeneration by aged and adult neurons.

The intention therefore was to shed light on the neurotrophic requirement of mature neurons and their capacity to retain plasticity in culture with respect to both nerve fibre regeneration and phenotypic expression. The relevance of these findings to the ever increasing problems related to aging will be considered. Attention will also be given to the value of *in vitro*, in addition to *in vivo* studies, in helping to elucidate these questions.

Chapter II

MATERIALS AND METHODS

2.1 Animals

2.1.1. Animal Source: Male CBA mice were inbred in a light-reversed room in the Departmental animal house. They were maintained by standard hygienic procedures and kept separated from female mice. The behaviour and weight of animals were monitored routinely to exclude animals with disease, particularly neuromas.

2.1.2. Animal Selection: In many studies of NGF effects on the survival, differentiation, and regeneration of mature DRG neurons, the rat has become a favoured species, possibly because the microsurgery involved in removing DRGs may be more easily carried out on larger animals. Although the mouse has been studied less frequently, investigations from this laboratory have found this a convenient animal (Smith, 1991). The present study continues to extend the data from this species by using 6 months old adult mice [in the weight range of 30-40grams (g)] and also aged (2 years old) mice (weighing 25-30g).

2.2. Microsurgery for Removal of DRG

Balanced salt solutions were used during the preparation of DRG neuronal cultures.

2.2.1. Balanced Salt Solution: Hank's buffer has a relatively weaker buffering ability compared to Earle's buffer, but this system has the advantage of being balanced by air. The buffer's pH was easily monitored by colour changes of the phenol red present. Aliquots of fresh Hank's buffer were prepared by addition of the following to each sterile universal bottle:

Sterile Second distilled, deionised water:	**9.0ml**

10X Hank's Buffer (Gibco, BRL):	**1.0ml**
7.5 % Sodium bicarbonate (Gibco Ltd., U.K.):	**0.1ml**

To reduce cell metabolism and further damage during the dispersal of DRGs, Hank's buffer was cooled to 4°C before use.

2.2.2. Operation: Mice were humanely killed by cervical dislocation. The fur was sprayed with 70% ethanol to aid with sterilization procedures. The skin and body wall were cut along the midline, from the neck to pubis to open the abdominal and thoracic cavities and the front of neck. The organs, soft tissues, blood vessels, and particularly sympathetic ganglia were removed to expose the entire vertebral column. The dissection was frequently rinsed with Hank's buffer throughout all stages. The vertebral column and attached ribs were removed by cutting at the top of neck and at the lumbosacral joint, and along a dorsolateral line of the thorax. This preparation was pinned onto a sterilized plate and, under sterile conditions, the vertebral bodies removed by making two anterolateral cuts under sterile conditions to reveal the spinal cord and attached spinal roots. The preparation was transfered and pinned in a petri-dish containing Sylgard, and filled with Hank's buffer. These procedures were completed within 15-20 minutes (min) of killing the mice.

The rest of the operation was carried out under a stereobinocular dissecting microscope in a laminar flow cabinet. The spinal cord was lifted to one side to expose the nerve roots and the DRGs within the intervertebral fossae. A total of 45-55 DRGs were excised by the use of iridectomy scissors and forceps and the nerve stump and meninges carefully removed by using the points of sterile needles (Falcon, U.K.). The entire operation was completed within a maximum of 2 hours (hrs). The isolated DRGs were transfered into

enzyme solution to digest the connective tissues surrounding the neurons (see below).

2.3. Neuronal Culture

2.3.1. Cell Preparation: Two modifications to the methodology were employed to isolate neurons once the DRGs had been removed from the mouse: (a) overnight enzymic digestion and gentle teasing (Smith and McInnes, 1986a), which produced neuronal co-cultures with NNC present (see 2.3.3.-a); (b) A reduced enzymic digestion period followed by mechanical dispersion, which was used to initiate NNC-reduced neuron-enriched cultures (see sections 2.3.1.-b and 2.3.3.-b).

(a) Enzymic digestion and gentle teasing

DRGs were transfered into a Petri dish containing 3ml of 0.125% Type IV collagenase (Sigma, USA) in Dulbecco's Modified Eagle Medium (DMEM, Life Tech. Ltd. U.K.) and incubated overnight at 37°C. 0.125% collagenase was prepared as follows:

<u>0.25% Type IV collagenase stock solution:</u>

25mg collagenase powder was weighed and dissolved in 10ml DMEM. The solution was millipore-filtered and stored at -20°C.

<u>Freshly prepared 0.125% collagenase working solution:</u>

0.25% collagenase	**1.5ml**
DMEM	**1.5ml**

Next morning, the DRGs were transfered into Nunc Multiwell dishes (3 ganglia/well). Neurons were separated by gently teasing apart the softened DRGs with the points of two sterile needles.

(b) Reduced enzymic digestion and mechanical dispersion

DRGs were bisected along the longitudinal fibre axis in Hank's buffer, transfered to 1ml of 0.25% Type IV collagenase solution and incubated at 37°C for 45min, followed by 20min 0.025% trypsin (Sigma, U.S.A.) in DMEM. After rinsing in Hank's buffer, they were placed into a dish with 1ml of culture medium supplemented with $5x10^{-6}M$ cytosine arabinoside (Ara-C, Sigma, U.S.A.), and triturated by 10 passages through flame-constricted pipettes with gradually reducing diameters (1.00, 0.75, 0.50 and 0.25mm diameters for orifices). The cell suspension was diluted 10 times with full culture medium and counted under an inverse phase-contrast microscope in the 4 corner and middle squares of a hemacytometer. The number of cells per ml was calculated following the formula: $n \times 10^4/5$ (n = all cells in the 5 squares). Neuronal numbers were adjusted to 8,000 cells/ml for neuronal survival and immunocytochemistry examination, and 4000 cells/ml for the investigation of morphological phenotypes and geometry tracing.

<u>0.025% Trypsin solution was made as follows:</u>

2.5mg trypsin in 10ml of DMEM, millipored and stored at -20°C.

2.3.2. Coating Dishes: Before cells prepared by mechanical dispersion were plated, culture dishes and/or Thermanox tissue culture coverslips were coated with 0.2ml of sterile 10μg/ml bovine fibronectin (FN, Sigma) in the laminar flow cabinet, shaken to ensure the entire surface was covered and incubated at

37°C for 3hrs. Excess FN solution was discarded and the dishes or coverslips washed with three 10min rinses of Hank's buffer before using.

FN solutions were prepared as follows:

1mg/ml Stock Solution:

1mg FN with DMEM added to the 1ml scale in a 5ml cylinder. The resulting solution was aliquoted as 100µl per bottle and stored at -20°C.

10µg/ml Working Solution:

FN stock solution	**0.1ml**
DMEM	**9.9ml**

The working solution was stored at -20°C.

In the case of neurons prepared by gentle teasing, this step was omitted since these were cultured in the presence of non-neuronal cells which formed a network which permitted an anchorage for neuronal attachment (Smith and McInnes, 1986a).

2.3.3. Culture Initiation and Maintenance: DMEM, with the addition of various supplements, was selected as the culture medium in present study. It was stored at 4°C before use.

Standard Culture Medium

10ml aliquots of the standard medium were freshly prepared as follows:

DMEM (Life Tech. Ltd., U.K.)	**8.65ml**
Fetal calf serum (FCS) (Life Tech Ltd., UK)	**1.00ml**

Glucose stock solution (Sigma, U.S.A., 180 g/L)	**0.25ml**
Gentamycin (Flow Labs.) (10mg/ml)	**0.10ml**

Glucose Stock Solution was made as follows:

1.8g glucose was added to 10ml DMEM with stirring. Once the glucose powder was completely dissolved, the stock solution was millipore filtered into a sterile Universal bottle and stored at -20°C.

Two culture systems were employed:-

(a) Co-culture system

In this case, each well of the Nunc Multiwell dishes received the cells of 3 DRGs (one cervical, one thoracic and one lumbar ganglion). The cultures were incubated in a humidified air mixture containing 5% CO_2 and 95% atmosphere at 37°C.

Cultures were maintained for 14 days in vitro (div) for the experiments of NGF concentration effects on survival, in which the volume of medium was kept at 0.5ml/well, and up to 29div for the experiments on the age-associated changes of NGF-dependence on survival and neuronal soma morphology where 1.2ml medium was added per well. Medium was replaced every 3 days.

(b) NNC-reduced culture

In this, Ara-C was introduced into the medium to inhibit NNC proliferation.

Ara-C solutions were prepared as follow:

2mM Ara-C Stock Solution:

4.86mg Ara-C powder was placed in a 10ml cylinder and DMEM was added. The stock solution was millipore filtered and stored at -20°C.

5 X 10^{-6} M (5μM) Working Solution:

Ara-C stock solution	**25μl**
Culture solution	**10ml**

A 10μM Ara-C working solution was also used in some experiments.

2.3.4. Treatment and Analysis:

(a) For co-culture

The medium in test wells was supplemented with 2.5S NGF (Sigma, USA) at final concentrations of 25, 50, 100 and 200ng/ml for the experiment of NGF concentration effect; and at 100ng/ml NGF throughout the majority of other experiments. Control wells, i.e. without exogenous NGF, were included.

10μg/ml NGF Stock Solution:

A 10μg/ml NGF stock solution was aliquoted as 50μl per bottle and stored at -20°C.

NGF Working Solution:

Initially, the 50μl stock solution was diluted 50 times by addition of 2.5ml DMEM to obtain 200ng/ml NGF. 100, 50 and 25ng/ml NGF solutions

were prepared by dilution with DMEM, following the law of multiple proportions.

Neuronal survival was monitored by an inverted phase-contrast microscope with numbers counted, including blind assessments, in 3 random standard reticule fields at various culture periods, and expressed as an absolute number per field (Orr and Smith, 1988). Statistical analysis by Analysis of Variance (ANOVA) (Statgraphics, STSC Inc., Rockville, MD) was carried out. Mean neuronal soma diameters (square root of the shortest X longest diameter) and shape index (shortest/longest diameter) were measured from photographs and distributions compared for adult and aged neurons (Fukuda, 1985).

(b) For NNC-reduced culture

For the experiments of NGF concentration effects on SP, CGRP, NPY and SOM expressions, the medium in test wells contained 2.5S NGF at final concentrations of 10 and 100ng/ml. 100ng/ml NGF was employed throughout the majority of the other experiments. Equal numbers of wells without addition of exogenous NGF were included in all experiments..

Neuronal survival was monitored and statistical analysis by ANOVA carried out for NNC-reduced cultures as described for co-cultures. In the experiments for time-course studies of SP expression, cultures were maintained for 19 days in vitro (div). 9div represented the time of peak expression however, so that in most experiments, cultures were maintained for this time period. Medium was replaced each 72 hours. Cells were further processed for immunocytochemistry (see 2.5.), scanning electron microscopy (2.6.) and image analysis (see 2.7.)

2.4. Fixation and Sectioning

2.4.1. Fixative and Fixation: A good fixative is essential to obtain optimal preservation of structure and antigenicity, retention of accessibility of the antigen to the antibody molecules and immobilization of the antigen (Van Noorden, 1986). Formaldehyde, which penetrates tissues very quickly, was used as a temporary stabilizer, whereas permanent cross-linkage can be achieved by glutaraldehyde. Formaldehyde can be employed alone for the localization of neuropeptides, although glutaraldehyde has been used successfully in immunocytochemistry (Richards *et al.*, 1986). Karnovsky's fixative composed of 1% formaldehyde and 2.5% glutaraldehyde buffed with 0.1M sodium cacodylate was used in the present study for the fixation of DRGs.

Karnovsky's fixative:

	Volume	Final conc.
Formaldehyde	**5ml**	**1%**
25% Glutaraldehyde	**50ml**	**2.5%**
5.4% Glucose	**15ml**	**0.16%**
0.2M Na Cacodylate buffer	**250ml**	**0.1M**
Second distilled water	**180ml**	

Whole cervical (C5), thoracic (T2) and lumbar (L3) ganglia from aged and adult mice were excised and immediately fixed with Karnovshy's fixative to examine neuropeptide expression in vivo, at the time of culture initiation. DRGs were fixed in Karnovsky's fixative at 4°C overnight, and stored in 0.1M cacodylate buffer at 4°C before sectioning.

2.4.2. Frozen Sectioning: Frozen sections were prepared for immunocytochemical studies in vivo since sectioning at low temperature is an advantage for preservation of antigenicity. Water is the most brittle component in frozen material and it is ice crystal formation which damages tissues (Richards *et al.*, 1986). In order to reduce ice crystal size and enhance the structural integrity, specimens were infused with chemically inert hydrophilic substances of low molecular weight, e.g. sucrose. In addition, liquid nitrogen is notoriously slow at freezing materials, and may result in the formation of large ice crystals. To minimise this, isopentane was introduced to the procedure (Richards *et al.*, 1986).

The permeability of DRGs was improved by incubation in 15% sucrose in 0.1M PBS at 4°C overnight; frozen in a container filled with isopentane, left in liquid nitrogen until the nitrogen ceased to boil, and mounted on a stock in freezing OCT Embedding Medium (Miles Inc, Elkhart, IN, USA) to form a hard block. The blocks were trimmed, in the shape of a trapezium, and cut as 10μm thick sections at 90μm intervals, on a Bright Cryostat at -30°C. A droplet of water was dripped on a glass slide previously coated with BIOBOND (Cardiff Business Tech. Cent., U.K.). The slide was inverted and touched gently to the frozen section, so that the sections could be collected and stored at -20°C.

2.5. Immunocytochemistry

Immunocytochemistry is aimed at high sensitivity and specificity. The avidin-biotin system was first applied to immunocytochemistry for fluorescence microscopy in 1977 (Heggeness and Ash, 1977). The reaction between avidin and biotin by non-covalent bonds is very rapid, sensitive and stable. This non-covalent binding is thought to be via one of the strongest bonds known in nature. Biotin is biotinylated to the second antibody by an indirect method and

the antigen signals amplified. When the avidin-biotin-peroxidase complex (ABC) is added, a second amplification of the antigen signals is completed. The ABC method ensures the maximal sensitivity of immunocytochemistry since an antibody can be coated with 150 biotin residues and each of them is able to conjugate with an ABC, e.g. a very high marker/antigen ratio (Coggi *et al.*, 1986). Peroxidase functions as a marker, which catalyzes the chemical reaction of hydrogen peroxide and thereby allows staining visualization in the presence of di-azo-aminobenzene (DAB). In view of this, the ABC method was chosen to examine neuropeptide expressions in present study.

2.5.1. Reagents Concerned: The reagents used in the ABC method were prepared as follows:

Phosphate Buffer (PB)

0.2M PB Stock Solution

Solution A: 18.72g NaH_2PO_4 in 600ml second distilled H_2O

Solution B: 42.45g Na_2HPO_4 in 1500ml second distilled H_2O

560ml Solution A was mixed with 1440ml Solution B.

0.1M PB working solution

0.2M PB diluted with an equal volume of second distilled water.

Phosphate Buffer Saline (PBS)

0.02M PBS

36g NaCl was mixed with 200ml of 0.2M PB and second distilled water added to 2L (litres).

0.01M PBS

0.02M PBS was diluted with the same volume of second distilled water.

4% Formalin in 0.1M PB

Formalin	**40ml**
0.1M PB	**960ml**

Triton X-100 solution

0.5% and 0.3% Triton X-100 in PBS were made by adding 9950μl and 9970μl 0.01M PBS to 50μl and 30μl Triton X-100 respectively.

0.1% Triton in 0.01M PBS

1 volume of 0.3% Triton supplemented with 2 volumes of second distilled water.

1% H_2O_2 in methanol

30% H_2O_2	**100μl**
methanol	**2900μl**

20% blocking serum

normal goat serum (NGS)	**400μl**
0.01M PBS	**1600μl**

Diluent for primary antibody (3% NGS)

NGS	**120μl**
0.5% Triton X100 in 0.01M PBS	**3880μl**

Primary antiserum

Anti-SP

1:100 anti-SP stock solution

(a). 1:2 frozen anti-SP (Incstar, USA)	**5.0μl**
NGS	**2.5μl**
1% sodium azide	**2.5μl**
0.01MPBS	**245.5μl**
(b). 1:4 frozen anti-SP (RIN7451 Rabbit antiserum, Peninsula, USA)	**5.00μl**
NGS	**1.25μl**
1% NaN_3	**1.25μl**
0.01M PBS	**117.50μl**

Solution was vigorously mixed and stored at 4°C.

1:20,000 (20K) anti-SP working solution

1:100 SP stock solution	**7.5μl**
Diluent for primary antibody	**1492.5μl**

Prepared freshly before using.

Anti-SOM, CGRP, NPY, NF-200 and GFAP

Commercially purchased primary antisera to neuropeptides had all been raised in rabbits. Following the method described above, the stock solutions for anti-SOM-14 (RAS8001N, Peninsula, USA), CGRP (poly-clonal, Affiniti, UK), NPY (Peninsula, USA), NF-200 (N-4142 Rabbit-Anti-bovine, Sigma, USA) and GFAP (DAKO, Denmark) were made at the final concentrations of 1:100, 1:1,000, 1:400, 1:100 and 1:100 in 0.01M PBS containing 0.01% sodium azide and 1% NGS, respectively. Working solutions were routinely prepared with diluent to give primary antibody at the final concentrations of 1:4K, 1:10K, 1:30K and 1:40K, prior to the examination of a wide concentration

range for each antiserum (see 2.5.4.). The working solutions of primary antisera were freshly prepared before use.

1:200 biotinylated second antibody (Vector, USA) (freshly prepared)	
NGS	**30.0μl**
0.01M PBS	**1960.0μl**
biotinylated second antibody	**10.0μl**

ABC (Vector, USA)	
Solution A	**40.0μl**
0.01M PBS	**1920.0μl**
Soluiton B	**40.0μl**

mixed immediately and allow to stand for 30 min.

0.05% DAB in 0.01% H_2O_2 /0.1M PB	
DAB	**1 aliquot**
0.1M PB	**50ml**

filtered and 15μl H_2O_2 added before using.

2.5.2. Procedure for Immunostaining: Fixed cultures or sections (i.e. the specimen) were rinsed with 0.1M PBS. 0.1% Triton X-100 in PBS and 1% H_2O_2 in methanol were each added for 10min to enhance membrane permeability and to block endogenous peroxidase respectively, and subsequently rinsed with PBS. Specimens were first incubated with 20% NGS in PBS for 60min to block non-antigenic sites at room temperature prior to incubation with the relevant primary antisera from rabbit against mouse antigens: SP, SOM, CGRP, NPY, NF and GFAP. Specimens were then incubated for 60min with 1:200 biotinylated goat anti-rabbit second antibody, followed by the 1h ABC incubation at 20°C. Two or 3 rinses were included

between incubations. Neuropeptide immunoreactivities were visualized by reaction with 0.05% DAB containing 0.01% hydrogen peroxide in 0.1M PB (pH 7.4). Finally cultures were washed 3 times with PB and distilled water and mounted in glycerol.

Staining controls were included as follows: (i) substitution of PBS and the diluent for primary antiserum, to omit one step during the entire staining procedure; (ii) the use of normal rabbit serum (NRS) instead of primary antiserum (at the same concentration), and (iii) pre-absorption of the antisera with the corresponding antigens (see 2.5.3.).

2.5.3. Pre-absorption of Primary Antisera: To test for specificity, primary antisera were incubated with relevant antigens at 4°C, for 48hrs, prior to incubation with the specimens. The antigens concerned were diluted as follows:-

(a) Antigens

<u>$1x10^{-4}$M SP (Sigma, USA)</u>	
SP (Peptide content 83%, MW=1347.6)	**1000.0μg**
second distilled water	**6172.8μl**
<u>$1x10^{-3}$M SOM (Sigma, USA)</u>	
SOM (Peptide content 90%, MW=1637.9)	**1000.00μg**
0.05M Acetic acid	**549.48μl**
<u>$1x10^{-3}$M CGRP (Sigma, USA)</u>	
CGRP (Peptide content 75%, MW=3806.3)	**100.00μg**
Second distilled water	**19.70μl**

$1x10^{-3}$M NPY (Sigma, USA)

NPY (Peptide content 83%, MW=4271.7)	**100.00μl**
Second distilled water	**19.43μl**

0.05M Acetic acid (MW=60)

Acetic acid	**3mg**
second distilled water	**1ml**

(b) Procedure for pre-absorption

Antigens were added to the corresponding antisera (1:20K SP, 1:10K SOM, 1:30K CGRP and NPY antisera) and shaken vigorously. They were stored at 4°C for up to 48hrs.

(c) Test concentrations

$1x10^{-4}$, $5x10^{-5}$M, $2x10^{-5}$M, $1x10^{-5}$M and $1x10^{-6}$M antigen solutions were examined to select the optimal concentration of antigens neutralizing the relevant antigens.

2.5.4. Antiserum Titer, Treatment and Analysis: Dilutions of 1:20K, 1:40K for anti-NF, 1:1K-1:80K for anti-SP and anti-SOM, 1:5K-1:40K for anti-NPY and 1:10K-1:30K for anti-CGRP were made following the law of multiple proportion, and examined to select the best titers for each antiserum.

The stained preparations were viewed under an inverted microscope. At least 7,000 neurons were counted from 4 experiments for each neuropeptide detection in both adult and aged cultures. The results were expressed as the

t-testing and ANOVA (and further investigated by least significant differences testing.

2.6. Scanning Electron Microscopy (SEM)

SEM was used in the present study to confirm neuronal identification under the phase-contrast microscope, to examine neuronal integrity status in the presence or absence of NGF, to investigate any morphological changes between adult and aged neurons, and to distinguish neurite numbers extending from individual neurons.

Culture medium was replaced with 3% glutaraldehyde in 0.1M sodium cacodylate with 3% glucose for 30min. Cultures were left in 0.1M sodium cacodylate with 3% glucose at 4°C, overnight before post-fixation with 1% osmium tetroxide for 15min, followed by a 5min buffer rinse. They were then dehydrated in a gradient of ethanols (50, 70, 90 95, 100, 100 and 100%), with 10min in each.

Coverslips or culture dishes were transfered into the boat of a CPD bomb and covered with 100% ethanol. This was replaced with liquid carbon dioxide and subsequently rinsed twice, 30min for each. Critical point drying was then accomplished at 35°C. The coverslips or dish substrata were taken or cut from culture dishes and adhered on the stocks. The stocks were transferred to a Gold Sputter Coater and the preparations coated with gold using 0.2Torr, 1.2KV for 5min, prior to viewing on a Jeol JS100 scanning electron microscope.

2.7. Estimation of Neuronal Geometry

Adult and aged neurons were monitored blindly for neurite growth both with or without exogenous NGF supplementation at 1, 3, 6 and 9div. The tracing work was carried out using a video-project computer-assisted program (Videoplan MOP-2) and digitizing tablet. The parameters chosen were as follows (Fig.**2.1.**):

Soma size--mean diameter of neuronal body $[(a.b)^{-2}$, a: major diameter; b: minor diameter); Neurite number--Number of neurites emerging from an individual neuron; Major neurite length--Maximal extension of the neurite (excluding the length of any branches), i.e. the distance from the neuronal soma to the distal margin of the neurite; Branch number--number of branches per neurite (>10μm). Entire length-- the major neurite length plus the lengths of all its branches; Total length--summation of all neurites and branches belonging to a neuron, i.e. the whole extent of one neuron's processes. Neurons and their processes were either photographed under an inverted phase-contrast microscope and printed at 290x enlargement, or else the neuronal geometry was directly traced from cells in the culture dish. In the first case, the images on the monitor were clearer, however montage preparation was time-consuming. Images taken directly from cultures were often of lower contrast but this proved a convenient and faster method of analysis. Data collected were subjected to statistical testing using the Minitab programme for two way ANOVA.

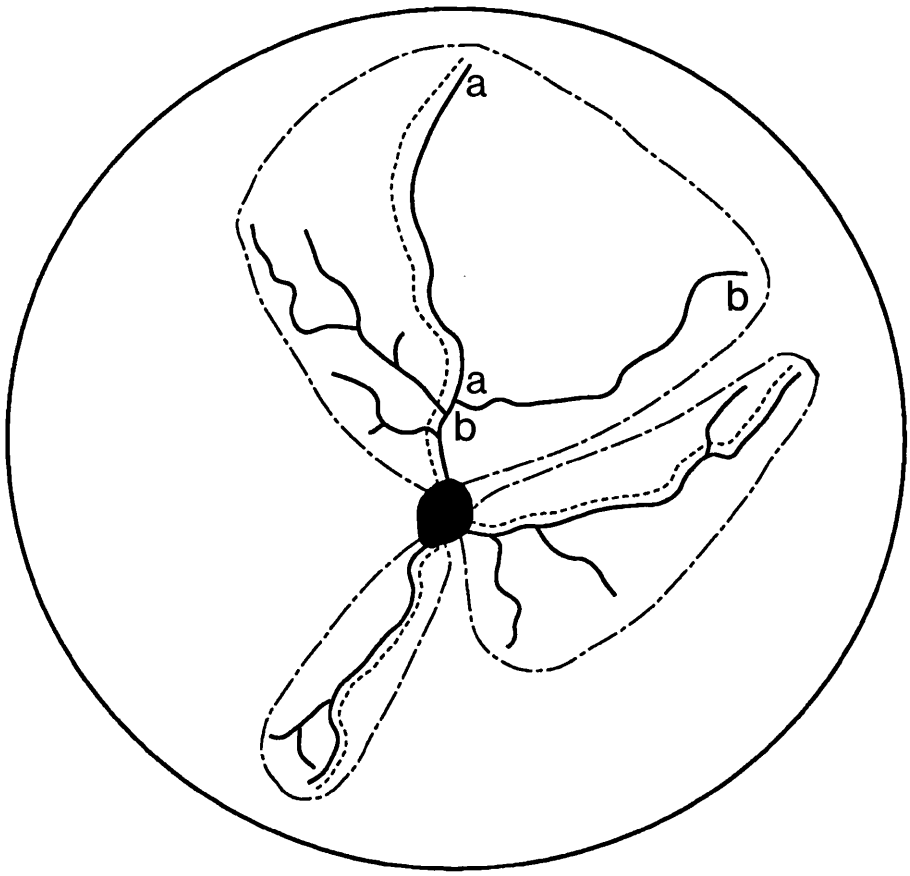

Fig.2.1. Diagramatic sketch to show parameters measured in neurite geometry tracings. The dotted line represents the major neurite length (3 major neurite lengths are shown on this neuron). The major neurite length and all branch lengths within each dotted circle represents the neurite entire length (3 neurite entire lengths would be recorded from this neuron). Summation of the 3 neurite entire lengths within the big circle gives the value of the neurite total length of a single neuron. Branch number is scored as the total number of branch points per neurite (neurites located at the top, right and bottom contain 6, 3 and 2 branches respectively, giving an average branch number of 3.7 for this neuron's neurites. Even if **bb** was longer than **aa**, **bb** was still taken as the branch and **aa** as the major neurite length since **aa** had another branch emanating from the opposite side from **bb**.

Chapter III

NGF EFFECTS ON NEURONAL SURVIVAL IN VITRO AND MORPHOLOGICAL SUBPOPULATIONS

Introduction

NGF has been shown by others to have a critical role in maintaining phenotypic expression and in the regeneration of neurites by adult sensory neurons (Lindsay, 1992). Controversy persists however as to whether NGF functions as a "surviving factor" for mature neurons. Adult sympathetic neurons died in NGF-immunized animals (Gorin and Johnson, 1980; Johnson *et al.*, 1983), whereas adult sensory neurons were not lost *in vivo* if deprived of endogenous NGF by use of an antibody (Schwartz *et al.*, 1982). However, NGF protected adult sensory neurons from cell death following nerve injury *in vivo* (Rich *et al.*, 1987). In *in vitro* studies with selective neuron-enriched cultures adult neurons were claimed to no longer require NGF for survival (Lindsay, 1988), whilst others showed that at least some adult neurons were still NGF dependent (Fukuda *et al.*, 1991; Manfridi *et al.*, 1992).

In order to investigate further the possibility of age-associated changes for the requirement of NGF in sensory neurons, the first part of the present investigations focused on the effects of exogenous NGF on the survival, population change and morphological phenotypes of primary cultured DRG neurons from adult (6 months) and aged (2 years) mice. A co-culture system with non-neuronal cells (NNCs) was initially used in this study; although endogenous NGF and NNC-mediated NGF effects can not be completely ruled out in such cultures, the system bridges between the *in vivo* and neuron-enriched and single-cell culture environments, which should help in resolving the contradictory findings of NGF's effects as a survival factor in both conditions.

In many studies to test the effects of *in vitro* trophic factors, single-cell cultures have been employed and represent a more elaborate method; however

many neurons do not survive the trauma of the complex separation protocols involved in single cell culture preparation, or die after a few days in culture (Yasuda *et al.*, 1990; Aguayo and White, 1992). For example, although 93% neuronal purity can be attained, neuronal losses as high as 97% have been reported during the preparation procedures (Yasuda *et al.*, 1990). In neuron-enriched cultures, "substantial (approximately 80%) neuronal losses during the dissociation and enrichment procedures" were reported (Grothe and Unsicker, 1987). Thus the possibility that NGF-dependent neurons did not survive the procedure can not be ruled out. Criticism can be made also of studies where only process-bearing neurons were scored as "surviving" (Lindsay,1988), since in these cases only 84% of the adult neurons remaining after the trauma of isolation met this criterion at 2div, and with reduced numbers by 7div Yet, the time for neurite regeneration should not be assumed to be synchronised within such cultures. In view of these drawbacks, a modified neuronal-enriched culture system was devised in a further set of experiments in the current study. This culture system retained the procedures of two enzyme digestion steps followed by mechanical dispersion to produce individual cells but without the need for centrifugation steps. Cytosine arabinoside (Ara-C) was introduced into the system from the onset of culture and pre-implantation steps avoided. By using this culture system, the full population range of DRG neurons was retained after preparation and complete cell dispersion achieved. In this system, NGF effects on the survival of adult and aged neurons were re-examined.

Results

1. Culture Characterisation

(A) Identification of Cell Types in Co-Culture

At the onset of culture, cells were dispersed around small fragments of non-dissociated ganglia. Spherical and phase-bright cells were identified as neurons (with a full size range of 10->60μm) (Fig.**3.1.**). Neurons were confirmed by immunostaining using the ABC method with anti-NF-200 (a characteristic neurofilament) as the primary antibody (Fig.**3.2.**). NF-200-ir neurons were evident in the full cell size range (A, B and C types), whereas a proportion of small sized neurons remained unstained. The immunoreaction product was present in neuronal perikarya and neurites. NNCs were predominantly NF-200-ir negative although fibroblast nuclei often stained weakly. Pseudounipolar neurons were rarely found, instead, neurons with 1-8 neurites were identified, mostly with 2-3 neurites/cell.

Non-neuronal cells (NNCs) were flat, darker and irregular in shape. By 2-3 days in vitro (div), the types of NNCs can be distinguished, following NNC proliferation and re-differentiation, as for example fibroblasts, Schwann cells, epitheioid-like and satellite cells (Fig.**3.1.**). In addition to morphological characteristics, glial cells were identified by using an antiserum against glial filament-associated protein (GFAP, a characteristic intermediate filament for glial cells). GFAP immunoreative (GFAP-ir) (Fig.**3.3.-d**) cells were equivalent to the flattened cells (and therefore distinguished from neurons), and had curved multi-processes (this distinguished them from Schwann cells, which were rarely found in culture but which had very thin spindle-shaped somata and fine, straight and wide processes extending from two soma poles). Fine, long

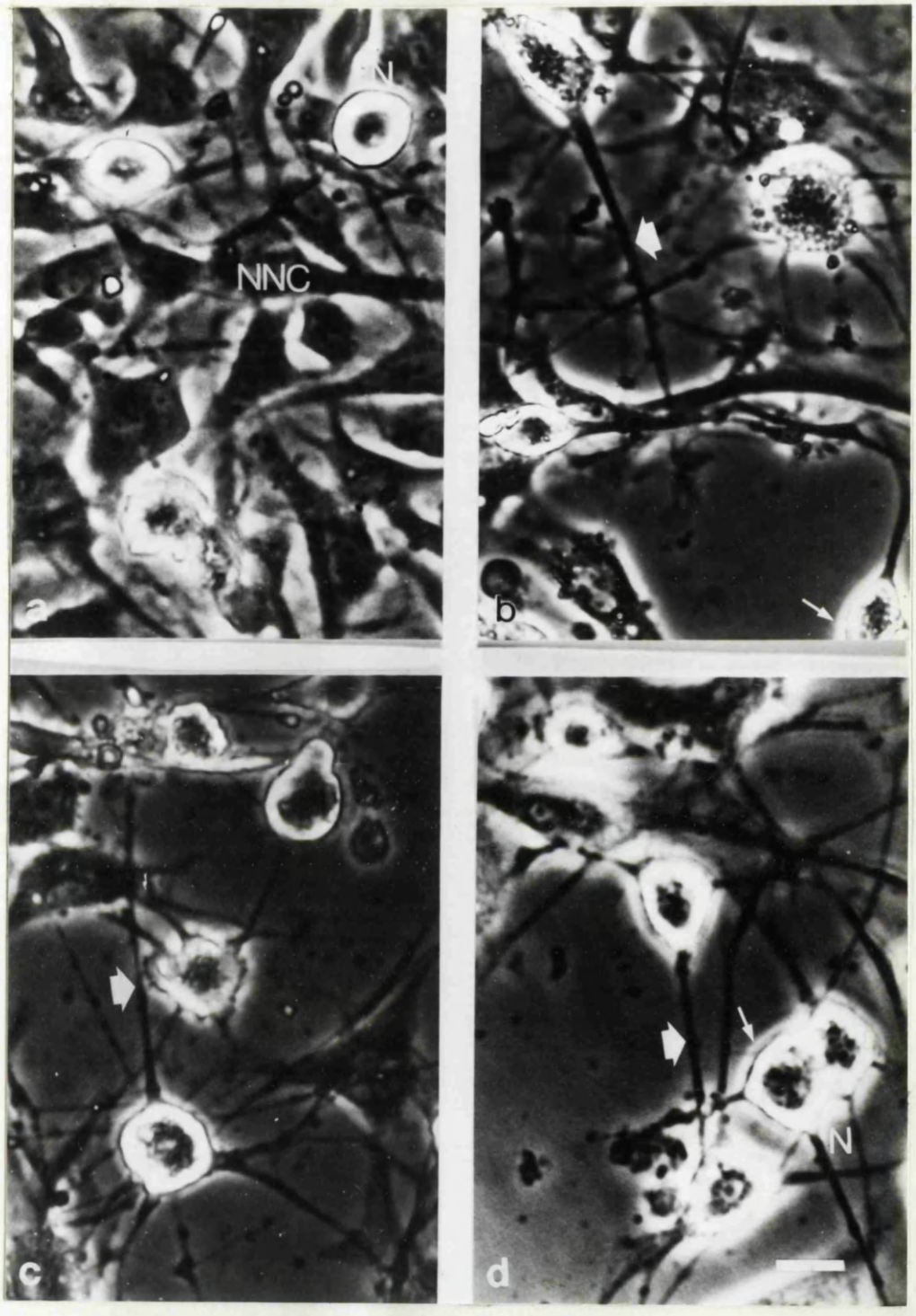

Fig.3.1. Neurons prepared from adult (**a, b**) and aged (**c, d**) mice maintained in co-culture for 9 div. Without addition of exogenous NGF (**a, c**), or with 200ng/ml (**b**) or 100ng/ml (**d**) NGF added. Neurons (N) were more numerous with NGF present than in cultures without NGF. NNC=non-neuronal cells. Note the nucleus with a distinct nucleolus (tiny arrow) in (**d**). Neurites are labelled by large arrows. Bar=20μm.

