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DEVELOPMENTAL CONDITIONING CONFERS VULNERABILITY IN THE ADULT AND AGEING NERVOUS SYSTEM

By

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ABSTRACT

This thesis looks at the condition under which sympathetic neurons (SCG) develop in order to understand the causes of selective vulnerability during ageing and therefore shows how pre-treatment in vivo with NGF at a specific point during development affects SCG neurons.

In summary, results show that following pre-treatment in vivo there is an increase in neuronal number, with differential effect on different subpopulations of neurons (MCA versus iris projecting neurons). MCA-projecting neurons (a vulnerable subpopulation of SCG neurons) increase in growth and innervation of specific target tissues following NGF pre-treatment in vivo, showing a maintained plasticity after termination of development and therefore a potential target site for future therapeutics.

NGF pre-treatment in vivo also increases neuronal survival time throughout life, showing that the limited supply of NGF in real life prime neurons to a reduced potential.

The results on survival also show a difference in the mode of action between the two major survival pathways (PI3-K and ERK), with PI3-K being the predominant in adult life and ERK acting mainly in early life. This shows a double survival mechanism which is plastic and capable of shifting predominance according to factors such as NGF stimuli and/or ageing.

Furthermore if the NGF pre-treatment in vivo is applied after termination of development, neurons show plasticity by developing an 'addiction' or dependance to NGF; pre-treatment termination results in death of the neurons.

Preliminary results show increase in Akt activity which is downstream of PI3-K, and is activated in NGF-dependent survival of SCG neurons (Pierchala *et al.*, 2004). Biological

consequences of Akt activation are survival, increase in cell number and growth, which are all characteristics relevant also to cancer-cell growth.

Further preliminary results show an inhibition of GSK-3β pathways, which is downstream of Akt and is determinant for cytoskeletal rearrangement, glucose metabolism and cell survival; regulation of GSK-3β has been widely studied in relation to Alzheimer's disease.

In conclusions this research shows that sympathetic neurons are plastic and by priming them with NGF, at a critical point during development, their survivability is increased. These results support the existence of a sensitive mechanism for adjusting neuronal capacity to resist cell death in response to neurotrophic factor deprivation.

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CHAPTER I

GENERAL INTRODUCTION

1.1 Anatomy and biology of superior cervical ganglia (SCG)

The SCG are positioned on each side of the neck and are an extension of the cervical sympathetic chain. SCG results form fusion of the upper four cervical spinal nerve ganglia, typically 3cm in length and spindle-shaped. It lies deep to the carotid sheath at the angle of the mandible; anterior to the lateral mass of the atlas and axis and is separated from them by the prevertebral fascia. The SCG receives preganglionic efferent fibres from the thoracic part of the sympathetic trunk; its branches have a complex distribution to supply blood vessels, glands and pilomotor muscles of the head and neck (see figure 1).

According to the neurotrophic hypothesis neurons are initially overproduced and their number subsequently refined by cell death through competition for trophic factors, which are synthesized in restricted amounts in target tissues. Sympathetic neurons transport NGF from their target tissues (Hendry *et al.*, 1974) to the cell body where they influence the expression of neuronal genes and hence production of new proteins. Analysis of mutant mice with targeted disruptions of the genes for NGF and its tyrosine kinase receptor, TrkA, confirm the importance of NGF and TrkA for sympathetic neuron survival (Smeyne *et al.*, 1994).

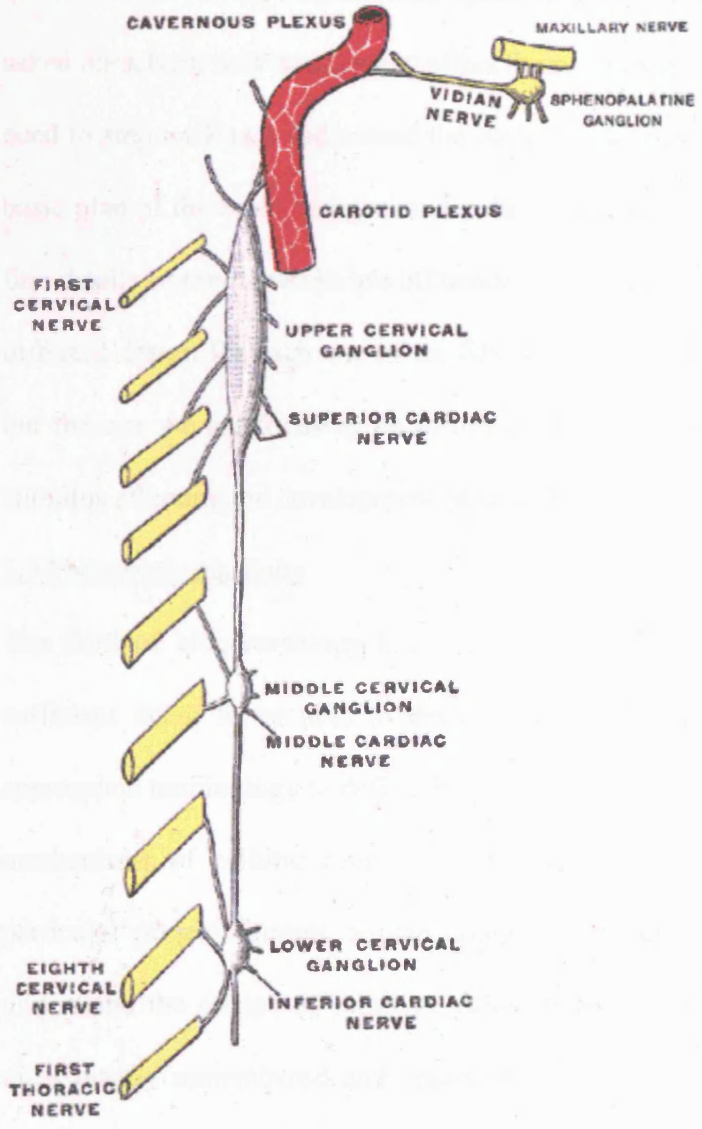

Figure 1.1: Anatomy and location of the superior cervical ganglia (by Young and Young, 1987).

1.2 Ageing and the nervous system

What is ageing at the molecular level and what are the cellular mechanisms that contribute to the characteristics we label ageing? Which changes can be avoided, compensated for, or postponed? Those, and many others, are the questions scientists and gerontologists normally ask themselves, and despite decades of research there is no clear answer as yet.

What we know is that the nervous system plays a central role in ageing and therefore we asked ourselves: how does ageing affect the nervous system? In order to tackle this issue we need to step back and understand the basics of the developing nervous system. Despite the basic plan of the brain being virtually identical from person to person, during early life the fine details of the networks are influenced by electrical activity which determines a slightly different design for each one of us. Several variables are responsible for such differences, but the one we are focusing on in this research is a definite extra cellular environmental stimulus affecting the development of specific groups of sympathetic neurons.

1.3 Phenotypic plasticity

The field of biogerontology has been classified as the one that best defines ageing in sufficient detail to be able to design experiments (Hayflick, 2000). It is therefore the appropriate terminology to define this research approach, which aims to explain the specific mechanisms of cellular ageing and phenotypic plasticity. Phenotypic plasticity in this particular project focuses on the conditions under which neurons develop in order to understand the causes of selective vulnerability during ageing. Selective vulnerability is most readily remembered and appreciated in the context of neurodegenerative disorders such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease (AD), where specific populations of neurons tend to be affected by degeneration more than others. AD specifically might be defined as a faster than normal deterioration of the capacity for plasticity (Teter and Ashford, 2002). Neuroplasticity is related to both a substrate of learning and memory as well as to responses to neuronal attrition and injury (compensatory plasticity); which involves modulation of structural and functional processes of axons, dendrites and synapses. The processes manifesting plasticity include: synapses (electrical,

biochemical, structural), neurite (axon, dendrite), neuron cell bodies, anterograde (toward distal neurites) and retrograde (from distal neurites) transport, cell interactions (neuron-glia), and neural networks. Signal of plasticity include interneuronal (anterograde and retrograde, transsynaptic and extra/parasynaptic) as well as intracellular signaling (Cotman and Nieto-Sampedro, 1984; Neill, 1995). This study is concentrating in neuron-target interactions, neurite, neuron cell body, retrograde and pathway signaling as well as neuronal survival.

1.4 Nerve growth factor (NGF) and other neurotrophic factors (NT)

One of the molecules playing a major role in plasticity of sympathetic neurons is nerve growth factor (NGF). NGF was discovered 50 years ago as a molecule that promoted the survival and differentiation of sensory and sympathetic neurons (Levi-Montalcini and Hamburger, 1951; 1953). The mature, fully processed form of biologically active NGF appears to be similar in all tissues and consists of a dimer of 13-kDa polypeptide chains, each of which has three intrachain disulfide bridges (McDonald *et al.*, 1991; Taiwo *et al.*, 1991). NGF is normally produced in the mouse submandibular gland in response to stress (Fahnestock *et al.*, 1991), and is part of the neurotrophin family of molecules sharing a high degree of structural homology and including brain-derived neurotrophic factors (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (Butte *et al.*, 1998; Ibanez, 1994; Robinson *et al.*, 1995; 1999). NGF has two known receptors, TrkA and p75 (Bothwell, 1995; Kaplan and Miller, 1997). TrkA is a single-pass transmembrane protein that serves as a receptor tyrosine kinase (RTK) for NGF signaling (Loeb *et al.*, 1991). Sympathetic neurons express both TrkA and p75 and nonneuronal target cells of sympathetic and sensory neurons throughout the body produce NGF during development. These includes

targets in the skin, vascular (middle cerebral artery, MCA) and other smooth muscle cells, iris cells, various endocrine tissues, such as the testis the ovary, pituitary, thyroid, parathyroid, and endocrine salivary glands. Most of these cells produce NGF throughout adult life and their production is modulated in response to neuronal stimuli (Levi-Montalcini *et al.*, 1995; 1996). See figure 2.

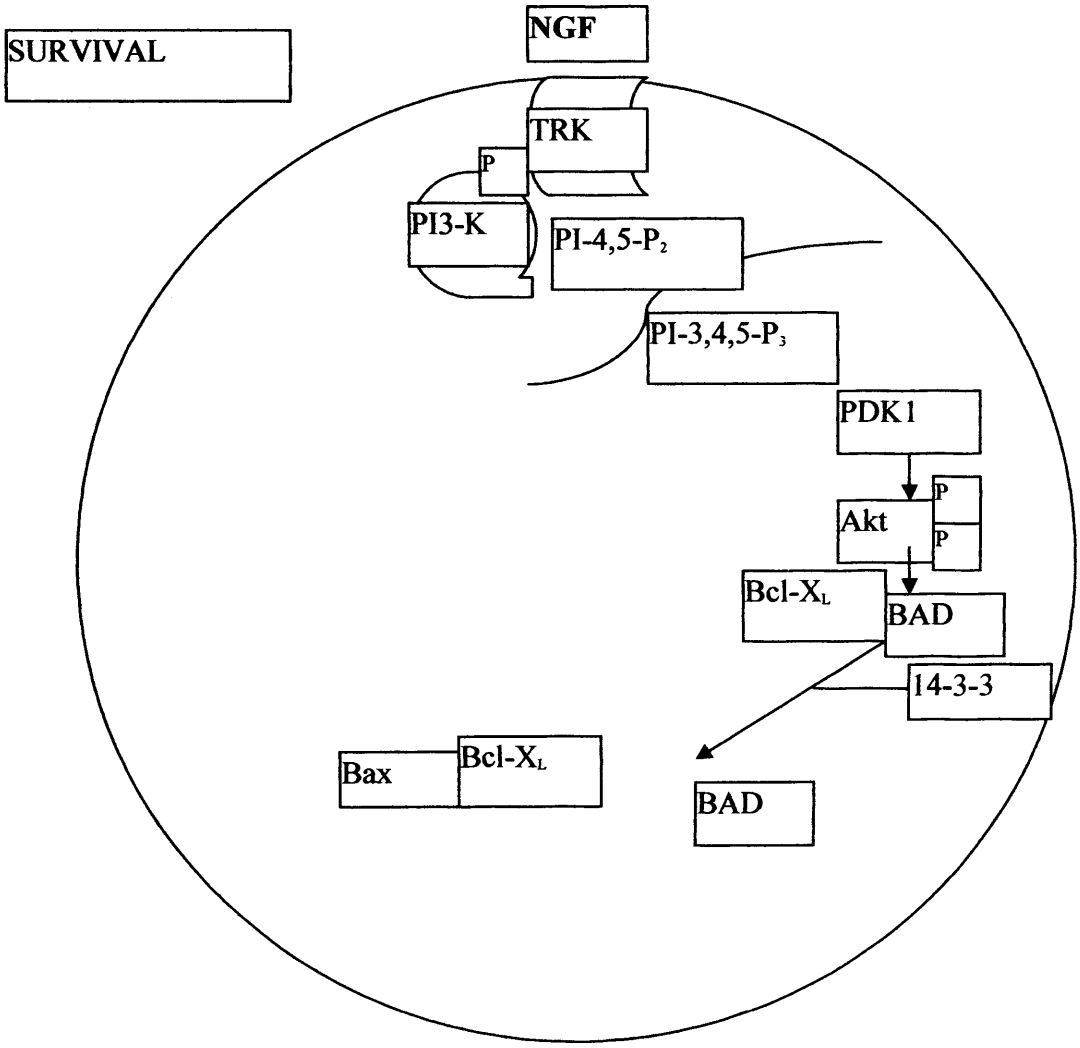

Figure 1.2: Diagram of NGF and PI3-K survival pathway.