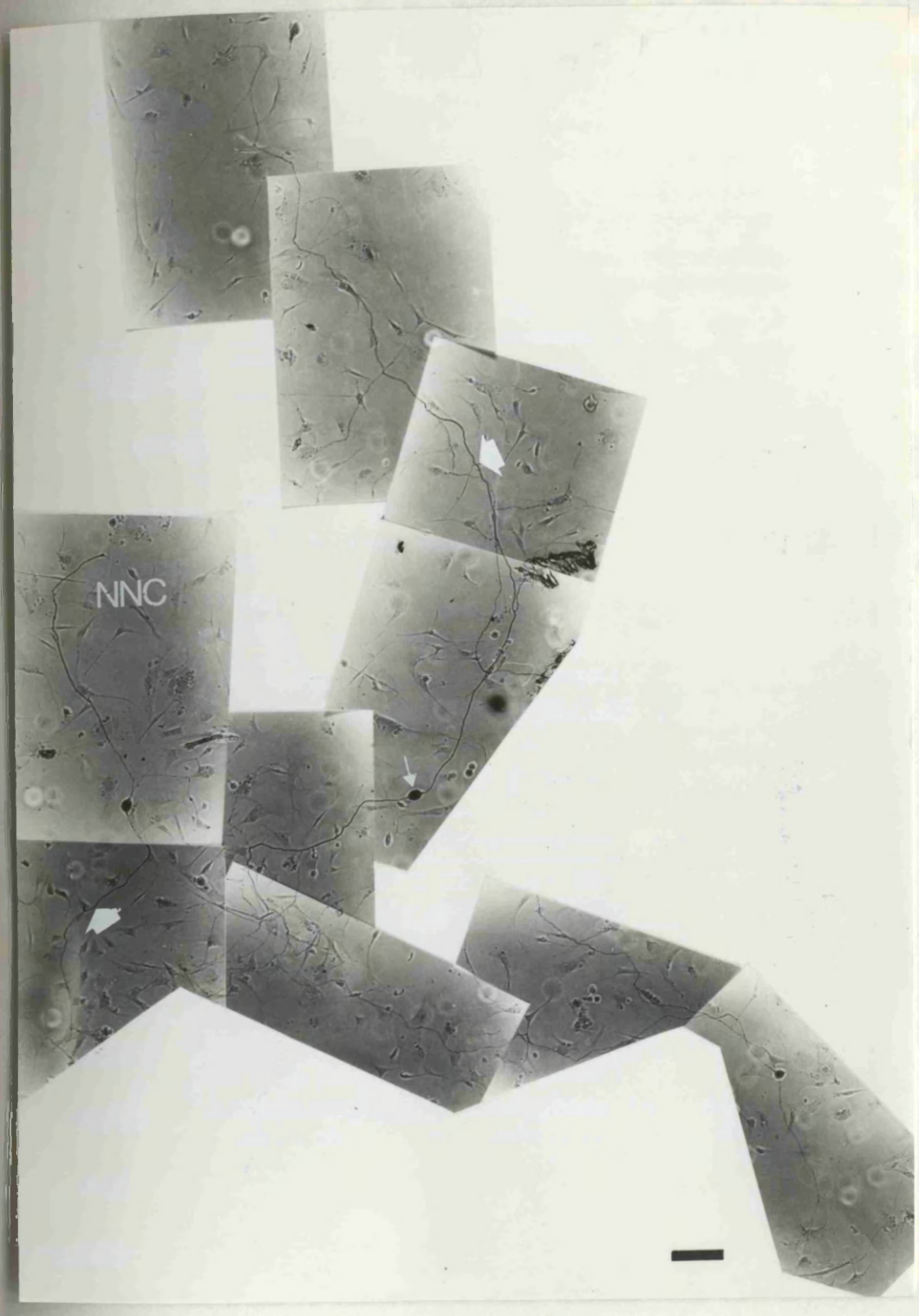

Fig.3.2. Montage of aged neurons cultured with exogenous NGF for 14div and stained with anti-NF. Neurons (filled arrows) were NF-ir, whereas NNCs and occasional small-sized neurons were negatively stained. Long neurites are evident in this co-culture. Bar=100μm.

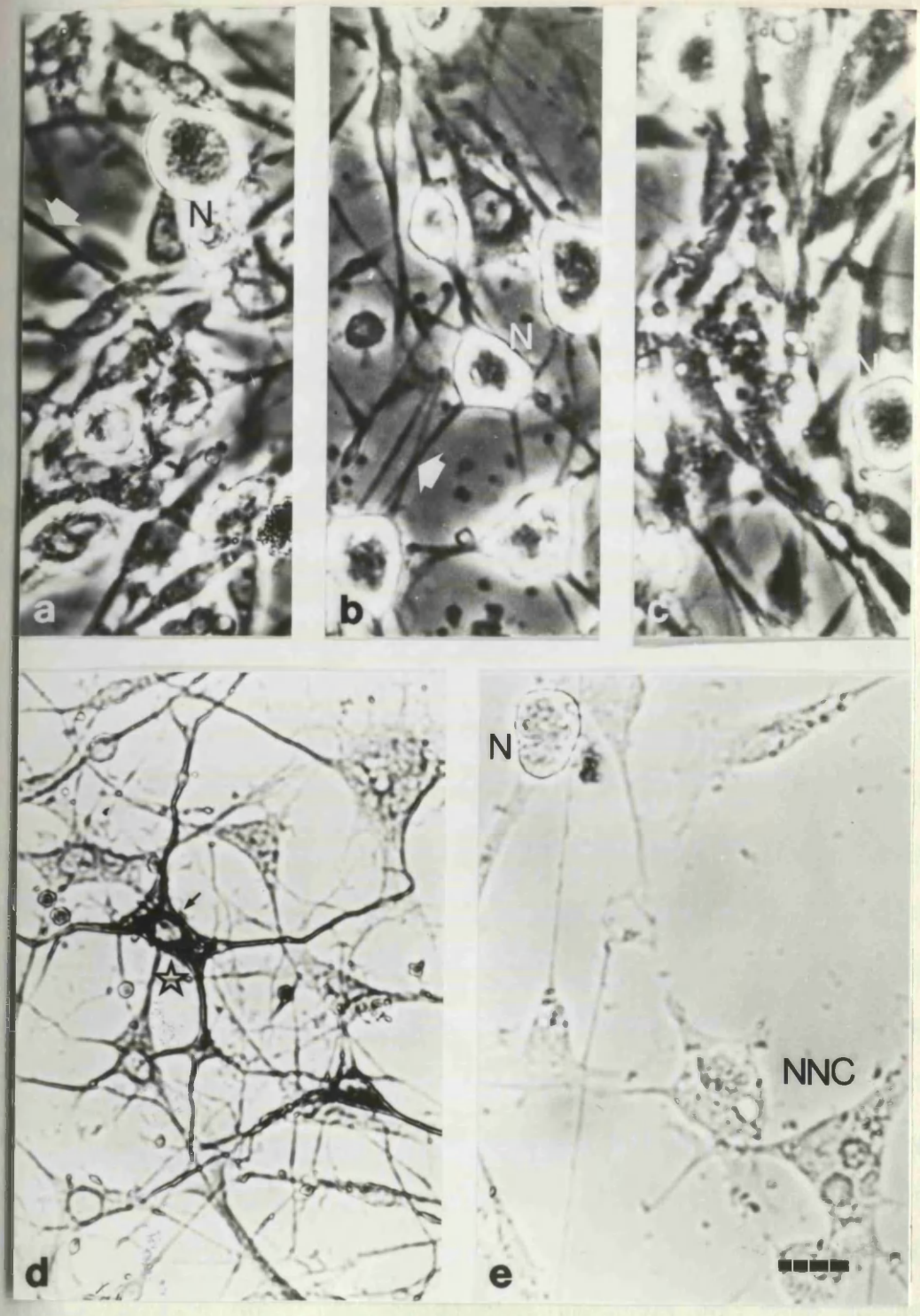

Fig.3.3. Adult neurons co-cultured for 9div, without NGF (**a**), or with 100ng/ml NGF (**b**), or with 1:100 anti-NGF (**c**). The density of surviving neurons was greater in **b** than in **a** or **c**. High neurite densities were evident (large arrow). **d** and **e**: co-cultures from adult mice stained with GFAP (**d**) or as staining control with diluent only (**e**). GFAP-ir (star) existed in soma glia II cells which had large nuclei with a distinct nucleolus (small arrow). Neurons (N) and NNCs were pale in **e**. Bar=20μm

GFAP-ir processes distinguished them from fibroblasts and epithelioid-like cells (which were unstained with GFAP). Fibroblasts, epithelioid-like and the glial cells all had flat cell somata. Fibroblasts were much smaller than epithelioid cells, with a triangular or spindle shape whilst epithelioid cells had large diameters (100-400μm). The glial cells were the smallest of the flattened NNCs, with somata which commonly had major and minor axes of 20μm and 10μm respectively. A larger elliptical nucleus was situated along the major soma axis with typically two nucleoli present. This glial cell type was classed as soma glia II to distinguish them from Schwann and satellite cells.

The cell types distinguished under light microscopy (LM) were further observed by scanning electron microscopy (SEM). Neurons could be identified by their large and roughly spherical or elliptical shaped somata, from which neuritic processes were extended (similar to in Fig.**3.5**.). Neurons with diameters as large as 70-72μm were found occasionally in both adult and aged culture preparations. Large neurons (which are considered equivalent to an A type) had a regular appearance, whereas small (C type) and medium (B type) neurons commonly had fine surface projections. No obvious difference was observed qualitatively for the soma size or appearance between adult and aged neurons in these preparations. In contrast to the processes of NNCs, neurites were rather uniform with 1-4μm diameters. Neurites extended onto the surface of NNCs, crossed other neurites or NNC-processes and even contacted neuronal somata, occasionally passing beneath other cells or processes.

(B) Growth Kinetics in Co-Culture

Cells attached directly onto the culture dish within 12-24 hours (hrs) of plating. By 4-5div, NNCs formed a confluent layer on which the neurons rested, and on which they extended neurites over the surface or into the cellular

network (Fig.**3.1**.a). Cell re-aggregation frequently occurred by 20div in culture, and clumps formed by about 24div. These consisted of centrally located neurons, surrounded by a barrier of NNCs. By 30div, a gap often encircled clumps separating them from other clumps and filled mainly with neurites. In the cultures for aged neurons, both NNCs and neurons were lower in number than in adult cultures so that the NNC networks had rather a looser apppearance covering smaller areas.

(C) Identification of Cell Type in Neuron-Enriched Culture

In addition to co-cultures, NNC-reduced neuron-enriched cultures were studied. That minimal neuronal loss occurred during culture preparation was confirmed by routinely monitoring the dishes following the DRG digestions after each enzymic and rinsing stage. Upon plating, cells were seen evenly scattered throughout the FN-coated culture wells. The full size range for neurons (large, intermediate and small) was easily identified by their phase-bright appearances (Fig.**3.4.**), whilst NNCs with small, flat and dark somata and the fragments of connective tissue were also initially evident.

The addition of Ara-C effectively inhibited NNCs, although occasional epithelioid-like and satellite cells were observed at 3div. Fibroblasts were rarely detected, and soma glial II and Schwann cells were efficiently inhibited by the Ara-C. By 6div, epithelioid-like cells had irregular shapes; their territories could cover several hundred micrometers in diameter. SEM at this stage further revealed the occasional satellite cell in cultures (Fig.**3.5.**); these were often located near neuronal somata, frequently giving off a single, fine and straight process, about 10μm in length, which was easily distinguished from neurites.

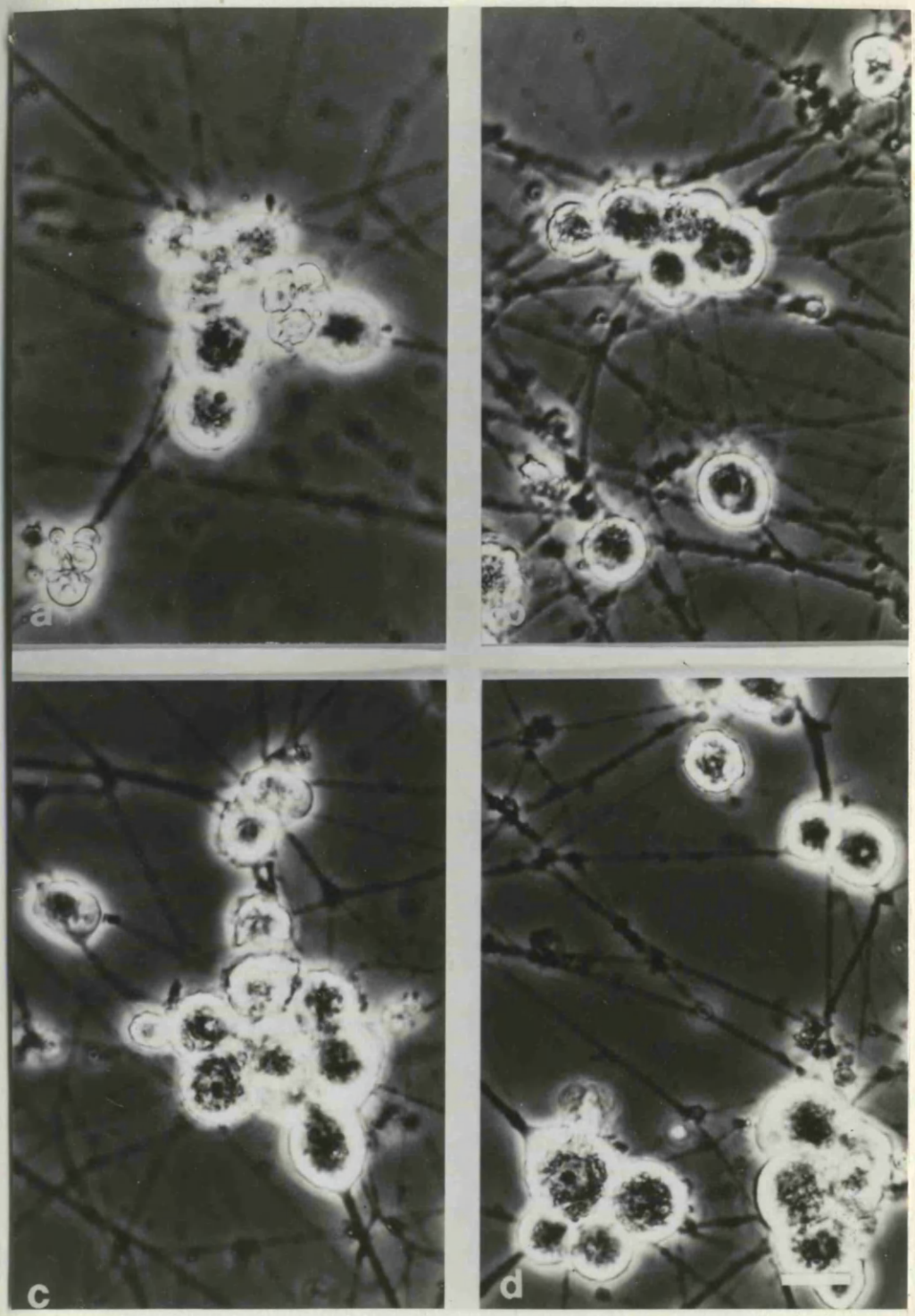

Fig.3.4. Adult (**a, b**) and aged (**c, d**) neurons in enriched cultures for 9div without exogenous NGF (**a, c**), or with 100mg/ml NGF (**b, d**). In the presence of NGF, numbers of neurons and neurites were greater than in cultures without NGF. In **a, c**, some small-sized neurons appeared unhealthy. Bar=20μm.

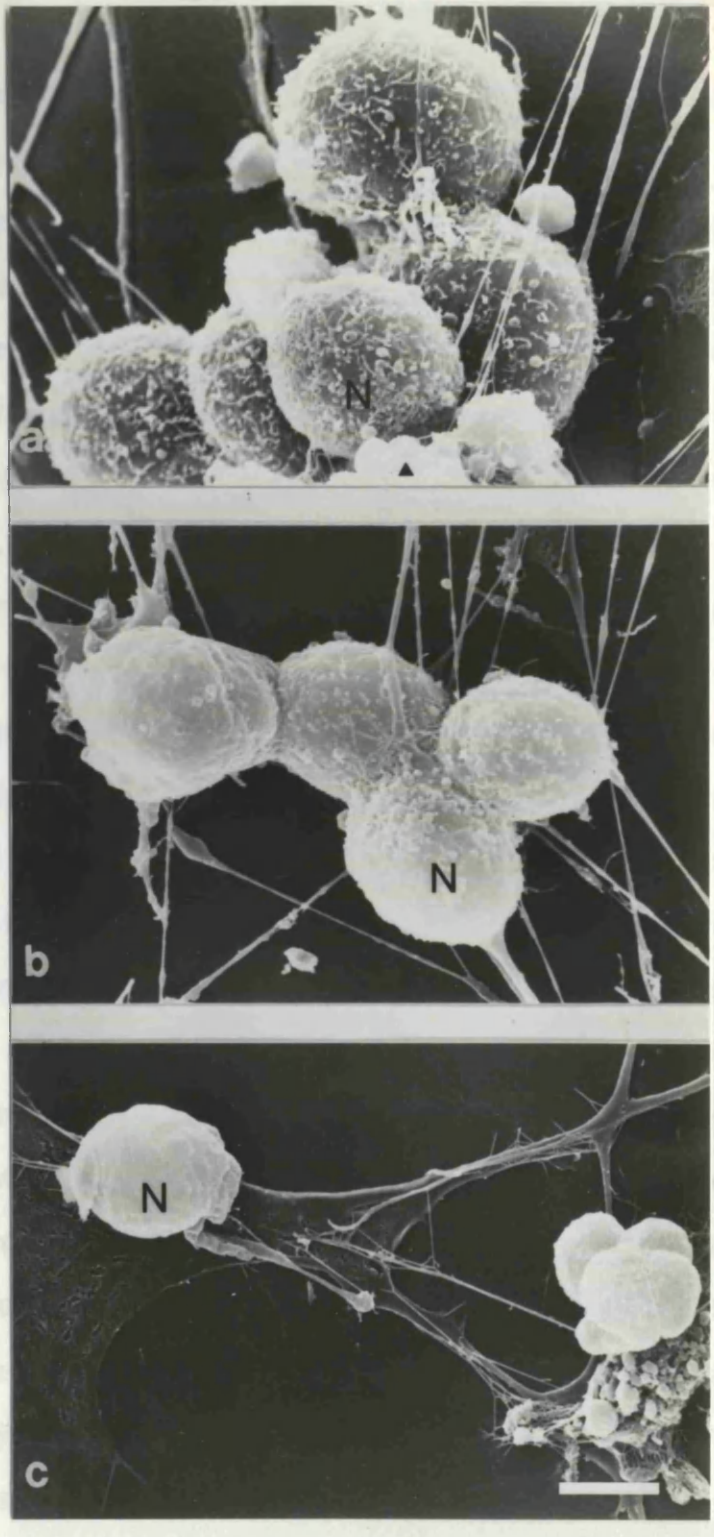

Fig.3.5. Scanning electron micrographs of aged neurons in enriched culture for 9div, with 100ng/ml NGF (**a**), without exogenous NGF (**b**), and with 1:100 anti-NGF present (**c**). Microvilli projections on the neuronal surface in (**a**) were greater than in (**b**), whereas neurons in (**c**) were often of a degenerating nature. Satellite cells (arrow head) were situated close to neurons in (**a**). Bar=10μm.

(D) Growth Kinetics in Neuron-Enriched Culture

Commonly, neurites extending over 100μm in length were often observed within 16hrs culture for both adult and aged neurons (Fig.**5.1.**, p-129), although the numbers of neurite-bearing neurons were lower in aged neuronal cultures than in adult cultures at 3div. Some neuronal re-aggregation occurred. These clumps were composed of a few small sized neurons; there was less cell overlapping, compared with that in the co-culture system. At this stage, neurites from adult and aged neurons often formed restricted, sparser networks. In contrast, the individual neurite territories of neurons often occurred out of the clumps or neurite networks, making it possible to trace neuronal geometry (Fig.**5.10., 5.11.,** pp-138,139). Neurite growth always extended towards other neurons and their neurites, or towards epithelioid-like and fibroblasts and their processes if these were nearby. Commonly, neurites branched on encountering NNCs rather than arresting on them, and in consequence when neurites passed over NNCs, they changed direction with the neurite curving (Fig.**5.11.-a, b**). Neurites were, of course, capable of extension when no other cells or processes were present nearby. In addition to large- and intermediate-sized neurite-bearing neurons, a proportion of small-sized neurons (<15μm) started to extend neurites in both adult and aged cell cultures by 3div. Intermediate-sized neurons extended fine neurites with small and branched growth cones (Fig.**5.10.-a**) whereas large neurons gave off thicker neurites with large and spindle-shaped growth cones (Fig.**5.11.-a, b**). In many cases, growth cones were less evident on the neurites extended from small neurons, irrespective of whether these were in adult or aged cultures (see Chapter 5 for further details).

The number of neurite-bearing small-sized neurons increased after 6div in both adult and aged cultures, commonly with the generation of one or two fine neurites. The local neurite networks started to form larger networks as

duration increased. By 9div, individual territories of neurites from single neurons were difficult to distinguish.

2. NGF Investigations on Survival and Neuritogenesis

(A) NGF Concentration Effects on Adult Neurons in Co-Culture

Initially the effect of NGF concentration was studied by using the co-culture preparations. Neurons from four adult mice were maintained for 14div either in the absence of exogenous NGF or with 25-200ng/ml NGF added to the medium, or with 1:100 anti-NGF antiserum present. Neurons were regularly counted under phase-contrast microscopy to evaluate survival ability. Surviving neurons had a phase-bright appearance and smooth soma membrane whereas non-viable neurons lost their phase bright appearance and normal membrane structure (Fig.**3.3.-a**). The evaluations were carried out at 6, 10 and 14div and the results are shown in Fig.**3.6.**. A slight increase in counts occurred for some groups after 1 week in culture due to the spreading of neurons from the small DRG fragments which remained at plating. By 14div, the neuronal numbers were reduced slightly in the cultures without exogenous NGF, and in those with the lower NGF concentrations. Controls were included where anti-NGF antiserum was added to the medium, to test the specificity of the exogenous NGF effect on neuronal survival. In these control cultures, neuronal counts were reduced compared to cultures without exogenous NGF and significantly less than those with NGF added. During the entire monitoring period, cultures with 25 or 50ng/ml exogenous NGF did not differ significantly from cultures without exogenous NGF whereas cultures supplemented with 100-200ng/ml NGF contained approximately twice the number of neurons, compared with cultures without NGF or in those containing anti-NGF. These observations

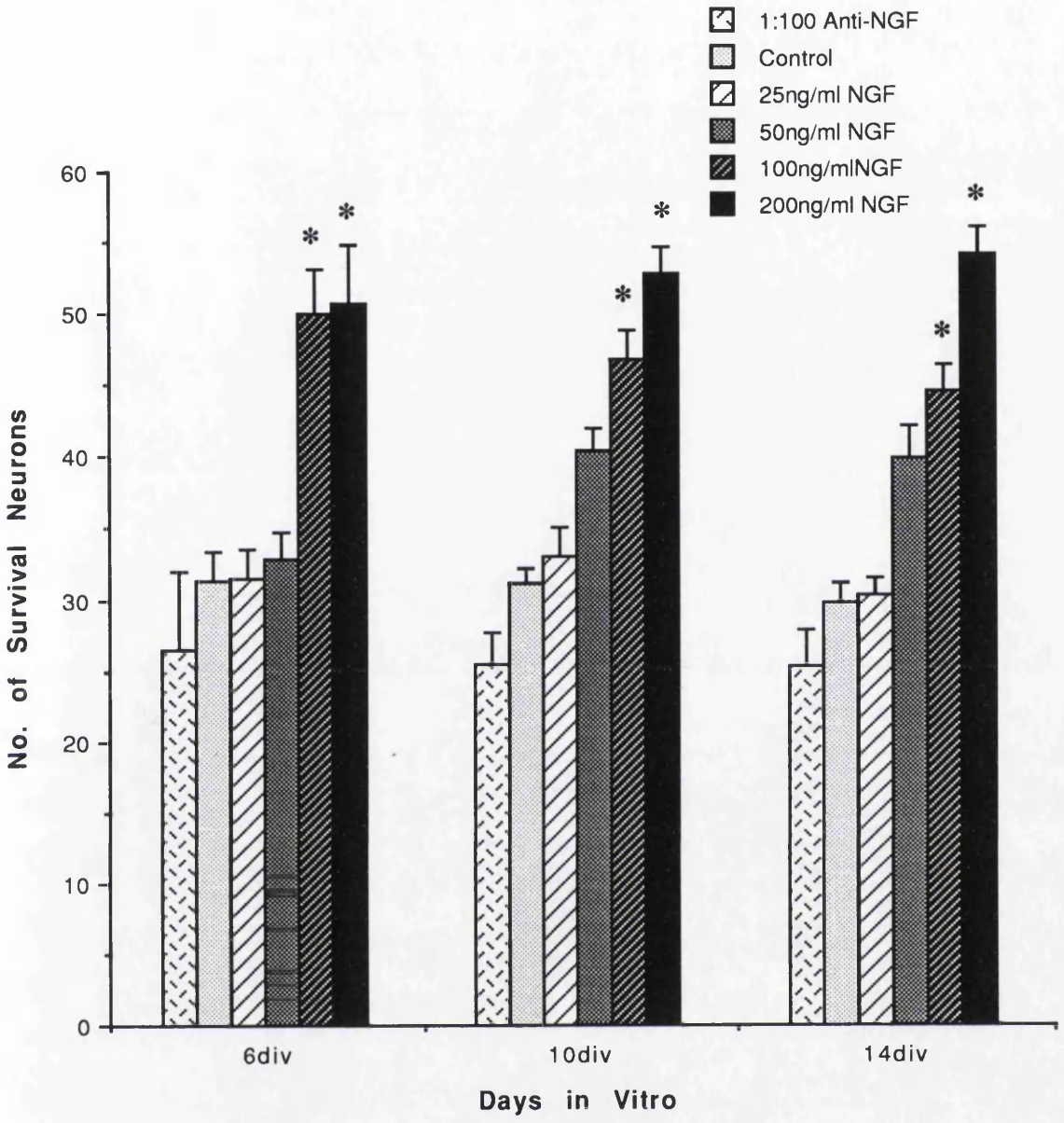

Fig.3.6. Effect of NGF Concentrations on the Survival of Adult Neurons in Co-Culture

Adult neurons co-cultured without exogenous NGF, or with 25-200ng/ml NGF, or with 1:100 anti-NGF added from initiation (from 5 experiments). Data were analysed by ANOVA. ⋆: $P < 0.005$, compared to cultures without exogenous NGF present, or with 1:100 anti-NGF.

demonstrate that 100-200ng/ml exogenous NGF concentrations can enhance adult neuronal survival *in vitro*, whereas cultures without exogenous NGF or with lower concentrations contained fewer neurons by 14div.

(B) Qualitative Effects of NGF on Adult and Aged Neuronal Survival in Co-Culture

In these major experiments, 100ng/ml NGF was used throughout to investigate effects on adult and aged neurons. Equal numbers of cultures without added NGF were monitored as experimental controls (Fig.**3.1.**).

(i) Adult:- Neurons of all sizes persisted in cultures supplemented with exogenous NGF and appeared very healthy having a perfect phase-bright appearance, distinct nucleus and an intact and smooth membrane (Fig.**3.1.-b**). This appearance remained for 1 month in vitro. Most (including small-sized) neurons, if not all, gave off neurites. Neurite numbers from each neuron were obviously greater than from neurons of cultures without NGF. By 7-9div, some neurons in adult cultures without NGF died, identified by an irregular membrane, loss of a phase-bright appearance, absence of the nucleus and somata collapse (Fig.**3.1.-a**). This was further confirmed by SEM. Cell death mainly occurred amongst small and medium-sized neurons; large neurons had a more healthy appearance. A small proportion of neurons (mainly large and medium neurons) extended neurites. Neurons commonly had 1-2 neurites, and occasionally 3-4 neurites in cultures without added NGF.

(ii) Aged:- In control groups without added NGF, aged neuronal survival was less than that of adult neurons. In the presence of NGF, aged neuron survival was greater, compared to that in aged control cultures without added NGF (Fig.**3.1.-c, d**). The extent of NGF-induced neurite outgrowth by aged

neurons also failed to reach the levels observed for adult neurons. In NGF-supplemented cultures maintained for up to 6 weeks, the number of neurons with neurites was obviously higher than in cultures without NGF added by which time almost all neurons were spindle-shaped. The density of fine surface projections seemed to be increased by exogenous NGF, irrespective of adult or aged neurons.

(C) Quantitative Effects of NGF on Neuronal Survival in Co-Culture

In an attempt to quantify the effects of NGF on survival indicated above, neurons were prepared from 5 animals for both adult or aged cultures. 100ng/ml NGF was used to study the effects on neuronal survival for 24div. In this case, 1.2ml medium was added to all cultures as this improved survival of the adult neurons, when compared to counts from the pilot experiments of NGF concentration effects (Fig.**3.6.**) where cultures were maintained in 0.5ml of medium/well. At 7div, more adult neurons existed in the group with exogenous NGF than in cultures without added NGF; this enhancement was observed throughout the entire culture period and was shown to be a statistically significant difference (Fig.**3.7.-a**).

Aged neuronal counts were, in general, lower than adult numbers by approximately 35%, irrespective of whether NGF was supplemented (Fig.**3.7.-b**). The numbers of aged neurons with 100ng/ml NGF, however, were consistently higher than the aged controls without NGF throughout the 4 week culture period. Although the absolute enhancement for aged neurons in the presence of NGF was less than existed for adult groups, it was significant when tested by ANOVA. The number of aged neurons remained constant after 17div, even without exogenous NGF, which differed from the observations of neurons prepared from adult mice where the survival was reduced.

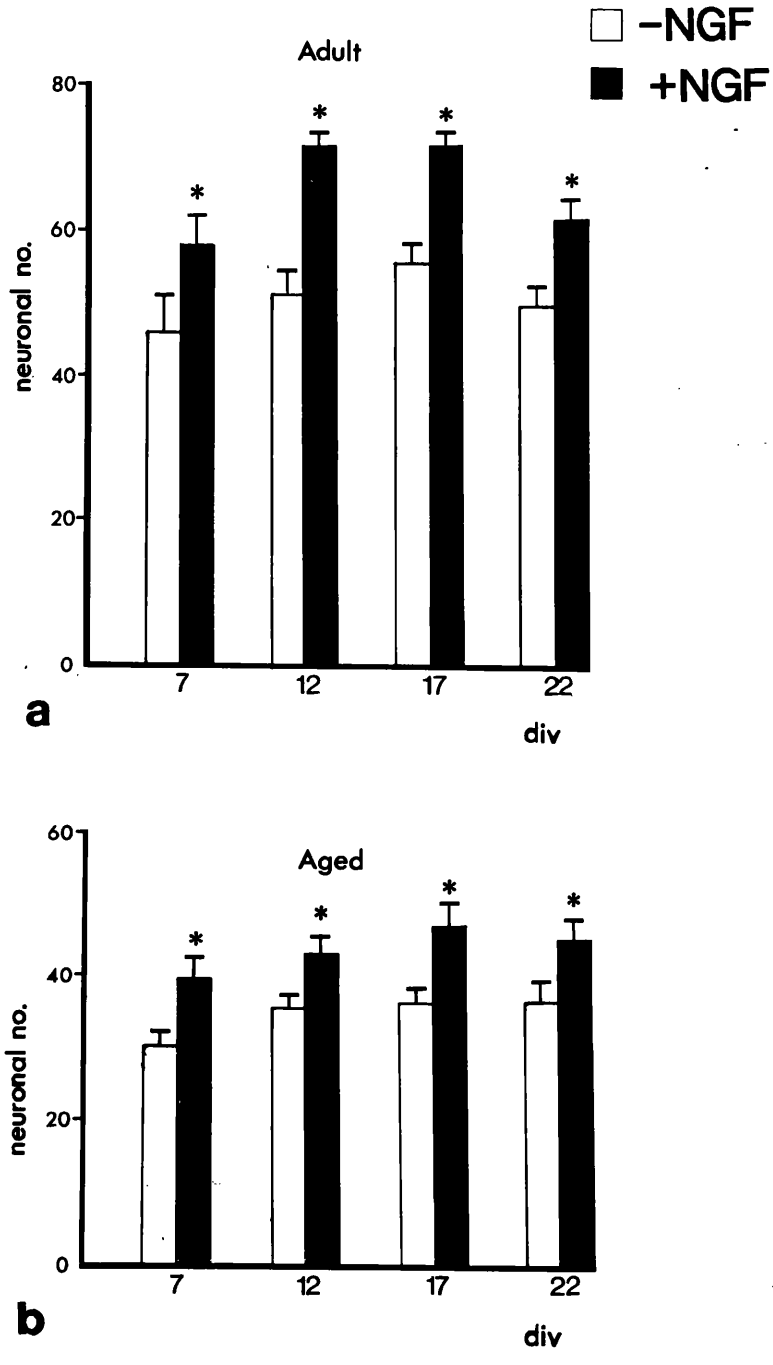

Fig.3.7. Effects of 100ng/ml NGF on Survival of Adult and Aged Neurons in Co-Cultures

a: Neuronal counts in cultures prepared from adult mice maintained over a 22 div culture period in the absence of exogenous NGF, and with 100ng/ml NGF added. **b:** Neuronal counts from aged mice in cultures without exogenous NGF and those with 100ng/ml added. $P<0.005$ compared to control by ANOVA.

(D) Anti-NGF Effect on Neurons in Co-Culture

Rabbit anti-2.5S NGF serum (a 1:500 dilution blocks the ability of 5ng/ml NGF to stimulate neurofilament like growth of E-8 chicken DRG) was administrated, at final concentrations of 1:200 and 1:100, into adult cultures from initiation. Medium-sized neurons were mainly affected by the presence of anti-NGF, with numbers reduced compared to control cultures (Fig.**3.6.**). Neuronal structure was often disrupted and fewer had neurites (Fig.**3.3.-c**). In both adult and aged cultures supplemented with anti-NGF, small and medium-sized neurons often lost their surface projections, with signs of membrane shrinkage or destruction. No obvious difference was observed for the effects between 1:200 and 1:100 anti-NGF.

(E) Qualitative Effects of NGF on Neuronal Survival and Regeneration in Neuron-Enriched Culture

NGF was added to half the enriched cultures from their initiation; the other cultures were maintained as controls without exogenous NGF. By 16hrs, both neurite number and length from individual neurons were much greater in cultures with NGF than in those without and the numbers of neurite-bearing neurons were also obviously higher. Although neurite-bearing neurons were less in this phase of culture for neurons prepared from aged animals compared to adults, the presence of NGF continued to increase their number in these experiments also. This qualitative difference was evident in cultures assessed blindly.

By 3div, neuronal death was less evident in the adult and aged cultures supplemented with exogenous NGF than in cultures without added NGF. Aged neurons appeared to have more neurite branches than adult neurons, although

there was no obvious difference in maximal neurite length of adult or aged neurons at this stage of culture, with NGF enhancing neurite length for both adult neurons and aged (see Chapter 5). A large area of neurite networks formed by 6div, with densities greater in the presence of exogenous NGF than in adult and aged culture controls without added NGF. In the 9 day culture period, SEM observations revealed that in the presence of NGF, many fine filaments on the neuronal surface were present, especially on intermediate-sized neurons, whereas neurons in culture controls without NGF frequently had a smoother appearance (Fig.**3.5.**). The number of surviving neurons, and their neurite densities, also was greater in the presence of exogenous NGF than in the controls for both cultures of adult and aged neurons (Fig.**3.4.**).

(F) Quantitative Effect of NGF on Neuronal Survival in Enriched Culture

In these experiments, adult and aged neuronal cultures were prepared from 3 and 4 animals respectively. Two wells per plate were supplemented with 100ng/ml NGF and the other two contained culture medium without exogenous NGF as culture controls. Neuronal survival was monitored by the inverted phase-contrast microscope for a 9 day period (Fig.**3.8.**). Within 4 div, the neuronal survival was similar for both adult and aged cultures. By 9div, the number of adult neurons remained constant in both controls and in the cultures with NGF added. For aged neurons, irrespective of whether exogenous NGF was added, numbers were reduced. Adult neuronal numbers in the cultures with added exogenous NGF were higher than in controls without NGF. Higher survival was also observed in aged cultures with added NGF, compared to those without.

(G) Withdrawal of NGF and Anti-NGF Effects on Neurons in Neuron-Enriched Cultures

When exogenous NGF was withdrawn from adult neurons at 6div, death occurred in the following 3div, with neuronal numbers reduced by 12% compared to those which continued to have NGF added, although the numbers surviving were slightly higher than controls without added NGF throughout the experiment. The effects of withdrawing NGF on neuronal survival were studied also in four experiments using aged neurons; exogenous NGF was withdrawn after 6div and the cultures maintained for a further 3 days in vitro (Fig.**3.8.**). Some neuronal death, particularly of small neurons, was evident compared with controls without NGF added. Neurite density remained in cultures following NGF withdrawal at levels greater than in cultures in which exogenous NGF had not been present (Fig.**3.9.**).

When anti-NGF serum was introduced into aged culture at initiation, many neurons had died by 4div. The remaining neurons shrank and were deformed (Fig.**3.5.-c**). A few neurites and their branches could be detected extending from these unhealthy neurons, which may suggest that anti-NGF had more of an effect on neuronal somata rather than on the potential for neurite extension, although it did also greatly inhibit neurite regeneration. The neuronal appearance was very similar to that observed when NGF was added to aged cultures at 3div and withdrawn by 6div; in this case neuronal damage was very evident. Cell death was predominantly in the intermediate-sized neurons, together with some small-sized neurons. Neurite density was also lower than the cultures supplemented with NGF.