1.5 NGF and ageing

Intrinsic age-related reduction of retrograde NGF signaling may contribute to degenerative changes, the consequences of failing in NGF signaling can be both direct, by inducing cell atrophy, and indirect, by increasing the vulnerability of the atrophic neurons to other insults; for this reason the ageing nervous system is peculiar in its highly selective nature of neurodegenerative phenomena affecting differently different groups of neurons (Cowen, 2002). In order to define and understand the determinants of selective vulnerability in neurodegenerative disease as well as in normal ageing, it will be important to determine neuron number according to specific classes of neurons, based on morphology and phenotype. However, how neurotrophic signaling contributes to adult neuronal plasticity and whether impairments in the system contribute to age-related neurodegeneration, is equally important.

Morrison and Hof (1997) studied how ageing tends to affect specific regions of the nervous system and subgroups of neurons; subset of neurons in the entorhinal cortex or gut neurons of the enteric nervous system (Cowen *et al.*, 2000). Two supporting evidence for the selective vulnerability theory (Cowen, 2002) are first the concept of antagonistic pleiotropy described by Kirkwood and Austad (2000) where phenotype and patterns of gene expression that are advantageous in early life become disadvantageous during ageing. A specific example linked to my research is the adaptation of MCA neurons to low level of NGF during early development, which in later life renders MCA neurons more vulnerable to cell death.

Secondly that lifespan maybe linked to the survival of key groups of cells, which, according to Wolkow *et al.* (2000) and Cowen (2001), might be neurons. Wolkow *et al.* (2000) showed that altered gene expression in particular neurons can affect lifespan.

Gavazzi and Cowen (1996) proposed a simple explanation of 'neurotrophic hypothesis of ageing' where the availability of NGF in different target tissues would determine selective vulnerability of sympathetic neurons, this was contrasted by results of equal axonal degeneration between iris and pineal gland compared to MCA (Cowen *et al.*, 1996; and Kuchel *et al.*, 1999 respectively), suggesting that there is no differential reduction during ageing of NGF synthesis or protein level.

Changes in neuronal plasticity contribute to neurodegeneration; in fact neuronal plasticity can be defined as the responsiveness of a neuron to its surroundings including the capacity to respond to injury. Decreased plasticity of ageing sympathetic neurons has been shown in 'in vivo' studies, including regeneration, collateral sprouting and synapse formation (Fawcett, 1992; Kuchel, 1993; Kuchel and Zigmond, 1991).

Plasticity in mature neurons is also shown by maintained responsiveness to NGF. According to Andrews and Cowen (1994) ageing sympathetic nerves sprout in response to NGF, even though signs of degeneration were present, indicating a high degree of plasticity. Little is known about why particular groups of neurons are more vulnerable than others to degeneration which is characterized by loss of synapses, dendrites, and axons. Cooper and Sofroniew (1996) looked at increased vulnerability of spatial cholinergic neurons to partial loss of target neurons in aged rats, finding an increased vulnerability with age. Several studies looked at selective vulnerability as an effect of neurotrophins (NT) availability on the recovery of nucleus basalis (Eriksdotter-Nilsson *et al.*, 1989; Dekker and Thal, 1992;

Dekker and Thal, 1993; Casamenti *et al.*, 1994; Dekker *et al.*, 1994; Chu *et al.*, 2001), substantia nigra (Camarata *et al.*, 1992; Anglade *et al.*, 1997; Yurek and Fletcher-Turnar, 2001), motor neurons(Greeson *et al.*, 1992; Bergeron *et al.*, 1998), enteric neurons (Chalazonitis *et al.*, 1998; Esteban *et al.*, 1998; Souberyre *et al.*, 2001; Chalazonitis *et al.*, 2001),

According to Cowen *et al* (2003) a characteristic of the ageing nervous system is a differential selective vulnerability between particular groups of neurons. In this study they used SCG projecting to the MCA and the iris; MCA projecting neurons are more vulnerable (Thrasivoulou and Cowen, 1995), while iris neurons seem to be more protected (Gavazzi *et al.* 1996) from atrophic changes during old age. Cowen *et al.* (2003) studied receptor expression of TrkA and p75 on those two populations of SCG neurons and found that for both receptors during early postnatal life messenger RNA increases, on the other hand differentiations between receptors occur in mature adulthood and old age, where TrkA remain stable while p75 expression decreases. Furthermore they looked at iris and MCA projecting neurons and their expression of NT receptors and mRNA which has a substantial increase, in p75 and TrkA, in iris for both young and old rats. Conversely no effect on either age was detected in MCA projecting neurons. They suggested that lifelong exposure to low levels of neurotrophin combined with impaired plasticity of neurotrophin receptor expression is associated with vulnerability to age-related atrophy.

The hypothesis is that during postnatal life, levels of extrinsic neurotrophic factors set the activity of a neuronal maintenance program, which in turn in later life determines vulnerability to age-related neurodegeneration ('developmental conditioning').

1.6 Ageing and selective vulnerability

Selective vulnerability during ageing has been observed in superior cervical ganglia (SCG) by Gavazzi *et al.* (1996), they looked at SCG neuronal projection to MCA which are characterized by loss of axons and dendrites, and, probably, neuron cell death, compared to SCG neurons projecting to the iris characterized by survival and continuing growth in old age. The two groups of neurons are physiologically and morphologically distinct. Differential behaviour of these two types of neurons could be associated with the type of innervations, which is 'multi-unit', for MCA (characterized by a slower response to stimuli) and 'single-unit' for iris (rapid response). Furthermore MCA- and iris- projecting neurons differ significantly in the neurotrophic factor expression in their target tissues and in their neurotrophic uptake characteristics. According to Andrews *et al.* (1996) phenotypic adaptation of these subpopulations of neurons is an adaptation to their particular function. According to Morrison and Hof (1997) ageing is not associated with widespread losses of neurons in either the central or peripheral nervous system (Cowen and Gavazzi, 1998). Gatzinsky *et al.* (2004) looked at the role of NGF uptake in selective vulnerability to cell death in ageing sympathetic neurons, and concluded that neuronal vulnerability is associated with life-long low or high levels of NGF availability; MCA projecting neurons showing a 37% loss with ageing and no significant loss in iris-projecting neurons.

1.7 The neurotrophic hypothesis

The original neurotrophic hypothesis first proposed by Purves, (1988) states that structure and connections of developing and mature neurons are governed by their ongoing interactions with target tissues. The hypothesis arose from work on NGF (Levi-Montalcini *et al.*, 1975), which is the founder member of the neurotrophin family of secreted proteins.

NGF, in the peripheral nervous systems (PNS), provides trophic support to sympathetic neurons. Neurons compete for a limited supply of neurotrophic factors, and therefore targets and neurotrophic availability regulate the population of innervating neurons (Korsching, 1993). According to Davies *et al.* (1987) neurotrophin levels in target tissues increase rapidly when growing axons reach their targets and this coincides with the time when neurons become responsive to neurotrophins (Miller *et al.* 1991, and Wyatt and Davies 1993). Furthermore in accordance with Davies (1996) results in the developing peripheral nervous system many neurons die shortly after their axons reach their target fields, this might be due to the fact that target fields requirement and size determines the number of neurons surviving. In fact, once neurons grow into their targets they either form a synapse and begin to establish the functional connections that characterize the mature nervous system, or they die. Studies on timing of neuronal death have shown that sympathetic neurons become dependent on the supply of two neurotrophins, NGF and NT3, at the time or just before they begin to innervate their targets (Crowley *et al.*, 1994; Wyatt *et al.*, 1997; Francis *et al.*, 1999). Neurons, when they reach the target tissues, become dependent on the trophic factor supplied by the target tissues for survival and differentiation. During neurogenesis there are initially more neurons sending axons to the target tissues than there are cells in the tissue, this initial overabundance is believed to ensure that all target cells receive innervation from axons, and competition of numerous neurons ensures that only those neurons that make functional connections will persist into the mature nervous system. Thus, although initially there is a lot of trial and error in proper matching between neurons and their targets, in adulthood no cells will be uninnervated and no surviving neurons will fail to make synapses. Neurons in the developing brain go through a period when they are

dependent on trophic factors in order to survive, and their targets go through a period when they can produce and secrete those factors. Different regions of the brain, or different kinds of neurons, become sensitive to one or a few neurotrophic factors, and make receptors for those factors, and their targets produce only one or few such factors, in limiting amounts (Davies, 1996; Bibel and Barde, 2000).

1.8 Superior cervical ganglia (SCG) dependence on NGF

While SCG neurons are dependent during perinatal development on NGF for survival and growth during early postnatal life they become independent of NGF for survival but remain NGF dependent for growth (Easton, *et* al., 1997; Orike, Thrasivoulou *et al.*, 2001; Orike, Middleton *et al.*, 2001). Because of the continued dependence of sympathetic, sensory and hippocampal neurons on neurotrophins for growth during adulthood, it is believed that specific trophic factors are instrumental in helping responsive neurons recover from injury or disease (Diamond *et al.*, 1992; Crutcher, 2002).

Synaptogenesis is a complex process involving a diverse set of molecular signals between the neuron and its target; the target cell send signals to the neuron that induces the growth cone to stop growing and convert into the stationary structure of the nerve terminal, that develop synaptic vesicles, active release sites, voltage-dependent calcium channels, and all the necessary machinery for production and release of neurotransmitter, as well as reuptake of transmitters and recycling of vesicle membrane.

Neuron sends signal to its target that induces the target cell to produce appropriate receptors for transmitters and neuropeptides, to consolidate the receptors at high density at the same location as the nerve terminal, and to assemble all the necessary signal transduction and second messenger pathways necessary for proper postsynaptic response to

transmitter; for the most part the nature of the molecular signals in the nervous system remains mysterious, and therefore constitutes an area for much research (Vrbova *et al.*, 1999).

1.9 Signaling and survival pathways

Neurotrophins bound to receptors are then internalized by retrograde transport to the perikarya, where they provide signals in the key processes affecting neuronal survival and differentiation. SCG neuronal responsiveness to trophic factors is mediated by the trk tyrosine kinase family and the low-affinity p75 receptors whose expression may change during development (Bothwell, 1995). Recent studies have suggested the role of PI3-K in SCG survival.

Belliveau *et al.* (1997) showed that NGF promotes sympathetic neuron survival by acting via TrkA. Ligand binding triggers dimerization and autophosphorylation on tyrosine residues forming docking sites for the adaptor protein, Shc, PLCγ, and SHP, resulting in activation of several signaling pathways:

- Shc/Grb2/Gab1-dependent activation of phosphoinositide 3-kinase (PI3-kinase) (Holgado-Madruga *et al.*, 1997);
- Shc/Grb2/Sos-dependent activation of Ras, leading to activation of ERK/MAP-kinase;
- PLCγ -mediated generation of DAG and inositol triphosphate (Kaplan and Miller, 1997).

Genetic studies on *Caenorhabditis elegans* (Hekimi *et al*, 1998) shows PI3-kinase as a central element in controlling lifespan; this is evident in the homology of the insulin-like signaling pathway that contributes to cell survival in mammalian neurons and to longevity regulation in *C. elegans*, *Drosophila* and other organisms. PI3-K and the Forkhead family

are homologous to age-1 and daf-16, and IGF-1 is orthologous to daf-2. According to Feldman *et al.* (1997) and Gorio *et al.* (1999) the pathway resembles that of survival and growth responses to neurotrophic factors present in central and peripheral neurons (Cowen 2002); with the only exception that down regulation of age-1 in *C. elegans* results in an increase in lifespan while a decrease of PI3-K in mammals results in a decrease of cell survival.

Numerous studies have considered the importance of class IA PI3-K for a multitude of cellular functions including cell survival, growth, proliferation, intermediary metabolism, and cytoskeletal rearrangements (Vanhaesebroeck and Waterfield, 1999; Shepherd *et al.*, 1997).

PI3-K catalyze the phosphate to transfer to the 3'-OH position of inositol lipids, producing phosphatidylinositol-4,5-biphosphate(PIP_2), and phosphatidylinositol-3,4,5-triphosphate (PIP_3), which act as second messengers by recruiting proteins containing pleckstrin homology (PH) domains to the plasma membrane to assemble signaling complexes (Vanhaesebroeck *et al.*, 2001). In vitro experiments have shown an intrinsic protein serine kinase activity of class I PI3-K (Vanhaesebroeck *et al.*, 1999). The form of IA PI3-K is typically a heterodimer with an 85-kDa regulatory subunit (identified as p85α and p85β) and a 110-kDa catalytic subunit (p110α, p110β, and p110δ), and is regulated by a range of mechanisms acting via the various modular domains of the subunits. P85α and p85β are products of different genes and several splice variants of p85α exist; a third gene product p55γ has also been identified. The p110α and β isoforms are widely expressed, whereas the δ is expressed predominantly in leukocytes. The functional consequences of this protein in vivo are different (Foukas *et al.*, 2004; Hunter, 1995). Phosphorylation of the sole PI3-K

phosphorylation site Ser608, by p85α, plays a role as a shutoff switch in growth factor signaling and contributes to the differences in functional properties of different PI3-K isoforms in vivo.

Crowder and Freeman (1998) first looked at the potential survival signals mediated by PI3-K to test the Akt functions during NGF mediated survival in rat SCG neurons; Akt is an effector protein kinase of PI3-K. PI3-K and Akt are both necessary for the survival of NGF-dependent sympathetic neurons. Furthermore in a wide range of tumour types PI3-K is over activated, causing Akt phosphorylation, which in turns triggers a cascade of responses, from cell growth and proliferation to survival and motility.

Akt, which is also known as PKB (protein kinase B) encodes a serine/threonine kinase that has an amino-terminal PH domain, a central catalytic domain and a short carboxy-terminal regulatory domain. There are three members of the Akt family (Akt1, Akt2, and Akt3); and Akt is activated by a dual regulatory mechanism that requires both translocation to the plasma membrane and phosphorylation at Thr308 and Ser473 (Andjelkovic *et al.*, 1997; Bellacosa *et al.*, 1998). The generation of PIP_3, on the plasma membrane, following by PI3-K activation, recruits Akt by direct interaction with its PH domain. At the membrane level 3-phosphoinositide-dependent protein kinase-1 (PDK-1) phosphorylates AKT at the Thr308, but maximal activation is obtained by phosphorylation of Ser473 by PKK-2 (Alessi *et al.*, 1997). Although models of Akt activation are fairly clear, there is very little insight into how Akt is down regulated after activation (Andjelkovic *et al.*, 1996; Maira *et al.*, 2001). The main biological consequences of Akt activation are survival, proliferation (increased cell number) and growth (increased cell size). Akt effect on survival pathway is by stopping PTEN (phosphatase and tensin homologue deleted from chromosome 10)

mediated apoptosis (Li *et al.*, 1998). The tumour suppressor PTEN is a dual-specificity phosphatase that plays a functional role in cell cycle arrest and apoptosis (Di Cristofano and Pandolfi, 2000; Simpson and Parsons, 2001). Constitutive activation of Akt is common event in cancer cell that have lost PTEN function because of either chromosomal deletion or mutation (Wang *et al.*, 1998). Activated Akt protects cells from apoptosis by phosphorylating and inactivating proapoptotic substrates such as BAD, procaspase-9, and forkhead family transcription factors (Nakamura *et al.*, 2000) Akt also prolongs cell survival by delaying p53-dependent apoptosis through Mdm2 phosphorylation (Zhou *et al.*, 2002). Expression of exogenous PTEN in mutant cells restores the endogenous pattern of Akt phosphorylation and therefore sensitivity to apoptosis induced by various proapoptotic stimuli (Frisk *et al.*, 2002). A recent study in *Drosophila*, by Stocker *et al.* (2002), reports that phenotype of PTEN loss in flies (lethality) is rescued by a PH-domain mutant Akt that lacks the ability to bind PIP_3; indicating that Akt might be the only important effector of PIP_3, even though there are still no evidence of the same effect in mammals.

Akt also affect proliferation by preventing degradation, regulating the activity of the glycogen synthase kinase-3β (GSK-3β) (Diehl *et al.*, 1998). GSK-3β was the first substrate of Akt to be identified, and its phosphorylation by Akt is inhibitory (Cross *et al.*, 1995), resulting in binding of the phosphorylated N-terminal residues of GSK-3β to its phosphate-docking motif which in turn inhibits phosphorylation of substrates (Dajani *et al.*, 2001; Frame *et al.*, 2001). Inhibition of GSK-3β plays a role in inhibiting apoptosis in neuronal cells. Ding *et al.* (2000) found that over expression of a GSK-3β inhibitor protein promotes neuronal cell survival in response to inhibition of PI3-K. Furthermore, selective and potent small-molecule inhibitors of GSK-3β have recently been developed, termed SB-415286 and

SB-216763 (Coghlan *et al.*, 2000) and these together with lithium (GSK-3β inhibitor) have been shown to protect both central and peripheral nervous system neurons in culture from death induced by reduced PI3-K pathway activity (Cross *et al.*, 2000). The proteins that GSK-3β phosphorylates to induce apoptosis remain to be defined. GSK-3β is of medical importance being involved in metabolic control, animal development, and oncogenesis (Harwood, 2001).

In addition to its role in proliferation, there is growing evidence that Akt also affects cell growth. Recent studies showed the protein mTOR (also known as FRAP1) as the central regulator of cell growth; it is a serine/threonine kinase functioning as molecular sensor regulating protein synthesis according to availability of nutrients, mTOR is a direct target of Akt (Nave *et al.*, 1999).

Summarizing NGF is necessary for survival during early stages of survival and perturbation of NGF levels later in life results in phenotypic changes, affecting SCG growth; Campenot (1977) demonstrated that local NGF regulates the advance of sympathetic neuron growth cones.

In addition to NGF there are other neurotrophic factors affecting neurons, including: Brain-derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF), insulin-like growth factor (IGF), and insulin (Sofroniew *et al.*, 2001; Hallbook ,1999).

BDNF doesn't seem to affect directly SCG neurons survival (Huang and Reichardt, 2001; Brady *et al.*, 1999); even though the BDNF-activated trkB/IRR (insulin receptor-related receptor), elicit activation of a signaling cascades, resulting in a PI3-K survival response, in SCG neurons (Kelly-Spratt *et al.*, 2002).

Members of the GDNF family of ligands do play essential early roles in development of SCG (Huang and Reichardt, 2001). According to results from a study carried out by Durbec *et* al., 1996), the requirement for GDNF precedes the dependence of SCG neurons on NGF and TrkA; mice lacking c-ret, the tyrosine kinase activated by GDNF, were shown to lack all neurons in the SCG without any obvious phenotype in the sympathetic chain at the trunk level. According to Tsui-Pierchala *et al.* (2002), NGF-dependent ret phosphorylation regulates soma size and metabolism but not survival of adult rodent SCG neurons.

Sympathetic neurons are known to possess insulin receptors of the peripheral type (similar to those of hepatic and fat cells, rather than the brain type which shows dual specificity for both insulin and insulin-like growth factor (IGF-1)) for both insulin and IGF-1 (Karagiannis *et al.*, 1997). According to Zackenfels *et al.* (1995), insulin and IGFs have a role in the control of neuron and neurite outgrowth in sympathetic neurons. According to Bitar *et al.* (1997), IGF-1 is not only implicated in development, survival and maintenance of sympathetic and sensory neurons, but neurons are affected during the course of diabetes. Diabetes caused a decrease in IGF-1 and its receptor proteins in SCG neurons, while insulin partially prevented such depletion. Similar results in the central nervous system where according to Fukudome *et al.* (2003) IGF-1 greatly promoted dendritic growth and survival of Purkinje cells in mammalian cerebellum. IGF-1 seems to up-regulate, among others, the signaling pathways involving PI3-K.

Ookuma *et al.* (2003) showed a different effect in the insulin-like ageing pathway of *c.elegans*, by identifying a target of DAF-16, the transcriptional regulator that extends lifespan when the insulin-related pathway is down regulated.

Dillin *et al.* (2002) looked at the age specific timing requirements for insulin/IGF-1 signaling in *C. elegans*; concluding that life-span regulation can be dissociated temporally from phenotypes.

1.10 Chapter division

The thesis aims to tackle several of the topics mentioned above in detail and some other topics are only forming a basis of preliminary results for further research. Chapter II expose the methodology used to develop the research; while chapter III is the first research chapter and studies the characterization of age-related neuron cell loss (cell counting) in vivo and how subpopulations with different phenotypes (MCA versus iris) are affected with age, before and after the in vivo pre-treatment regime, as well as when pre-treatment in vivo have been applied late (i.e. late treatment group). Neurite growth have also been measured (retrograde tracing and axonal arborizations) across ages and following either early or late pre-treatment in vivo with NGF. Furthermore this research looked at the effect of pre-treatment in vivo on nerve fibre degeneration (nerve density was measured using imaging and data analysis by counting the number of intercepts per millimetre (I.D. per mm)), specifically using subgroups of sympathetic neurons in the SCG: MCA and iris; initially three more targets have been considered: skin, sweat glands and SCG ganglia, but due to lack of initial results the last three targets have been abandoned.

Chapter IV looked at mechanisms mediating neuronal vulnerability to age-related neurodegeneration and considered the survival and mortality patterns in vitro before and after pre-treatment in vivo across ages, as well as when treatment have been applied late. Furthermore the effect of pre-treatment in vivo have been studied on subpopulation of neurons (MCA and iris), to observe possible alterations of survival and mortality pattern.

External neurotrophic factors may have an effect on survival of SCG neurons: insulin, IGF, BDNF, GDNF, those have been studied in vitro before and after pre-treatment in vivo with NGF.

In chapter V the signaling pathways affecting growth and survival in adult and ageing neurons have been studied by investigating effects of age and treatment. The research concentrated on the effect of inhibition of survival pathways (such as PI3-K and Erk) across ages with early or late pre-treatment in vivo with NGF. Furthermore p-Akt and GSK-3β upregulation was observed after early and late pre-treatment in vivo, and form preliminary results for further research. Chapter VI aims to analyze and discuss the entire research results into a broader biological context.

1.11 Aims

This PhD research intend to look at the developmental conditioning, by altering NGF availability to sympathetic neurons, at a critical time, at 3 weeks old, before maturation; the effect of the conditioning is then observed at different stages of maturation. Two major target tissues, MCA and iris, are considered in order to determine how selective vulnerability is affected by a developmental conditioning in early age. The research objectives focus on manipulation of trophic factor availability, using NGF and NGF specific antibodies during early postnatal life or after termination of development at three months old in rat SCG neurons.

Developmental conditioning affects survival and growth, based on the suggestion that high NGF levels are associated with neuronal protection against age-related degeneration. Key

regulator of signaling pathway are studied, PI3-K, Erk, Akt, GSK-3β in order to see the effect of exogenous factor during early postnatal life.

1.12 Hypothesis

The main hypothesis is that altering NGF availability during early postnatal life will increase survival and neuronal plasticity and decrease age-related neuron cell loss and fibre atrophy, in adult and ageing neurons. Treatment after the termination of development is not expected to have similar effects. Changes in NGF availability are predicted to affect some subgroups of sympathetic neurons (those projecting to the MCA) more than others (those projecting to the iris).

I hypothesize that increased plasticity resulting from altered availability of NGF will occur as a consequence of changes in the signaling pathway (mainly PI3-K).

CHAPTER II

MATERIAL AND METHODS

2.1 Animals

The rat (*Sprague Dawley*) is a standard mammalian model organism, widely used in neuroscience, ageing, and developmental studies. It has been studied and used extensively because it is easily obtainable, relatively inexpensive, readily maintained and cared for, and for having several characteristics similar to humans, being a mammal. Male rats have been regularly used in our laboratory for other studies and are kept in groups of 3 per cage and fed *ad libitum* on a dry pellet diet at constant 12hr light/dark cycle and temperature was controlled at 17°C. The average lifespan of a rat is 2 years, weaned at 3 weeks, and reaches maturity at about 3 months old. All animals were raised in the Royal Free Hospital School of Medicine comparative biology unit, all rats were subject to regular veterinary inspection and all the experimental procedures used were licensed by the Home Office.

2.2 Experimental design and in vivo pre-treatment regime

A target sample size of nine male animals per group per-treatment was judged to be the minimum required for this study. The age groups used are:

1. Two 'acute' groups (examined immediately after treatment, at about seven weeks old);
2. Four 'eight months' groups (examined 6 months after termination of treatment);
3. One 'eighteen months' group;
4. One 'twenty-four months' group;

5. One 'late treatment' group (i.e. treated after development);
6. One ELISA group (group size and treatment regime are explained in section 2.6).

There are two acute groups in order to provide a repetition to consolidate the data; the eight month group has been also repeated once. One more eight months group was prepared for examination of loss of subpopulations of neurons, this was analysed either in vitro (on dissociated cultured neurons in absence of external growth factor support), or ex vivo on frozen sections of SCG (using cell counting following retrograde tracing).

Two sub-populations of SCGs are examined: one projecting to the iris, characterised by single-unit innervation and considered the less susceptible to external insult (Cowen, 1993; Gavazzi and Cowen, 1996), and one projecting to the middle cerebral artery (MCA), characterised by a multi-unit innervation and regarded as being the more vulnerable of the two.

The treated tissue used from the animals was either superior cervical ganglia (SCG) for in vivo and ex vivo experiments, or iris and middle cerebral artery (MCA) for ex vivo experiments. Initial experiments were carried out with skin and sweat glands as well, but lack of significant data caused us to abandon these last two tissues.

The in vivo treatment is a manipulation of trophic factor availability, using exogenous growth factor (Nerve Growth Factor or NGF, 2.5S, Murine; from Promega) and specific antibodies (anti-NGF, kindly supplied by Dr Robert Rush at Flinders University, Australia) during early postnatal life; treatment started at three weeks old or after maturation in the case of the late treatment group which is starting at three months old.