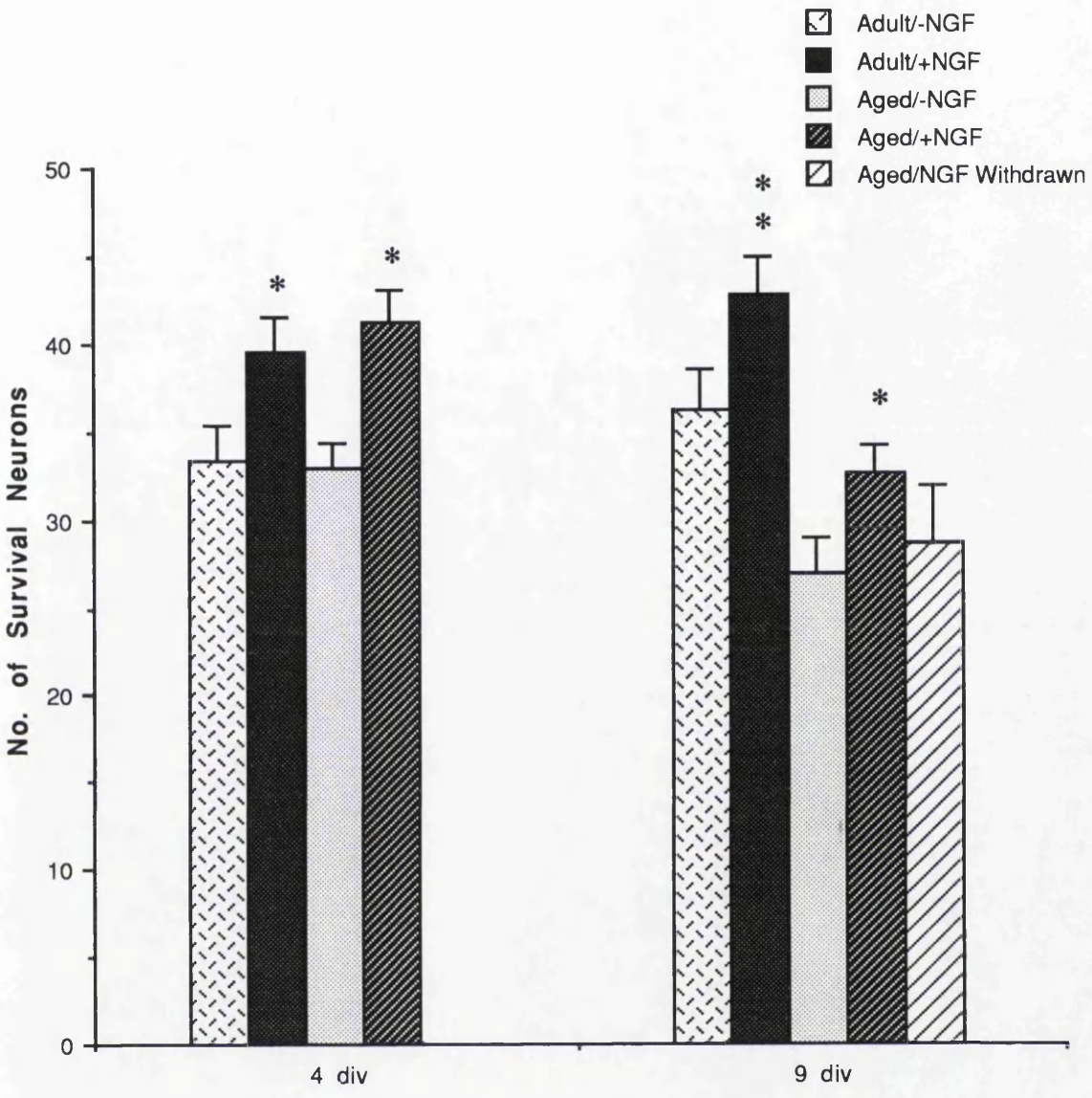

Fig.3.8. Effects of 100ng/ml NGF on the Survival of Neurons in Enriched Culture

Adult and aged neurons in enriched cultures without exogenous NGF or with 100ng/ml NGF present. Neurons were counted in 20 cultures for each group, prepared from 3 adult and 4 aged mice, and expressed as the absolute number per field. Data were statistically analysed by ANOVA. ⋆: $P < 0.05$; ⋆⋆: $P < 0.01$.

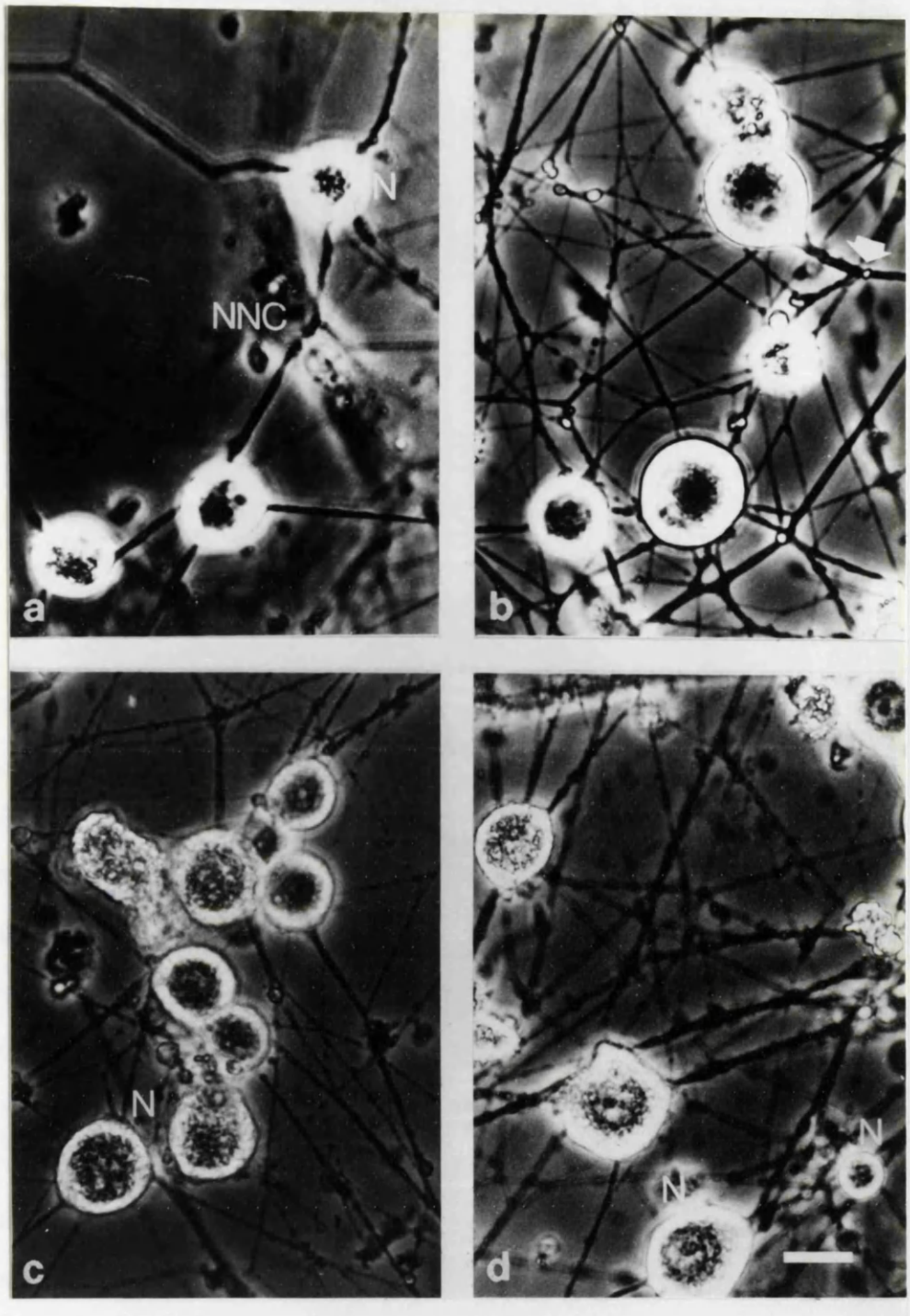

Fig.3.9. Aged neurons cultured for 6div, without NGF (**a**), or with 100ng/ml NGF (**b**), and for 9div, without (**c**), or at 9 div with NGF withdrawn from 6div (**d**) [**d** is from the same culture well as in (**b**)]. At 6div, the numbers of neurons and neurites in the presence of NGF in (**b**) were much greater than those in the culture without NGF (**a**). However, at 9div, some neurons in cultures following the withdrawal of NGF (**d**) had a less smooth and unhealthy appearance. NNCs only rarely occurred in enriched cultures (**a**). Bar=20μm.

3. NGF Effects on Neuronal Population Size and Shape Distributions

The preparations used to investigate neuronal survival were also monitored for studies of neuronal soma size or shape. An average of 400 neurons were measured on phase-contrast micrographs for each adult and aged group with and without NGF for the different culture intervals.

(A) Neuronal Soma Size in Co-Culture

Soma size distributions were compared for adult and aged neurons cultured with or without NGF for the entire 29div period (Fig.**3.10.**). The population mean for adult neuronal soma size in cultures without exogenous NGF added was greater in the earlier stages of cultures, but less by 22div when size variation was more evident. In contrast, the proportions of medium-sized adult neurons surviving in the presence of NGF gradually increased within the 4 week culture period, although neurons with diameters >36μm remained at relatively similar levels.

Aged neuronal somata had a marginally larger mean soma diameter than adult neurons, with a difference between the population means of 2μm for neurons cultured without exogenous NGF, and 3μm for neurons with 100ng/ml NGF included. In general, the aged neuronal population became more variable both with or without exogenous NGF when the culture period was extended. A difference was apparent, however, with higher proportions of smaller (<18μm) and large (>36μm) neurons in the absence of added NGF, and a higher proportion of 24-33μm medium-sized neurons over the entire 4 week period with NGF added.

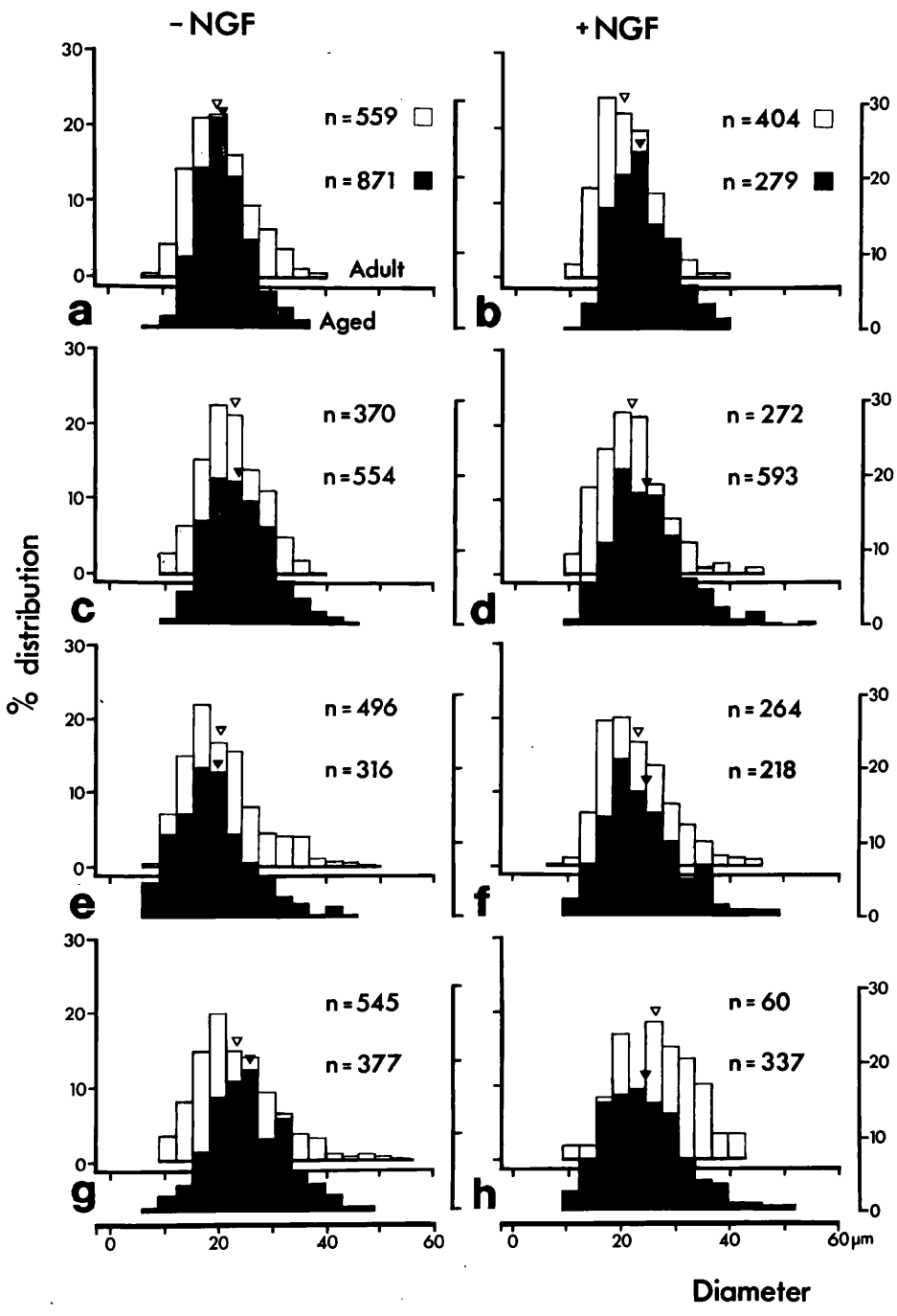

Fig.3.10. Size distributions of neurons in co-cultures without exogenous NGF (plots **a, c, e, g**) and for cultures supplemented with 100ng/ml NGF (plots **b, d, f, h**). **a, b**: 7div; **c, d**: 12div; **e, f**: 22div; **g, h**: 27div. n=number of neurons measured from adult (open bar) and aged (filled bar) mice.

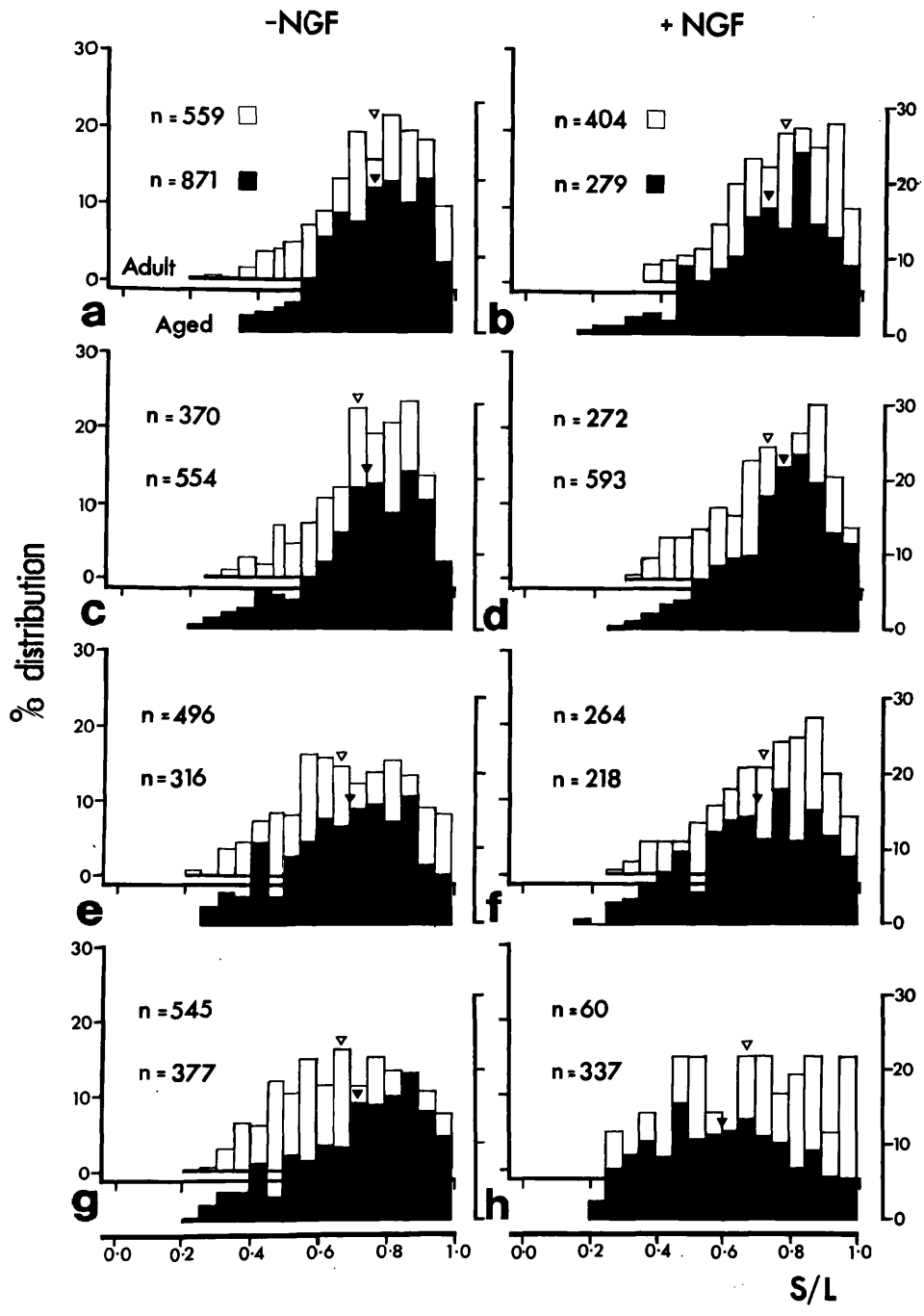

Fig.3.11. Shape index for neurons from adult (open bar) and aged (filled bar) mice co-cultured in the absence and presence of NGF. For further details, see the legend to Figure 3.10.

(B) Neuronal Shape in Co-Culture

Cultured adult and aged neurons were mostly spherical during the early culture phases (Fig.**3.11.**). They had a shape index, which approached 1 [where shape index was expressed as the longest diameter of the cell soma divided by the shortest diameter (Fukuda, 1985)]; a few neurons were more spindle shaped (with a shape index <1). As the culture period was increased, the proportion of spherical adult neurons declined, with a higher proportion of spindle-shaped neurons by 17div. This change in neuronal shapes occurred in both the presence and absence of NGF and, therefore, is probably a general culture effect, although there was more variation in neuronal shape from earlier phases of cultures if NGF was present. Proportions of aged neurons with more spindle shaped increased with prolonged culture duration. In aged cultures with NGF added, the shape variability was more noticeable than in the absence of NGF by 12div.

Discussion

1. Evaluation for the Culture Systems

The co-culture system, as an experimental model, was used initially in the present study, to mimic the local *in vivo* environment. Evaluation of NGF effects on the survival of mature DRG neurons in this system gives valuable data which is pertinent to clarifying the controversial results already obtained from *in vivo* and *in vitro* studies (e.g. Johnson *et al.*, 1987; Lindsay, 1988), since the co-culture system represents a position mid-way between *in vivo* and neuronal-enriched culture (especially single-cell culture) environments.

In addition to neurons, non-neuronal cells (NNCs) were also present in these cultures. NNCs are present in the local environment of neurons within the intact DRG. On the other hand, NNCs may supply endogenous NGF to the neurons and support their survival and differentiation. NGF mRNA was detected in fibroblasts and sciatic nerve-derived Schwann cells in culture (Longo *et al.*, 1993). However, Schwann cells were rarely found in the present cultures. In the case of sciatic nerve transection in vivo, macrophages infiltrate into the injured nerve and produce Interleukin 1 (IL-1), which stimulates the production of NGF by Schwann cells (Lindholm *et al.*, 1987). In cultured sciatic nerve fibroblasts, elevated levels of NGF mRNA were induced by IL-1 also (Lindholm *et al.*, 1988). So, it is not possible to rule out the possibility of endogenous NGF and/or other neurotrophic factor production by DRG-derived NNCs. In view of this, large numbers of culture controls without added NGF were included (the number of controls was equal to NGF test cultures), together with cultures supplemented with anti-NGF, to identify the specificity of exogenous NGF effects. Neuronal numbers in the presence of anti-NGF were shown to be lower than in culture controls, whereas in presence of 100ng/ml or more exogenous NGF, neuronal survival was consistently greater than in controls without exogenous NGF for both adult and aged cultures. The results are consistent with some endogenous NGF production in co-culture, but the effects of this on neuronal survival was significantly less than if 100ng/ml exogenous NGF was present.

It is possible that NGF requirements of DRG neurons for survival vary with different physiological and injury conditions. Nerve roots are completely transected, prior to culture, so that the NGF requirement of neurons *in vitro* may be much higher than in normal *in vivo* environments. In transection of sciatic nerve *in vivo*, the enhancement of endogenous NGF synthesis by Schwann cells (Lindholm *et al.*, 1987) may reflect a higher requirement by

injured neurons. The present *in vitro* studies are consistent with this, e.g. low concentrations of exogenous NGF (25-50ng/ml) did not significantly enhance neuronal survival in culture (Jiang and Smith, 1993a). The differences between culture controls (without added NGF) and cultures with anti-NGF may represent a blocking of endogenous NGF by anti-NGF. A significant difference for neuronal survival was demonstrated in both adult and aged cultures with the addition of 100ng/ml NGF.

In neuron-enriched cultures, NNC influences were limited. In evaluating the effects of neurotrophic factors *in vitro*, neuron loss during the procedures of cell dissociation and dispersal should merit particular attention for neuron-enriched cultures, since subsets of neuronal populations differing in their response to a given trophic factor have been revealed by many studies (Meakin and Shooter, 1992; for review see Lindsay, 1992). If subpopulations of NGF-dependent neurons are lost during preparation, the final results obtained from such experimental studies will be obviously greatly limited in the evaluation of the action of NGF *in vitro*. In other neuron-enriched culture systems, two enzyme digestions (trypsin and collagenase) followed by mechanical triturations demonstrated good cell dispersion but with much neuronal loss (Lindsay, 1988; Grothe and Unsicker, 1987; Yasuda *et al.*, 1990), and these were used to monitor neuronal growth, and neurite tracing following NGF treatments. In the present modified neuron-enriched culture system, the procedures of centrifugation and pre-implantation were omitted as neurons were dispersed directly into full culture medium with Ara-C added to inhibit NNCs (Smith and Orr, 1987). This had the advantage that almost the entire population, if not all, remained, as was verified by monitoring the digestion and rinse dishes. The system therefore was advantageous in examining the effects of NGF on neuronal survival and phenotype expression in an

environment with NNCs limited, and in monitoring neurite regeneration *in vitro*.

Dispersed neurons re-aggregated in enriched culture from as early as 3div, and were established by 6div; this preceded similar aggregations seen in the co-culture system by about 10-15div. Such a phenomenon for adult DRG neurons in neuron-enriched culture has previously been described by others (Grothe and Unsicker, 1987). Cell re-aggregation suggests that neurons require the existence of other neurons for sustained individual cell survival and differentiation in culture. During embryogenesis, cell migration and cell-cell adhesion are vital processes mediated by secreted soluble factors and tissue-specific cell-cell recognition systems, with vital homophilic and heterophilic bindings, and/or molecular-linker binding between cell-surface receptors of adjacent cells (Alberts *et al.*, 1989). In the present studies with both co-cultures and neuron-enriched cultures, neuronal re-aggregation also occurred in cultures from 2 year old mice, which may suggest that neuronal dependence on other neurons persists in aged stages. It is suggested that the single-cell culture systems of others therefore can not satisfy this essential requirement for long-term neuron survival, although maintenance for 7div may be possible.

The numbers of aged neurons in enriched cultures were reduced in later culture phases, irrespective of whether or not NGF was added. This may result from aged neurons in the limited NNCs environment having a lower ability to resist injury at dissociation than adult neurons, as aged neuronal numbers were enhanced in co-culture and reduced in neuron-enriched culture.

2. NGF as a Survival-Dependent Factor for Aged DRG Neurons

Reports exist that NGF-dependent survival can extend to early postnatal life, as was evidenced by the finding that 50% of sensory neurons in neonatal rats were lost following axotomy (Arvidson *et al.*, 1986), but the administration of exogenous NGF effectively prevented the death of injured neurons (for review see Lindsay, 1992). Furthermore, administration of NGF antiserum resulted in the reduction of DRG neurons in neonatal rats (Yip *et al.*, 1984). In recent years, it has been demonstrated that NGF enhanced the survival of mature (adult and even aged) sensory neurons in vitro from various species, including trigeminal sensory neurons of shrew (Fukuda *et al.*, 1991), and DRG neurons of rat (Manfridi *et al.*, 1992) and mouse (Jiang and Smith, 1993b). On the other hand, addition of NGF to neuron-enriched or single-cell cultures of adult neurons failed to increase surviving neurons within 7div (Grothe and Unsicker, 1987; Lindsay, 1988). The co-culture system (Smith and McInnes, 1986a, b) in our research is likely to resemble more closely the *in vivo* environment than the more elaborate single cell system or neuron-enriched cultures of others (Lindsay,1988). In injured sciatic nerve, the enhancement of NGF mRNA levels seen in the proximal stump and distal nerve segment (Heumann *et al.*, 1987a,b) only represented one aspect of the injury reaction, e.g. increased NGF production may have a role in repairing injured cells. The *in vivo* findings, where enhanced NGF mRNA levels remained in the absence of reinnervation (i.e., nerve transection instead of crush injury) are consistent with this. In contrast, a fall in NGF mRNA levels corresponded to the phase of reinnervation (Review see: Longo *et al.*, 1993). Furthermore, adult DRG neuronal death following sciatic nerve transection could be completely prevented by administration of exogenous NGF at the transection site (Rich *et al.*, 1987), which suggested that a higher than normal dosage of NGF satisfied the requirements of both survival and neurite regeneration of injured DRG

neurons. The NGF concentration effect in the current study produced similar results to that those *in vivo*, and 25-50ng/ml NGF was not sufficient to enhance the survival of cultured DRG neurons. Lindsay (1988) concluded that NGF was not required for survival of adult sensory neurons, although results from co-cultures in his paper showed that NGF at concentrations of 50 to 200ng/ml did increase survival of adult neurons in 2div cultures. In the neuron-enriched and single-cell culture systems of Lindsay (1988) and Grothe and Unsicker (1987), only 50ng/ml NGF was added with singly cultured neurons surviving for 7div only. The low dosage of NGF may not rescue such neurons which have first been axotomised and then maintained in adverse conditions. Furthermore, injured sensory neurons show some characteristics of embryonic neurons, such as re-aggregation *in vitro*, or changes of protein metabolism, e.g. increased tubulin systhesis (Heacock and Agranoff, 1976) and reduced transmitter synthesis (Frizell and Sjostrand, 1974). NGF therefore, may act as a survival factor for injured adult DRG neurons, similar to its maintenance of pre-developmental sensory neurons.

NGF dosages in other *in vitro* studies have varied from 2.5ng/ml (Aguayo and White, 1992) to 200ng/ml (Winter *et al.*, 1988), with many studies employing mid-range doses of 50-100ng/ml (Yasuda *et al.*, 1990; Lindsay *et al.*, 1989). 200ng/ml concentrations continue to be employed (Winter *et al.*, 1993). In the current study with NNCs present, neurons can be monitored for up to 6 weeks without significant loss (Smith and McInnes, 1986a). Certainly since the labelling sensitivity for NGF receptors of adult DRG neurons is at approximately the 50% level (Richardson *et al.*, 1986), one might predict that in this culture system there would be a higher concentration requirement than in the lower density cultures to react with available receptors. In addition, NGF has only been shown to bind with a high-affinity receptor $p140^{trk}$ and with $p75^{LNGFR}$, with no evidence of NGF binding to other high-affinity NGF

receptors. Cross reaction with other trophic factor receptors by the high NGF concentrations is unlikely since trkB and trkC only bind with BDNF, NT-3, 4, 5. There are a number of plausible explanations; for example, a requirement for higher NGF levels due to the higher neuronal density in the present cultures by injured neurons as would be the case *in vivo* in injured sciatic nerve. In the present modified neuron-enriched culture system, the effects of NGF on DRG neuronal survival were further demonstrated. Neuronal survival differences of neurons in cultures supplemented with NGF compared to those without exogenous NGF were evident as early as 4div for both adult and aged neurons, and persisted throughout the entire culture period. NNC-mediated NGF effects on neuronal survival were limited in neuron-enriched cultures.

The mechanism by which NGF acts on neurons has not fully been elucidated. However some explanations for NGF-dependent survival by mature neurons have been proposed. Neuronal death *in vivo* was initially considered as a chronic process, resulting from the accumulation of sub-lethal damage or error-containing molecules (for review see Davies, 1984). Further studies demonstrated that neuronal death was an active process (programmed cell death, apoptosis or suicide) (for review see Rich, 1992). Martin and co-workers (1988) proposed that neurotrophic factors supported neuronal survival by depressing an active and endogenous death programme within the cell, since cycloheximide or actinomycin D (inhibitors for protein synthesis or RNA production) protected the death of sympathetic neurons after withdrawing NGF from culture medium. There is evidence that neuronal survival can involve the low affinity NGF receptor: neuronal cells died when $p75^{NGFR}$ was unbound in temperature sensitive immortalised neuronal cells or in R2, a neural cell line immortalised from cerebellar neurons and transfected with $p75^{NGFR}$, whilst binding by NGF or monoclonal antibody against $p75^{NGFR}$ inhibited cell death (Rabizadeh *et al.*, 1993). Furthermore loss of sensory neurons was seen in

transgenic mice carrying a mutation of the gene encoding for $p75^{NGFR}$ (Lee *et al.*, 1992). In developing rats, low affinity NGF receptor is expressed by most DRG neurons (Yan and Johnson, 1988), and in peripheral nerve, $p75^{NGFR}$ was 10- to 20-fold in excess of $p140^{trk}$ (Sutter *et al.*, 1979; Vale and Shooter, 1985) as demonstrated retrogradely (Taniuchi and Johnson, 1985; Johnson *et al.*, 1987). Ibáñez and colleagues in 1992 showed that the peptide A region of NGF was involved in regulating binding to $p75^{NGFR}$ but not to $p140^{trk}$. Low affinity NGF receptor has a low binding ability to NGF, compared to $p140^{trk}$. The high concentration of NGF (>100ng/ml) required for adult neuronal survival in the present study may be due to the low binding ability of $p75^{NGFR}$ to NGF and its lower density on the mature neuronal surface, which may be inferred from the present results to be reduced further with prolonged aging.

Recently, BDNF mRNA has been detected in a subset of neurons in adult rat DRGs (Ernfors *et al.*, 1990). Some cultured embryonic DRG neurons may be supported by BDNF (Barde *et al.*, 1982; Davies, 1986), the BDNF acting as an autocrine and/or paracrine factor (Davies, 1992). BDNF has been shown to have an affinity binding to $p75^{NGFR}$ equal to NGF (Rodriguez-Tébar *et al.*, 1990), and according to Lindsay "The most striking feature of the primary structure of BDNF is similarity to NGF" (Lindsay, 1993). Murphy and colleagues (1993) demonstrated that mouse NGF, human recombinant BDNF (hrBDNF) and hrNT-3 are immunologically related proteins, with mouse NGF antibodies reacting with the other members of the neurotrophin family and inhibiting their effect on chick (E8-E10) DRG neuronal survival. It is possible that antiserum against 2.5S NGF in the present study blocked an endogenous BDNF effect on neuronal survival via $p75^{NGFR}$.

The survival dependence of DRG neurons on trophic factors appears to be gradually lost with aging. In embryonic stages, rat and guinea pig DRG

neuronal numbers were reduced by approximately 80% after administration of anti-NGF in utero (Johnson *et al.*, 1980); In neonatal animals, a combined axotomy of peripheral and central nerve roots resulted in >70% DRG neuronal loss (Yip and Johnson, 1984). However, in adult guinea pig, a maximun loss of DRG neurons of only about 40% was observed by using similar experimental manipulation (Johnson and Yip, 1985). In the present study, the increased numbers of surviving aged neurons with exogenous NGF present was similar to the effect upon adult neurons, although the absolute numbers of aged neurons were less than in cultures from adult mice, irrespective of whether exogenous NGF was present or absent. Similar results were obtained with both co-culture and in the neuron-enriched cultures. A continued dependence of aged DRG neurons on exogenous trophic factor was further demonstrated by withdrawing NGF or by using anti-NGF, where neuronal loss promptly occurred. These and the results of other's (Manfridi *et al.*, 1992) suggest that an NGF-dependent subpopulation may be retained in older animals.

3. Subpopulation Analysis of NGF-Dependent Survival in Co-Culture

Different subpopulations of sensory neurons have been shown with respect to NGF responsiveness. Small neurons in the chick (E21) DRG (approximately 60% of the total population) accumulated NGF, as did smaller neurons from adult (6 month) ganglia, whilst larger neurons were NGF-negative (Keller *et al.*, 1990). For young adult rats, the highest percentage of neurons positive for NGF receptors was in the size range of 20-26μm diameter (Richardson *et al.*, 1986). With adult shrew (4-5 months) trigeminal ganglia both NGF-dependent and NGF-independent cells were observed, with 20% of the population consisting of L-type cells (24-32μm) and 80% of an S-type (15-25μm) (Fukuda *et al.*, 1991). A statistical difference was shown for the percentage distribution of surviving L-type neurons with long neurites in the

presence or absence of NGF (Fukuda *et al.*, 1991). Furthermore, the sensitivity of trigeminal sensory neurons to NGF was reduced following aging by this animal. The distribution for adult and aged neuronal diameters from cultures supplemented with exogenous NGF were greater than in controls without NGF in the present DRG study also. A skew distribution for soma size from 24-33μm was apparent for adult and aged DRG neurons, whereas in cultures without NGF, the proportions of these intermediate neurons were less. For aged neurons in the absence of exogenous NGF, a higher proportion of neurons with a diameter of 9-18μm survived 17div, with a corresponding reduction in the proportion of neurons with diameter <24μm, whilst in the presence of NGF the number of aged neurons with 22-33μm diameter was enhanced. Smaller sensory neurons (<18μm) may therefore represent cells which are less sensitive to NGF effects, whereas neurons of 24-33μm diameter, are more NGF-dependent.

These results from the co-culture system may present valuable data for extrapolation to the *in vivo* studies and those of nerve injury, rather than the data of other neuron-enriched or single-cell cultures. However, synergistic effects of NGF and other possible factors produced by NNCs, and NNC-mediated NGF effects are not completely eliminated. Hence, the effects of exogenous NGF on the survival of mature neurons were further investigated by a neuron-enriched culture where NNCs were limited. In population analysis, the recruitment of smaller sized neurons to the intermediate groups due to an NGF-related growth stimulus could also contribute to the effect if cell size does not remain static throughout the culture period. The studies in next two chapters may shed further light on whether these are relevant possibilities.

Chapter IV

NGF EFFECTS ON NEUROPEPTIDE PHENOTYPE AND BIOCHEMICALLY-DEFINED POPULATIONS

Introduction

Neuronal size distributions, and their relationship to neuropeptide-, enzyme- or carbohydrate content has given rise to a proposed DRG neuronal classification (Lawson, 1992); this may be used to investigate further if neurotrophic factor effects are interrelated to specific functional subsets of DRG neurons. Price in 1985 demonstrated different size distributions *in vivo* for tyrosine hydroxylase (TH), fluoride-resistant acid phosphatase (FRAP), SP and SOM containing neurons, although all fell within the small and intermediate-sized neuronal populations. It is now known that small and intermediate-sized DRG neurons are the subpopulations upon which NGF acts predominantly (for review see Lindsay, 1993). Within these, given neuropeptide-containing neurons have been considered to have specific functions. In the present study, substance P (SP), calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY) and somatostatin (SOM) were selected for investigation of whether NGF had an effect on neuropeptide expression in aged as well as adult neurons.

SP, a member of the tachykinin family, is an 11-amino acid peptide restricted mainly within small, or in a few intermediate-sized neurons (Hökfelt *et al.*, 1976; Tuchscherer and Seybold, 1985). 50% of neurons with C-fibres and 20% with Aδ-fibre showed SP immunoreactivity (ir), but neurons with fast-conducting A-fibres did not possess SP-ir (McCarthy and Lawson, 1989). SP may be transported to the dorsal horn of the spinal cord and mediate noxious mechanical and cold stimuli (Kuraishi *et al.*, 1985; Tiseo *et al.*, 1990). It is released peripherally causing vasodilation and plasma extravasation, and also induces the proliferation of fibroblast and smooth muscle cells (Nilsson *et al.*, 1985), and stimulates mast cells and macrophages producing tumor necrosis factor α (angiogenic) (Lots *et al.*, 1988).

CGRP is a 37-amino acid peptide, localised in mainly small, and in some intermediate and large DRG neurons (for review see Lawson, 1992). Like SP, CGRP-ir fibres terminate extensively throughout laminae I and II outer (IIo) and penetrate to laminae V and X (Rosenfeld *et al.*, 1983). CGRP-containing neurons induced expression of dopamine in the mouse olfactory bulb (Denis-Donini, 1989). In the PNS, most splanchnic afferents and higher proportions of skeletal muscle and joint afferents than skin afferents had both SP- and CGRP-immunoreativities (for review see Lawson, 1992). Binding sites for SP and CGRP were found on blood vessels and in dermal tissue (for review see Hunt *et al.*, 1992). CGRP potentiated the action of SP in extravasation (Gamse and Saria, 1985). CGRP, together with SP, is involved in pain and inflammation reactions, and has been demonstrated both in *in vivo* (Donnerer *et al.*, 1992) and in *in vitro* (Vedder and Otten, 1991) studies. In addition, CGRP stimulated the proliferation of endothelial cells (Haegerstrand et al., 1990) and caused vasodilation, therefore having an important role in angiogenesis, inflammation and wound healing (Hunt *et al.*, 1992).

Neuropeptide Y is a 36-amino acid peptide first isolated from porcine brain (Tatemoto, 1982), and subsequently found in many regions of the central nervous system (Adrian *et al.*, 1983; Allen *et al*, 1983). Neuropeptide Y was found in cultured rat adult DRG neurons (Schoenen *et al*, 1989), and was shown to decrease Ca^{2+} channel activity of DRG neurons (Ewald *et al.*, 1988). NPY mRNA was also present in megakaryocytes of bone marrow (Ericsson *et al.*, 1987), and the peptide has been reported in platelets (Myers *et al.*, 1988). Since NPY is released in the second phase of platelet aggregation, it may act by causing contraction of vascular smooth muscle in haemostasis (Myers *et al.*, 1988; Daimon, 1991).