Animals have been subsequently examined, either immediately after treatment (i.e. acute group) six months after termination of the treatment (8 months group), 16 months after treatment (18 months group) and 22 months after treatment (24 months group). The NGF antibodies (anti-NGF) were kindly supplied by Dr. R. Rush who published several articles on the effect of anti-NGF on SCG neurons (Zhou and Rush, 1996; Zhou *et al.*, 1994; Lees *et al.*, 1981). Anti-NGF injections produced pain and localized inflammation, therefore to assess such effect a second control was introduced, pre-immune serum. The control for NGF was cytochrome-C and for the initial acute group a further untreated control was included, and showed no difference compared to cytochrome-C.

The only information available on similar administration of NGF is from Tria *et al.* (1994) where they have carried out some studies on pharmacokinetics of murine NGF. The treatment used in this experiments consists of three subcutaneous injections per week of 4.5μg of NGF, anti-NGF, or cytochrome-C (used as a control in all the groups), for a duration of four weeks (0.09 μg/gm). NGF has been dissolved in sterile water and proper concentration for injection has been diluted in PBS; cytochrome-C has been diluted in PBS as well. Initially two different concentration of NGF have been tested in order to understand the most suitable (see figure 2.1).

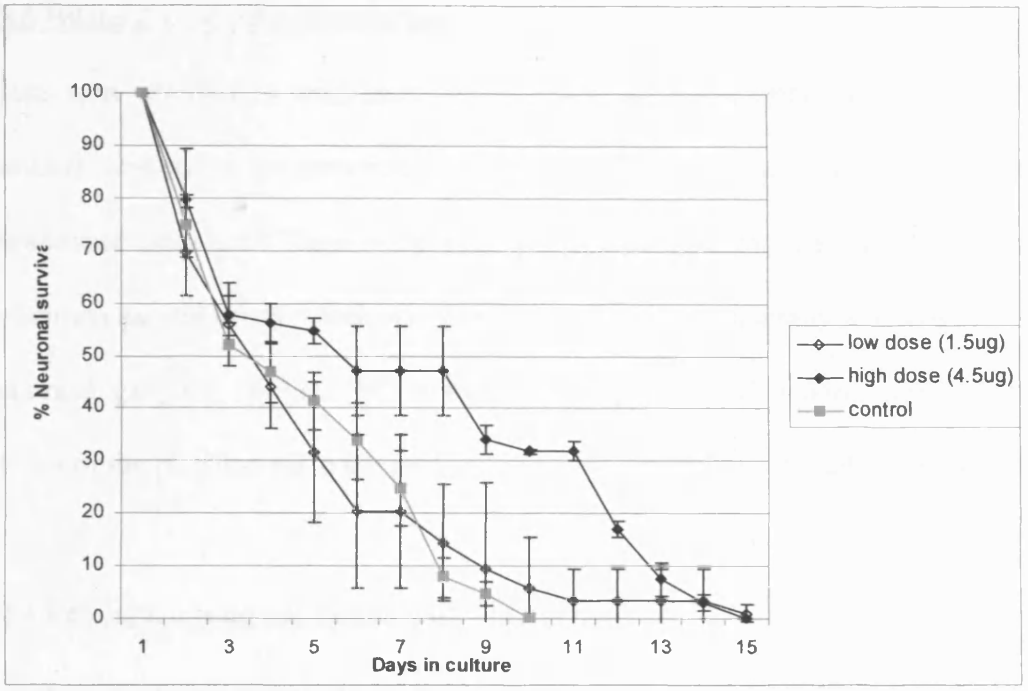

Figure 2.1: NGF dose trial of in vitro serum free cell culture following high (4.5 µg) versus low (1.5ug) doses of murine NGF subcutaneous injections on SCG neurons. High dose is symbolized with the solid black diamond line; low dose is a black empty diamond; control is a solid grey square line.

More specifically we characterized (1) neuron cell loss and nerve fibre degeneration (using morphometry on ex vivo tissues), (2) loss of subpopulations of neurons (using cell counting following retrograde tracing and in vitro dissociated neurons), (3) in vitro survival and growth of adult and ageing SCG neurons including subpopulations of neurons and the factors and pathways involved (using dissociated cell culture) in serum-free medium.

As the treated rats may constitute a model for studying ageing in SCG neurons we also started to consider further mechanisms (factors, and intracellular signalling pathways) mediating neuronal vulnerability to age-related neurodegeneration.

2.3 Bilateral surgical sympathectomy

Rats were scarified, a mild incision was made and tissue retracted. The sternomastoid muscle was cut at the insertion and reflected medially to expose the sternohyoid and omohyoid muscles. These were reflected medially to expose the bifurcation of the common carotid artery which was exposed and retracted laterally to expose the superior cervical ganglion (SCG). SCGs were carefully removed making sure to remove all lobes of the ganglion up to the post ganglionic internal and external carotid trunks.

2.4 Retrograde neuronal tracing with FluoroGold

Rats were anaesthetized with halothane and their heads fixed firmly in a stereotactic head frame. For MCA projecting neurons, a mild-line incision was made in the scalp and tissue retracted to expose the cranium. The temporalis muscle was gently teased away at its superior aspect where it is attached to the temporal bone and undermined to expose the temporal bone beneath. After retracting the temporalis muscle, a small hole was drilled into the temporal bone, lateral to the sagittal suture with a dental burr. A small cut in the dura mater overlying the MCA allowed a small piece of foam pre-soaked in 2µl of 2% FluoroGold (from Fluorochrome, Inc.) to enter and was placed over the MCA under the dura mater. A small piece of antibiotic gauze was placed over the dura mater, the temporalis muscle was returned to its original position and the operation site sutured. For iris-projecting neurons an injection in the anterior chamber of each eye with 1µl of 2% FluoroGold was performed under anaesthesia as above.

2.5 Immunostaining

Growing SCG neurons used for growth assay were fixed for 20 minutes in 4% performaldide and washed 2 times in sterile PBS, immunostained using, as primary

antibodies, mouse monoclonal Tuj (neuron-specific tubulin-1; from Biochem) 1:500 overnight and washed twice in PBS and as fluorescent secondary, goat anti-mouse IgG conjugated to FITC (Molecular Probes Alexa Fluor 488) at a concentration of 1:400 for 1 hour all at room temperature and washed twice in PBS. Antibodies proper concentration was reached using a diluant of Hepes containing 1% goat serum, 0.1% sodium azide , 0.1% DL-Lysine and 0.1% triton when used for GSK-3β measurements, after Tuj staining, BUGS (courtesy of Dr. P. R. Gordon-Weeks) antibodies were applied for 2 hours and washed twice with PBS. BUGS is an affinity purified rabbit polyclonal against a glycogen kinase-3β phosphorylation epitope on MAP1B. In neuronal cultures BUGS labels cell bodies and axons and, when they reach a certain length, the staining of axons is graded with growth cone having the highest levels; it was used routinely at a concentration of 1:200 on cultures with goat anti-rabbit fluorescent secondary (Molecular Probes Alexa Fluor 568) at 1:400 concentration for 1 hour following by two washes in PBS. SCG neurons on coverslips used for measuring phospho-Akt where stained using mouse monoclonal primary antibodies at a concentration of 1:200 for 2 hours following by two washes in PBS, and fluorescent goat anti-mouse secondary antibodies (Molecular Probes Alexa Fluor 568) concentrated 1:400 for 1 hour and washed twice.

Iris and MCA whole mounts where immunostained with Tyrosine Hydroxylase (TH rabbit pAb, polyclonal antibodies). The whole mounts where first fixed for 2 hours in 4% performaldide, and then washed twice for 10 minutes in Hepes buffer. Half an hour wash in 0.1% Triton and goat serum and 3% Hepes buffer in order to increase antibodies penetration into the tissues. TH was applied in concentration of 1:50 overnight; followed by three times ten minutes washing in PBS (was used in these last 2 sets of washing in order to create affinity with the mounting Citiflour which PBS

based). The secondary antibodies used are goat anti rabbit Alexa red, in concentration of 1:200 for an hour and an half, followed by three times ten minutes wash in PBS and mounting in Citiflour.

2.6 Cell-Death ELISA

Potential criticism in the treatment regime might be that by treating SCG neurons during development with NGF we might be killing the vulnerable neurons, and therefore using a subset of the entire population for these studies. In order to overcome this problem we decided to test levels of apoptosis at different times during treatment by using a cell-death detection ELISA kit (Promega, UK), a photometric enzyme-immunoassay for the quantitative and qualitative in vitro determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) after induced cell death. The kit used 96 plates and is a 3 hours procedure; the samples, solutions and reagents were prepared according to the 'Roche Applied science protocols'. The data analysis was as follows:

- average of the value from the double absorbance measurements of the samples (measured using a luminescence microplate reader Dynex Technologies, MRX);
- subtracted the background value of the immunoassay from each of these averages;
- calculated the specific enrichment of mono- and oligonucleosomes released into the cytoplasm from these values using the following formula:
    - enrichment factor = mU of the sample (dying/dead cells)/mU of the corresponding negative control (cells without CAM treatment)
        - mU = absorbance [10^{-3}]

Three groups of six animals per treatment, as well as an untreated control were set aside, resulting in three repeats and 2 replicates per treatment. One group was treated with

either NGF or CYT-C (Cytochrome-C) for one week and tested. The second group was treated for four weeks, standard regime, and tested and the third group, composed of four animals, was not treated at all.

2.7 Imaging, morphology, densitometry and physical dissector

Nerve fibre degeneration was measured using morphometry on ex vivo tissues (i.e. MCA and iris), neuronal outgrowth was measured on in vitro dissociated cultured SCG neurons; GSK-3β and p-Akt activation was estimated by densitometry. Neuronal cell loss was calculated on preparations of ex vivo tissue, 15μm thick frozen sections of SCGs.

Animals scarified for neuronal cell loss calculation were killed by carbon dioxide asphyxiation at 48 to 72 hours after tracing operations were performed. Animals were perfused with 150 to 300ml (depending on age and size) of 4% paraformaldehyde (PFA) in PBS through the left ventricle after placing a haemostat on the thoracic aorta. SCG.s were dissected, taking care to preserve the rostral pole of the ganglia where the majority of neurons are found, the ganglia are subsequently pinned to a silgar block and fixed for two hours on a shaker. Following fixation ganglia have been washed in phosphate-buffered saline (PBS), kept at 4°C overnight in 15% sucrose PBS and frozen at –70°C in moulds with O.C.T. cryoprotectant until sectioning using a cryostat (Bright Instrument Company Ltd.) and all sections were positioned on Superfrost slides (Polysine, Manzel-Glaser). The area of every section was traced and measured and complete average volumes for each ganglion calculated. The total number of neurons per ganglia was counted on a Nikon fluorescence microscope and a x20 planapo objective and estimated using the average volume data calculated from the completely sectioned ganglia as follows:

Total number= (average volume of whole ganglion / volume of counted section) x mean number of labelled neurons counted per section.

The physical disector (Gundersen, 1986) method was used for correction of overestimation of cell numbers in order to be able to count the number of neurons cell body every fourth section and use the adjacent section as a look up one, following the physical disector method used in Williams and Rakic (1988).

Microscopical image analysis for acquisition of images and subsequent analysis of in vitro tissue was achieved using the established methods of image analysis Cowen and Thrasivoulou (1992) using the Kontron, KS400 computer programme with an attached Zeiss AxioCam. Image analyser interfaced to a NIKON fluorescence microscope via a low light video camera. An image was taken in, with standardised optimal gain setting and integration time of the camera. The measurements taken for tissues analysis (i.e. MCA and iris) were:

- Nerve density (expressed as area % of the field covered by nerves);
- Intercept density (measured as the number of nerve bundles that transacted a grid of lines and expressed as intercepts per mm);

The measurements taken for neurite growth are:

- P1 = cell body;
- P2 = perimeter at half-maximum radius;
- P3 = circumference;
- TNL = total neurites length;
- AREA_P1_P3 = area covered by neurites;
- AREA CELL;
- Pid 1 = number of intercepts at half-radius;

- GV cell = integrated grey value of the whole cell (for measuring growth of BUGS and p-Akt);
- GV mean = mean grey value per μm^2 for whole cell.

Densitometry for measuring growth BUGS and p-Akt differential activation due to pre-treatment in vivo have been analysed using KS400 programmed written appositely (see figure 2.2).

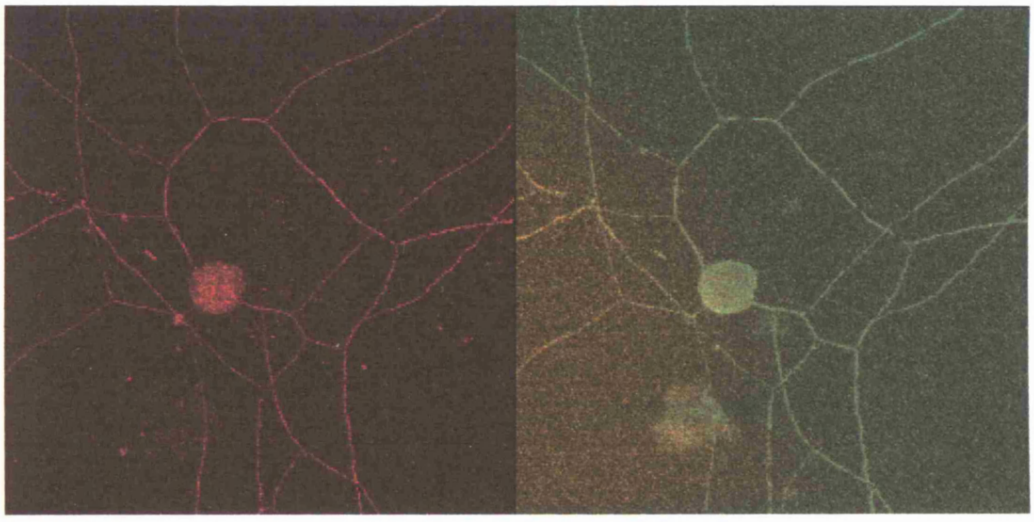

Figure 2.2: Example of BUGS immunostaining (left red panel) and Tuj (right green panel) on the same cultured SCG neuron.

2.8 Cell culture and survival/mortality counting

SCG were dissected, dissociated and cultured as described by Orike *et al.* (2001). Dissected SCG were collected in Hanks' balanced salt solution (HBSS- Gibco BRL, UK; cat.no. 14170-088). The SCG were desheathed carefully under a dissecting microscope to remove all surrounding connective tissue, ganglia were than cut into 8 to 12 pieces (depepnding on the age of the donor). The pieces of ganglia were treated with enzymes; first they were incubated in 2ml HBSS + 10mM HEPES buffer (Sigma, UK; cat. no. H0887) containing 800iu collagenase (Sigma, UK; cat.no. C9891) and 12mg bovine serum albumin (BSA; Sigma, UK; cat.no. A8806); secondly, after two washes in

HBSS, the pieces of ganglia were incubated in 2ml HBSS containing HEPES and BSA as before plus 2mg trypsin (Sigma, UK; cat.no. T2271); following by two washes in HBSS. Times of incubation veried according to the age of the tissue: collagenase between 20 and 35 minutes, and trypsin between 30 and 45 minutes. Neurons were than dissociated with sequential mechanical trituration in a small volume (0.5ml) of culture medium (Ham's F-14, Imperial Labs, UK, pH 7.4) supplemented with 2mM glutamine, 0.35% BSA, 60ng/ml progesterone, 16g/ml pultrescine, 400ng/ml L-thyroxine, 38 ng/ml sodium selenite, 340ng/ml tri-iodothyronine, 60g/ml penicillin, 100g/ml streptomycin and 10mg cytosine arabino-furanoside (all Sigma, UK). Trituration was performed using fire-polished glass pipettes with a bore diameter at the tip of 0.05-0.1mm. A maximum of two ganglia were triturated at any one time. Two factors are critical in the production of viable dissociated neurons, firstly the ganglia pieces have to be of the right size to fit the bore of the pipette quite tightly, thus being subject to some shearing stress as they passed through the tube; secondly a maximum of 2 or 4 triturations were performed following by removal of cells by decanting the supernatant medium. Fresh medium was then added and a further 2 or 4 triturations were carried out before the cells were again removed. These sequential triturations were repeated as many times as it took to remove the majority of the neurons, which again varied according to the age of the donor. Before plating, the decanted supernatant aliquots cantaining dissociated cells were pooled and non-neuronal cells removed by differential sedimentation (Davies, 1986). Differential sedimentation consists of allowing the cell suspension to sedimentate for 1 hour through a dropping funnel containing Ham's F-14 medium plus 10% heat-inactivated horse serum (Gibco). Separate 5ml fractions were removed and a 0.5ml sample from each was examined under phase contrast microscopy for the presence of non-neuronal cells. Fractions containing neurons only were pooled , and spun at 1000rcf

fro 3min. After removal of the supernatant, neurons were gently resuspended in culture medium and counteded in a small droplet of medium of known volume. From the counts estimates the total number of dissociated neurons , and hence the approximate proportion of the total numbers of SCG neurons dissociated from each ganglion.

Neurons were cultured on coated coverslips (19mm round coverslips; Corning) in culture plates (NUNC, Corning) in serum-free medium with no addition of growth factors and the density of plating was kept constant at about 500 cells per coverslip.

Coverslips were first treated with acetone, ethanol and 1 molar hydrochloric acid, and then marked specifically for counting survival/mortality of neurons. Coating of coverslips was performed in two phases, a primary coating with 0.5mg/ml poly-d-lysine (Sigma, UK; cat.no. P6407) overnight and a second with 20μg/ml laminin (Sigma) for 4 hours.

The survival/mortality data were gathered by counting one horizontal strip of microscope fields on the coverslip starting from the mark on one side to the opposite side (see Figure 2.3), the strip contains about 70 to 100 cells, counting intervals were of about 12 or 24 hours depending on whether it was a mortality or a survival assay respectively; the number of live (phase-bright) cells was recorded using a Nikon Eclipse inverted microscope TE300 with phase optics and a motorised stage controlled by joystick with electronic coordinates. Cultured neurons survived from a minimum of one week to about 3 weeks after dissociation.

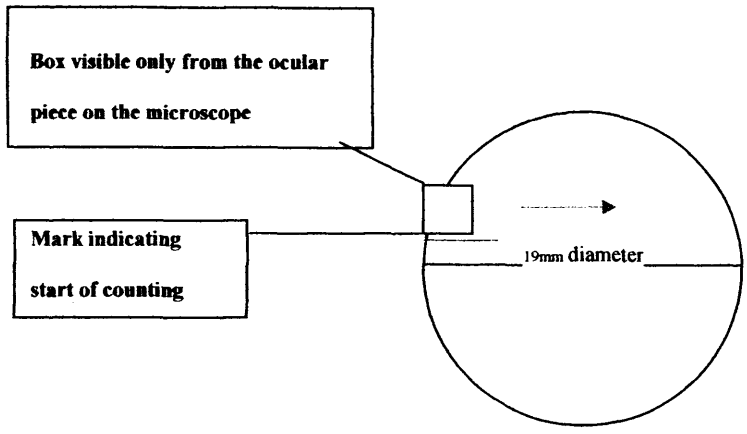

Fig. 2.3: Characteristic of the coverslips used for growing neurons and mode of counting for survival/mortality assay; the grey arrow indicates the direction of counting.

2.9 Survival vs. mortality plotting and analysis

Mortality and survival are both important because they explain different cellular changes. Mortality isolates only one of the two important components of ageing; the physiological degeneration even in the absence of age-specific changes in mortality, the other important element is the effect of treatment which is explained better with the survival plots. Biologists, ecologists and gerontologists have used estimates of age-specific mortality as a critical component in studies of various biological processes. In organisms performance and physiological conditions progressively deteriorate, and therefore mortality rates progressively increase, with age. However the analysis of age-specific mortality rates has some statistical challenges caused by sampling error, Promislow *et al.* (1999) presented simple maximum likelihood models that effectively remove biases even at relatively small sample sizes, they state that the most powerful approach, used commonly in medical survival analysis, but infrequently by population biologists, is that of maximum likelihood estimation (MLE). In my experiments physiological changes due to age don't represent potential statistical error as we divide experiments in age groups, although we have to be careful when comparing mortality of

treated cells with that of controls. Inter-experimental variation due to cell culture changes between experiments is also taken into consideration. Therefore two different plotting regimes have been used in order to distinguish specific effect of treatment on neuronal lifespan, the survival curve and the mortality plot.

The survival curve is produced using changes in neuronal number with time, and is calculated as the percentage of N at t_x compared to the percentage of N at t_0 (where N represents the number of live cells at every count in one horizontal strip of the coverslip; t_x is the counts on the day and t_0 is the first count 12 to 15 hours after plating). The formula is as follows:

$$\% \text{ survival} = (t_x \times 100)/t_0$$

The mortality plotting is considered as the percentage of mortality calculated as the logarithm of the age-specific mortality minus the logarithm of the age-specific survival (Tatar, 2001). The formula is as follows:

$$\% u_t = \text{LN } q_t$$

$$q_t = -\text{LN } p_t$$

$$p_t = \%Nt_x / \%N(t_x - 1)$$

where:

u_t = mortality rate;

q_t = age-specific mortality;

p_t = age-specific survival which is equal to 1- q_t;

LN = natural logarithm;

t_x = the count on a specific day ;

$t_x - 1$ = the count of the previous day;

t_0 = the count at time 0 (i.e. first day of counts).

The comparison between the survival curve and the mortality plot is particularly interesting when looking at the specific effect of treatment at different stages of the life of the cell in vitro and the time when mortality increases, reaches a plateau, and start increasing again to reach a final plateau; we have described this triphasic behaviour as a curve divided into three 'phases'. Phase one (day 1 to day 3-5), phase two (day 3-5 to day 11-15), phase three (day 11-15 to day 25 or more).

2.10 Signalling pathway in culture

Neurotrophins and other external factors, including NGF, brain-derived neurotrophic factor (BDNF), and insulin suppress neuronal apoptosis, while glial-derived neurotrophic factor (GDNF) and IGF-1 promotes apoptosis in SCG neurons.

NGF suppresses neuronal apoptosis through the receptor tyrosine kinases (Trk) A by activating the Erk1/2 and PI3-K pathways. PI3-K activates the serine/threonine kinase Akt, a component of many receptor signal transduction pathways, which amongst other can prevent cell death by deactivating GSK-3β.

In the Erk pathway NGF phosphorylates and activates MAP-kinase which suppress apoptosis by inhibitory phosphorylation of BAD (Hetman and Xia, 2000).

Pharmacological inhibition and external growth factor interaction have been employed in this study to describe the signalling pathway activated in survival mechanisms following in vivo treatment. The pharmacological inhibitors of PI3-K used in culture is 50μM of LY294002 (Vlahos *et al.*, 1995), while UO126 (Duncia *et al.*, 1998) was used

at the same concentration for Erk/MAPK inhibition. A pilot experiment with several doses (see figure 2.4) of LY294002 was carried out in order to adopt a physiological level of the pharmacological inhibitor and at the same time avoid cell apoptosis due to toxicity rather than PI3-K inhibition.

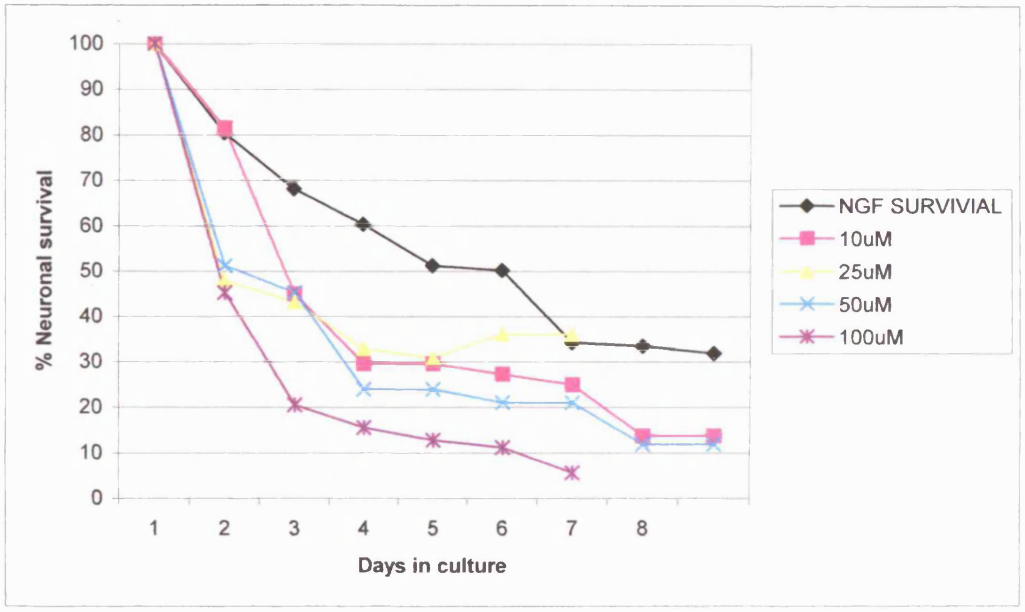

Figure 2.4: Trial of in vitro serum free cell culture following NGF pre-treatment in vivo on 8 months SCG neurons and application of differential doses of PI3-K inhibitor (LY 294002) in culture; n=500.

Inhibitors were applied at plating time and the first count was carried out between 5 and 12 hours after plating, and repeated at 24 hours intervals until termination of live cells. External growth factor experiments have been carried out in order to understand whether survival is activated by external factors, such as BDNF, insulin, GDNF and IGF-1 (see figures 4.18 and 4.19 in chapter 4). External factors concentration has been maintained at physiological levels (50μM), and mode of application as well as plating density was kept constant.

All data sets have been statistically tested with ANOVA analysis of variance followed by Bonferoni post oc test.