SOM is a tetradecapeptide, widely distributed in the mammalian central nervous system (Cooper *et al.*, 1981; Emson *et al.*, 1981; DiFiglia *et al.*, 1982; Takagi *et al.*, 1983). In the PNS, it was localised in small and intermediate neurons (Molander *et al.*, 1987; Ju *et al.*, 1987; Seybold *et al.*, 1989). Lack of overlap with RT97 indicated that SOM-ir existed in the neurons with C fibres (Lawson and Waddell, 1991). In the CNS, SOM was released in the spinal dorsal horn to mediate noxious heat stimuli (Kuraishi *et al.*, 1985; Tiseo *et al.*, 1990) and projected mainly to skin and to a less extent to muscle (Perry and Lawson, 1990). It has a role in attenuating immune responses (O'Dorisio *et al.*, 1985), and synergistically facilitates the effect of a conditioning stimulus on the flexor reflex, together with SP, VIP or CGRP (Xu *et al.*, 1990).

Schoenen and colleagues in 1989 systematically studied a large number of neurotransmitter phenotypes of cultured and freshly dissociated adult rat DRG neurons and demonstrated the occurrence of phenotypic plasticity in mature neurons with changes in environment. Similarly, *in vivo* studies revealed that disconnection of DRGs from their spinal targets induced numerical enhancement of some neuropeptide-containing neurons (Delree et al., 1992). There is growing evidence that NGF can modulate neuropeptide expressions (for review see Lindsay, 1992). NGF levels were elevated during peripheral inflammation with a parallel enhancement of SP and CGRP expressions in the afferent fibres innervating the inflammed tissue (Lembeck *et al.*, 1981; Kuraishi *et al.*, 1989). Antiserum to NGF reduced SP levels in adult DRGs (Rich *et al.*, 1984). NGF up-regulation of SP and CGRP expression was also shown in *in vitro* studies (Lindsay and Harmar, 1989; Vedder and Otten, 1991). Conflicting results were obtained concerning whether DRG neurons which expressed SOM required NGF (Ross *et al.*, 1981; Goedert *et al.*, 1984; Verge *et al.*, 1989a). NPY and SOM have been found to co-exist in many forebrain neurons

(Vincent., 1982; Vincent and Johansson, 1983), and NPY gene expression was shown to be up-regulated by NGF in PC12 cells (Allen *et al.*, 1987).

SP, CGRP, NPY and SOM phenotypes were examined in aged as well as adult DRG neurons in the present study [see Jiang and Smith (1993b) for preliminary report], where the following aims were defined:-

1. To monitor the time-course for SP expression *in vitro*;
2. To determine if the proportions of neuropeptide-containing neurons were restricted to a specific size range in the presence of exogenous NGF;
3. To determine if the proportions of neuropeptide ir-neurons differed, in the presence or absence of exogenous NGF;
4. To consider any NGF concentration effects on the proportions of the neuropeptide ir-neurons;
5. To correlate neuropeptide phenotypes and neurite regeneration with NGF effects.

The present studies focused on age and trophic factor's effects on individual neuropeptide phenotypes; neuropeptide co-localisation was not included, although it may would give additional detailed information in future studies. Further, mRNAs for the neuropeptides involved were not investigated in the present study although this approach would also be of interest in future. The current results however shed some light on the biochemically defined neuronal subpopulations which remain dependent on NGF into old age.

Results

1. General Observations for Immunocytochemistry (ICC)

In the main experiments described, adult or aged neurons were maintained in neuron-enriched cultures for 9div. Cultures were stained with antisera against SP (Fig.**4.1.**), CGRP (Fig.**4.2.**), NPY (Fig.**4.3.**) and SOM (Fig.**4.4.**) antigens (the optimal concentration for each was 1:20,000; 1:30,000; 1:30,000 and 1:10,000 respectively). Antisera to SP, obtained from two commercial sources (Incstar and Peninsula), were compared: the proportions of SP ir-neurons were found to be very similar (22.7±0.93 and 23.0±0.91 respectively). CGRP and NPY ir-neurons showed strong positive staining; whilst SP, and particularly SOM ir-neurons generally had weaker immunoreactivities (Fig.**4.1., 4.4.**). Staining was primarily perikaryal with coarse granular staining around the nucleus in larger neurons (Fig.**4.2.-c, 4.7.-b**). With anti-CGRP, neurites, and even growth cones were also extensively stained (Fig.**4.5.**). Ir-neurites were also occasionally found in cultures stained with the other antisera, especially when the concentration of primary antiserum was elevated. Staining was evenly distributed throughout the perikarya of small neurons (Fig.**4.1.-c, d**). Neuropeptide-containing neurons were predominantly of small or intermediate sizes; although some large ir-neurons did exist, and particularly for NPY staining where positive neurons with diameters of >40μm were observed (Fig.**4.3.**). Occasional NNC's stained very weakly with high concentrations of anti-NPY. In cultures supplemented with exogenous NGF, staining intensity was greater for CGRP and NPY ir-neurons than in cultures without added NGF, whereas the staining intensity was the same for SOM ir-neurons in the presence or absence of NGF (Fig.**4.4.**). Neurons remained pale in staining controls using the saline diluent instead of the primary antibody, and following pre-absorption

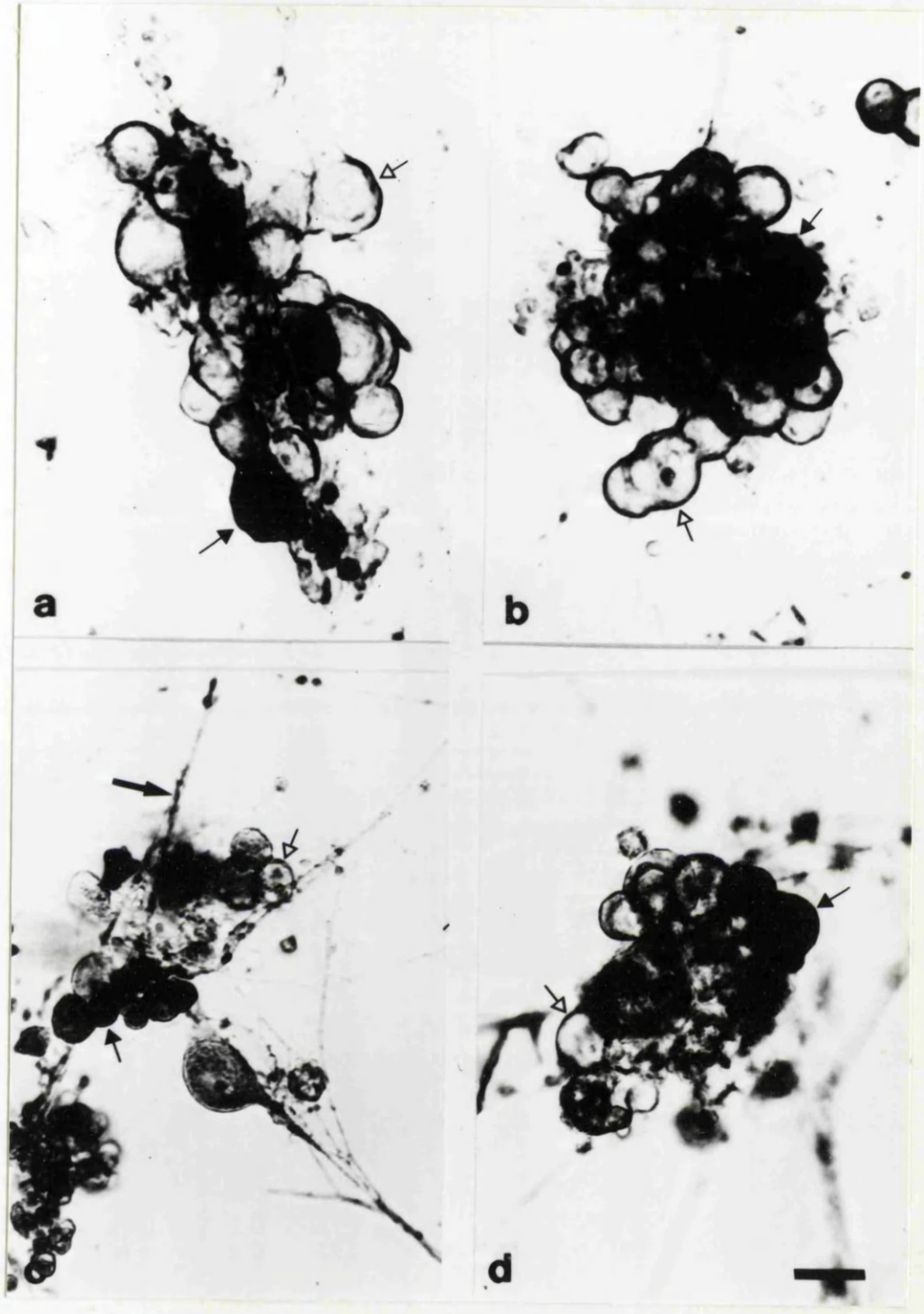

Fig.4.1. Adult (**a, b**) and aged (**c, d**) neurons cultured for 9div without NGF (**a, c**), or with 100ng/ml NGF added (**b, d**), and stained with anti-SP. Filled arrows=ir (immunoreactive) neurons; open arrows=non-ir neurons. Some neurites were SP-positive (large arrow). In the presence of exogenous NGF, the proportions of SP ir-neurons were greater than in cultures without NGF for both adult and aged neurons. Most SP-ir neurons were intermediate- and small-sized. Bar=20μm.

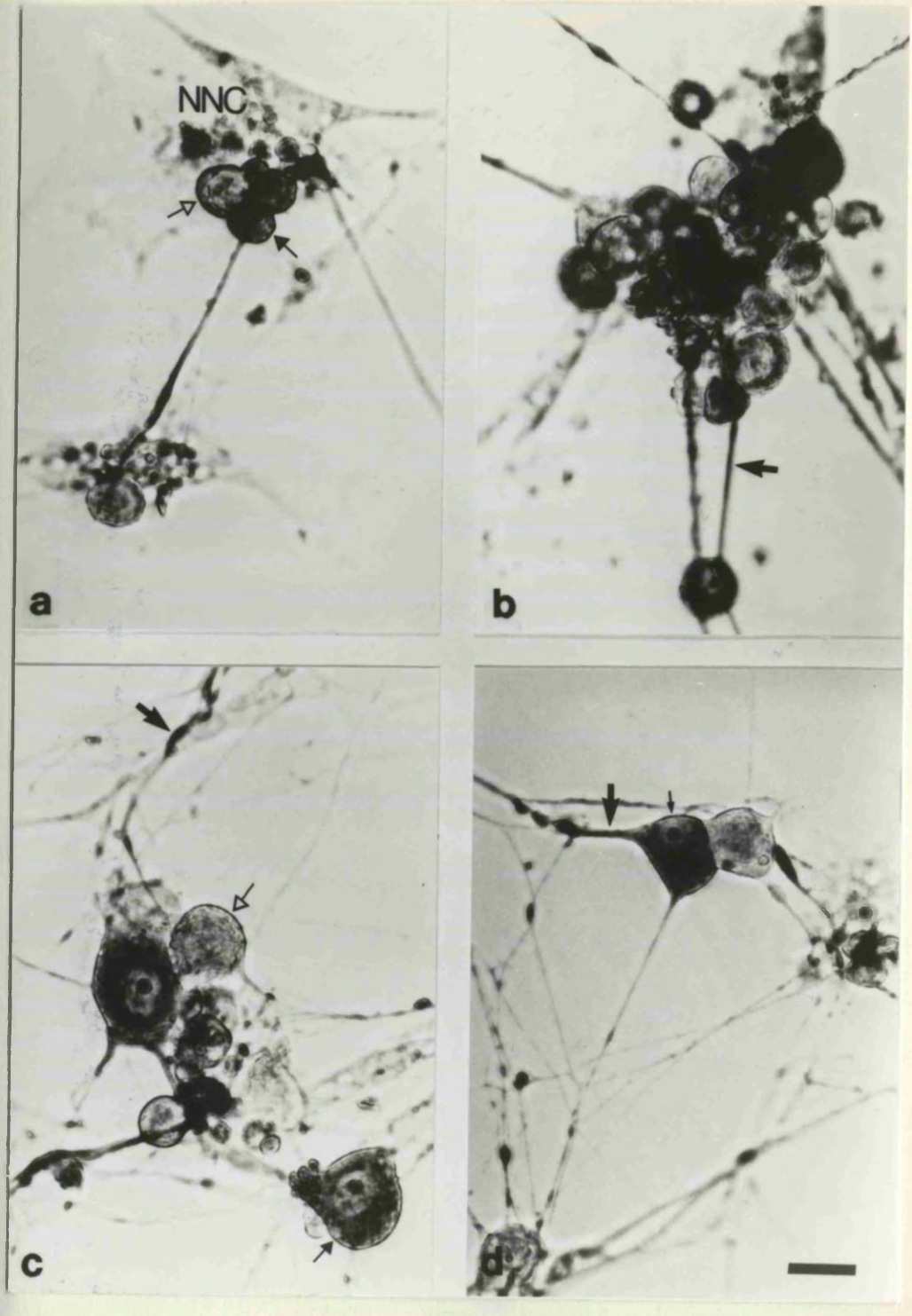

Fig.4.2. Anti-CGRP stained adult (**a, b**) and aged (**c, d**) neurons cultured for 9div without NGF (**a, c**) or with 100ng/ml NGF added (**b, d**). CGRP-ir (filled arrows) mainly occurred in small- and intermediate-sized neurons; and the occasional large neuron (note granular perikaryal staining in **c**). Nucleoli were distinct (tiny arrows). NNCs and unstained neurons (open arrows) were pale. In the presence of NGF, both CGRP-ir proportions and staining intensity were greater than in cultures without NGF. Stained neurites were seen (large arrows). Bar=20μm.

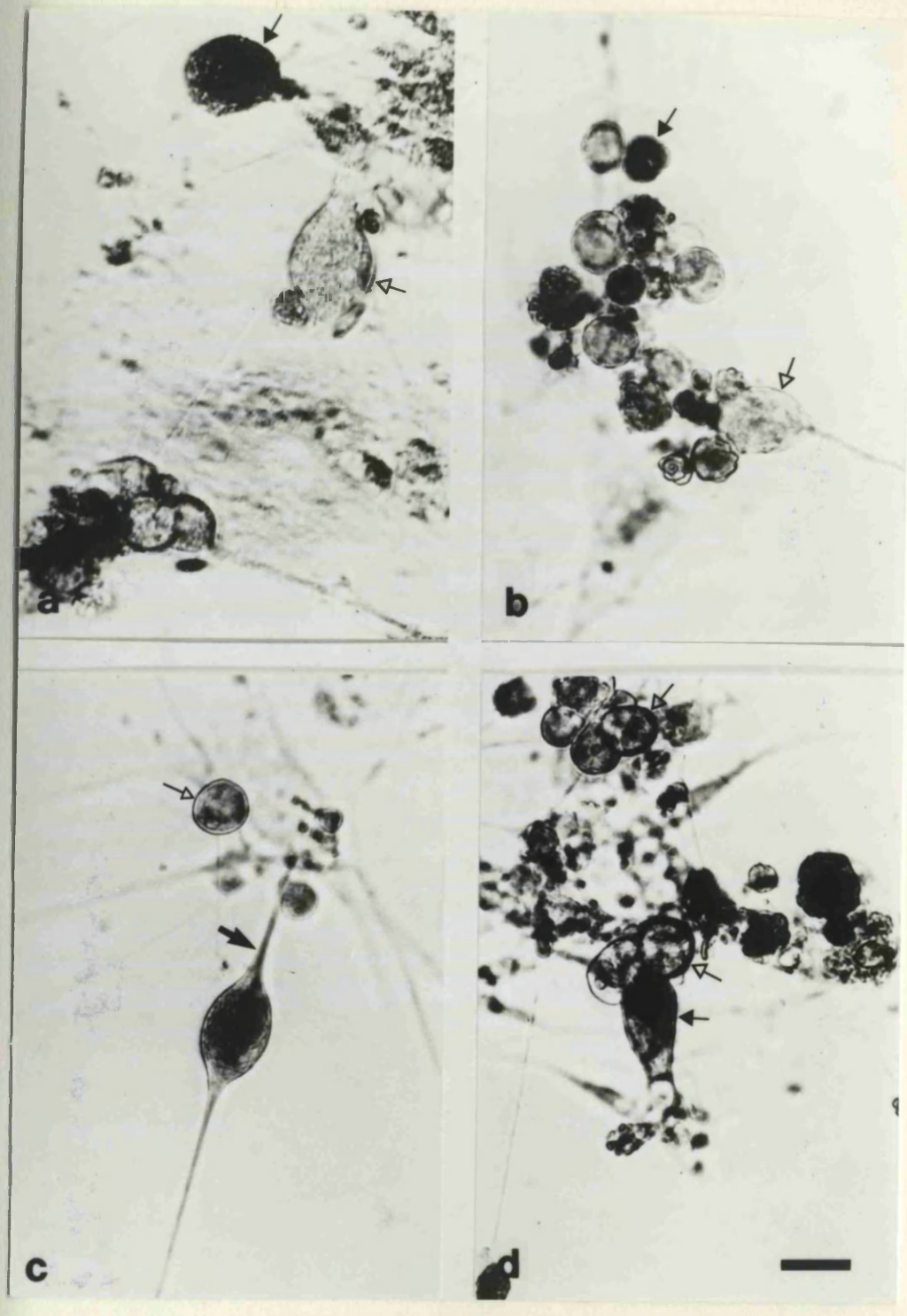

Fig.4.3. Anti-NPY stained adult (**a, b**) and aged (**c, d**) neurons cultured for 9div without NGF (**a, c**) or with 100ng/ml NGF added (**b, d**). Filled arrows = ir-neurons; open arrows = non-ir neurons. Most NPY-ir occurred in small- and intermediate-sized neurons. In the presence of NGF, NPY-ir proportions and staining intensity were greater than in cultures without NGF. Occasionally, staining extended into neurites (large arrow). Bar=20μm.

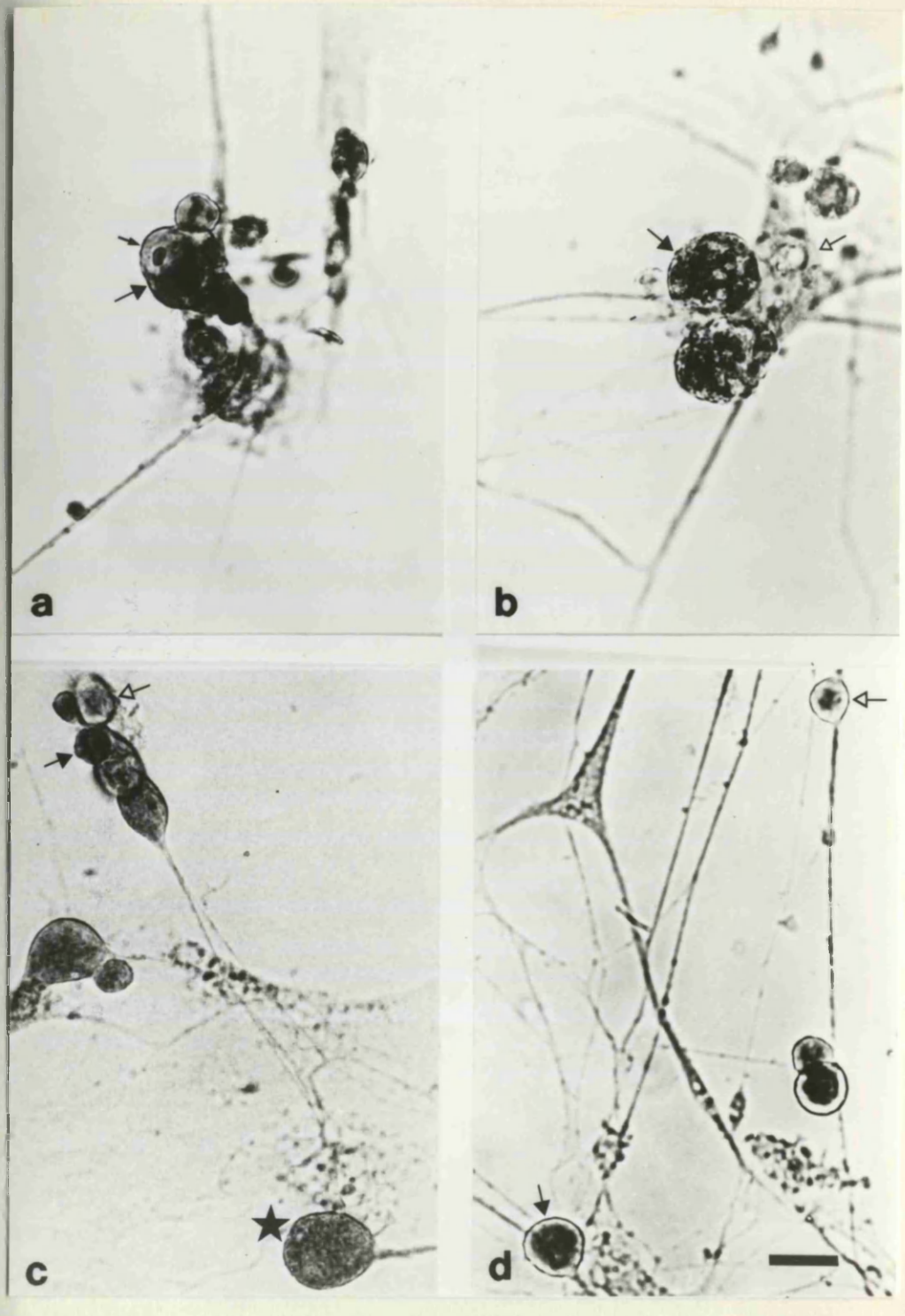

Fig.4.4. Anti-SOM stained adult (**a, b**) and aged (**e, d**) neurons cultured for 9div without NGF (**a, c**) or with 100ng/ml NGF added (**b, d**). SOM-ir (filled arrows) was mainly in intermediate- and small-sized neurons, and in the perikarya of large neurons (the tiny arrow shows an unstained nucleus with nucleolus). Some neurons were only weakly stained (filled star). Proportions and staining intensity were similar in cultures with or without NGF added for both adult and aged neurons. Bar=20μm.

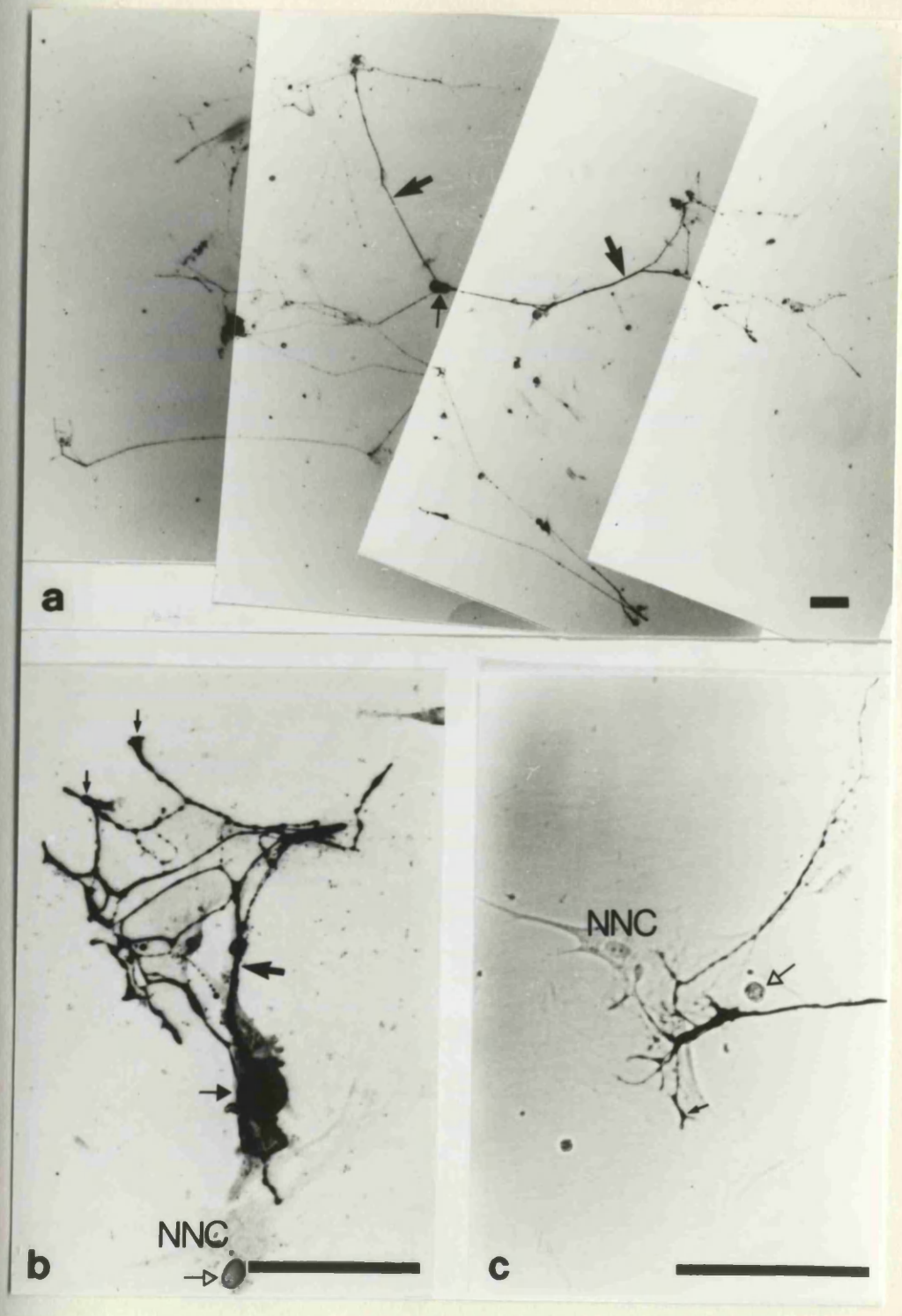

Fig.4.5. CGRP-ir neurons to show neurite geometry. Adult (**a**) and aged (**b, c**) neurons in 9div cultures with 100ng/ml NGF. CGRP production in the neuronal soma (filled arrow) was suggested by the intense neurite staining (large arrows) near the cell soma but weaker staining more distally. CGRP staining often filled the entire neurite outgrowth and growth cones (tiny arrows). Non-ir neurons (open arrow) and NNCs (NNC) were pale. Bar=100μm.

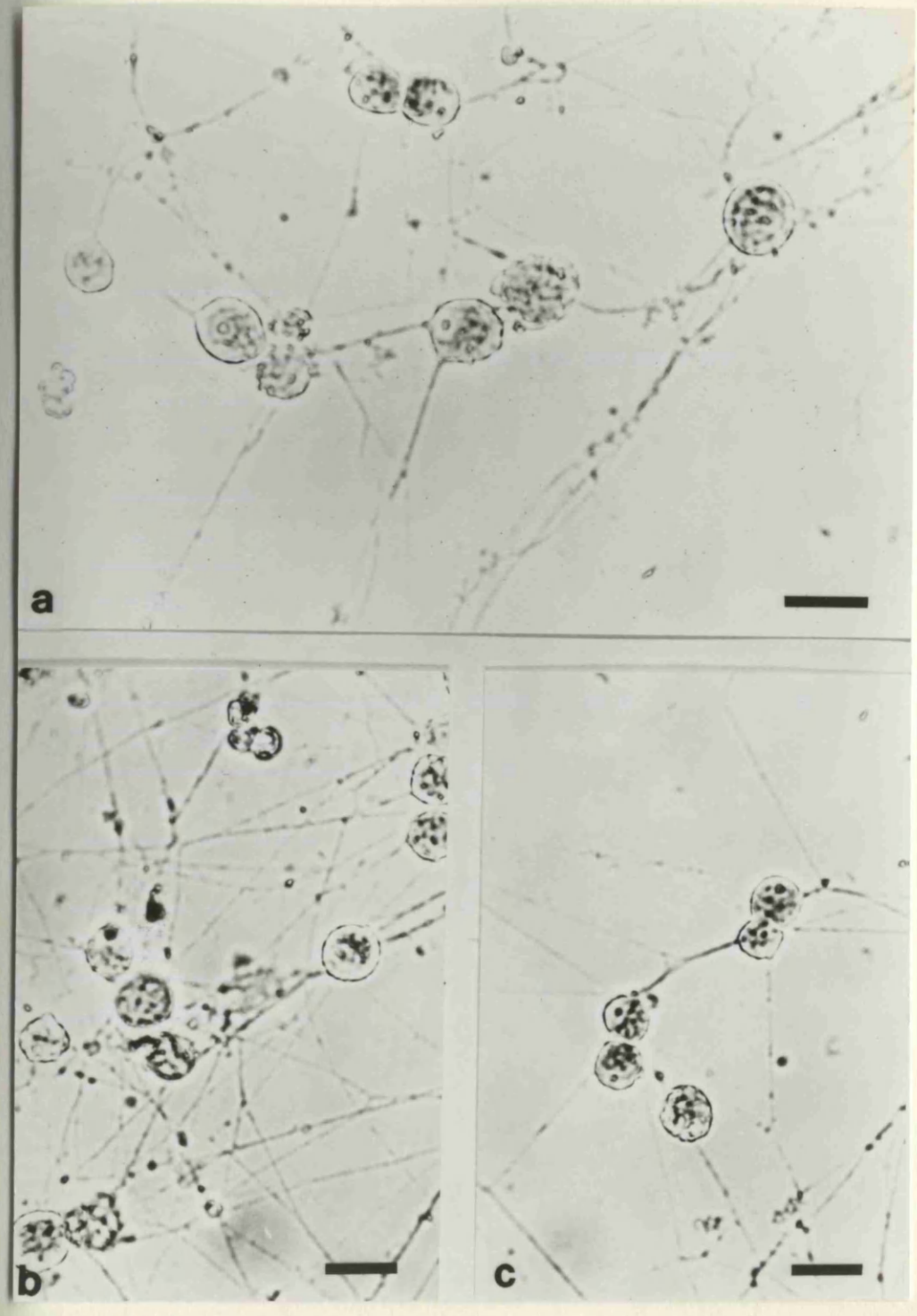

Fig.4.6. Staining controls. **a**: pre-absorption of anti-SP with $5x10^{-5}$M SP, prior to the second antibody; **b**: substitution of diluent, instead of primary antibody; **c**: use of NRS, instead of primary antibody. Both neurons and neurites were pale in these controls. Bar=20μm.

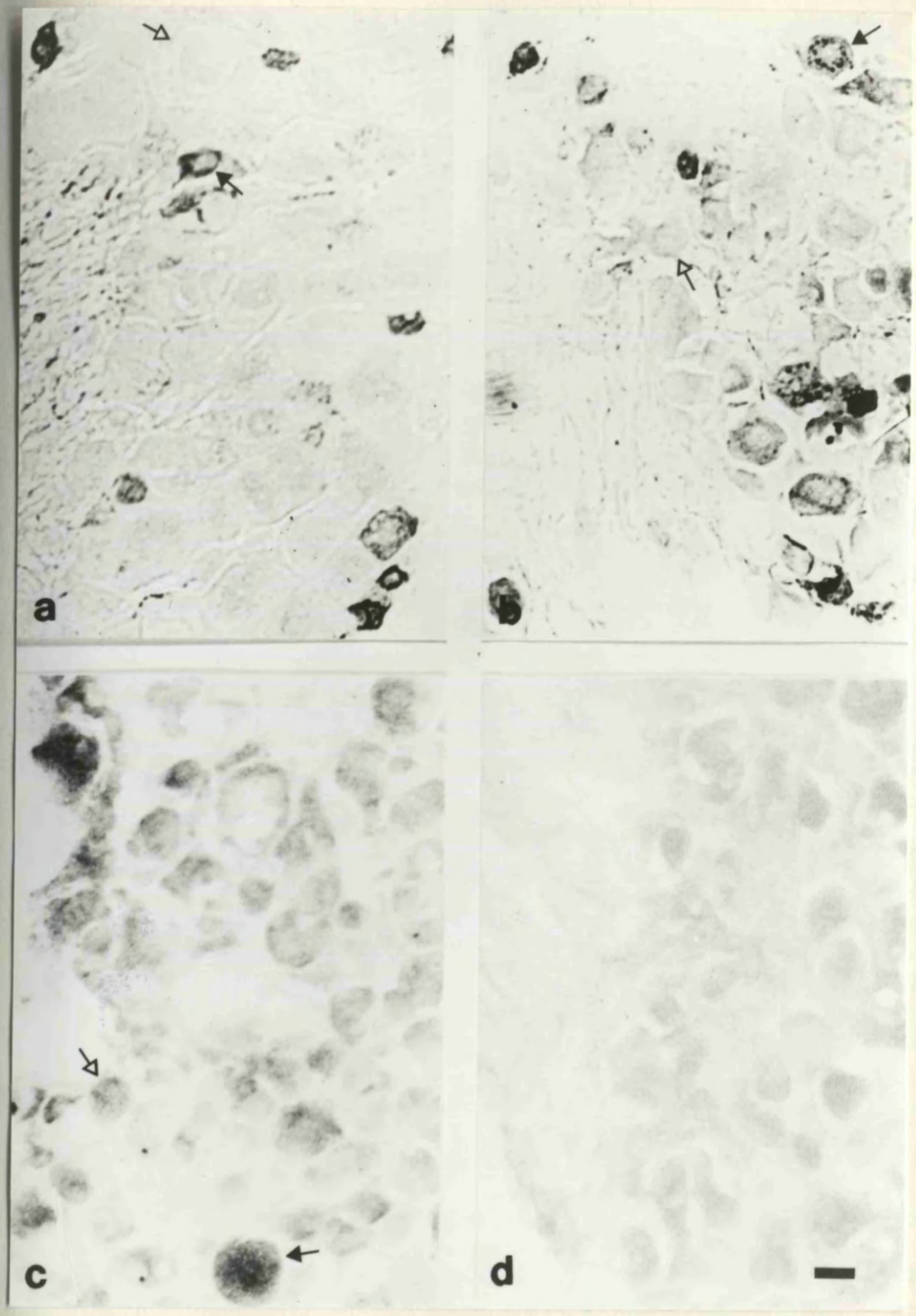

Fig.4.7. Frozen sections of aged (**a**) and adult (**b, c, d**) DRGs, stained with antisera against SP (**a**), CGRP (**b**) and SOM (**c**), or with NRS as a staining control in (**d**). Fewer ir-neurons (filled arrow) are seen for each (when compared with those *in vitro* in Figs.4.1.-4.4.). Open arrows = non-ir neurons. Filled arrow in **c** = coarse granular staining. Neurons where NRS was used, instead of primary antibody remained pale in **d**. Bar=20μm.

of the relevant antibody with its neuropeptide, or where serum was substituted for the primary antibody (Fig.**4.6.**).

Immunocytochemical staining *in vivo* was studied by using sections of lumbar 3 (L-3) DRGs. SP, CGRP and SOM ir-neurons were predominantly of small and intermediate sizes, and identical with those *in vitro*. The proportions of ir-neurons were lower *in vivo* (Fig.**4.7.**). Percentages of SP, CGRP and SOM containing neurons from adult mice were estimated as 12, 17 and 19, respectively from counts of 1,000 neurons for each neuropeptide. In L-3 DRGs from aged mice, SP and SOM ir-neurons had similar values as for adult ganglia (Table **4.1.**).

Table 4.1. Neuropeptide-contained Neurons Comparison *in vivo*

	SP (n)	SOM (n)	CGRP (n)
Adult	12.3±0.67 (4)	17.1±1.13 (9)	19.1±0.61 (3)
Aged	12.3±0.50 (8)	15.6±0.85 (5)	----------

2. Time-Course of SP Expression in vitro and NGF Effect

SP expression in aged neurons was studied both in the presence or absence of NGF (equal culture numbers for the plus and minus NGF groups). Cultures from 3 experiments were fixed after 4, 9, 14 and 19div, respectively and over 3,300 neurons in each group assessed to determine the proportion of SP ir-neurons (Fig.**4.8.**). At 4 div, SP expression was observed in 18% of the neurons examined from cultures without exogenous NGF. The numbers of SP ir-neurons in cultures without added NGF peaked at 9div, and this level was sustained for a further 5 days. After 14div, the proportions of SP ir-neurons declined slightly.

The proportion of SP ir-neurons in cultures supplemented with exogenous NGF (100ng/ml) was higher but not statistically different from cultures without NGF during the first 4div. In the next 5div, however, the numbers of SP ir-neurons in the presence of exogenous NGF were statistically different as shown by 2 way ANOVA ($P<0.01$) from those of cultures without NGF. Significant enhancement remained during the rest of culture period (Fig.**4.8.**).

3. NGF Concentration Effects

Neuropeptide-containing neurons were examined in aged neuronal cultures without or with the addition of 10ng/ml or 100ng/ml NGF. Approximately 5,000 neurons in each group were counted from 3 experimental cultures at 9div (Fig.**4.9.**). The proportion of SP ir-neurons were enhanced with both 10ng/ml and 100ng/ml NGF supplementation and were 23% greater than in cultures controls without NGF. CGRP ir-neurons proportions in the presence of 10ng/ml NGF were enhanced by 15%, compared to cultures without NGF; with a further enhancement of 15% with 100ng/ml NGF. Interestingly, the low NGF concentration did not increase the numbers of NPY ir-neurons whilst with 100ng/ml NGF obvious increases of 19% were seen. In cultures with 10ng/ml or 100ng/ml NGF added, the proportions of SOM ir-neurons were little changed from those in cultures where no NGF was added.

4. Effects of 100ng/ml NGF's on the Proportions of Neuropeptide ir-Neurons from both Adult and Aged Animals

Cultured adult and aged neurons were fixed after 9div and immunostained for SP, CGRP, NPY and SOM. Approximately 7,000 neurons

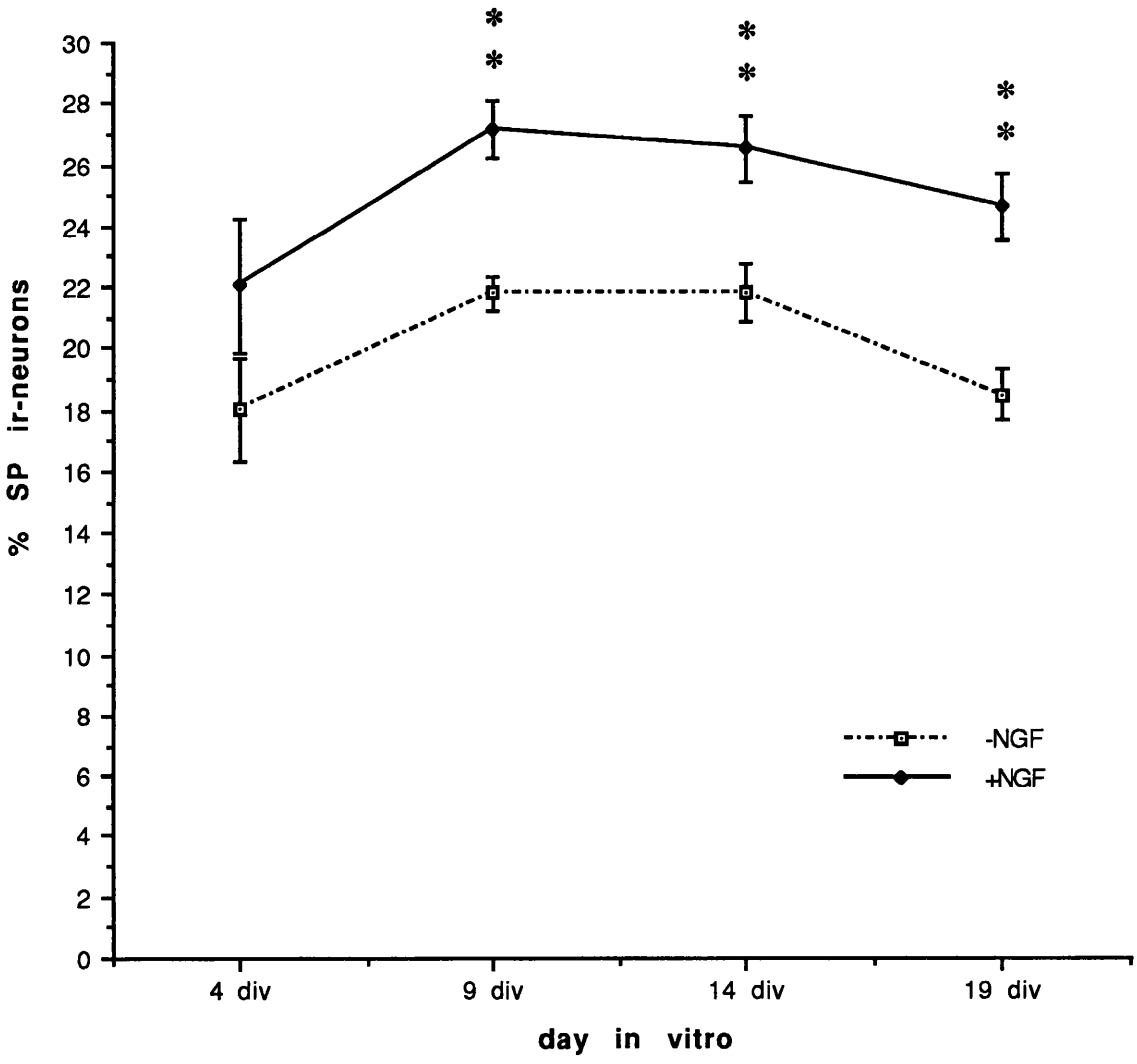

Fig.4.8. Time Course of SP Expression and NGF Effect

Aged neurons in enriched cultures without exogenous NGF, or with 100ng/ml NGF present. Cultures were fixed and stained with 1:20 anti-SP. Over 3300 neurons were counted (in 6 cultures prepared from 3 aged animals for each point). Immunoreactive (ir) neurons are expressed as a percentage. Higher proportions of SP-ir neurons were present in cultures in the presence of NGF which differed statistically from cultures without exogenous NGF by 9div. **: $P < 0.01$ (by 2-way ANOVA).

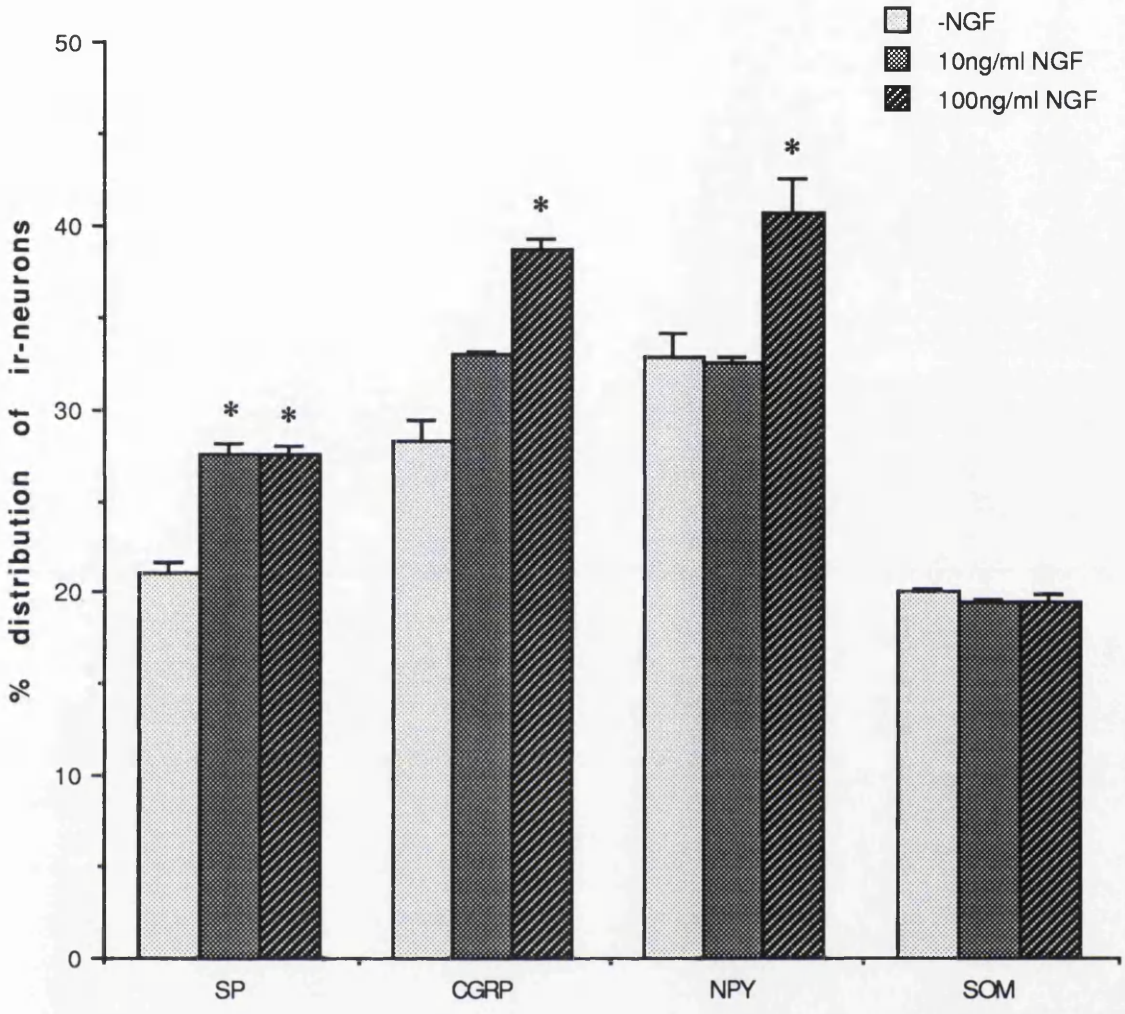

Fig.4.9. Effect of NGF Concentrations on Subsets of Neuropeptide-Containing Neurons (Aged)

Aged neurons in enriched cultures for 9div, without exogenous NGF, or with 10 or 100ng/ml NGF added from culture onset, and stained with antisera against SP, CGRP, NPY and SOM respectively. An average of 5,000 neurons/group was counted (in duplicate cultures prepared from 3 aged mice), and ir-neurons were expressed as a percentage. In the presence of 100ng/ml NGF, SP-, CGRP- and NPY-ir proportions are greater than in cultures without NGF added. However in cultures with 10ng/ml NGF added, only SP- and CGRP-ir proportions were enhanced. $*$: $P < 0.05$ (by one way ANOVA).

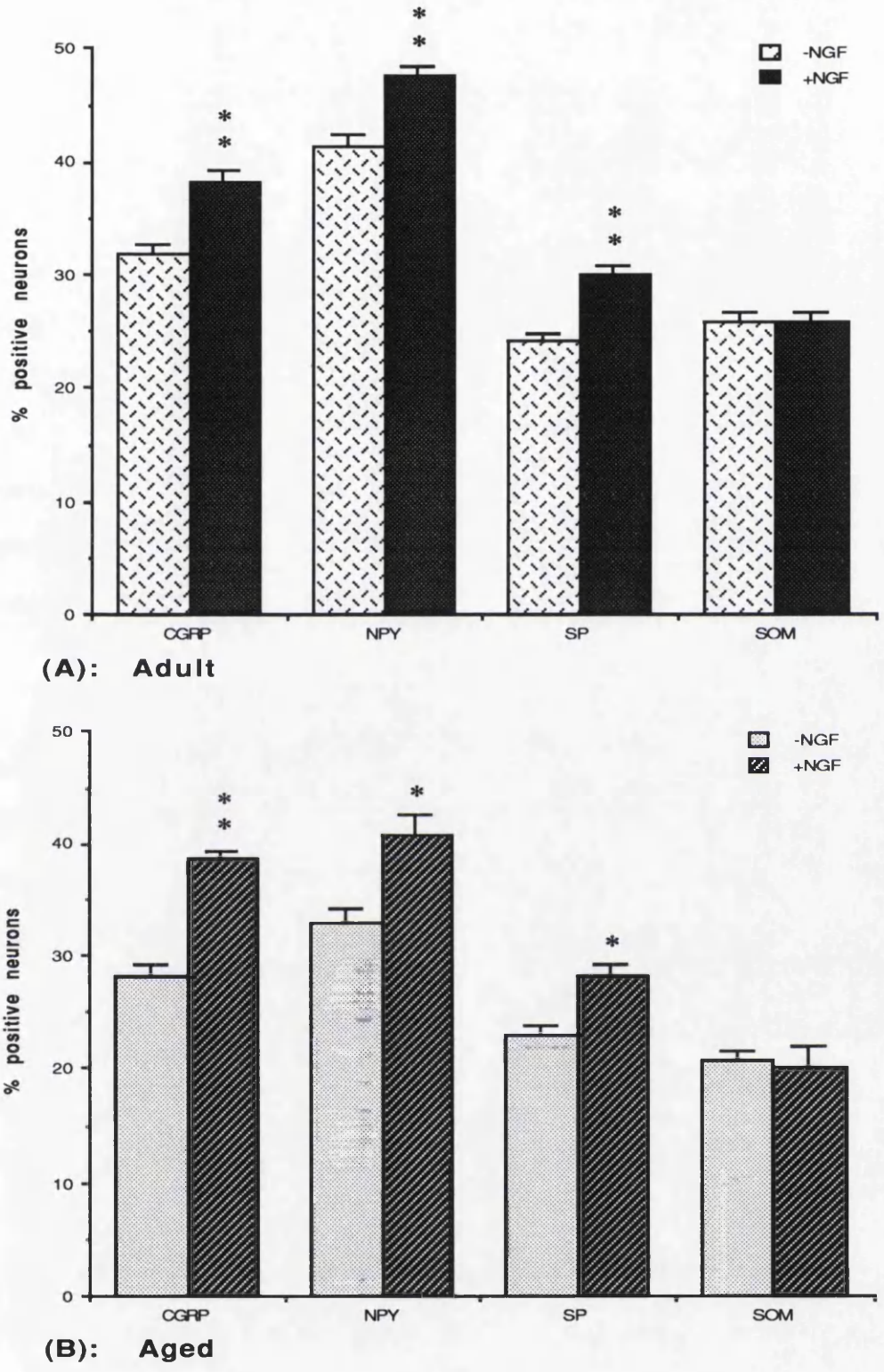

Fig.4.10. Effects of NGF on Numbers of Ir-Neurons

Neurons (over 7,000/group counted from 9-11 culture dishes prepared from 3 adult and 4 aged mice) maintained for 9div without NGF, or in the presence of 100ng/ml NGF, and stained with antisera against SP, CGRP, NPY or SOM. ⋆: $P < 0.01$; ⋆⋆: $P < 0.001$ (by t-test analysis).

from 4 experiments were counted for each group. The percentages of ir-neurons are shown in Fig.**4.10.**.

Without added exogenous NGF, SP, CGRP, NPY and SOM ir-neurons represented 24%, 32%, 42% and 26% of the neuronal population of cultures from adult mice DRG. The proportions for the four neuropeptides were less in aged neuronal cultures with a statistical difference in the proportions of CGRP ir-neurons by t-test ($P<0.05$), and for NPY and SOM ir-neurons ($P<0.01$) when comparing neurons of adult and aged cultures. The proportions of SP ir-neurons were not significantly lower from aged animals.

In adult neuronal cultures supplemented with 100ng/ml NGF, the proportions of SP ir-neurons were enhanced by 20%, and for CGRP and NPY, the proportions were ~17% and 13% greater respectively. No change was observed for the SOM ir-subsets, . For cultured aged neurons, the addition of 100ng/ml NGF resulted in a significant 27% increase in CGRP ir-neurons, reaching the level seen in the case of adult cultures with NGF added. The enhancement for NPY ir-neurons was also greater than for this subset in adult cultures. The proportions of SP ir-neurons in aged cultures were similar to the numbers from adult neurons in the presence of NGF. Only marginal changes were seen in NGF supplemented aged neurons reacted with SOM-antiserum.

5. NGF Effects on Soma Size Distribution of Biochemical Defined Subsets

The diameter of ir-neurons for the four neuropeptides were measured from photographs collected from all ICC experiments. The size distributions for neurons in enriched cultures were mainly confined to a diameter up to 33μm for adult neuronal cultures and extended to 41μm for aged neuronal cultures. The mean diameter for the neuronal populations in these experiments was ~12μm.

SP ir- (Fig.**4.11.**) and SOM ir-neurons (Fig.**4.12.**) up to 36μm in diameter were present with peaks in their distributions at 11 and 20μm. CGRP ir- (Fig.**4.13.**) and NPY ir-neurons (Fig.**4.14.**) ranged up to 33μm diameter, although neurons with 40μm diameter were occasionally present in both adult and aged cultures. In aged neuronal cultures, SP, CGRP and NPY containing neurons were less in the size range of 10-27μm; aged SOM ir-neuron did not show lower numbers in this part of the population distribution. The size ranges of SP, CGRP, NPY and SOM containing neurons in the presence of exogenous NGF were similar to those of relevant subsets in culture without exogenous NGF for both adult and aged neurons.

6. Correlation of Neuropeptide Phenotype and Neurite Regeneration, with NGF's Effect

Neurites extending from neuropeptide-containing neurons were recorded on photographs taken from all ICC experiments, and the percentage distributions of neurite bearing subsets were estimated (Fig.**4.15.**). By 9 div, no great differences for the number of neurites existed for a given neuropeptide-containing subset in adult or aged neurons. Addition of NGF increased neurite regeneration however by NPY, CGRP and SP ir-neurons in both adult and aged cultures. In the presence of NGF, SP ir-neurons with 1-2 neurites predominated, neurons with 2-3 neurites were more common in cultures of aged neurons (Fig.**4.16.**). In adult and aged cultures supplemented with NGF, CGRP ir-neurons with 2-3 neurites were more prevalent, but those with 1 neurite were still the major geometry type as was the case for NPY-ir neurons also (Fig.**4.17., 4.18.**). Adult SOM ir-neurons were mainly restricted to 1 neurite/neuron and was not greatly affected by NGF (Fig.**4.19.**).

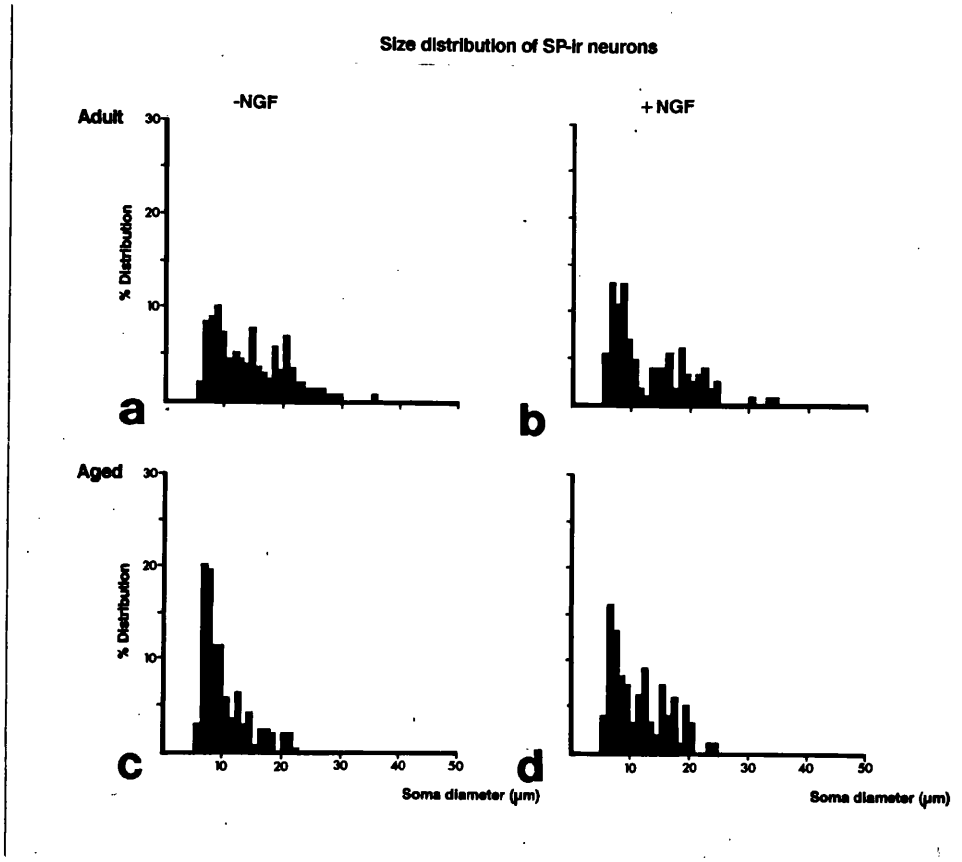

Fig.4.11. Size-Distributions of SP-ir Adult and Aged Neurons cultured for 9div and NGF Effects

Adult (**a, b**) and aged (**c, d**) neurons in enriched cultures without exogenous NGF (**a, c**) or with (**b, d**) 100ng/ml NGF added. SP-ir predominantly occurred in small and intermediate sized neurons, irrespective of whether or not exogenous NGF was present in cultures. Note: Distribution range of SP-ir neurons in the presence of NGF was similar to that of cultures without NGF.

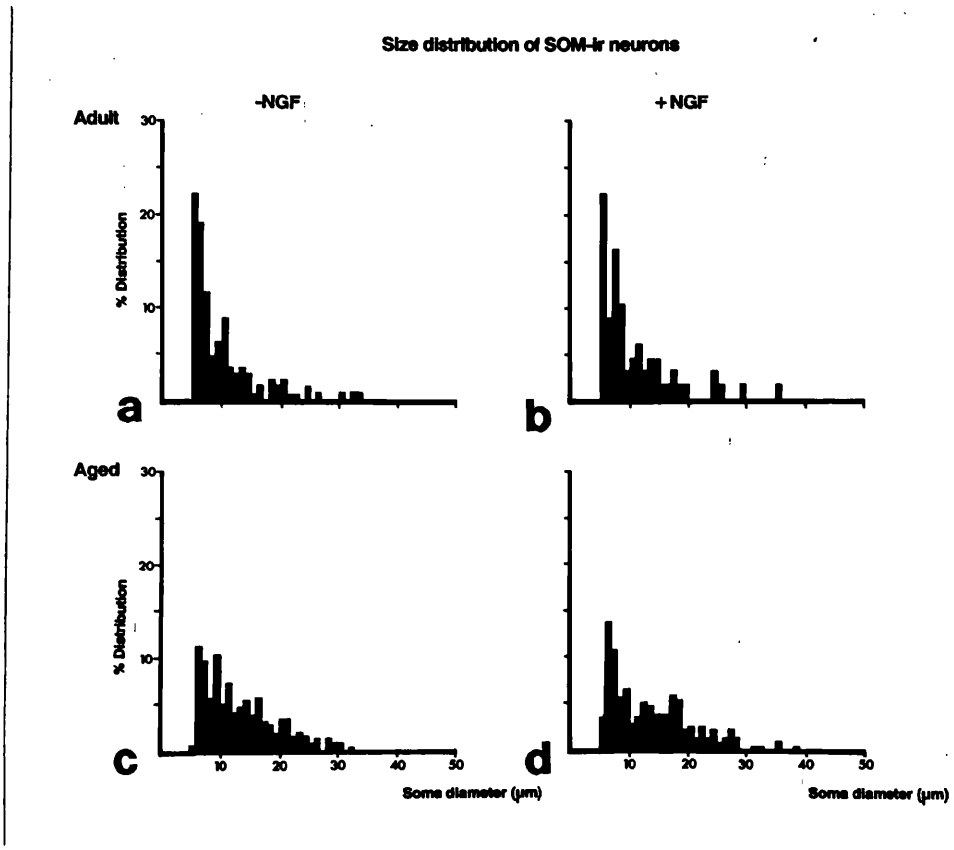

Fig.4.12. Size-Distributions of SOM-ir Adult and Aged Neurons cultured for 9div and NGF Effects

Adult (**a, b**) and aged (**c, d**) neurons in enriched cultures without exogenous NGF (**a, c**) or with (**b, d**) 100ng/ml NGF added. SOM-ir predominantly occurred in small and intermediate sized neurons, irrespective of whether or not exogenous NGF was present in cultures.

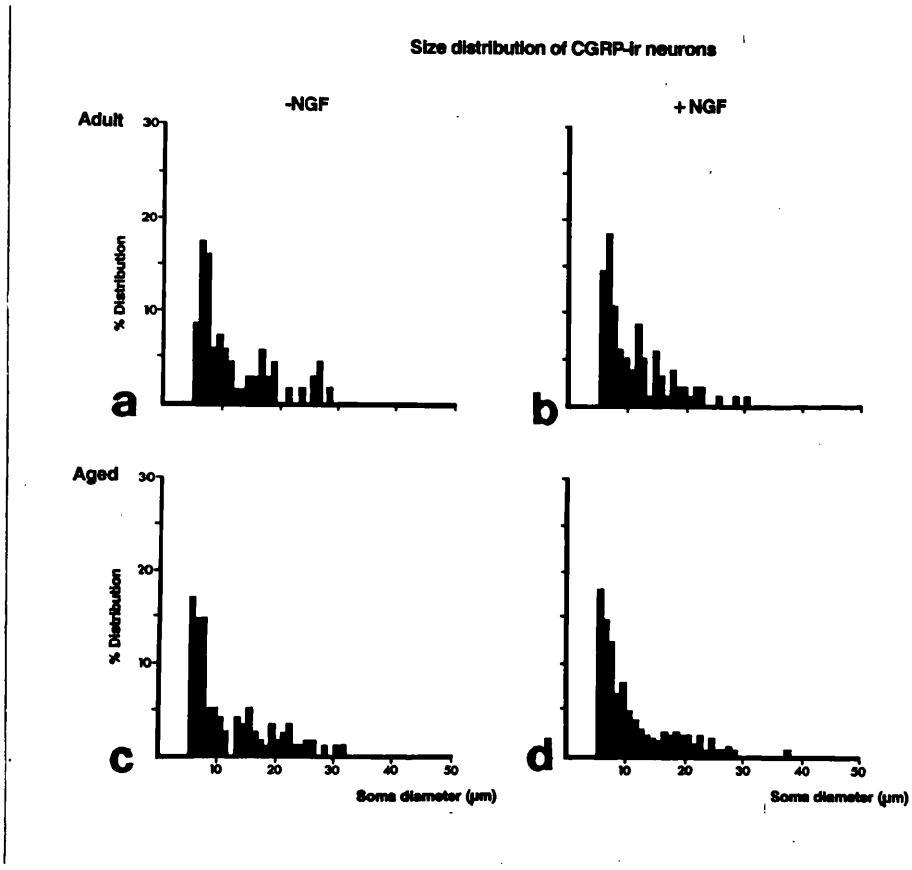

Fig.4.13. Size-Distributions of CGRP-ir Adult and Aged Neurons cultured for 9div and NGF Effects

Adult (**a, b**) and aged (**c, d**) neurons in enriched cultures without exogenous NGF (**a, c**) or with (**b, d**) 100ng/ml NGF added. CGRP-ir predominantly occurred in small and intermediate sized neurons, irrespective of whether or not exogenous NGF was present in cultures. Note: Distribution range of CGRP-ir neurons in the presence of NGF was similar to that of cultures without NGF.

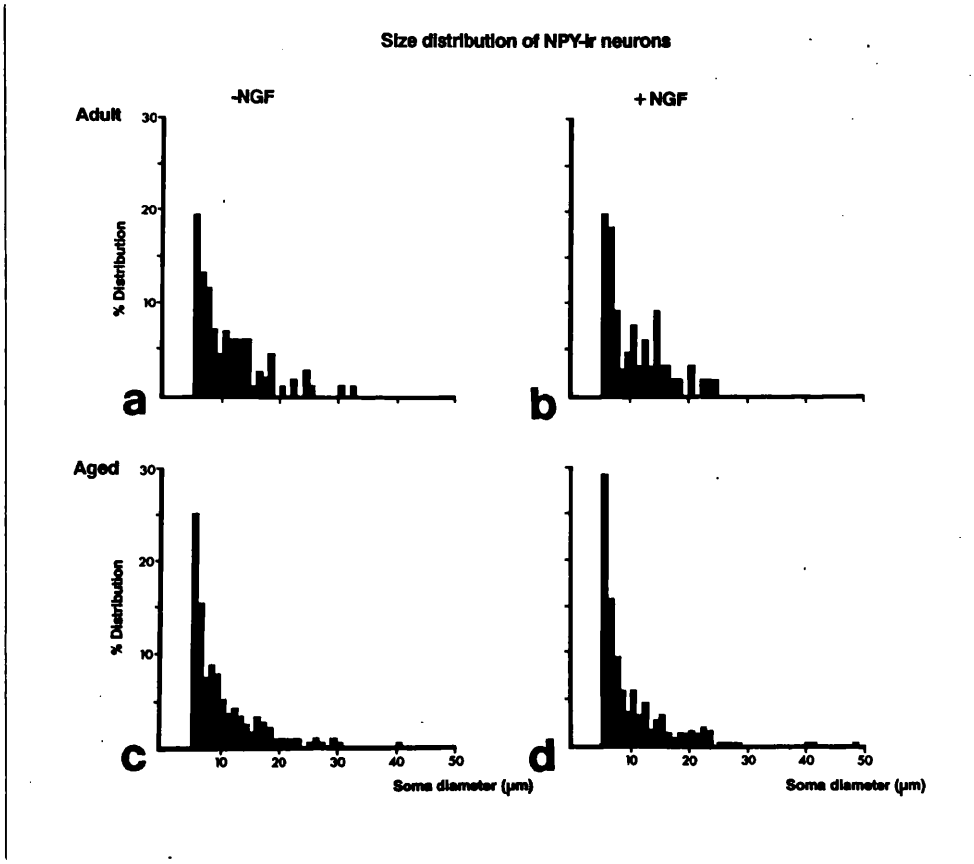

Fig.4.14. Size-Distributions of NPY-ir Adult and Aged Neurons cultured for 9div and NGF Effects

Adult (**a, b**) and aged (**c, d**) neurons in enriched cultures without exogenous NGF (**a, c**) or with (**b, d**) 100ng/ml NGF added. NPY-ir predominantly occurred in small and intermediate sized neurons, irrespective of whether or not exogenous NGF was present in cultures. Note: Distribution range of NPY-ir neurons in the presence of NGF was similar to that of cultures without NGF.

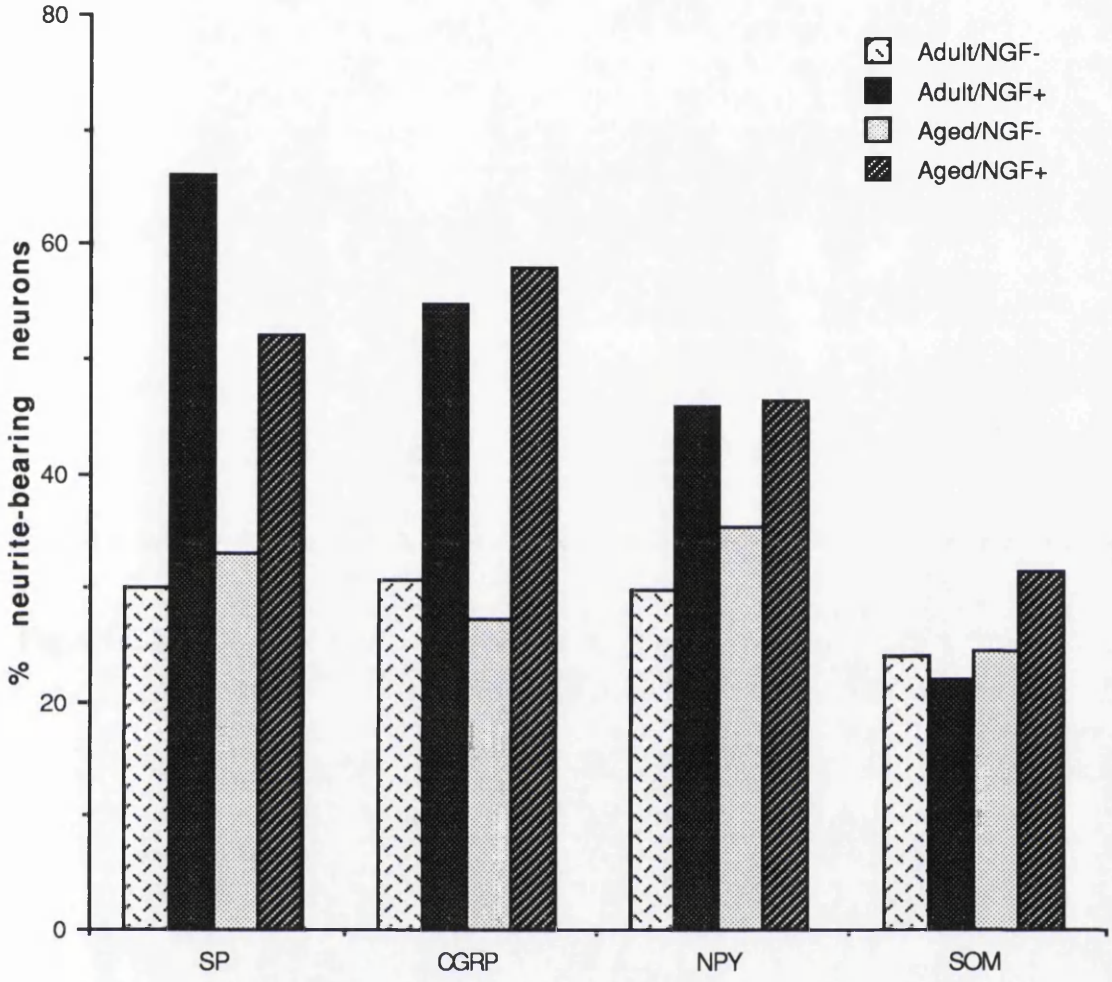

Fig.4.15. Correlation of Neuropeptide Subsets with Capability to Extend Neurites in the Presence or Absence of NGF

Adult and aged neurons from 9div enriched cultures without exogenous NGF, or in the presence of 100ng/ml NGF, and stained with antisera against SP, CGRP, NPY or SOM. NGF increased the proportion of neurite-bearing adult and aged neurons containing SP, CGRP and NPY, whereas it had a limited effect on the SOM-ir subset. Data not subjected to individual statistical analysis.

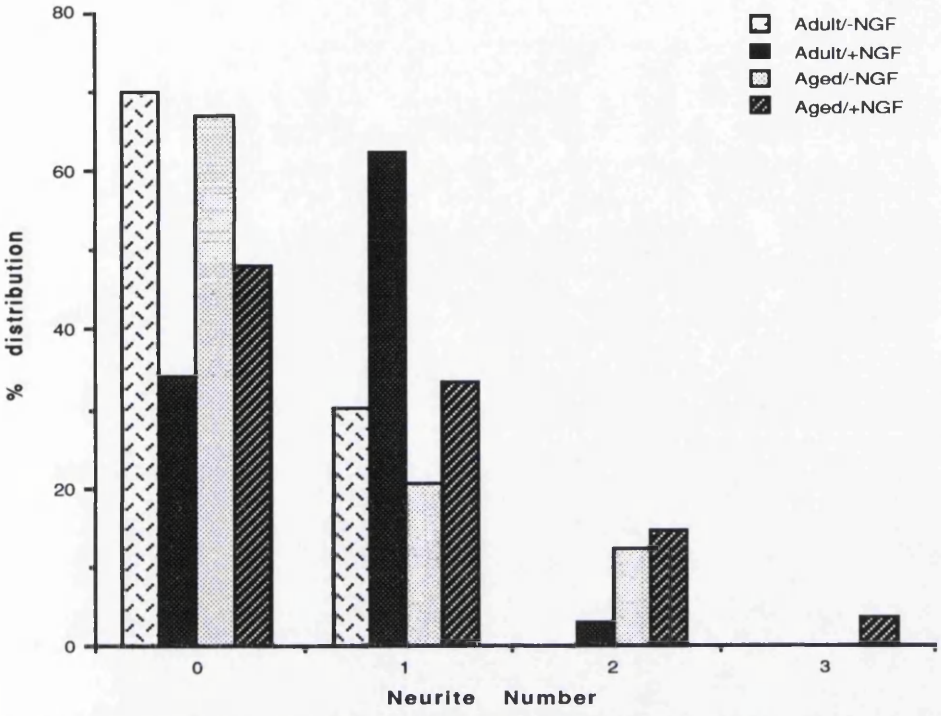

Fig.4.16. Effect of NGF on Neurite Numbers/SP ir-Neuron

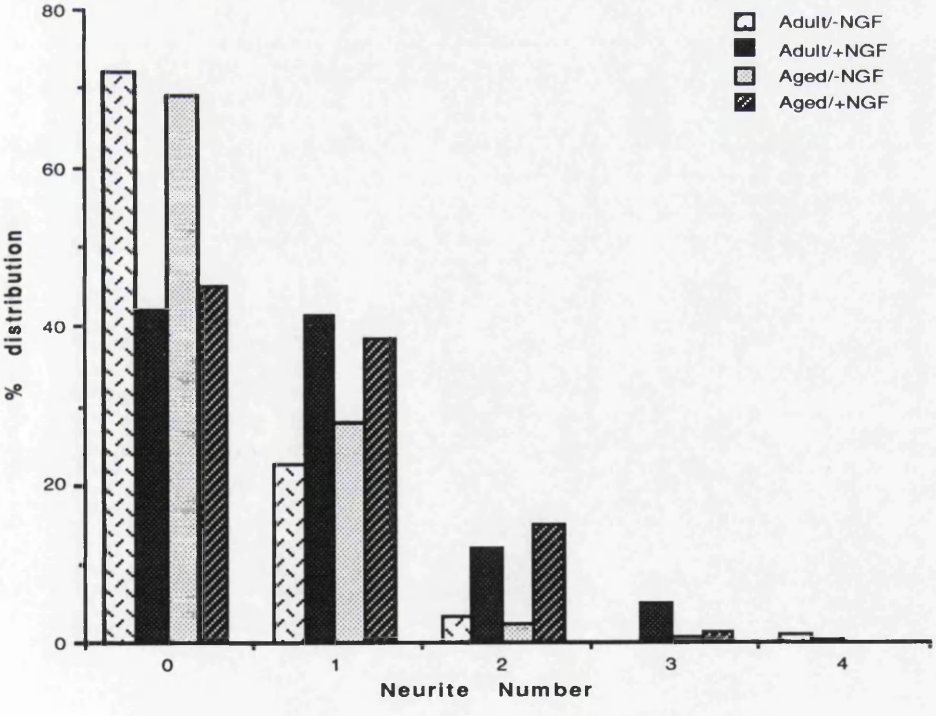

Fig.4.17. Effect of NGF on Neurite Numbers/CGRP ir-Neuron

Fig.**4.16.** and Fig.**4.17.**: Numbers of neurites extended by adult and aged SP-ir and CGRP-ir neurons in enriched cultures for 9div in the presence of 100ng/ml NGF or without exogenous NGF.