CHAPTER III

THE ROLE OF EARLY LIFE PRE-TREATMENT WITH NGF OR NGF ANTIBODIES ON SELECTIVE VULNERABILITY TO CELL DEATH AND NEURITE OUTGROWTH IN ADULT AND AGEING SYMPATHETIC NEURONS

3.1 Introduction

The mechanisms underlying selective vulnerability, in ageing and in neurodegenerative diseases, remain obscure; there is evidence that development does affect later life neurodegeneration (Gatzinsky *et al.*, 2004; Gardner *et al.*, 2005). Gatzinsky *et al* (2004) first related neurotrophic factors (NT) levels in targets to local pattern of age-related SCG neurodegeneration, in this study a significant difference in neuronal number between ages has been detected, with a decreased neuronal number with increasing age. When neurons grow into their targets they either form synapses, begin to establish the functional connections that characterize the mature nervous system, or they die (Heumann *et al.* 1984; Shelton and Reichardt, 1984; Johnson *et al.*, 1987; Davies, 1996). These processes are regulated by molecular crosstalk between the ingrowing neurons and cells of the target tissues, and particularly by target-derived proteins called 'trophic factors; this model is called the neurotrophic hypothesis (Oppenheim, 1989; Haverkamp and McManaman, 1990; Sohal *et al.*, 1991; Cowen, 1993; Johnson and Oppenheim, 1994; de la Rosa and de Pablo, 2000). The hypothesis states that neurons, at about the stage that their neurites reach the target tissue, become dependent on trophic factors supplied by the target tissue, for survival and differentiation. The targets are thought to make limited quantities of these factors, not enough to sustain all the neurons that grow into the region, thus neuronal axons must compete for the limited

supply of trophic factor. The neurons that by chance obtain a threshold amount of the factor will survive and prosper to form synapses, the others will die. An 'overabundance' of neurons is formed during neurogenesis, and they send in more axons to the target tissue than there are cells in the tissue. This is in order to ensure that all target cells receive innervation from axons, while the limited supply of factors from the target tissue ensures that only those neurons that make functional connections will survive into the mature nervous system. Furthermore the overabundance of neurons is also necessary in order to establish proper matching between neurons and their targets, which initially is characterised by trial and error mode.

In the experiments described here, the focus is on the effect of exogenous NGF on SCG neurons applied systemically in vivo before termination of development (i.e. 'early' pre-treatment in vivo), or after termination of development (i.e. 'late' pre-treatment in vivo). It is therefore important to identify the characteristics of development of SCG neurons and how they are affected by NGF as well as the mode of action of NGF.

In mammals soluble peptide growth factors play indispensable roles in inter and intra cellular communication; NGF in particular is the molecule that regulates the survival and maturation of developing neurons in the peripheral nervous system (Huang and Reichardt, 2001). NGF biology does not only centre around the concept of target-derived neurotrophic factors where NGF is released by postsynaptic targets and acts on presynaptic neurons to build and/or maintain functional contacts. This is not the sole role of NGF actions, NGF and its receptors are produced throughout adult life and during ageing by many different cell types. According to a review from Sofroniew *et al.* (2001) the dynamically regulated expression of NGF and its receptors suggests multiple functions of NGF signaling, many of which are poorly understood. During neuronal

development NGF promotes survival and maturation of neurons expressing TrkA and $p75^{NTR}$ (Conover and Yancopoulos, 1993; Snider, 1994), by participating in the classical target-derived neurotrophic relationships which is evident, according to Crowley *et al.* (1994), in the experiments where the NGF gene is knocked out resulting in loss of most sympathetic neurons. Smeyne *et al.* (1994) also found a depletion of SCG in TrkA knockout mice. Gene disruption of $p75^{NTR}$ causes changes in sympathetic innervation, specifically of the pineal gland and sweat glands (Lee *et al.*, 1994), as well as on the number of sympathetic neurons (Brennan *et al.*, 1999). NGF signaling through $p75^{NTR}$ might either restrain the normal survival and development of sympathetic neurons, or alternatively $p75^{NTR}$ disruption may allow NT-3 to signal through TrkA to enhance neuronal survival (Clary and Reichardt, 1994); this remain a controversial issue and further studies are needed to clarify the interaction. To summarize the effect of NGF on developing neurons, firstly neurons are not continuously dependent on the supply of a single target-derived factor throughout life and a multitude of molecules from different sources influence developmental survival and maturation (Davies, 1994). Secondly, according to Enokido *et al.* (1999) transiently required growth factors may derive from sources other than the final region, such as local interactions around the cell bodies or intermediate targets that axons encounter and than grow past to final destinations (Wang and Tassier-Lavigne, 1999). Thirdly, NGF signaling can also mediate axon sprouting, as well as growth cone turning and local guidance (Campenot, 1977, Gallo *et al.*, 1997; Patel *et al.*, 2000; Rice *et al.*, 1998; Tuttle and O'Leary, 1998). Fourth, NGF can induce the death of certain developing neurons by signaling through $p75^{NTR}$ in the absence of TrkA, as in the retina (Frade and Barde, 1998; Frade *et al.*, 1996).

Finally, if neurons survive successfully the stages of cell birth and migration, neurite extension to appropriate targets, avoiding apoptosis at each stage, then they may form synapses with their target tissue. Synaptogenesis involves diverse set of molecular signals between the neuron and its target, and sometimes involving glial cells as well. After synaptogenesis started, target cells send signals to the neurons that induce the growth cone to stop growing and convert into stationary structure of the nerve terminal, which develops synaptic vesicles, active release sites, voltage-dependent calcium channels, production and release of neurotransmitter, and reuptake of transmitters and recycling of vesicle membrane. The neurons send signals to their targets inducing them to produce appropriate receptors for transmitters and neuropeptides, to consolidate the receptors at high density at the same location as the nerve terminal, and to assemble all the necessary signal transduction and second messenger pathways necessary for proper postsynaptic responses to neurotransmitters (Van den Pol *et al.*, 1998).

3.1.1 Role of receptors and signaling pathways

Almost two decades ago Yan *et al.* (1988) reported the potential functional significance of NGF receptors of spinal motoneurons and how they can bind, internalize, and retrogradely transport NGF.

A decade ago numerous studies reported axonal regeneration such as Gold *et al.* (1993) documenting axonal regeneration over long distances following axotomy in the C57BL/Ola mouse mutant. Nowadays neurotrophins prevention of cell death in the peripheral nervous system is well-established; and according to studies carried out by Tucker *et al.* (2001), the physiological involvement in nerve growth of mixed nerves is also dependent upon neurotrophins during development.

NGF in vivo treatment also influences neuronal growth; according to Emanueli *et al.* (2002) endogenous NGF stimulates neurovascularization and vascular endothelial cell

proliferation in mice. Furthermore Mills *et al.* (2003) showed the link where intergrin-linked kinase (ILK) is an important effector in NGF-mediated neurite outgrowth. ILK is a, PI3-K dependent, serine-threonine kinase that regulates adhesion, migration, differentiation, and survival (Giancotti and Ruoslahti, 1999; Dedhar, 2000; Wu and Dedhar, 2001). Stimulation of ILK after exposure to NGF results in activation of AKT and inhibition of GSK-3β (Delcommenne *et al.*, 1998; Attwell *et al.*, 2000; Dedhar, 2000; Persad *et al.*, 2000). Therefore to summarise the importance of these findings NGF seems to affect growth which in turn is linked with neurovascularization via a ILK mediated pathway which is a PI3-K dependent causing activation of Akt and inhibition of GSK-3β (refer to chapter VI).

The aim is to investigate how 'early' and 'late' pre-treatment with exogenous NGF affects selective vulnerability, and specifically changes in neuronal numbers, growth, and peripheral innervation.

The hypotheses investigated in this chapter are firstly that there will be changes in the numbers of neurons in the adult and aged SCG following pre-treatment with NGF, and changes in the ratio of vulnerable versus protected neurons. Secondly, I hypothesise that growth of adult and aged SCG neurons in vitro will be affected by pre-treatment (example neurons immunostaining in figure 3.1).

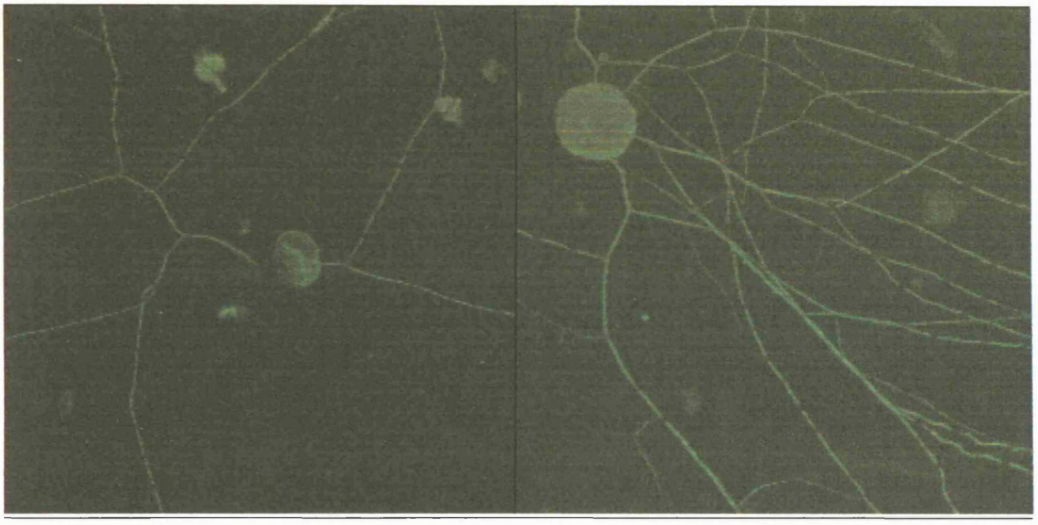

Figure 3.1: Example of neurons immunostained with Taj for growth assay.

3.2 Results

3.2.1 Investigation of a possible side-effect of NGF pre-treatment in vivo on neuron survival

NGF is also known to cause inflammation, and there was the possibility that pre-treatment in vivo might be causing neuronal toxicity and therefore partial neuronal death during treatment; this would have caused a subset of neurons survived to treatment rather than the entire population. In order to avoid such possibility animals have been treated for either one week or four weeks (the latest is equivalent to the full treatment) and the SCG removed and tested immediately after termination of treatment for cell death with a Cell Death ELISA kit (Promega, UK). Results from the ELISA kit showed no significant difference in the extent of cell death between NGF and CYT-C pre-treated neurons after one week of in vivo treatment ($p = 0.5$), or after four weeks of in vivo treatment ($p = 0.9$). Figure 3.2 shows no difference between treatment and some difference between ages.

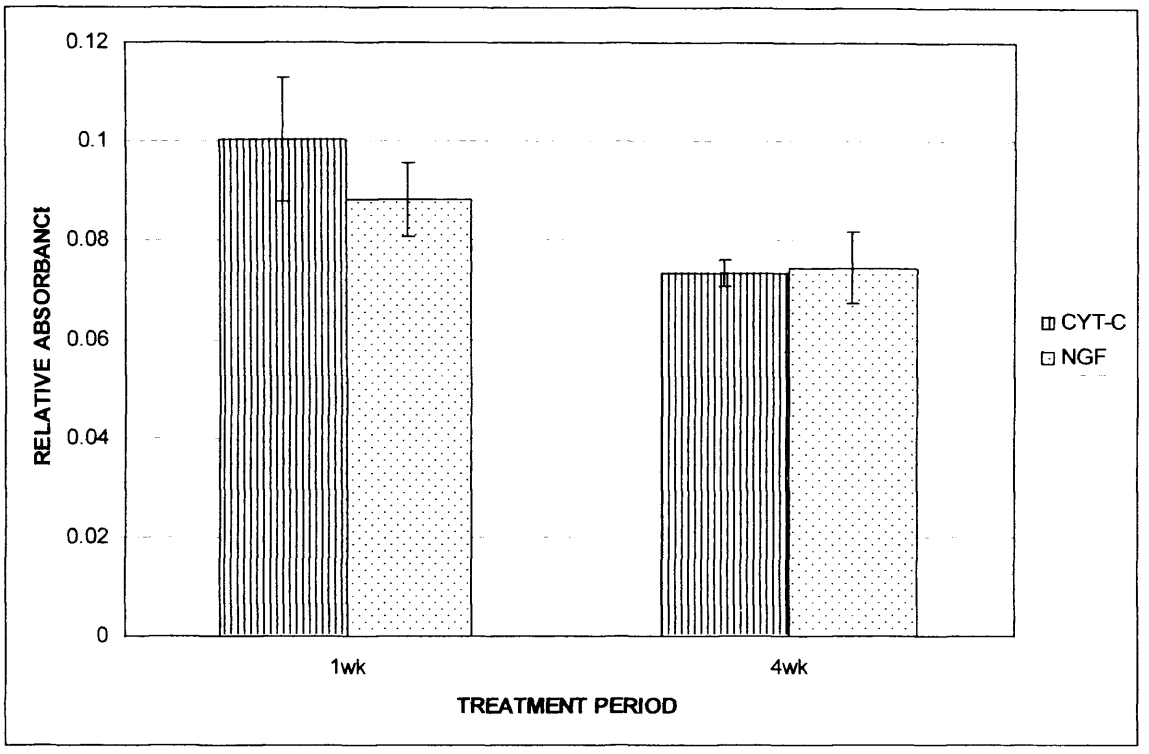

Figure 3.2: Cell death ELISA showing neuronal death following in vivo pre-treatment with either NGF (dotted columns) or CYT-C (control, striped columns) at three weeks of age and following a period of either one week (1 wk) or four weeks (4 wk) of treatment.

3.2.2 Counts of total number of neurons following retrograde tracing with Fluorogold; on ex vivo samples

Counts have been carried out after using conventional retrograde tracing techniques (in vivo), combined with stereological counting methods (ex vivo). The retrograde tracing dye, Fluorogold, was used followed by estimation of total neuron numbers using a physical disector (Tandrup, 1993) (see Chap II for details). MCA and iris projecting neurons were found mainly in the rostral pole of the superior cervical ganglion, often exhibiting labelled neurites. Iris neurons were generally larger than those projecting to the MCA, confirming previous observations using similar techniques (Andrews *et al*, 1996).