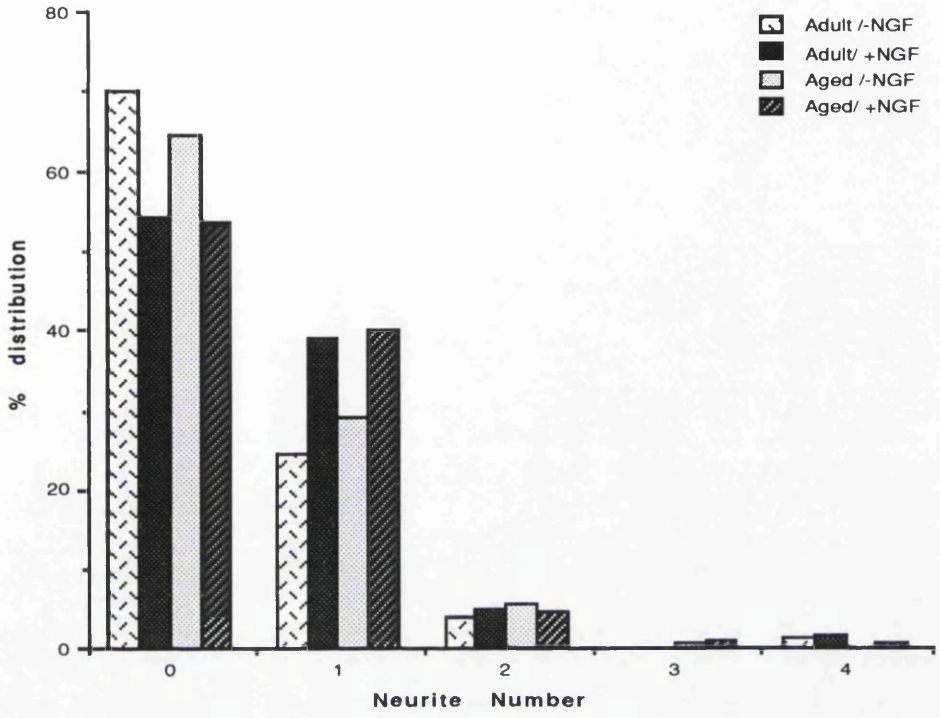

Fig.4.18. Effect of NGF on Neurite Numbers/NPY ir-Neuron

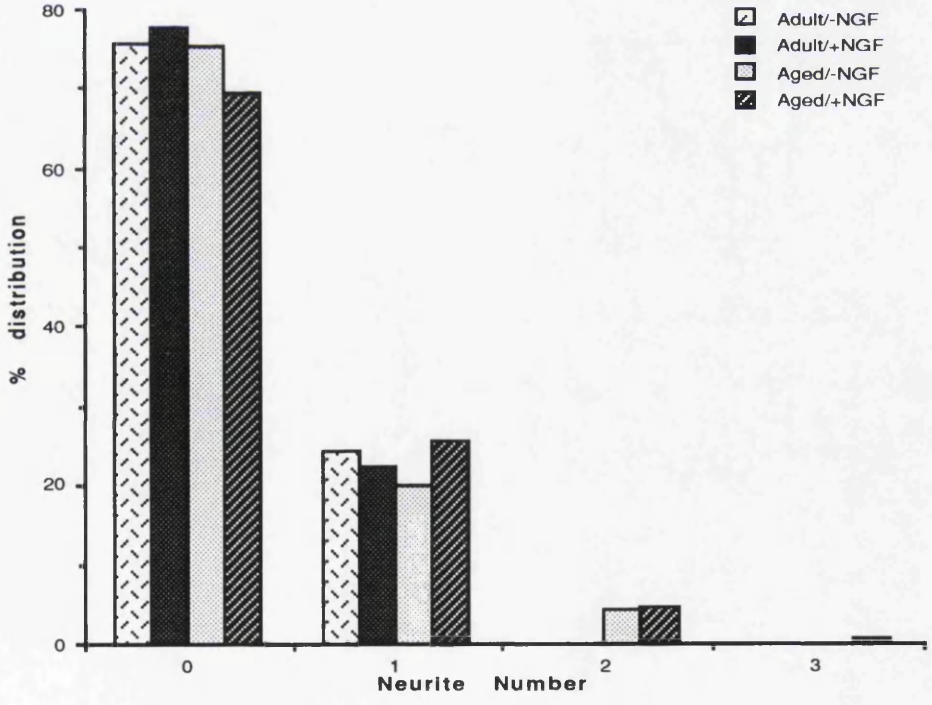

Fig.4.19. Effect of NGF on Neurite Numbers/SOM ir-Neuron

Fig.**4.18.** and Fig.**4.19.**: Numbers of neurites extended by adult and aged NPY-ir and SOM-ir neurons in enriched cultures for 9div in the presence of 100ng/ml NGF or without exogenous NGF.

Discussion

1. Neuropeptide Phenotype in Adult and Aged Neurons

Neuropeptide immunoreactivities in larger neurons were primarily seen as coarse granules around the nucleus or with a more peripheral localisation, which correlates with the interspersed distribution of Nissl substance (rough endoplasmic reticulum) in these neurons (Lawson, 1992). Staining was more uniform in smaller neurons where a denser distribution of cytoplasmic organelles exists. The neuropeptides are considered to be primarily synthesized in the neuronal perikaryon and then transferred distally into the neurites as seen in some instances in the present study.

Schoenen and colleagues (1989) previously demonstrated the presence of CGRP, NPY, SP and SOM in dissociated rat DRG neurons (3-6 month old); with the proportions of positively stained neurons after 3div being 37%, 17%, 12% and 1% respectively (only 200 neurons were counted for each by these workers). Studies *in vivo* by others showed that the proportion of CGRP, SP and SOM ir-neurons were respectively 30-50%, 20% and 5-15% in rat DRG neurons, whilst CGRP ir-neurons represented 10-20% in the guinea pig (for review see Lawson, 1992). These investigations were not related to animal aging when considering the proportion of DRG neurons containing peptides.

In the present study, these neuropeptide-containing DRG neurons were examined *in vivo* for both adult and aged mice. In adult mice DRG (L-3), the proportions of SP and SOM ir-neurons were 12% and 17% respectively, which are similar to reports for rat DRG neurons, whereas the CGRP ir-neurons represented 19% of population of L-3 DRG, similar to the finding in guinea pigs. In aged mice DRG (L-3), the proportions of SP ir-neurons were similar to

those of adults; SOM ir-neurons were slightly reduced. The results demonstrated that these neuropeptide phenotypes were sustained in mature DRG neurons and extended to aged mice.

2. Phenotype Plasticity in Mature Neurons

All neuropeptides examined in this study showed obvious enhancement in culture, compared to the *in vivo* levels, irrespective of whether the neurons were from adult or aged animals. In the 9 day culture period, CGRP, NPY, SP and SOM ir-neurons represented 32%, 42%, 24% and 26% respectively for adult mice DRGs, with 28%, 33%, 23% and 21% for aged DRG neurons. This enhancement was very similar to that reported in the investigation of Schoenen and colleague's (1989) where most of neuropeptides were expressed in significantly higher proportions of neurons in 3div cultures than in freshly dissociated preparations.

In the present study, the proportions of SP ir-neurons from aged mice DRGs were followed *in vitro* in the presence or absence of NGF, for a period from 4 to 19 days. The percentage of SP ir-neurons showed enhancement in the early phase of culture when neurons underwent the transition from a dissociated to cultured status. In the presence of NGF, the proportions of SP ir-neurons were significantly higher, peaking at 9div. Evidence from an *in vitro* radioimmunoassay study also demonstrated that SP content was enhanced in an early phase of culture, but was slightly reduced in later phases (Vedder and Otten, 1991).

The down-regulation of SP and CGRP *in vivo* by peripheral nerve transection was thought to result from these neuropeptide-containing neurons ceasing neurotransmitter activities to their targets (Barbut *et al.*, 1981; Noguchi

et al., 1989, 1990). In contrast to the studies *in vivo*, the proportions of SP-ir neurons (and also the other neuropeptides in the present study) were enhanced even though axotomy of central and peripheral nerve roots had occurred during the DRG dissociations (Schoenen *et al.*, 1989; Lindsay and Harmar, 1989). Neuropeptide expression in DRG neurons *in vivo* was shown to be regulated via their central and peripheral targets, and also by supporting cells (Delree *et al.*, 1992). The expression of tubulin mRNA in DRG neurons increases following peripheral axotomy, whereas central axotomy caused a reduction in its expression (Wong and Oblinger, 1990a). In contrast to the regulation of structural protein synthesis, chronic section of rat sciatic nerve reduced CGRP and its mRNA levels in DRG neurons, whereas section of the dorsal roots gave increased levels (Inaish et al., 1992). When the sciatic nerve had been cut 1 week previously, dorsal rhizotomy no longer increased CGRP levels, whilst NGF infused into the central stump of the cut sciatic nerve still elevated the level of CGRP (Inaish *et al.*, 1992). The percentage of CGRP-containing DRG neurons was also reduced by sciatic nerve transection or by application of higher doses of vinblastine (which blocked axonal flow without causing neuronal damage) in the studies of Kashiba and co-worders (1992). Likewise, 5-hydroxytryptamine (5-HT), thyrotropin-releasing hormone (TRH) and CGRP were expressed in significantly higher proportions of cultured adult DRG neurons than in freshly dissociated preparations (Schoenen *et al.*, 1989). Feeder layers of astrocytes, Schwann cells or fibroblasts inhibited the serotoninergic phenotype of adult DRG neurons, and the proportion of CGRP ir-neurons was also reduced when on fibroblast feeder layers (Delree *et al.*, 1992). In the same study, rhizotomy of DRGs from their spinal targets induced a significant increase in the percentage of 5-HT and TRH-ir neurons.

In summary from the findings of others, factors from the CNS and supporting cells reduce most neuropeptide expressions, whereas NGF from

PNS has been shown to enhance expression. Neuropeptide enhancement for neurons in culture as in the present study may reflect a neuronal reaction to injury following central and peripheral rhizotomy prior to setting up DRG cultures, e.g. an innate characteristic which may be promoted or depressed by exogenous trophic factors. Furthermore, up-regulation of peptides resulted in culture and following dorsal rhizotomy *in vivo* (Inaish *et al.*, 1992) with NGF administration, and down-regulation of neuropeptide expressions if NNCs were present in the cultures thereby demonstrating plasticity of neuropeptide phenotypes in adult, and in the present study, even in aged DRG neurons.

3. Age Differences in the Proportions of a Neuropeptide-Containing Neurons

The proportions of neuropeptide-immunoreactive neurons were lower for aged cultures without added NGF than for adult cultures, with CGRP-, NPY- and SOM-ir neurons reduced by 12%, 21% and 20% respectively. There may be two possibilities for this: (i) differential neuronal death in certain subpopulations during the aging process *in vivo* and/or (ii) a reduction in the potential for phenotypic expression with age. A means of distinguishing if loss was due to neuronal death or to lower phenotypic expression in cultured aged neurons should be evident from whether or not neurotrophic factors can up-regulate the proportion of the relevant neuropeptide-containing aged neurons to the level observed for adult neurons. Proportions of aged NPY ir-neurons were approximately 20% less than for adult ir-neurons and this proportion could not be up-regulated by NGF to the extent which occurred with adult neurons. Proportions of CGRP ir-neurons from aged animals were also lower than for adult cultures, but in this case the proportion of ir-neurons in the presence of NGF reached the level observed for adult neurons in identical culture conditions. Hence, it is proposed that some NPY-containing neurons have

naturally died *in vivo* during the aging of the mice. Also, NPY and SOM co-localize in many cases (Vincent et al., 1982; Vincent and Johansson, 1983), and SOM ir-neurons in aged mouse DRG was 20% less than for adult neurons *in vitro*. For CGRP ir-neurons however in aged mouse DRG, the proportions surviving are more similar to those in adults and undergo a greater regulation by NGF than was the case for NPY. A 10% decline of human brain in both weight and volume occurs with aging up to 90 years of age, mostly attributed to cell death leading to tissue shrinkage, but suggested to be due in part to atrophic changes in the neurons (Davies, 1984). Age-dependent structural alterations restricted the amount of transcriptionally active chromatin in both neurons and non-astrocytic glial cells of adult rat. An increase in acetylation of neuronal chromatin (Lux *et al.*, 1983), and low deacetylase activity (Sarkander, 1983) have been found in aged neurons so that transcription may last for a longer period. In aged neuronal cultures, exogenous NGF may modulate inactive regions of CGRP genes to become active, and constantly expressed, so that CGRP are more evidently up-regulated in aged neurons, compared to those in adult cultures. The surviving neurons from aged DRGs apparantly compensate for absent functions caused by the loss due to naturally dying neurons.

An alternative interpretation for lower ir-neurons in aged culture is that the expression of these neuropeptides in vivo is little changed during the period from adult to aged; but that after the axotomy occurring during neuronal isolation, the ability to increase CGRP, NPY and SOM expressions is lower than for the equivalent adult neurons. The SP ir-subpopulation remains responsive in aged neurons as it does in adult.

4. Subpopulation of NGF Dependent-Survival

Intermediate sized neurons (24-33μm) maintained in the presence of exogenous NGF survived in greater numbers than those in cultures without NGF in the co-culture system (Jiang and Smith, 1993a). These neurons were presumed to be equivalent to mouse type B DRG neurons classified *in vivo* by combining ultrastructural with cytochemical features and making up about 63% of the total DRG population (Sommer *et al.*, 1985). SP and SOM ir-neurons fall into the distribution range of a B type or "small dark" (15-35μm) subpopulation in rat DRGs (Anderton *et al.*, 1982), with SP ir-neurons peaking around a mean of 20μm, and SOM around a mean of 28μm and being significantly different (Price, 1985). However, Lawson (1992) demonstrated in an *in vivo* study of Wistar rats that size distributions of SP and SOM ir-neurons in L-5 DRGs ranged between 6-16μm (peaking at 10μm) and 7-19μm (peaking at 12μm) respectively; also CGRP ir-neurons were restricted to a size range of 6-20μm and peaked around 11μm. Neuropeptide-containing neurons were predominantly found in a small sized subsets and distributions for SP and SOM ir-neurons did not obviously differ from each other (Lawson, 1992). Enhancement of SP or its precursor mRNA in the presence of NGF has been reported in both *in vivo* (Donnerer *et al.*, 1992; Inaishi *et al.*, 1992) and *in vitro* (Lindsay and Harmar, 1989; Lindsay *et al.*, 1989).

Consistent with Lawson's study, both SP and SOM ir-neurons were distributed to small and intermediate sized neurons, with diameters of 5-36μm in the present *in vitro* study. By correlation analysis of peptide-containing neurons and neurite-bearing cells, SP and SOM ir-neurons predominantly had 1-2 neurites and 0-1 neurite respectively. In the presence of exogenous NGF, proportions of SP ir-neurons from adult and aged mice were 20% higher than those in cultures without exogenous NGF, whereas NGF did

not up-regulate either the adult or aged SOM ir-neuron proportions. The results were consistent with previous studies for adult neurons where the genes for SP and CGRP were only strongly expressed if the neurons were capable of responding to NGF (Nicoll *et al.*, 1980; Otten and Lorez, 1983; Verge *et al.*, 1989; Lindsay, 1989; Vedder and Otten, 1991). 95% of CGRP ir-neurons and 100% SP ir-neurons had high-affinity NGF receptors (Verge *et al.*, 1989a, b). SP ir-neurons were consistently heavily labelled by radioiodinated NGF and almost all SP ir-neurons contained CGRP (Lee *et al.*, 1985; Lindsay, 1988; Verge *et al.*, 1989). In contrast, neurons containing SOM invariably lacked the high-affinity NGF receptor (Verge *et al.*, 1989a). In adjuvant-induced arthritis, the content of SP and CGRP, but not SOM, were increased in the DRG neurons that innervated the affected joints (Smith *et al.*, 1992).

In summary, neuropeptide phenotypes remain in aged as well as adult DRG neurons. By aging, some specific subpopulations, such as NPY ir-neurons may be naturally reduced due to death. Aged neurons showed lower expressions of NPY and CGRP with a higher sensitivity to NGF, reflecting a functional compensation. SOM ir-neurons were reduced by ageing and remained non-responsive to NGF as in adult cultures. It is possible that SOM ir-neurons may represent a subpopulation receptive to other neurotrophic factors. Recently, SOM immunoreactivity in cultured ciliary ganglion neurons has been shown to be induced by activin, a member of the TGFβ family (Ling *et al.*, 1986), in choroid cell-conditioned medium (Coulombe *et al.*, 1993). In addition, bFGF was present in most SOM-containing DRG neurons (Unsicker *et al.*, 1993). SP ir-neurons were not lost *in vivo* by aging and have a similar reaction to exogenous NGF in adult and aged cultures.

Chapter V

EFFECT OF NGF ON NEURITE OUTGROWTH IN NEURONAL-ENRICHED CULTURE

Introduction

Neuronal regeneration involves complex mechanisms, comprising of a number of sequential steps: initiation, elongation, direction guidance, arborization and synapse reconstruction. In the regeneration of peripheral sensory nerve, many molecules are thought to regulate the entire procedure for both peripheral and central projections (for review see Smith and Jiang, 1994).

NGF has been shown to promote and increase the extent of neurite outgrowth/regeneration and differentiation of neurites by adult and aged sensory neurons *in vitro* (Fukuda and Yamaguchi, 1982; Scott, 1982; Fukuda, 1985; Davies *et al.*, 1987b; Lindsay, 1988). In single-cell cultures, evidence of enhancement of neurite arborization by adult rat DRG neurons (4 months old), but not neurite length was reported upon addition of NGF; in contrast, NGF up-regulated neurite length for neonatal neurons (1 day old), but not neurite ramification (Yasuda *et al.*, 1990). The extent of NGF's effect on neonatal neurons was much greater than for adult, although the absolute total neurite length from neonatal neurons was only 1/4 of that from adult neurons, following NGF treatment for the 7 day culture period. However, others have shown that NGF at a concentration of 100ng/ml markedly enhanced the length and number of regenerating neurites from both young adult (3 months old) and aged (26 months old) mice in transected DRG cultures within 5 days (Horie *et al.*, 1991). In low density cultures, NGF effects on neurite regeneration of 4-5 month old shrew trigeminal ganglion (TRG) neurons were shown to be reduced with aging; the TRG neurons consisted of both NGF-dependent and NGF-independent subpopulations as identified by their soma size, neurite number and length (Fukuda *et al.*, 1991).

To clarify these various results with respect to the potential for neurite regeneration, adult and aged DRG neurons were examined in neuron-enriched culture at a low plating density (4,000 neurons/ml/well) in the present study. Equal numbers of culture wells without or in the presence of exogenous NGF were included and neurite regeneration monitored for up to 9div. A number of inter-related, though distinct, parameters were considered in order to fully define neuritogenesis in the present study. These were (i) the number of neurites extended by each neuron, (ii) the extent of major outgrowth by an individual neurite (=major neurite length), (iii) the number of branches per neurite, (iv) a neurite's entire length (the major length plus the lengths of all its branches), and (v) the total amount of neurite outgrowth by a neuron (the summation of all entire lengths for that neuron). Although not exhaustive in characterising neurite regeneration this list adequately covers the definition of neurite elongation (major neurite length), and also highlights the level of outgrowth achieved (entire neurite length) and the total regenerative capacity (total neurite length) for each neuron analysed.

Results

1. Neurite-Bearing Subsets and NGF Effects

Neurite regeneration was evident from intermediate-sized (20-35μm in diameter), large (>35μm) and from some small (<20μm) neurons within the first 24hrs in cultures from both adult and aged mice (Fig.**5.1., 5.2.**). By 6div, the proportion of small and larger neurite-bearing neurons increased in both the absence or presence of exogenous NGF (Fig.**5.2.**). In the presence of NGF, the proportion of intermediate-sized neurons with neurites was, however, greater than those in cultures without exogenous NGF.

2. Neurite Numbers and NGF Effects in Neuron-Enriched and Co-Cultures

At 9div, most neurons extending processes had 1-2 neurites, irrespective of whether NGF was present. Adult neurons with 1-2 neurites and aged neurons with 2-4 neurites (180 neurite-bearing neurons/group counted) predominated in the presence of exogenous NGF (Fig.**5.3.-a, b**). The mean number of neurites per neuron (both for adult or aged) was reduced with culture duration (by counting 50 neurite-bearing neurons/group), irrespective of whether or not NGF was present (Fig.**5.4.**). NGF increased numbers of neurites per neuron up to 6div for adult neurons, but only initially (1div) for aged neurons. Comparisons of the proportions of neurite-bearing neurons in neuron-enriched cultures with those in co-cultures (Fig.**5.5.**), showed no difference for adult or aged neurons at 9div (by counting 150 neurons/group). However, the proportion of neurite-bearing adult neurons in co-cultures supplemented with 100ng/ml NGF reached 65% by 15div. No further increases were observed by administration of 200ng/ml NGF (Fig.**5.6.**). 50ng/ml NGF did not increase the proportion of neurite-bearing neurons. Less neurite-bearing cells were present if 1:100 anti-NGF was introduced into the co-cultures. With prolonged culture periods (up to 28-35div), the proportion of adult neurons bearing neurites was reduced (only approximately 10%), whereas the proportion of aged neurons with neurites was constant (by counting 250 neurons/group) (Fig.**5.7.**). The percentage of aged neurons bearing neurites was only 6% within 1-2div in cultures without exogenous NGF (counts of 270 neurons/group), whereas in the presence of NGF, 20% of the aged neurons had neurites at this stage of culture (Fig.**5.8.**). By 5-7div, the number of neurite-bearing neurons reached 46% in cultures without exogenous NGF and remained at this level for the rest of the culture period. In the presence of NGF the numbers of neurons bearing neurites increased throughout the entire culture period and reached 60% by 9div (Fig. **5.8.**).

3. Branch Numbers Per Neurite and NGF Effects

The amount of neurite branching by aged neurons in culture, although slightly higher, did not differ significantly from that by adult neurons in cultures without exogenous NGF. Initially, branch numbers increased up to 6div for neurites of adult neurons, and up to 3div for aged neurons (by counting all neurites from 50 neurons/group), but then decreased in both if cultured without exogenous NGF (Fig.**5.9.**). In cultures supplemented with exogenous NGF, branch numbers per neurite were consistently higher than those for neuronal cultures without added NGF. In the absence of NGF, neurites frequently had <20 branches both for adult and aged neurons, whereas in addition to neurites with <20 branches, neurons with neurites containing 40-60 branches were also common in the presence of NGF. NGF up-regulation for branch number per neurite of adult neurons (Fig.**5.10.**) were greater than for aged neurons (Fig.**5.11.**).

4. Major Neurite Length and NGF Effects

Extension of the major neurite (excluding its branches) for adult and aged neurons was about 120μm within 1 day (by counting 50 neurite-bearing neurons/group), in both the absence or presence of exogenous NGF (Fig.**5.1.**, **5.12.**). By 3 or 6div, major neurite lengths measured in adult neurons cultured with or without NGF were similar to those for aged neurons in the same culture regimen. By 9div, mean major neurite lengths were 24% longer for adult compared to aged neurons without added NGF, and by 32% in the presence of NGF. Up to 3div, the maximal major neurite lengths from adult or aged neurons were not significantly different in cultures with or without exogenous NGF added (Fig.**5.12.**). A skew distribution was evident however by 6div for adult and by 9div for aged with some neurite lengths of >2,000μm in the presence of

NGF and with significant differences ($P < 0.0005$) in neurite lengths in cultures with NGF compared to those without (Fig.**5.13.**).

5. Entire Length of Neurite and NGF Effect

The entire length of a neurite was measured and defined as the sum of its length plus the lengths of all branches ramifying from the process. Entire lengths of neurites (by counting 50 neurite-bearing neurons/group) for adult and aged neurons increased with days *in vitro*, both in the absence or presence of NGF (Fig.**5.14.**). The entire lengths extended from adult neurons cultured without exogenous NGF were similar to those of aged neurons initially, but slightly greater after 9div. In cultures supplemented with NGF, neurite entire lengths were greater than in adult neurons cultured without NGF by 1div, and for aged neurons by 3div. By 6 and 9div, the significant differences were more pronounced in cultures with NGF for both adult and aged neurons (Fig.**5.13.**, **5.15.**). In the presence of exogenous NGF, entire neurite lengths for some adult and aged neurons were 6,000-8,000μm by 3div and over 10,000μm by 6-9div.

6. Total Length of Neuron and NGF Effect

Total neurite lengths represented the sum of all neurite outgrowth extending from a given neuron. Within 16-24hrs *in vitro*, the total neurite lengths of adult and aged neurons were similar; by 3div, the total length from aged neurons were greater than from adult neurons cultured without added NGF, but no other differences were seen in further culture periods (Fig.**5.16.**, also **5.15.**). In the presence of exogenous NGF, the total neurite lengths for adult and aged neurons were greater than those in cultures without NGF as early as 1div (also see Fig.**5.1.**). The total length of adult neurons reached a

maximum within 6div in the presence of exogenous NGF, whereas it took aged neurons another 3div to reach this level.

In the early phase of culture (1div), the maximum total neurite length for adult neurons in the presence of NGF was approximately 800μm greater than that from neurons cultured without NGF (Fig.**5.16.**). The range for total neurite lengths was more variable (6μm-3,500μm) for cultures with NGF, compared to those without NGF (14-2,700μm). Similar differences were also evident for aged neurons in the presence of NGF compared to those in the absence of NGF. By 3div, a skew distribution occurred mainly at >4,500μm for total lengths of neurite from adult and aged neurons in the presence of exogenous NGF. The total neurite length of adult and aged neurons were mainly distributed within a 30-4,500μm range, together with some distributed in the 5,000-10,000μm region during the entire culture period, although some neurons with 12,000μm total neurite lengths could be occasionally found for adult and aged culture by 6 or 9div. However, adult and aged neurons with total neurite lengths of 5,000-20,000μm in cultures supplemented with NGF were often seen, compared to minus NGF controls in the late culture phase.

To summarise, NGF effects on neuronal geometry for adult and aged neurons were as shown in Fig.**5.17.** and Table **5.1.**.

Discussion

1. Population and Onset of Neurite Regeneration

Intermediate and large sized neurons most frequently extended neurites, irrespective of whether these neurons were from adult or aged animals. Neurites

Table 5.1. % Enhancements with NGF Present Compared to Culturing Without Exogenous NGF for Adult and Aged Neuron

		Neurite Number	Major Length	Branch Numbers	Entire Length	Total Length
Adult	6div	36.8%	19.4%	61.4%	29.7%	54.6%
	9div	2.4%	29.2%	46.8%	35.4%	40.2%
Aged	6div	-1.0%	29.5%	9.6%	38.8%	37.3%
	9div	17.3%	20.6%	58.3%	44.3%	54.3%

were only occasionally extended from smaller sized neurons by 6div, although numbers were slightly higher by 9div. These results are similar to those of Fukuda and colleagues (1991) who observed that large mature sensory neurons (24-32μm) in low density cultures extended 2-7 long neurites with dense arborizations, whilst smaller neurons (15-25μm) had only 1-3 short neurites with less branches. In the present study cultures supplemented with exogenous NGF for 9div, most neurons extended total neurite lengths of approximately 5,000μm, although some still existed with lengths of only 20-40μm, suggesting that the onset of neurite regeneration was not synchronised *in vitro*.

Approximately 25% of cultured sensory neurons from aged shrew did not have neurites, although these cells were viable as identified by their phase-bright contrast and by trypan blue exclusion (Fukuda *et al.*, 1991). In the 9 day culture period of the present study, approximate 40% of the sensory neurons from aged mice cultured with exogenous NGF did not possess neurites, although these cells were phase-bright, had smooth membranes and a perfect nuclear outline. Hence, only taking neurite-bearing neurons as an indication of survival in short-duration cultures, especially for neuron-enriched or single-cell cultures, such as in the study of Lindsay (1988), is an unreasonable criterion for the evaluation of a trophic factor's effect on neuronal survival.

Neurite extension still occurs in culture, although the existence of central and/or peripheral targets is lacking, suggesting that neurite regeneration is an intrinsic character of DRG neurons. Intermediate and large DRG neurons have a greater ability for neurite regeneration than smaller neurons.

2. Reconstruction of Neurite Geometry with Culture Age

As the culture period increased, neurite reconstruction occurred in both adult and aged neurons as demonstrated by a reduction in the numbers of neurites and their branches, with a concomitant enhancement of major, entire and total neurite lengths (due to increases in the length of branches, in addition to the neurite's absolute length). Neurons had an obvious ability therefore to modify their neurite geometry. Initially during early culture stages, many branched neurites were extended, reflecting random neuronal regeneration. In older cultures, fewer neurites were present due to further development, and suggesting that neurite regeneration may have responded to directional cues, as has been observed for DRG neurons in single-cell culture also (Yasuda *et al.*, 1990). A reduction in neurite number with elongation by 2-3div was reported previously for aged mouse DRGs in cultures supplemented without or with 100-1,000ng/ml NGF (Horie *et al.*, 1991).

Neurite reconstruction in the present studies may involve destruction of the cytoskeleton in many neurites with active synthesis at the molecular level in selective or given neurites. Down-regulation of neurofilament proteins and their mRNAs following nerve injury may contribute to the destruction in regressing neurites since these function in maintaining integrity, continuity and stability of neurites (Obinger *et al.*, 1991). In contrast, the extending neurites may possess an up-regulation of tubulin, actin and peripherin following nerve injury since these are fundamental for cytoskeletal formation (Fine and Bray, 1971). Further work would be necessary to test this hypothesis.

3. Comparison of Adult and Aged Neurons for Neurite Regenerations

No statistical differences existed when tested by ANOVA in either entire or total neurite length for adult and aged neurons maintained without the addition of NGF at the 4 culture intervals studied. However, distinct models for the geometry of neurite regeneration were evident. Neurite length elongation predominated in adult neuronal cultures, evident by 6div, whereas, for aged neurons, branching was a more dominant feature, especially in early culture phases. Neurite length reduction of cultured aged DRG neurons, compared with adult, has also been shown by others (Horie *et al.*, 1991), and also in aged sympathetic neurons in serum-free culture (Fukuda *et al.*, 1985), and in cultures with serum (Argiro and Johnson, 1982; Uchida and Tomonaga, 1986). In general, the reconstruction ability was reduced with neuronal age, although aged DRG neurons still retained a capability for neurite regeneration.

4. NGF Effects on Neurite Regeneration of Adult and Aged Neurons

Tracing neuronal geometry is a complex and time-consuming exercise and difficult *in vivo*. It can be done more easily *in vitro*, and a few reports have described the effects of NGF effects on this during neurite regeneration. NGF was shown to increase the total axonal length from adult sympathetic (Uchida and Tomonaga, 1985), DRG (Yasuda *et al.*, 1990) and TRG neurons (Fukuda *et al.*, 1991) *in vitro*. In the study of Fukuda and coworkers (1991), NGF did not increase total neurite length for aged shrew TRG neurons, although increases did occur in adult TRG neurons. However, enhancement by NGF was demonstrated for DRG neuronal cultures from 26-month-old mice (Horie *et al.*, 1991). In the present study, NGF promoted neurite regeneration, enhancing total neurite length of both adult or aged neuron as early as 24hrs. This was identical with Lindsay's observation (1988) where NGF promoted the initial

growth of neurites from adult DRG neurons. High-affinity NGF receptor completely co-localized with GAP-43 mRNA (Verge *et al.*, 1990), and the latter contributed to initiation of neurites (Meiri and Burdick, 1991).

The present study also revealed that distinct NGF effects on neurite regeneration occurred in early or late culture phases for both adult and aged neurons, although total length continued to increase throughout the entire culture period. Initially, NGF caused an increase in the numbers of neurites per neuron and the numbers of branches per neurite, as was also demonstrated in adult neurons in single-cell cultures within 7div (Yasuda *et al.*, 1990). In later culture phases however, NGF predominantly increased the lengths of neurites and/or branches. By 6div, neurite lengths of adult or aged neurons with NGF added was significantly greater than those in cultures without exogenous NGF, whilst neurite numbers reduced. These opposite changes in neurite number and length were also observed for cultured aged DRG with NGF as high as 1,000ng/ml (Horie *et al.*, 1991). Reconstruction of neurite geometry appeared to be an inherent property of neurons during regeneration with NGF accelerating both neurite initiation and the reconstruction procedure. Furthermore, NGF was seen, in the present study, to increase neurite elongation in the late culture phase and not only affected neurite initiation.

NGF has been shown to be a significant factor in repairing peripheral nerve, with NGF mRNA levels in the proximal stump and distal nerve segment enhanced following sciatic nerve crush or transection (Heumann *et al.*, 1987a, b). Addition of anti-NGF to lesioned adult sciatic nerve inhibited regeneration (Gorin and Johnson, 1980; Schwartz *et al.*, 1982). In a separate study, deprivation of endogenous NGF reduced post-lesion collateral sprouting (Diamond *et al.*, 1987). Axonal sprouting has been suggested as the most characteristic feature for regenerating nerves and contributes to various

peripheral neuropathies (Said and Selva, 1983; Berciano *et al.*, 1986; Sobue *et al.*, 1986, 1988). In addition, NGF enhanced axonal numbers of optic nerve (Turner and Glaze, 1977; Yip and Grafstein, 1982), the nerve to medial gastrocnemius (Hulsebosch *et al.*, 1984) and the facial nerve (Chen *et al.*, 1989) *in vivo*.

NGF effects on entire and total neurite lengths of aged neurons were greater than on adult neurons by 9% and 14% respectively by 9div. These enhancements of aged neurons in the presence of NGF mainly represented extensive growth of branch lengths. However, statistical differences for entire neurite lengths of adult neurons in cultures with of without NGF present occurred as early as <1div, but not until 3div for aged neurons. In addition, the total neurite length for adult neurons peaked by 6div, whereas aged neurons took another 3 days to reach the peak level with exogenous NGF added. These findings demonstrated that during neurite regeneration, adult neurons reacted to NGF more rapidly than aged neurons although aged neurons still respond to exogenous NGF up to 9div. These findings correlate with the results obtained from the immunocytochemistry study.

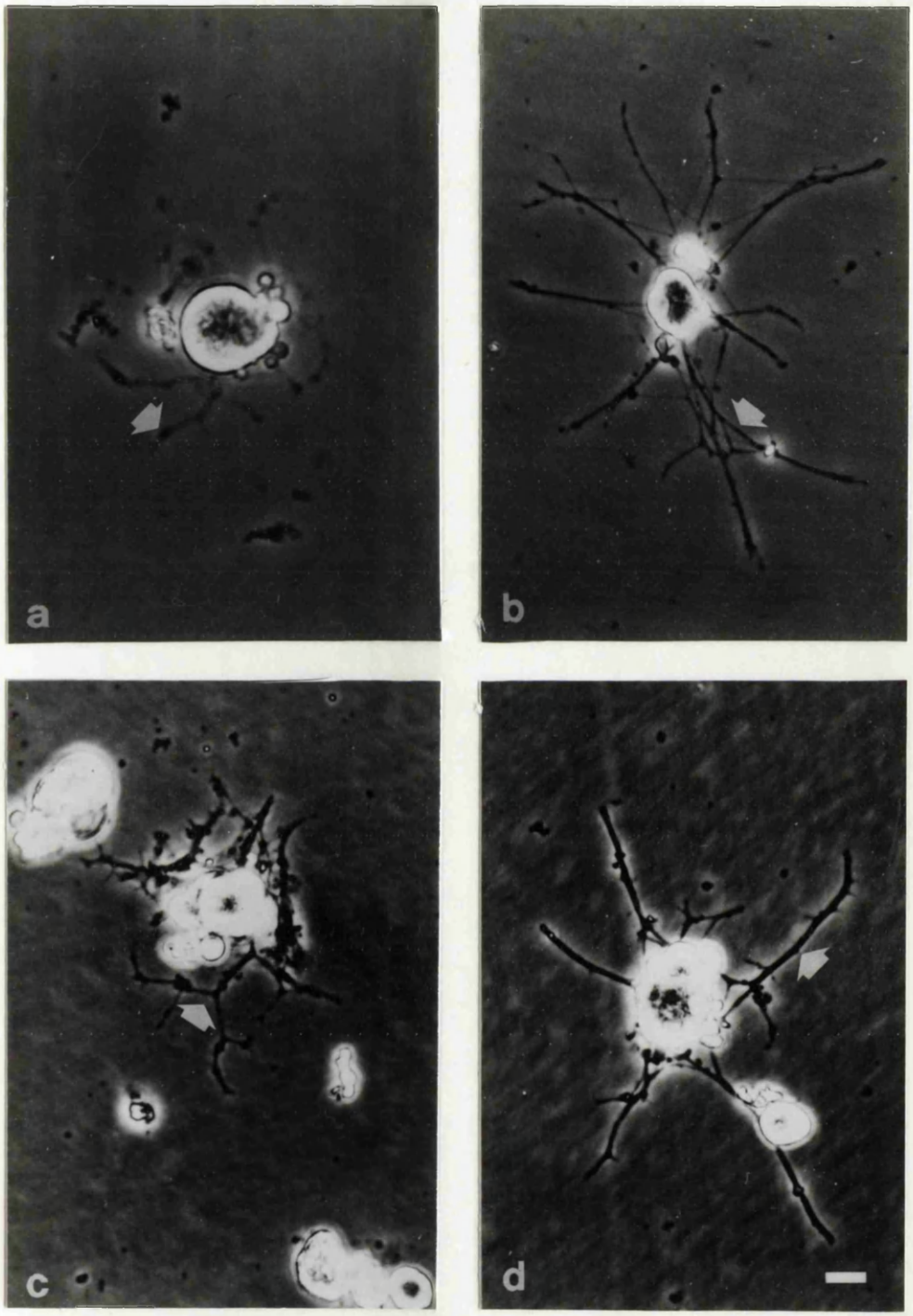

Fig.5.1. The effect of NGF on neurite (large arrow) geometry. **a, b**: 1div adult neurons; **c, d**: 1div aged neurons. **a, c**: cultured without exogenous NGF; **b, d**: treated with 100ng/ml NGF. In this early culture phase, neurites mainly extended from large- (**a, d**) and intermediate-sized (**b, c**) neurons; a small-sized neuron without neurites is labelled (arrow in **c**). Bar=20μm.

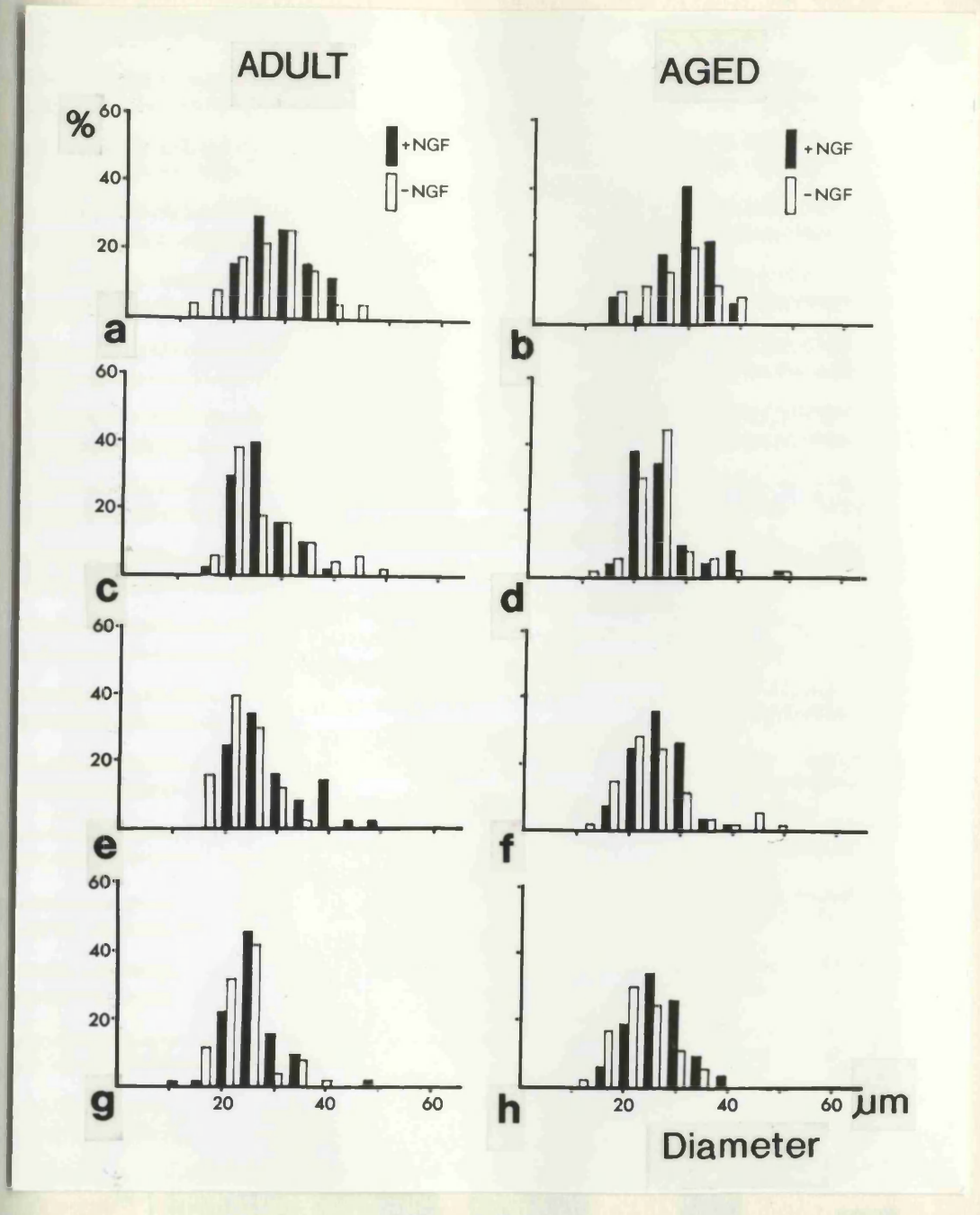

Fig.5.2. Soma size distribution of neurite-bearing adult (left hand columns) and aged (right hand columns) neurons in cultures supplemented with 100ng/ml NGF(filled bar) or without (open bar). **a, b**: 1div; **c, d**: 3div; **e, f**: 6div and **g, h**: 9div. Neurites predominantly extended from intermediate-sized (20-30μm) neurons, with large neurons (>30μm) or small size neurons (<20μm) extending less, especially in the early culture phase. With NGF, intermediate-sized neurons (adult or aged) were higher than those in cultures without NGF.

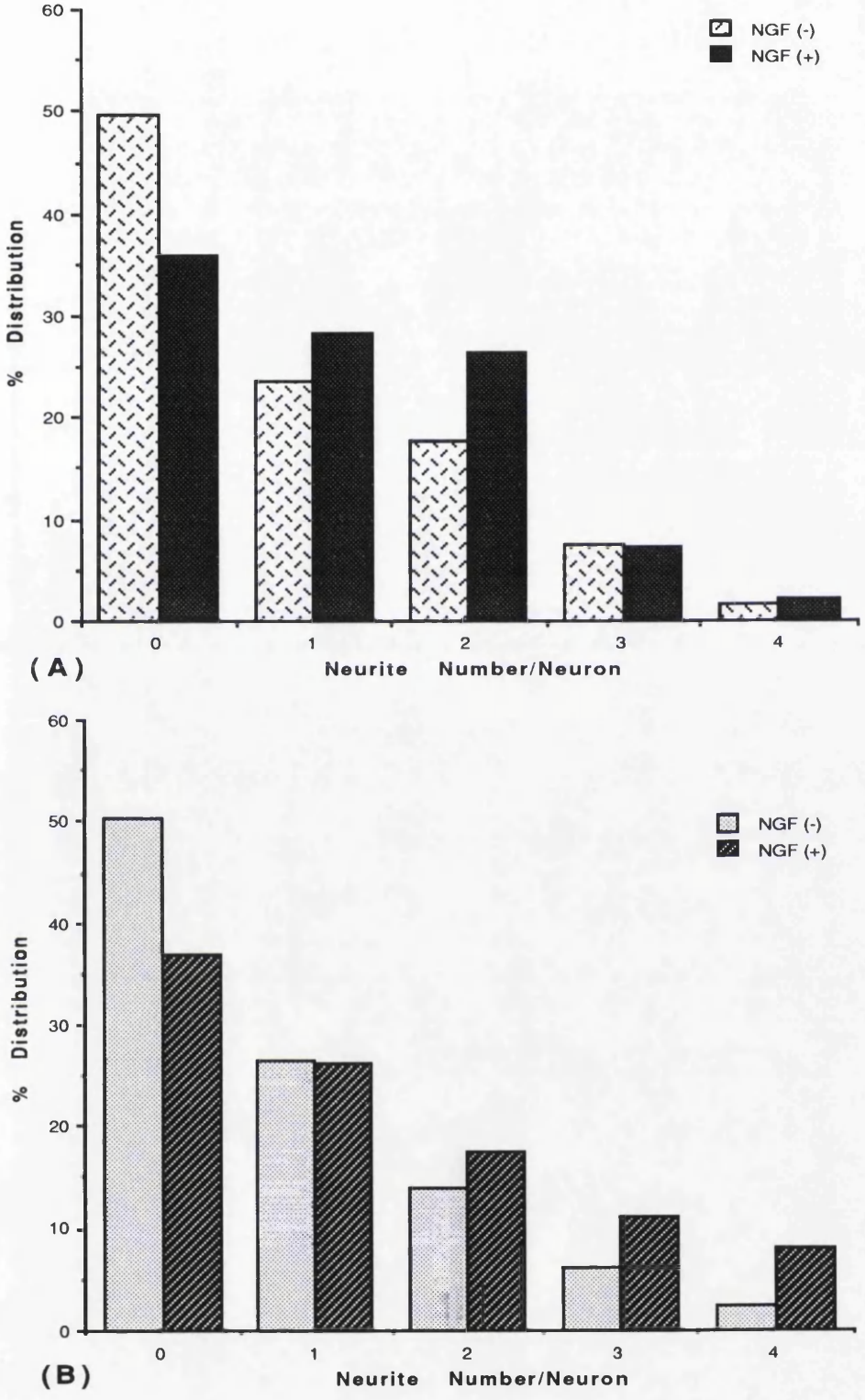

Fig.5.3. Effect of NGF on Neurite Numbers per Neuron (Adult and Aged) at 9div.

9div adult **(A)** and aged **(B)** neurons, without exogenous NGF, or with 100ng/ml NGF added. (n=180/group). Data not subjected to statistical analysis.

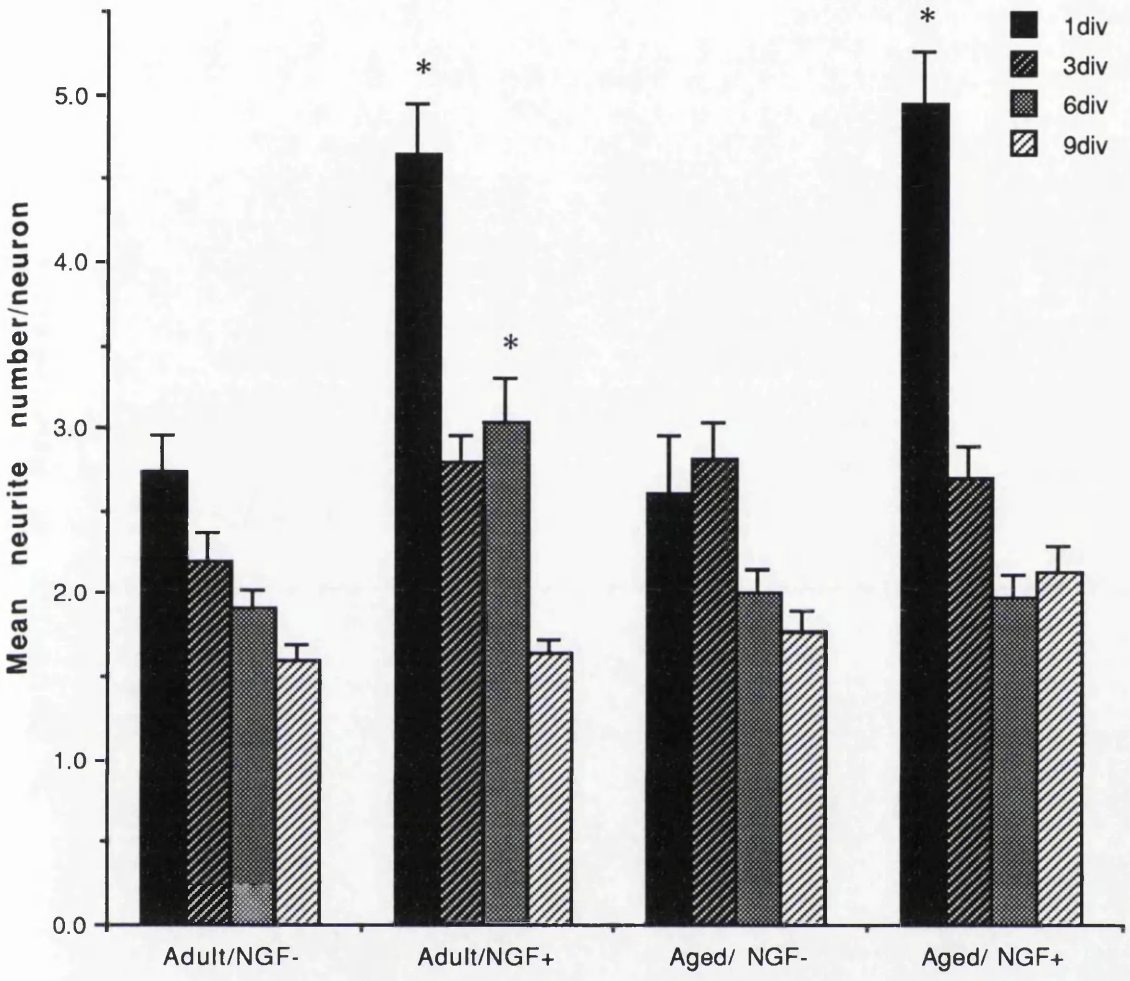

Fig.5.4. Effect of NGF on Neurite Numbers per Neuron

Adult and aged neurons in enriched cultures without exogenous NGF, or with 100ng/ml NGF added. The number of neurites from 50 neurons/group (neurons prepared from 5 adult and 5 aged mice) is expressed as the mean neurite number per neuron ± standard error. * $P < 0.001$ (by 2-way ANOVA).

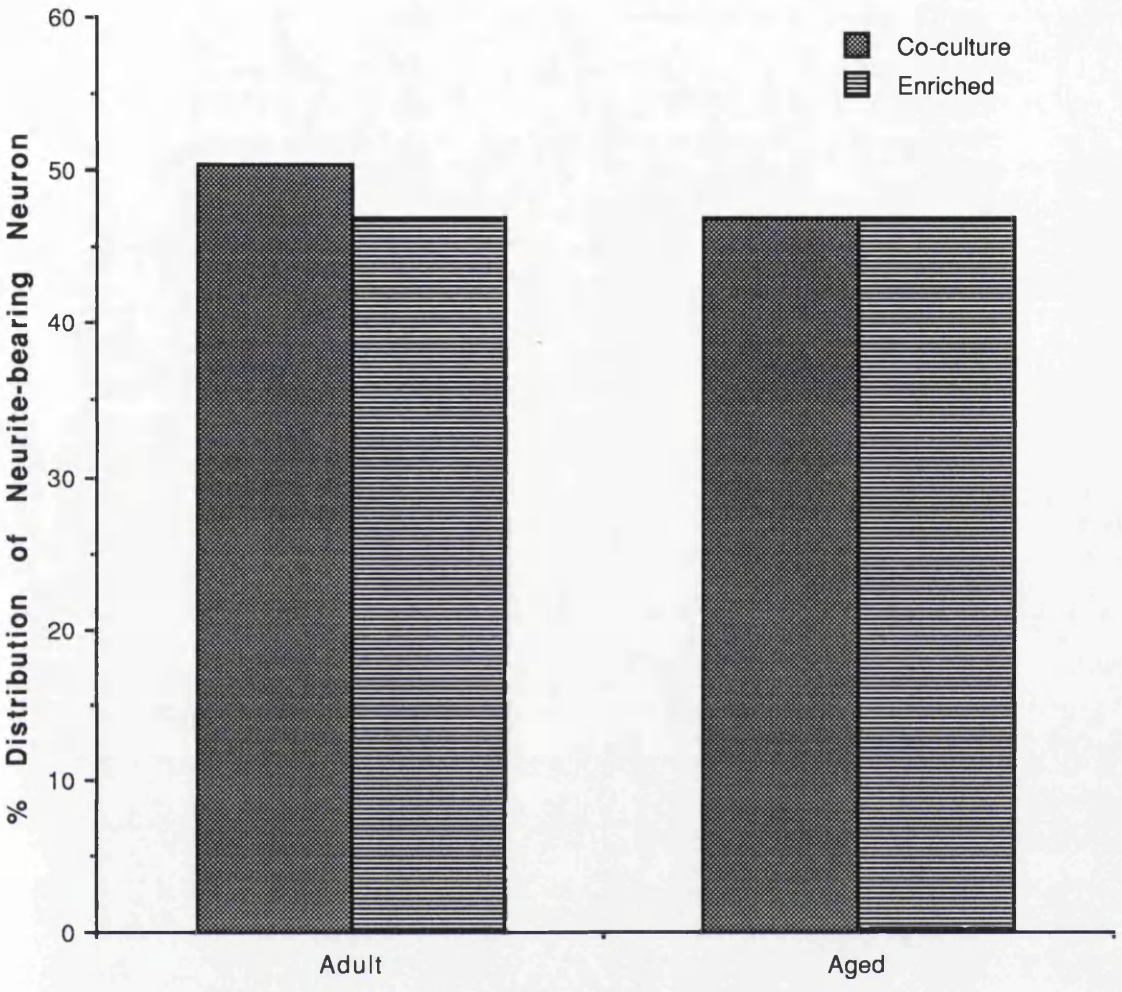

Fig.5.5. Comparison of the Percentage of Neurite-Bearing Neurons in the Two Culture Systems

Adult and aged neurons in either co-cultures, or in enriched cultures for 9div. Data were collected from micrographs taken from 5 adult and 5 aged mice (n=150 neurons/group). Neurite outgrowth did not appear to differ between the two culture systems for adult or aged neurons. Data not subjected to statistical analysis.

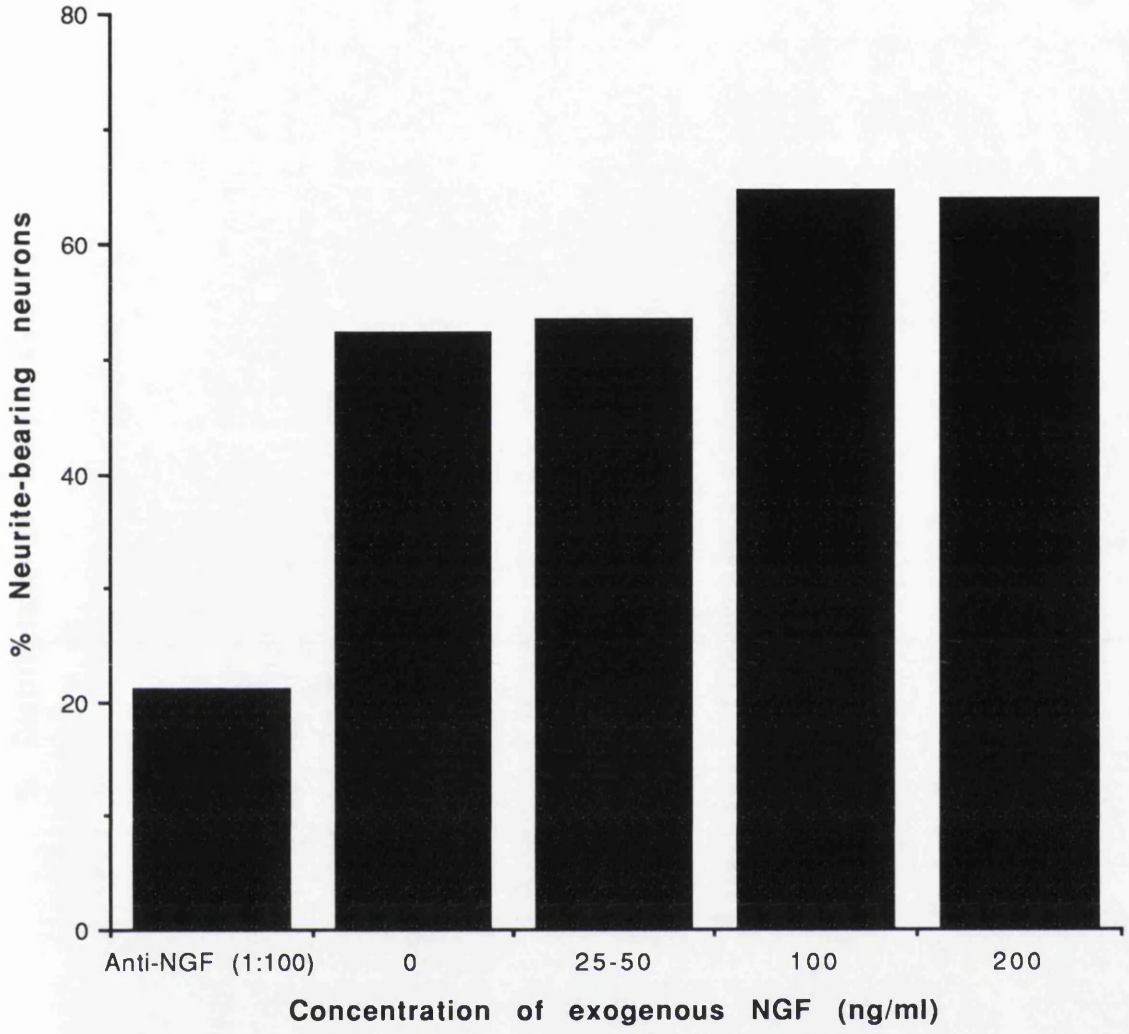

Fig.5.6. Effect of NGF Concentrations on Neurite-bearing by Adult Neurons in Co-Cultures for 15div

Adult neurons cultured for 15div, either without added NGF, or in the presence of 25-200ng/ml NGF, or 1:100 anti-NGF from culture initiation. Neurons were counted on scanning electron micrographs taken from 3 adult mice. (40 neurons/group). Data not subjected to statistical analysis.

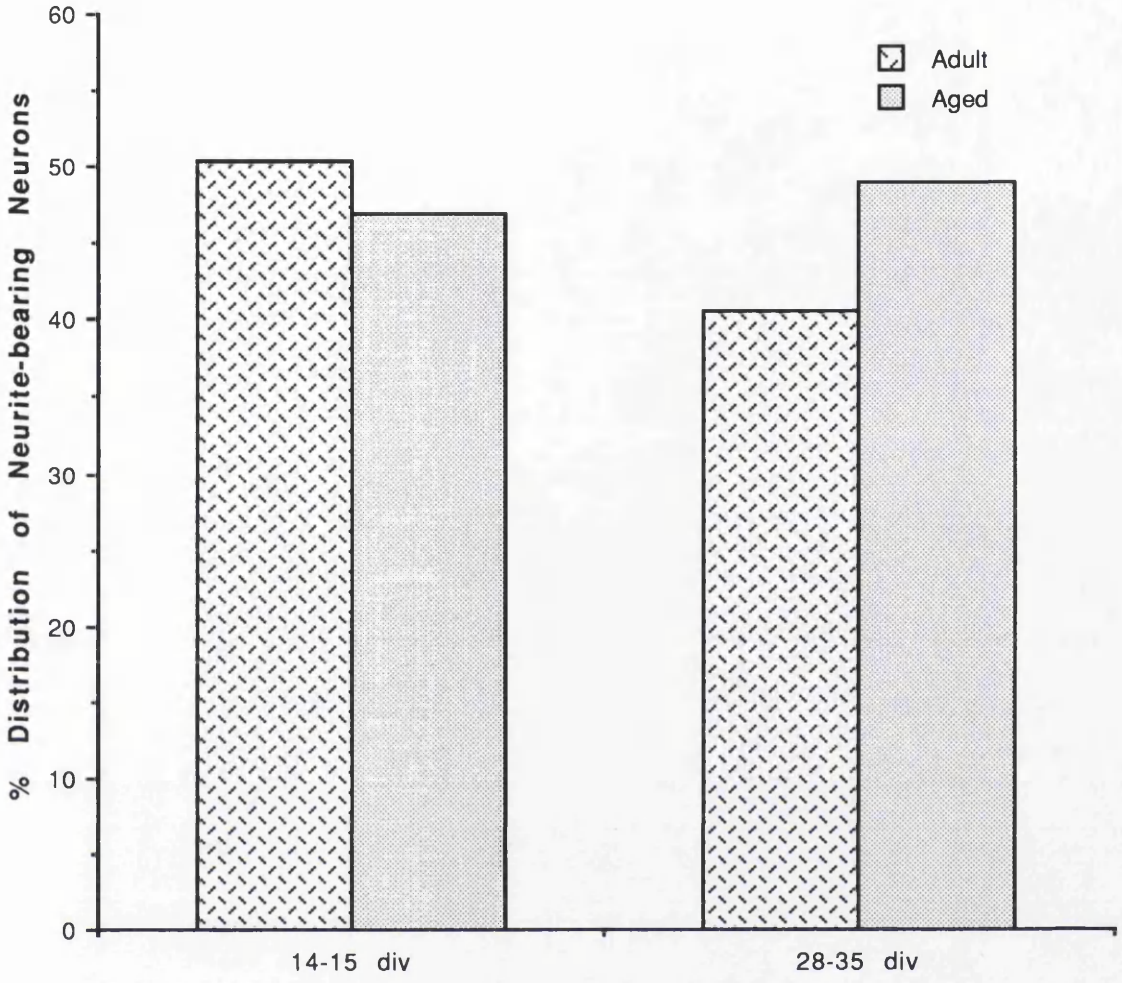

Fig.5.7. Effects of Culture Duration on the Numbers of Neurite-Bearing Neurons in Co-Culture

Neurons from 3 adult and 3 aged mice were cultured with NNCs present for 35div. Data were collected from micrographs of phase-contrast and SEM (250/group/day) . The number of adult neurons extending neurites was less in the late culture phase, whereas this number remained for aged neurons. Data not subjected to statistical analysis.

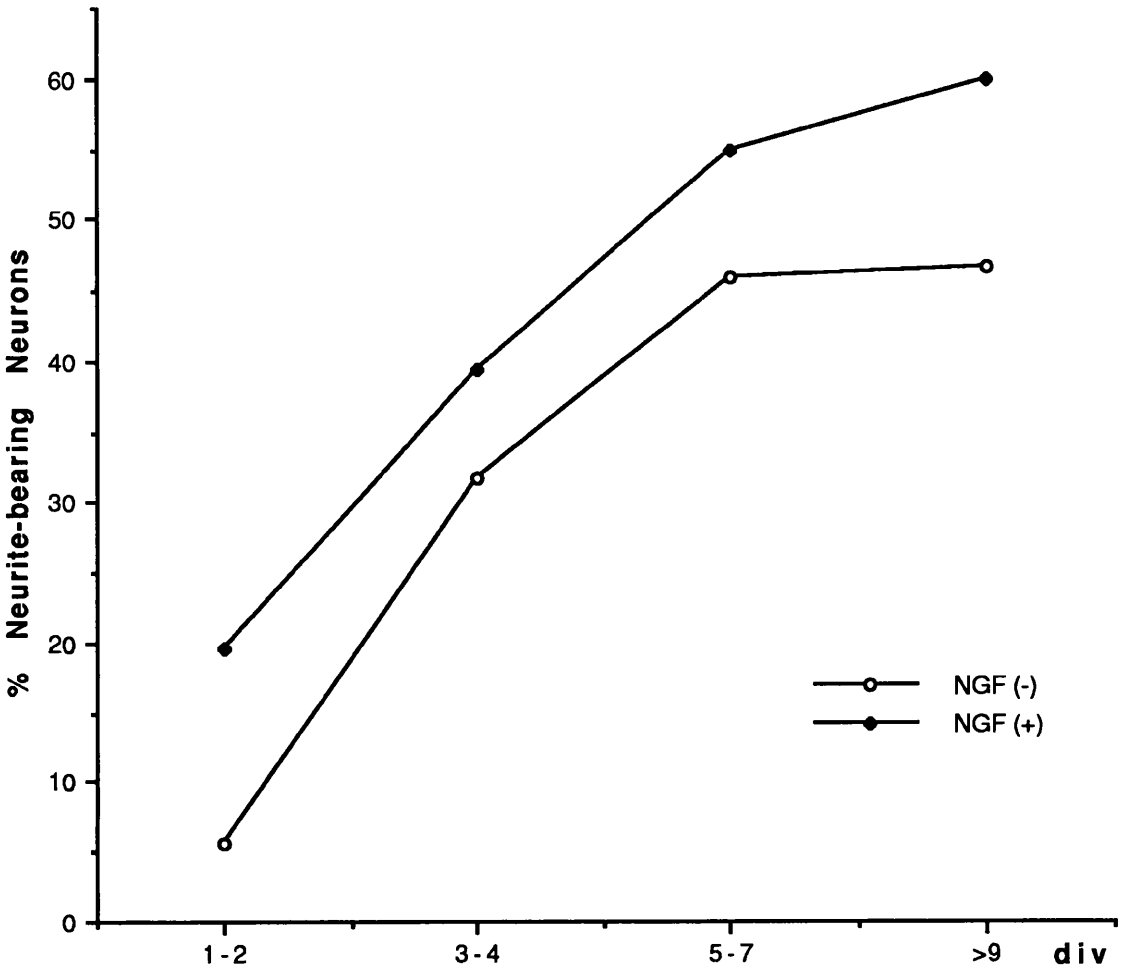

Fig.5.8. The Effects of Days in Vitro and NGF on the Proportions of Neurite-Bearing Aged Neurons

Aged neurons in enriched cultures without exogenous NGF, or with 100ng/ml NGF added. An average of 270 neurons per group, taken from 5 aged mice was counted on phase-contrast micrographs for each div. Enhancement of neurons extending neurites was evident during the entire culture period. In the presence of exogenous NGF, it was greater than in cultures without NGF. Data not subjected to statistical analysis.

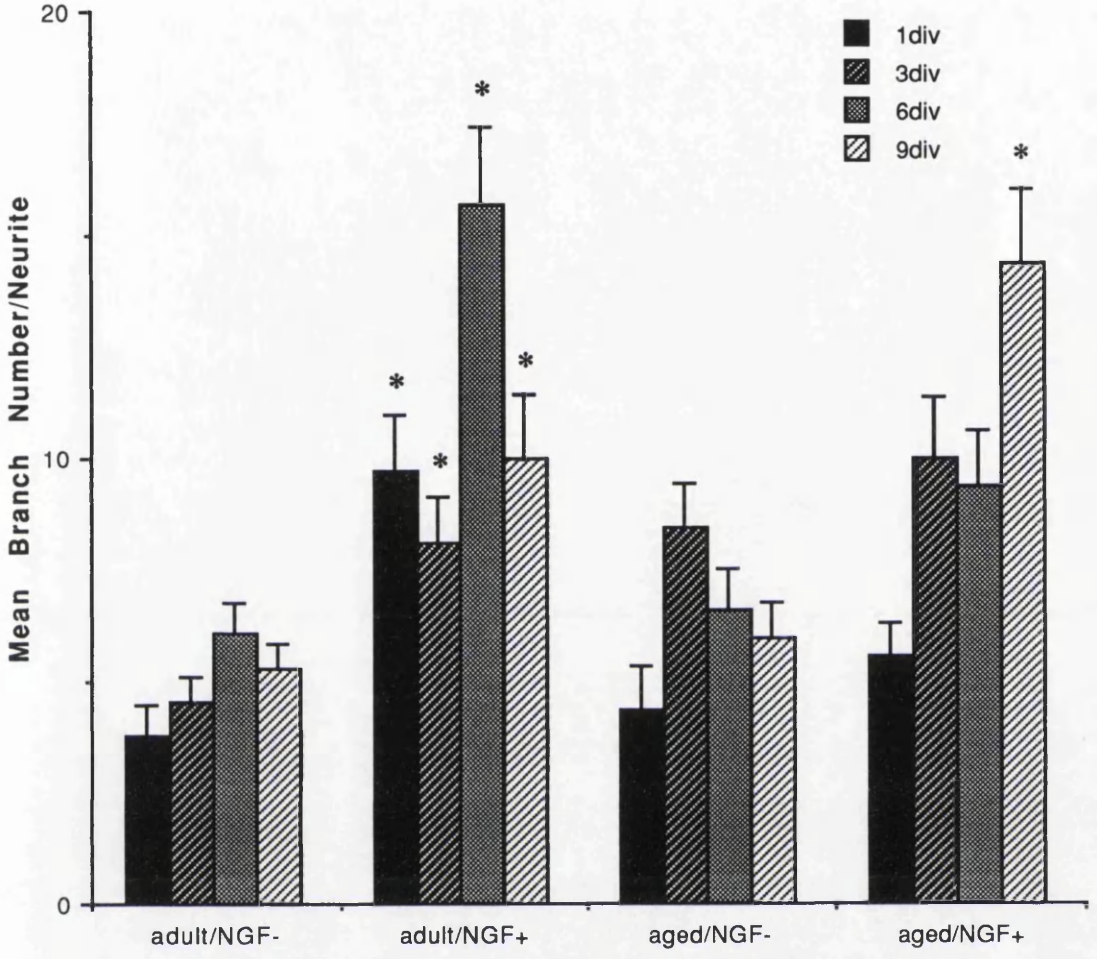

Fig.5.9. Effect of NGF on Branch Number/Neurite

Adult and aged neurons in enriched cultures without (-), or with (+) 100ng/ml NGF added. All branches from the neurites of 50 neurons per group were counted by a video-project computer-assisted programm, and digitizing tablet, and expressed as mean number per neurite for each group. In the presence of exogenous NGF compared to cultures without NGF, a significant difference was seen as early as 1div for adult, but only at 9div for aged neurons. ∗: $P < 0.0005$ (by ANOVA).

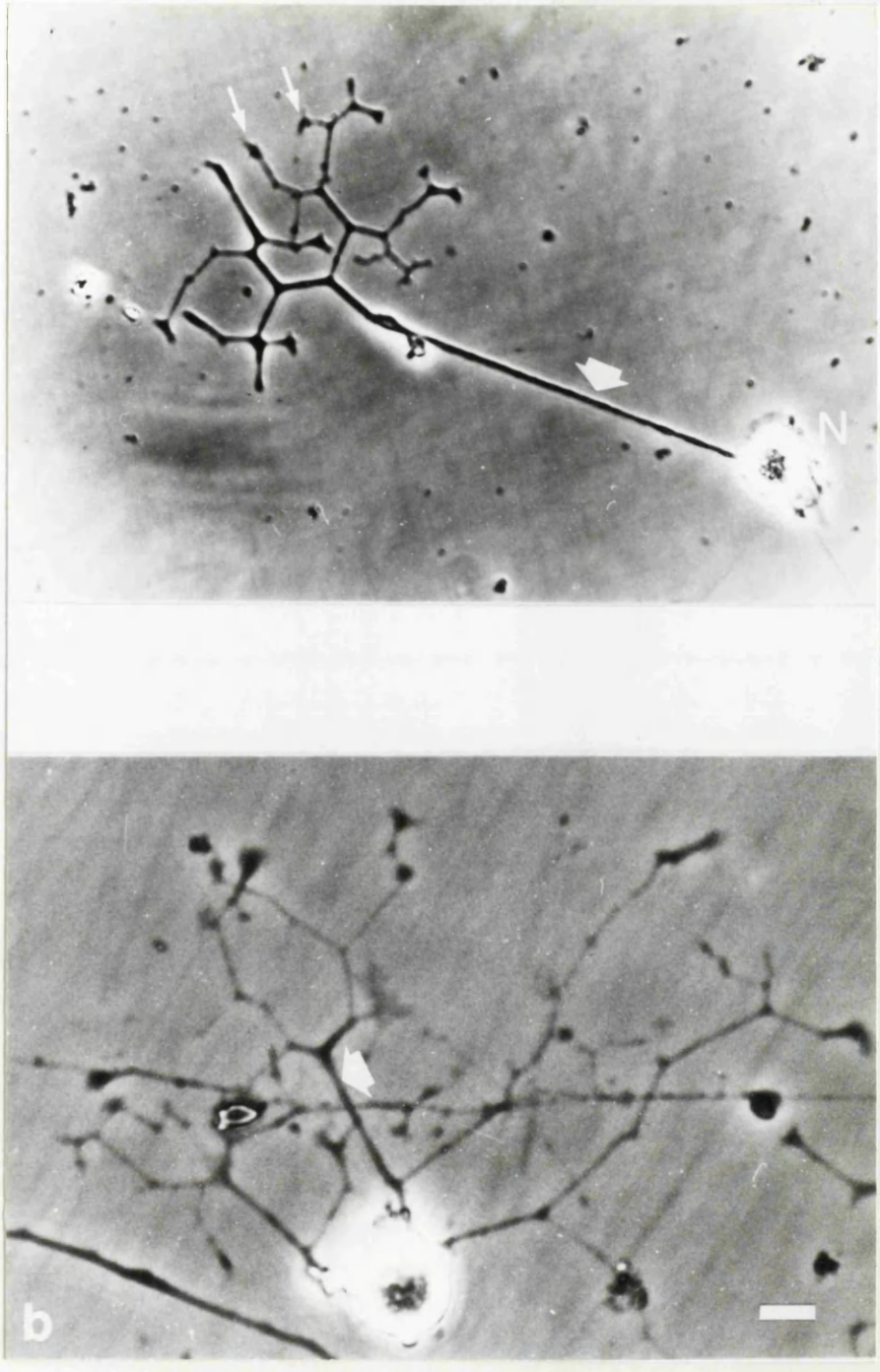

Fig.5.10. The effect of NGF on neurite (large arrow) geometry at 3div for adult neurons (N) cultured with 100ng/ml NGF (**b**) compared with without NGF added (**a**). Neurite number and total neurite length for the neuron in **b** was greater than for **a**. Tiny arrows show growth cones. Bar=20μm.