The age-groups used are: young adult (6wk), middle aged (15m) and old aged (20-24m).

Although the retrograde tracing technique used is a conventional method, well known and regularly used in our lab, we experienced some difficulties with the oldest group, which included in some samples leaking of Fluorogold into adjacent cells (mainly glia) and tissues made counting of labelled cell bodies difficult and hence second observer repetition was necessary. The second possible difficulty with the old group was the risk that neurons might be affected by age-related alterations in retrograde transport (Cooper *et al.* 1994). For this reason, counts were made about 3 days (short term, ST) or 10 months (long term, LT) after tracing in vivo. The data showed very similar results for the ST compared to the LT groups. See figure 3.3.

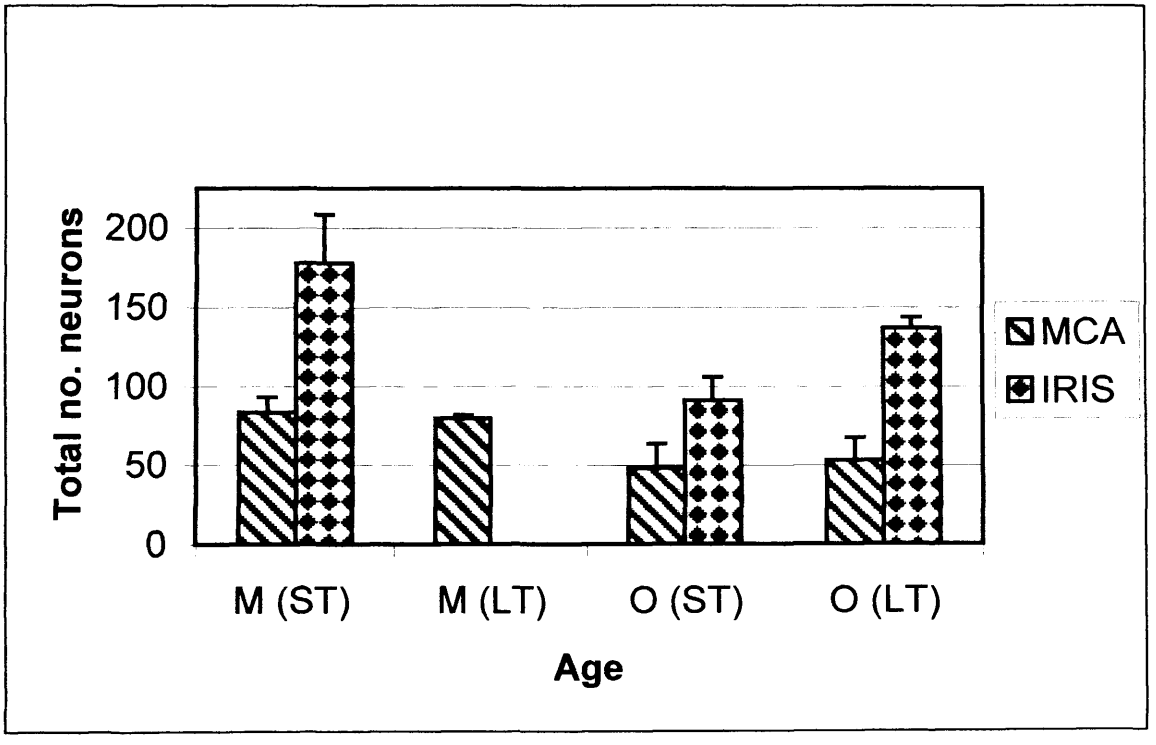

Figure 3.3: Number of neurons retrogradely traced with Fuorogold in MCA versus iris SCG ganglia. Ganglia of middle aged neurons (M) and old neurons (O) have been harvested 48 hours after tracer have been applied (ST) or several weeks after application of tracer (LT)

Besides when comparing MCA-projecting neurons, both showed a reduction in neuron number with age, and therefore no age-related reduction in retrograde transport of the Fluorogold tracer; as a result ST and LT groups were amalgamated for subsequent analysis. On the contrary only ST iris projecting neurons showed a small but significant (27%; $p<0.01$) reduction both between age-groups as well as between ST and LT groups, indicating a reduction of retrograde transport, therefore only LT iris data (labelling carried out before any age-related deficits in retrograde transport) were used for subsequent analysis. Considering neuronal number across ages, the results show a clear decrease with age in number of MCA-projecting neurons but no comparable decrease in iris-projecting neurons, confirming the selective vulnerability of MCA- compared to iris-projecting neurons. Our results also demonstrate an age-related decrease in retrograde transport for the iris projecting neurons. See figure 3.4.

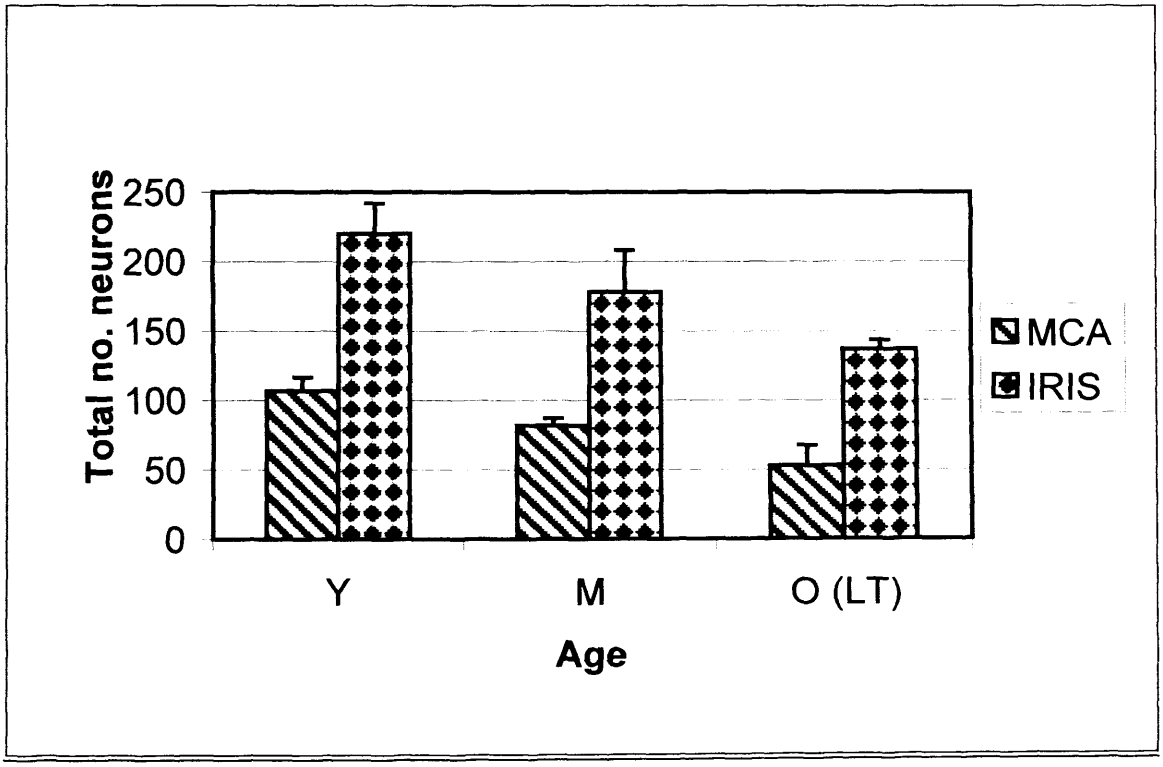

Figure 3.4: Number of neurons retrogradely traced with Fuorogold in MCA versus iris SCG ganglia. Ganglia of young neurons (Y), middle aged neurons (M) and old neurons (O) harvested 48 hours after application of tracer or several weeks after application (LT).

3.2.3 Effect of 'early' (age 3-7wks) pre-treatment with NGF, A-NGF, CYT-C or serum on total numbers of neurons and of subpopulations of MCA- and iris-projecting neurons counted either immediately after treatment (acute group) or 6 months later (8m group); on ex vivo samples

These experiments present results regarding the number of neurons in the acute (7 weeks old animals) and the 8 month old groups, following in vivo retrograde tracing with Fluorogold (see figure 3.5).

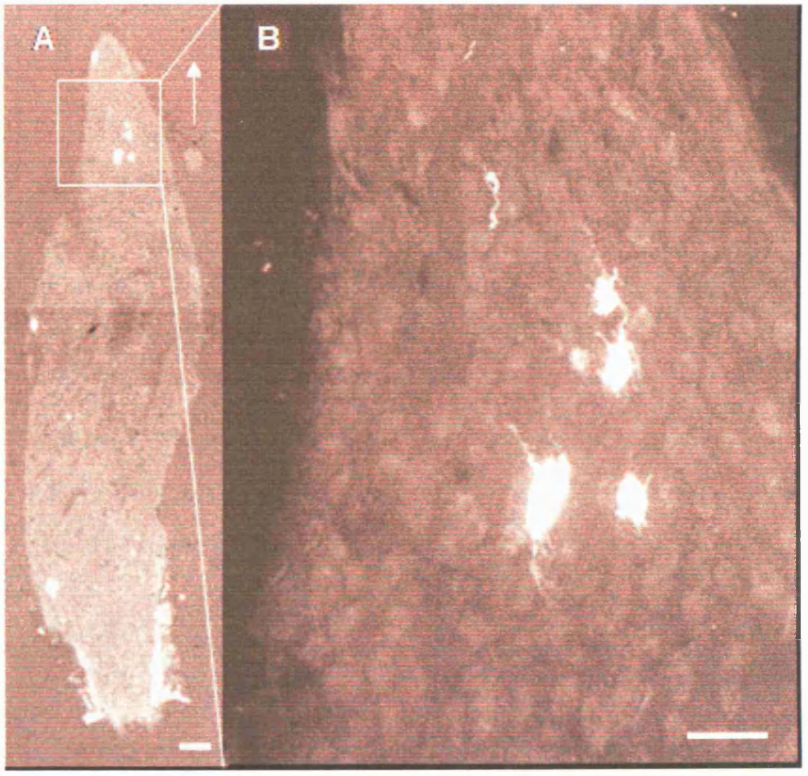

Figure 3.5: 20µm SCG section retrograde traced with Fluorogold. Figure A shows the entire section; while figure B shows a magnified section with traced SCG neurons.

Four acute groups were pre-treated in vivo with either NGF or CYT-C (control for NGF), or anti-NGF or serum (control for a-NGF); two 8 months old groups have been treated with either NGF or CYT-C. The acute group present a significant higher number

of neurons when pre-treated with NGF in vivo ($p < 0.05$), while at 8 months the overall number of neurons is not significantly higher. See figure 3.6.

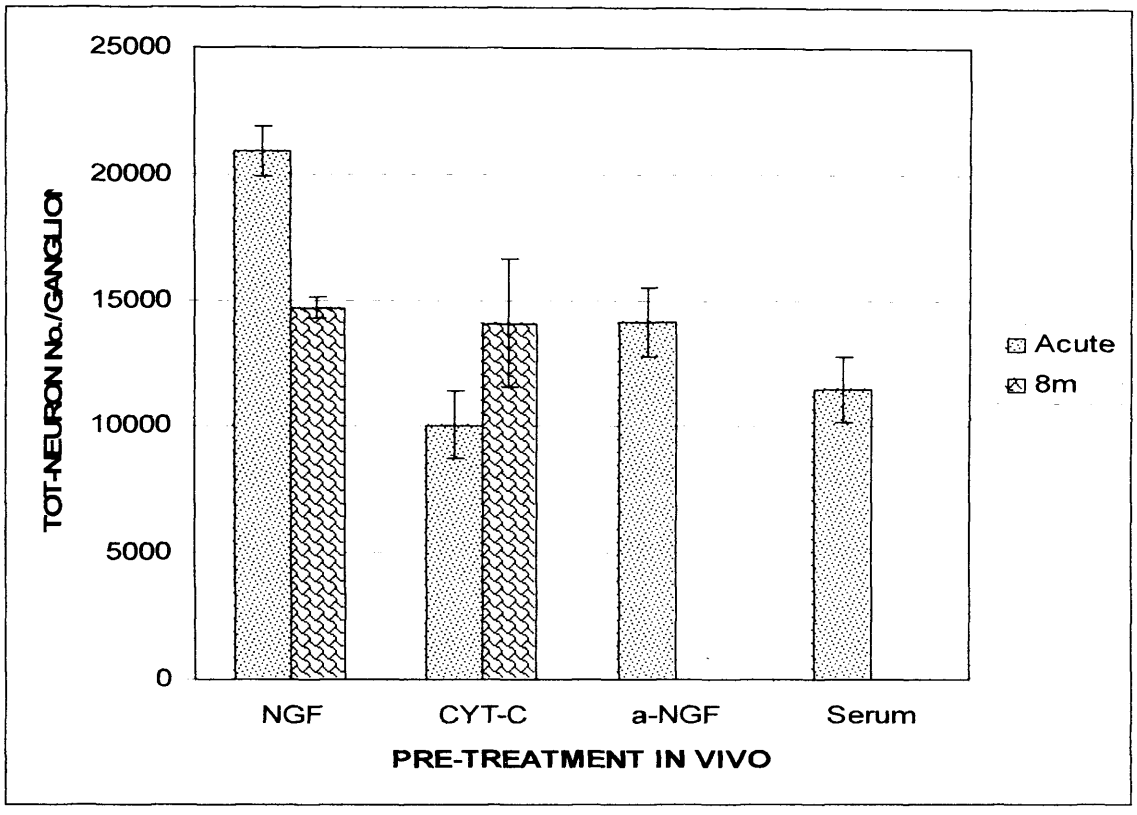

Figure 3.6: Total number of acute and 8 months SCG neurons pre-treated in vivo with either CYT-C (control for NGF), NGF, a-NGF, or serum (control for a-NGF. The bars represent the means mean +/- sem of the total number of neurons per ganglion, and the neurons have been retrogradely traced in vivo with Fluorogold.