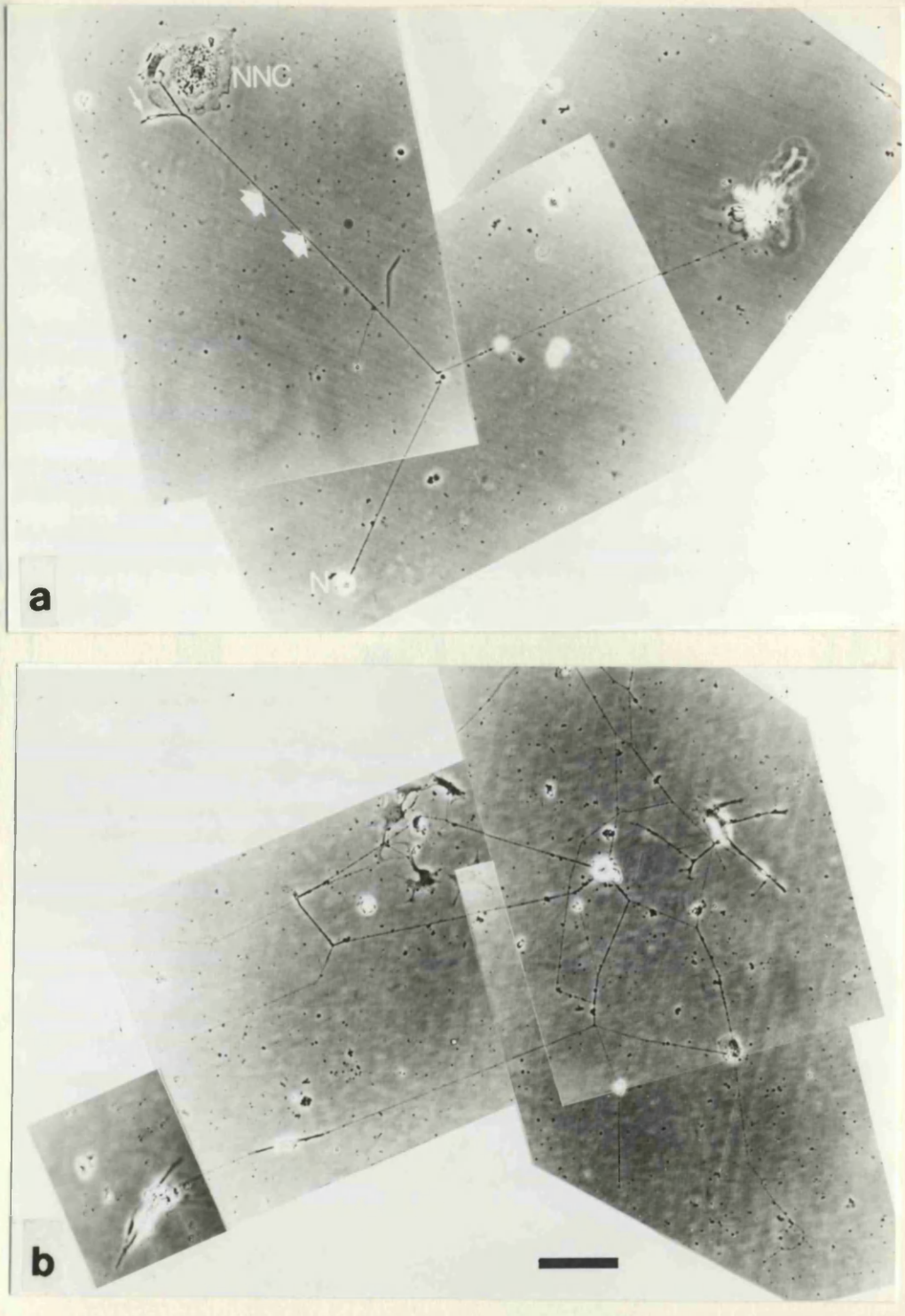

Fig.5.11. Aged neurons (N) in 3div cultures without (**a**) or with 100ng/ml NGF (**b**). Neurite and branch numbers, and neurite total length in **b** were much greater than in **a**. Large arrows show neurites and tiny arrows indicate growth cones. When a neurite elongated towards a epithelioid-like cell (NNC) and contacted it, a change in direction of growth occurred with branches left in contact with the NNC. Bar=100μm.

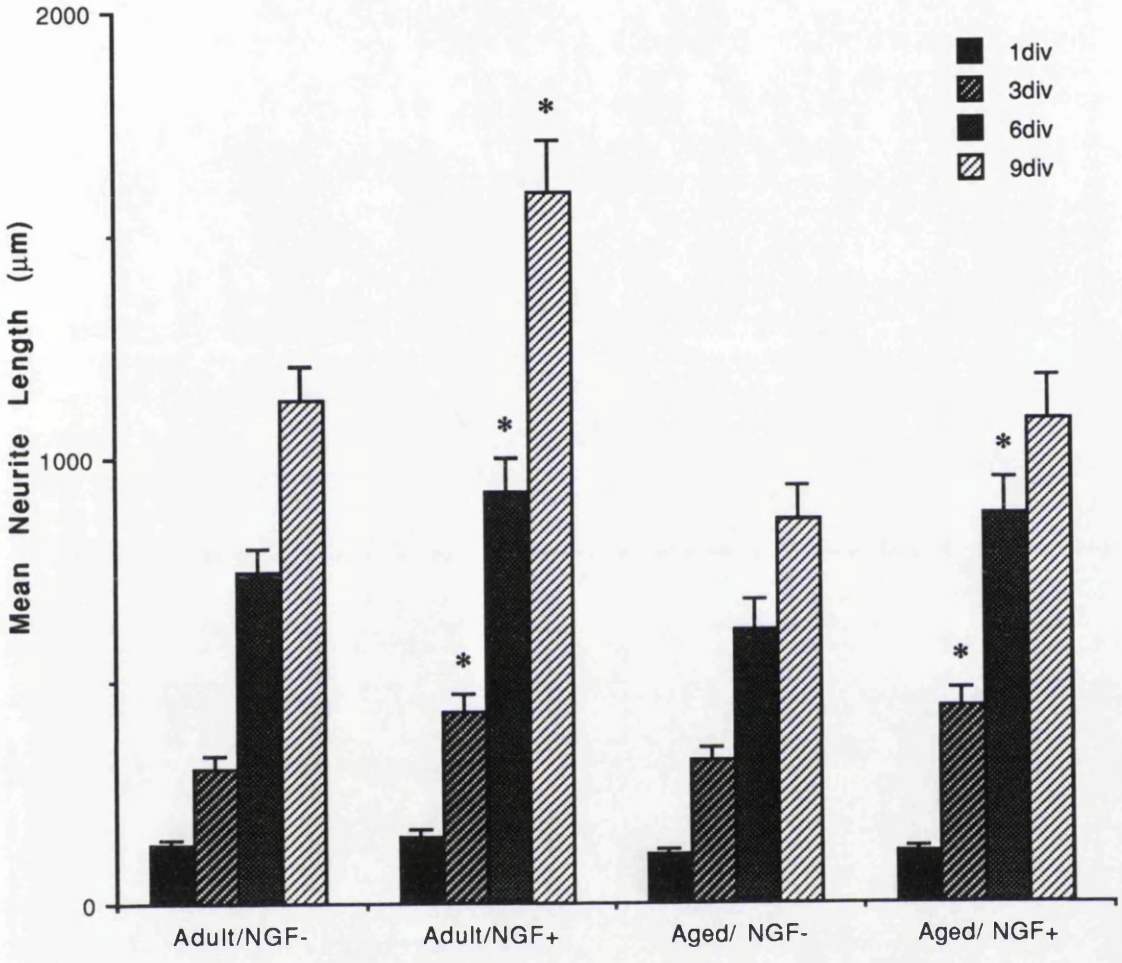

Fig.5.12. Effect of NGF on Major Neurite Length

Adult and aged neurons in enriched cultures without (-), or with (+) 100ng/ml NGF added. Major neurite lengths recorded from 50 neurons per group were expressed as the mean length per neurite. This length increased with culture duration, and was greater in the presence of exogenous NGF than in cultures without NGF added, being significantly different by 3div for both adult and aged neurons. ∗: $P < 0.0005$ (by ANOVA).

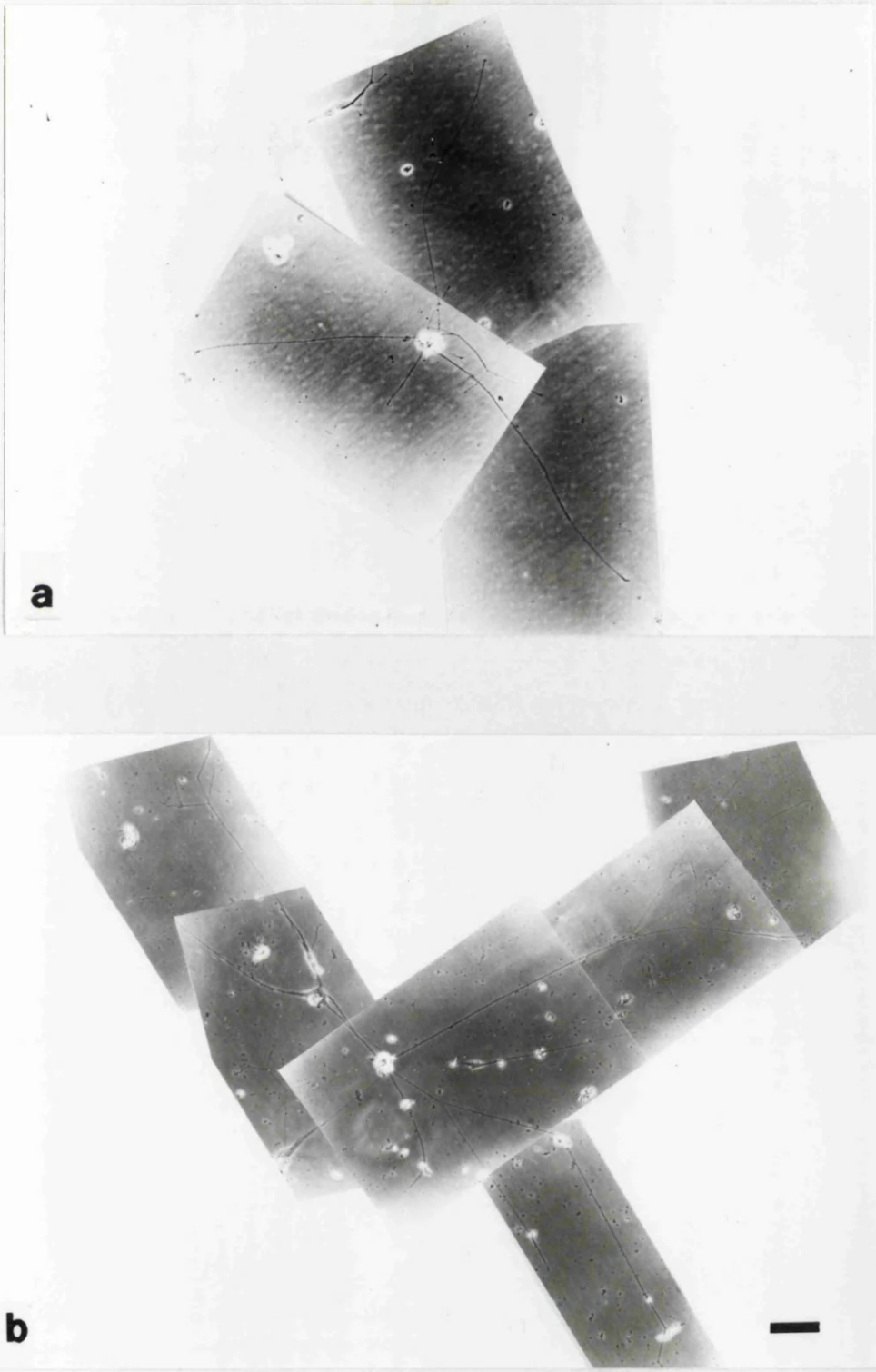

Fig.5.13. Adult neuron (N) in 6div enriched culture without added NGF (**a**) or with 100ng/ml NGF added (**b**). In the presence of NGF, neurite and branch numbers, and major, entire and total neurite lengths were much greater than those in culture without NGF. Bar=100μm.

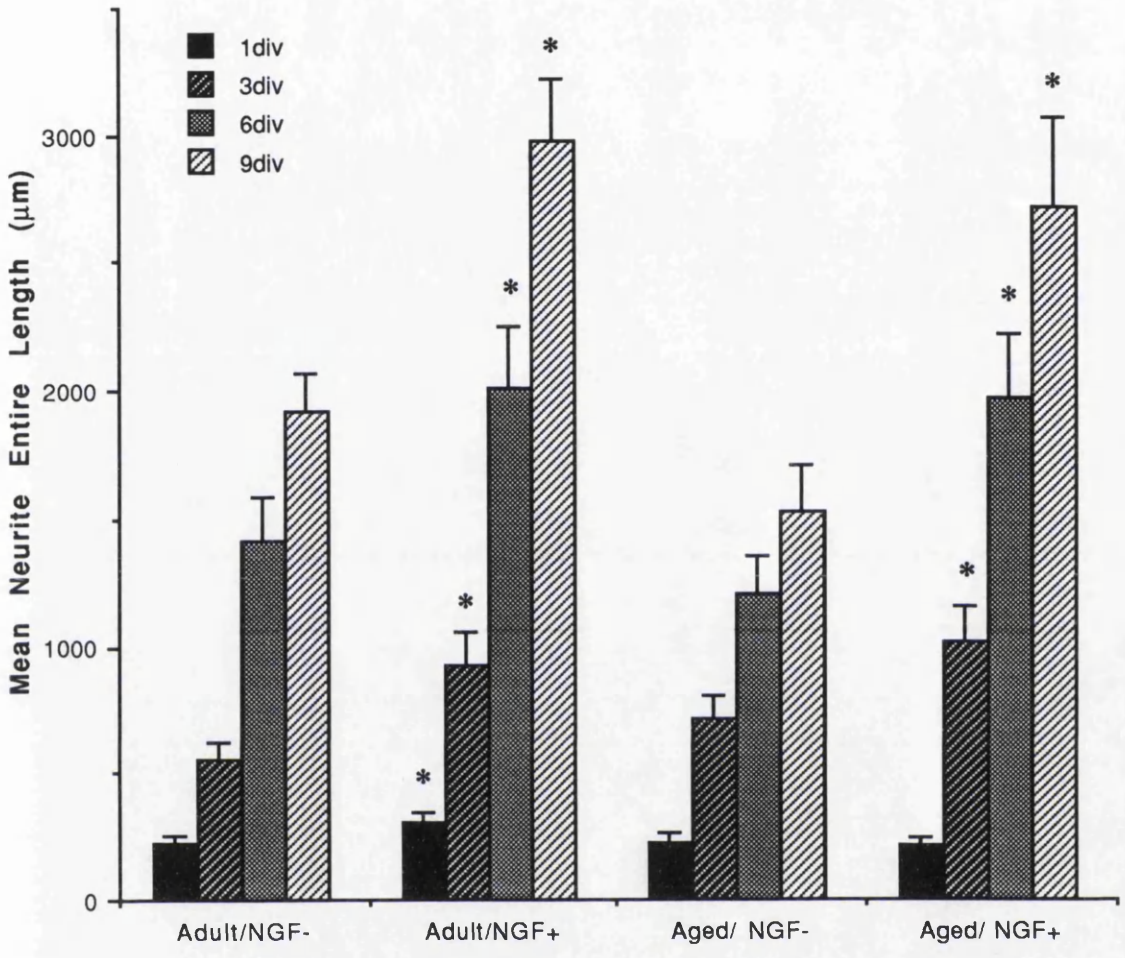

Fig.5.14. Effect of NGF on Neurite Entire Length

Adult and aged neurons in enriched cultures without (-), or with (+) 100ng/ml NGF. Entire neurite lengths were traced from 50 neurons per group. This mean length increased greatly with culture duration, and in the presence of NGF was significantly different from in cultures without NGF as early as 1div for adult, and 3div for aged neurons. ⋆: $P < 0.0005$ (by ANOVA).

Fig.5.15. Montage of aged neuron in enriched culture with 100ng/ml NGF added for 9div. Bar=250μm

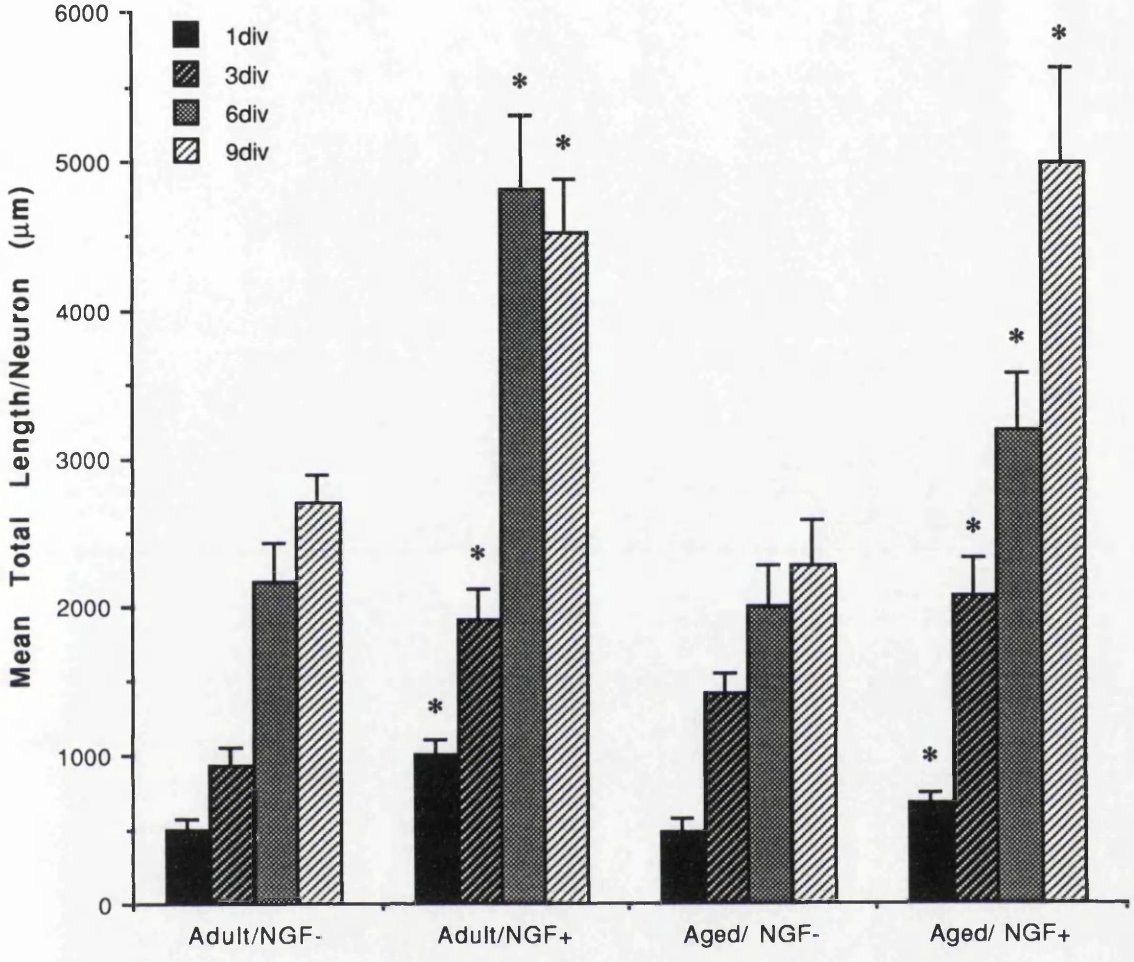

Fig.5.16. Effect of NGF on Total Length per Neuron

Adult and aged neurons in enriched cultures without (-), or with (+) 100ng/ml NGF added. Total neurite lengths were recorded from 50 neurons per group were increased with culture duration, and in the presence of exogenous NGF was significantly greater than in cultures without NGF as early as 1div for both adult and aged neuron. ∗: $P < 0.0005$ (by ANOVA).

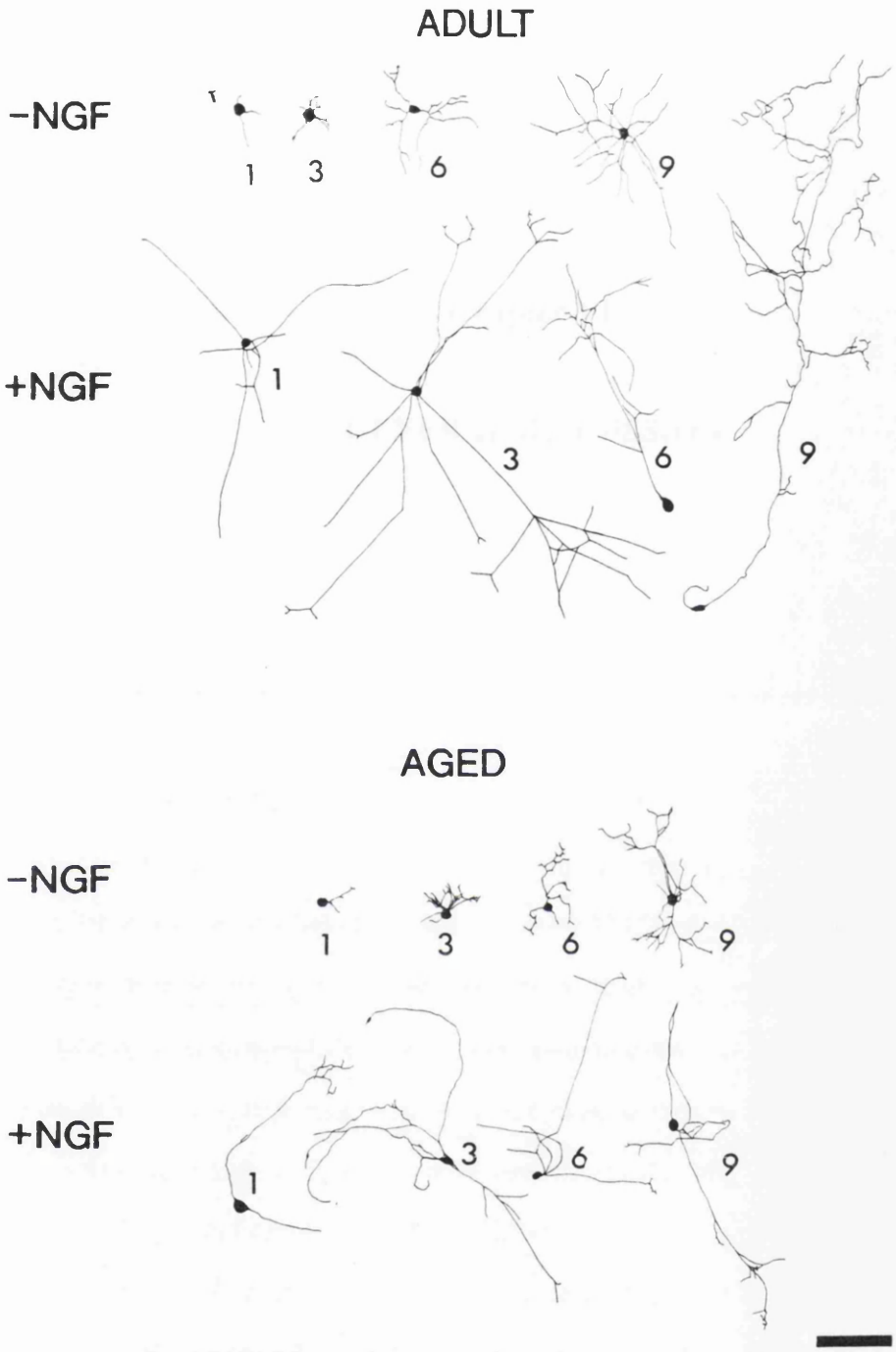

Fig.5.17. Camera lucida drawings of typical neurite extension from adult (a) and aged (b) DRG neurons maintained for 1, 3, 6, and 9 days in culture without exogenous NGF (-NGF), or with 100ng/ml NGF added to culture from onset (+NGF). Note: increase in total neurite length and fasciculation in +NGF cultures. Bar=100μm.

Chapter VI

GENERAL DISCUSSION

Research on the regeneration of mature neurons is an attractive field, particularly since nerves are more frequently injured in post-developmental stages, whether caused by viral, chemical or mechanical trauma. Elaborate surgical reconnections have only really been possible for peripheral nerve, and with limited success, and a growing interest is now turning to the possible therapeutic effects of trophic factors in maintaining adult neurons. The influence of neurotrophic factors and target guidance has understandably been extensively studied mainly on embryonic neurons, although the embryonic nervous system is rarely injured by mechanical force when *in utero* where NGF has been shown to be significant for both the survival of, and neuritogenesis by, sympathetic and most sensory neurons. Some studies of trophic factor effects, especially NGF, have been carried out on mature neurons, but the results of these are very ambiguous. The present study aimed to undertake a thorough re-examination of the effects of NGF on adult, and also aged neurons, thereby evaluating any NGF-dependence of mature DRG neurons and investigating any changes related to age. In this *in vitro* study, neuronal survival, neuronal morphology, neuropeptide phenotype, and neurite outgrowth were monitored. In addition, the proportions of neuropeptide-containing neurons *in vitro* were compared to those *in vivo* to evaluate plasticity of peptide phenotypes. The main findings and results were as follows:-

NGF continued to influence the survival of at least some mature DRG neurons, even those of old mice. In the co-culture system, adult and aged neuronal survival in the presence of NGF was approximately 20% greater than in cultures without NGF. This significant difference between cultures with and without exogenous 100ng/ml NGF was evident at 7div. To exclude the possible effect of endogenous NGF and/or any indirect effect of exogenous NGF via non-neuronal cells, anti-2.5S NGF was introduced to some co-cultures.

Enriched cultures were also prepared to examine whether the effects of NGF on the survival of adult and aged neurons in co-culture were specific. In the first case, neuronal numbers in cultures supplemented with anti-NGF were lower but not significantly different from in cultures without exogenous NGF present. In the neuron-enriched cultures, both adult and aged neurons survived in the presence of 100ng/ml NGF at significantly higher levels than in cultures without NGF. Hence, NGF's effect on the survival of adult and aged neurons is considered to be direct.

In some *in vivo* studies, administration of exogenous NGF or anti-NGF did not appear to affect the survival of adult DRG neurons (Rich *et al.*, 1984), and mature DRG neurons were considered therefore to lose their NGF-dependence for survival. However, in normal adult rat skin, a major target of peripheral sensory neurons, continued NGF synthesis has been demonstrated (Diamond *et al.*, 1992a), and retrograde transport of endogenous NGF has been shown for mature neurons under physiological conditions (Korsching and Thoenen, 1983a; Palmatier *et al.*, 1984). Endogenous NGF may be sufficient therefore for the survival of certain NGF-dependent subpopulations of DRG neurons and in this instance, exogenous NGF does not have an additional effect. Administration of anti-NGF (Diamond *et al.*, 1992b) may however, introduce other difficulties in evaluating the effects of endogenous NGF *in vivo*. *In vitro* approaches have been successful in many studies concerned with investigating the effects of trophic factors and will continue to do so (for review see Smith and Jiang, 1994). The data from *in vitro* studies probably relate more closely to those of the *in vivo* response to neuronal injury since axotomy is necessary in order to prepare the cultures. In relation to this, exogenous NGF has been shown to maintain the survival of adult neurons *in vivo*, following sciatic nerve transection (Rich *et al.*, 1987).

From earlier *in vitro* studies, it was concluded that NGF did not affect the maintenance of adult neurons in neuron-enriched and/or single-cell cultures (Grothe and Unsicker, 1987; Lindsay, 1988; Yasuda *et al.*, 1990). However, although 93% neuronal purity could be obtained in these culture models, neuronal loss as high as 97% was reported during the preparation procedure (Yasuda *et al.*, 1990). Hence, one cannot eliminate the possible loss of NGF-dependent neuronal sub-populations, and therefore the results obtained from such culture systems are very limited in determining NGF's effect on adult neuronal survival. In recent years, others have now shown that at least 20% of adult, and even aged DRG neurons, retained an NGF-dependence for survival (Fukuda *et al.*, 1991; Manfridi *et al.*, 1992; Jiang and Smith, 1992, 1993a). By analysing neuronal soma size distributions in the present co-cultures, an NGF-dependent sub-population of neurons with mean diameter of 24-33μm was indicated; this is similar to the results of Fukuda and his colleagues (1991). Furthermore, the proportions of SP ir-neurons from adult and aged mice in enriched-cultures supplemented with 100ng/ml NGF were greater than those without NGF added (Jiang and Smith, 1993b). *In vivo,* the proportion of SP ir-neurons did not decrease with the aging process (from 6 month to 2 years). This may indicate SP ir-neurons retain an NGF-dependence for survival in mature mice. Although CGRP and NPY may co-exist with SOM (Vincent *et al.*, 1982; Garry *et al.*, 1989), and probably other neuropeptide which are markers for NGF-independent survival neurons, higher proportions of CGRP and NPY ir-neurons did occur in both adult and aged cultures supplemented with NGF. NGF-dependent neurons therefore were apparently predominantly small, intermediate-sized, including the SP-ir sub-population and possibly those representing CGRP and NPY containing neurons.

In the immunostaining study, it is important to consider two possibilities, i.e. phenotypic expression and continuing survival, since both may influence the enhancement of ir-neurons. The enhanced proportions of SP, CGRP and SOM ir-neurons *in vitro*, compared with those *in vivo* in the present study, and also compared with those of freshly dissociated cells (Schoenen *et al.*, 1989), indicate a potential for neuropeptide plasticity in mature neurons. This may partly be due to constant neuropeptide transport and release *in vivo*, resulting in too a low basal level in some neurons *in vivo* to be visualized by immunostaining. When DRG neurons are transfered into cultures, either neuropeptide expression is enhanced or neuropeptide levels rise due to storage, and more neurons therefore become visualized. Others have favoured the first explanation (Schoenen *et al.*, 1989).

In addition to affecting the survival of a proportion of mature neurons, NGF up-regulated neuropeptide expressions *in vitro*, as demonstrated by monitoring the enhancement of staining intensities of SP, CGRP and NPY in the presence of exogenous NGF. NPY is not detected by immunostaining *in vivo*, although NPY expression was greatly enhanced in rat DRGs following partial peripheral axotomy (Wakisaka *et al.*, 1991). Furthermore, NPY ir-neurons represent over 30% of cultured DRG neurons which was significantly higher than the proportion in freshly dissociated preparations (Schoenen *et al.*, 1989). In the present *in vitro* study, the proportions of the NPY-ir subpopulation were greater than the proportions of ir-neurons for SP, CGRP and SOM. In the presence of NGF, the staining intensity with anti-NPY was greater than in neurons from cultures without exogenous NGF. NGF did not however affect either the proportions or staining intensity of SOM ir-neurons, a finding which correlates with other studies on adult animals, where neurons containing SOM lack high-affinity NGF receptors (Verge *et al.*, 1989). SOM ir-

neurons may represent a subpopulation which is responsive to other neurotrophic factors, e.g. activin (Ling *et al.*, 1986), in adulthood. NGF also affected the morphlolgy of mature neurons with abundant microvilli projections upon the perikaryal surface in cultures supplemented with 100ng/ml NGF, whereas neurons lost their normal smooth appearance or appeared deformed in cultures with 1:100 anti-NGF added.

NGF initiated and promoted neurite regeneration of adult and aged DRG neurons, with an enhancement of total neurite length evident as early as 1div. By 3div, higher values of total neurite lengths for adult and aged neurons cultured with exogenous NGF were evident, compared with those in cultures without exogenous NGF. That NGF promoted neurite elongation in the present study is consistent with the results of other *in vitro* studies (Yasuda *et al.*, 1990; Horie *et al.*, 1991). Recently, NGF was shown to induce a 5-fold increase in the number of neurons extending neurites (termed as dendrites), an increase in the length of each process and a 17-fold increase in total outgrowth from postnatal rat nodose sensory neurons cultured for 3 weeks (Koninck *et al.*, 1993). Molecular studies have shown that neonatal rat nodose neurons express high-affinity NGF receptors in culture (Mandelzys *et al.*, 1990), whilst in other studies, NGF, via its receptor, induced phosphorylation of microtubule-associated protein-2 (MAP2) (Landreth *et al.*, 1990; Schanen-King *et al.*, 1991), which may have an essential role in the growth and maintenance of neurites (Hirokawa *et al.*, 1988; Matus, 1988; Goedert *et al.*, 1991). NGF stimulated MAP2 expression (Koninck *et al.*, 1993) via high-affinity NGF receptors which remained on a proportion of adult rat nodose neurons (Richardson *et al.*, 1986). The mechanisms underlying NGF effects on neurite regeneration by mature neurons are now becoming clearer from a number of studies. For instance an effect on neurite regeneration was indirectly

demonstrated by complete co-localization of high-affinity NGF receptors with GAP-43 mRNA (Verge *et al.*, 1990). GAP-43 is present in regenerating neurites of adult DRG neurons *in vitro* and *in vivo* (Woolf *et al.*, 1990), and influences neurite elongation and synaptic formation (Benowitz and Routtenberg, 1987). In addition to increasing the total neurite length of regenerating mature neurons, neurofilaments with larger calibers in the NGF-treated nerves result from an actual increase in NF content (Gold *et al.*, 1991). The neuronal cell adhesion molecule L1, which specifically supports neurite outgrowth (Bixby *et al.*, 1988; Seilheimer and Schachner, 1988), is up-regulated by NGF in neurons and Schwann cells (Seihermer and Schachner, 1987). Schwann cells are a major site for NGF synthesis when sciatic nerves are injured (Taniuchi *et al.*, 1986a; Heumann *et al.*, 1987a). Syngeneic Schwann cells derived from adult nerve seeded in semipermeable guidance channels have been shown to promote neurite regeneration across an 8mm gap in transected adult rat sciatic nerves (Guénard *et al.*, 1992). In the present study, entire neurite lengths of over 3mm for adult and aged neurons in the presence of NGF were commonly seen, with some over 10mm by 6-9div. Total neurite lengths in the presence of NGF could reach up to 20mm.

In the present study aged neurons survived *in vitro* at lower levels than adult neurons and had lower neuropeptide expressions and reduced potential for neurite regeneration. Aged neurons however retained an ability to react to exogenous NGF. Cell survival and programmed cell death are thought to be activated or suppressed by signals from other cells; an increase in the synthesis of specific mRNAs has been shown to precede cell death, which can be suppressed by the inhibition of other RNAs or proteins, e.g. *ced*-9 gene product in *Caenorhabditis elegans* (review see Raff, 1992). Aged neurons were more vulnerable than adult neurons with respect to surviving in culture without

exogenous NGF. Higher expression of CGRP was seen however in aged neurons in the presence of exogenous NGF which may be explained by long-term transcription products in aged neurons (Lux et al., 1983; Sarkander, 1983). Another possibility is that some transcriptionally inactive regions are activated in the presence of NGF, since in adult rats (12 months), 21% of the total neuronal chromatin was transcriptionally active, whereas the value was reduced to 13% in older animals (30 months) in *in vivo* studies (Sarkander, 1983).

NGF probably affects neuronal survival and differentiation via distinct mechanisms. Most neurons affected by NGF with respect to survival, neuropeptide phenotype or neurite outgrowth/regeneration were mainly intermediate in size in the present study and in the work of others also (Miyata *et al.*, 1986; Fukuda *et al.*, 1991). Low-doses (25-50ng/ml) of NGF did not maintain higher survival of adult neurons, whereas numbers in the presence of 100-200ng/ml NGF were greater than in co-cultures or in enriched cultures without exogenous NGF for both adult and aged neurons. This dose-dependent effect is unlikely to be due to a cross reaction with other trophic factors of the trk family, i.e. trkB or trkC, since these high affinity receptors readily discriminate NGF, BDNF and NT-3. BDNF-dependent TMN neurons for example are not supported by 1000-fold higher NGF concentration (Davies *et al.*, 1993), and 5000-fold molar excesses of NGF were necessary to prevent 50% of the binding of NT-3 to its high-affinity receptor on both sensory and sympathetic neurons (Rodríguéz-Tebar *et al.*, 1992; Dechant *et al.*, 1993). Furthermore, when anti-NGF was introduced into the present enriched cultures, neuronal loss was evident; this could be due to a blocking of an endogenous BDNF effect via low-affinity NGF receptors (Ernfors *et al.*, 1990). NGF is probably more likely to support adult and aged neuronal survival via the low-affinity NGF receptors rather than high affinity receptors, but affect phenotypic

markers. Study of the two types of NGF receptors could reveal the mechanisms of regulating NGF's effects on the survival and differentiation of DRG neurons. Monitoring the specific mRNAs which determine cell survival or death by *in situ* hybridization or other molecular methods should give further information on neuronal interactions with other cells or their factors, and could generate significant data for the understanding of neuronal survival, aging and death.

In summary, the results of this study to date however demonstrate:-

(i). NGF continues to act as a survival factor for mature, and even aged, mouse DRG neurons *in vitro*, with the maintenance of intermediate-sized neurons being most influenced;

(ii). NGF continues to affect neuropeptide phenotype of aged as well as adult neurons, as shown by an up-regulation of SP, CGRP and NPY expressions;

(iii). Aged neurons have comparatively lower abilities than adult neurons however for both their survival and their peptide expression;

(iv). Phenotype plasticity of aged as well as adult DRG neurons has been shown by comparison of SP, CGRP and SOM from *in vitro* with *in vivo* studies;

(v). Neuritogenesis and reconstruction *in vitro* are intrinsic characteristics of mature neurons; but NGF continues to promote neurite outgrowth of aged as well as adult DRG neurons *in vitro*;

(vi). It is suggested that NGF may affect neuronal survival via its low-affinity receptors, whereas increases in phenotypic differentiation of mature neurons may be via its high-affinity receptors.

(vii). Previous neuron-enriched culture methodology was further modified in this study and may represent a useful system for future analysis of subsets and trophic factor effects *in vitro*.

Recently, NGF has been demonstrated to have a role in maintaining the functions of septal neurons: a deficit of NGF, induced by infusion of an NGF monoclonal antibody, caused memory impairment and neuronal dysfunction of adult rats (Nitta *et al.*, 1993). This suggests a potential clinical value of NGF in preventing neuronal death, in neuronal regeneration and possibly in the functional recovery of adult, and even aged subjects. The current *in vitro* results have relevance to this wider area of aging, neurodegenerative disease and the effects of trophic factors and potentially adds to the data in this expanding field of interest.

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