The acute group presents a larger number of MCA-projecting neurons compared to iris-projecting one, with a significant difference between the two groups ($p < 0.0.5$) once the neurons have been pre-treated in vivo with NGF. The effect of NGF pre-treatment on the acute MCA-projecting neurons reveals a non-significant increase in the number of neurons compared to controls (see figure 3.7); a-NGF in vivo pre-treatment, on the contrary, significantly reduces the number of neurons ($p = 0.05$). The iris-projecting acute group of neurons shows no significance at all for any of the treatment; showing that previous results on vulnerability of MCA neurons is correlated with plasticity (in this case, the capacity to respond to NGF pre-treatment).

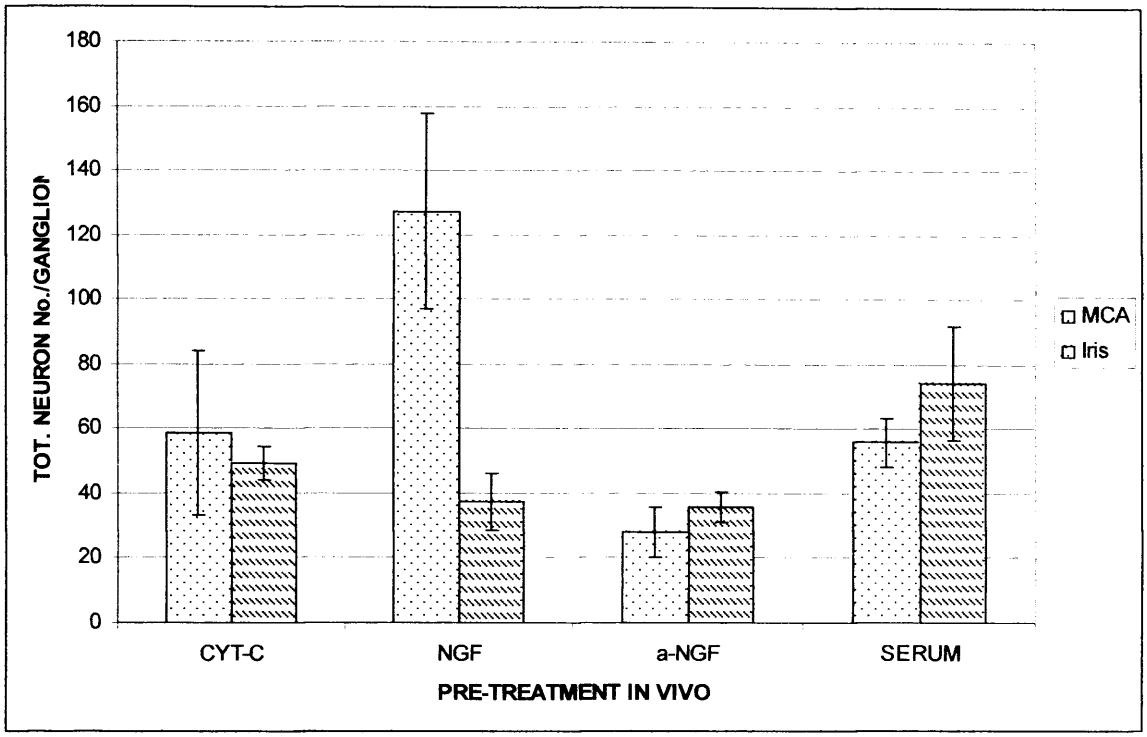

Figure 3.7: Number of acute MCA- and iris-projecting SCG neurons pre-treated in vivo with either CYT-C (control for NGF), NGF, a-NGF, or serum (control for a-NGF). MCA projecting neurons are identified with the dotted bars, while iris-projecting neurons are represented with short diagonal bars. The bars represent the means mean +/- sem of the total number of neurons per ganglion, and the neurons have been retrogradely traced in vivo with Fluorogold.

The 8 months old group (see figure 3.8) results shows the numbers of MCA-projecting neurons as significantly greater ($p < 0.05$) once treated with NGF compared to the control, as well as iris-projecting neurons are significantly more ($p < 0.05$) when treated with NGF compared to the control.

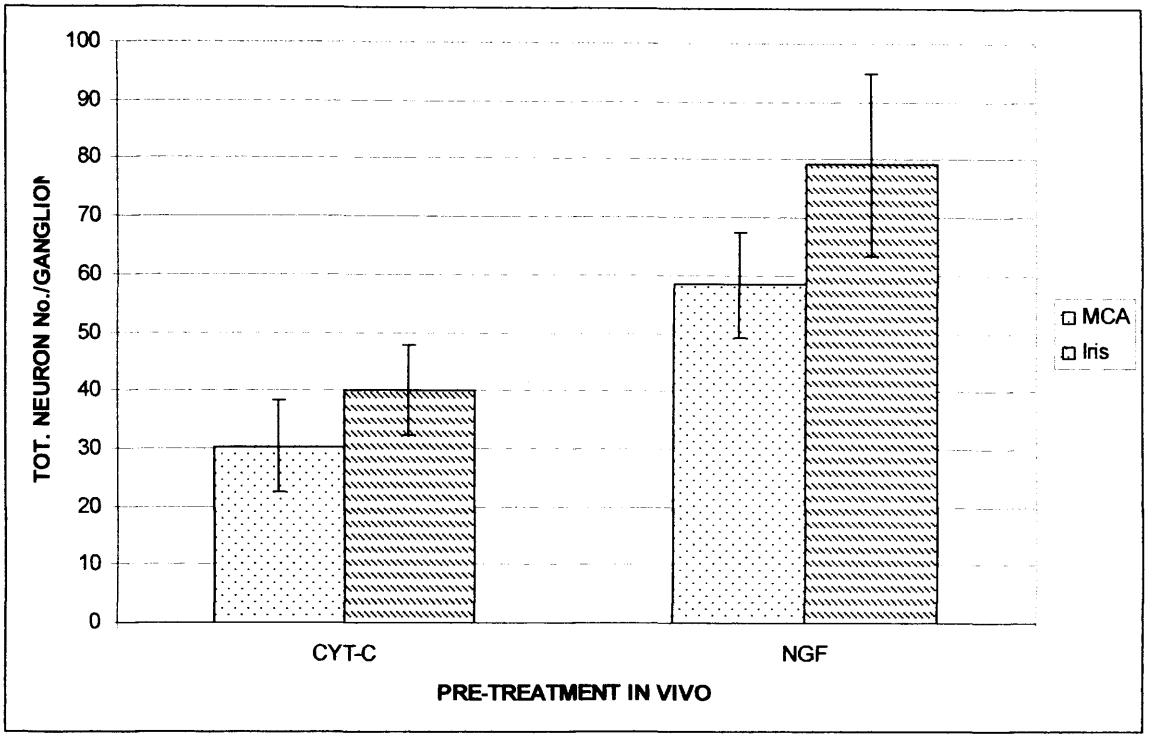

Figure 3.8: Number of 8 months MCA- and iris-projecting SCG neurons pre-treated in vivo with either CYT-C (control for NGF), or NGF. MCA projecting neurons are identified with the dotted bars, while iris-projecting neurons are represented with short bars. The bars represent the mean +/- sem of the total number of neurons per ganglion, and the neurons have been retrogradely traced in vivo with Fluorogold.

Comparing the number of neurons across ages, results show a difference between the controls and the NGF pre-treated neurons (see figure 3.9). CYT-C (control) pre-treated neurons tend to decrease with age (for both MCA- and iris-projecting neurons); while once they have been treated with NGF, MCA and Iris react differently to the pre-treatment in vivo. While MCA-projecting neurons tend to decrease with age, passing from being significantly more than iris to less, iris-projecting neurons increase, not only compared to MCA but as overall number. It seems that both an availability of NGF early in life and an anatomical difference between the two sets of neurons (single- versus multi-unit system) are determining the survival of neurons.

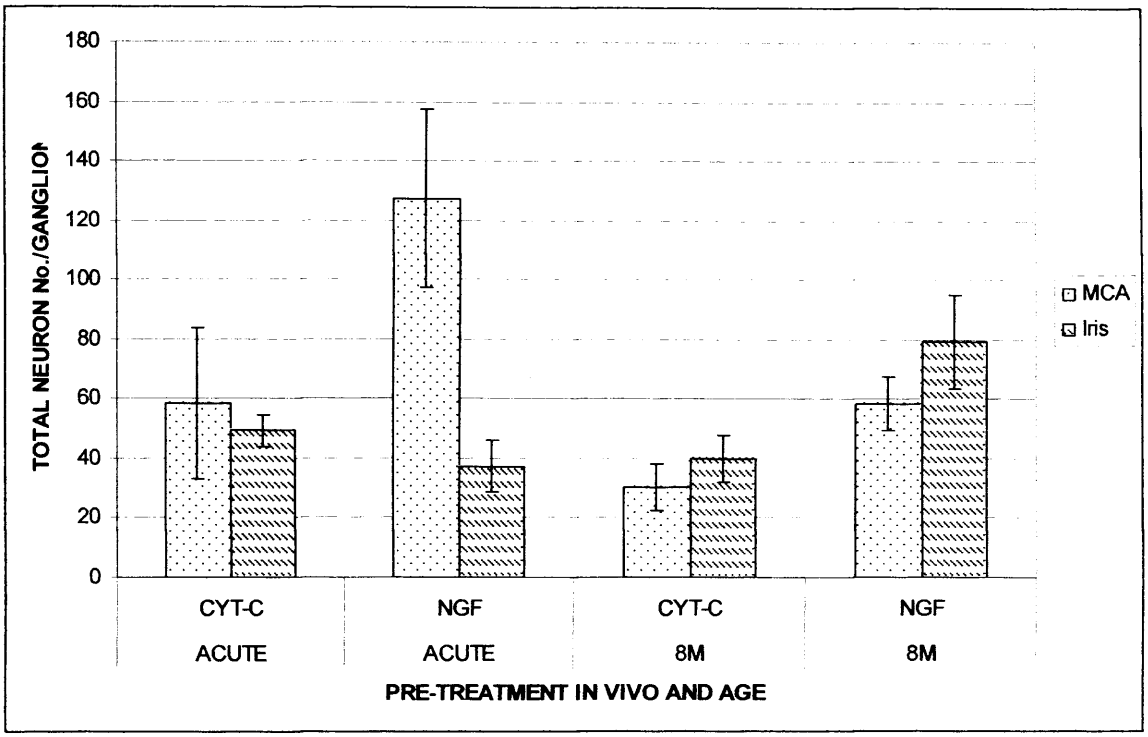

Figure 3.9: Number of acute and 8 months MCA- and iris-projecting SCG neurons pre-treated in vivo with either CYT-C (control for NGF), or NGF. MCA projecting neurons are identified with the dotted bars, while iris-projecting neurons are represented with short bars. The bars represent the total number of neurons per ganglion, and the neurons have been retrogradely traced in vivo with Fluorogold.

These results showed in table 3.1 shows a summary of the significant results. There is a significant result on the acute MCA-projecting neurons showing plasticity with a decreased number of neurons following pre-treatment in vivo with a-NGF. The 8 months group is positively affected by NGF pre-treatment in vivo, causing a significant increase in the number of both MCA- and iris- projecting neurons. There is still significant difference, between acute MCA- and iris-projecting neurons, after NGF pre-treatment in vivo.

	MCA	IRIS	MCA VS. IRIS
ACUTE	CYT-C vs. A-NGF $p < 0.05$		
8M	CYT-C vs. NGF $p < 0.05$	CYT-C vs. NGF $P < 0.05$	
NGF	Acute vs. 8m $P < 0.05$	Acute vs. 8m $P < 0.05$	$P < 0.05$

Table 3.1: Summary of significant p-value for the numbers of MCA- and iris-projecting neurons at different ages (acute and 8 months) with different in vivo pre-treatment (CYT-C, NGF or A-NGF).

3.2.4 Effect of 'early' (age 3-7wks) pre-treatment with NGF or CYT-C treatment on growth of SCG neurons in vitro: 8 MONTH GROUP

The 8 months old group were treated between 3 and 7 weeks old of age and analysed 6 months after termination of treatment. Neurons were grown in the absence of serum and in the presence of NGF (see chapter II for details).The cell body size seems to be affected by pre-treatment in vivo (see figure 3.10), NGF pre-treatment increased the size of the cell body, but the increase is not significant.

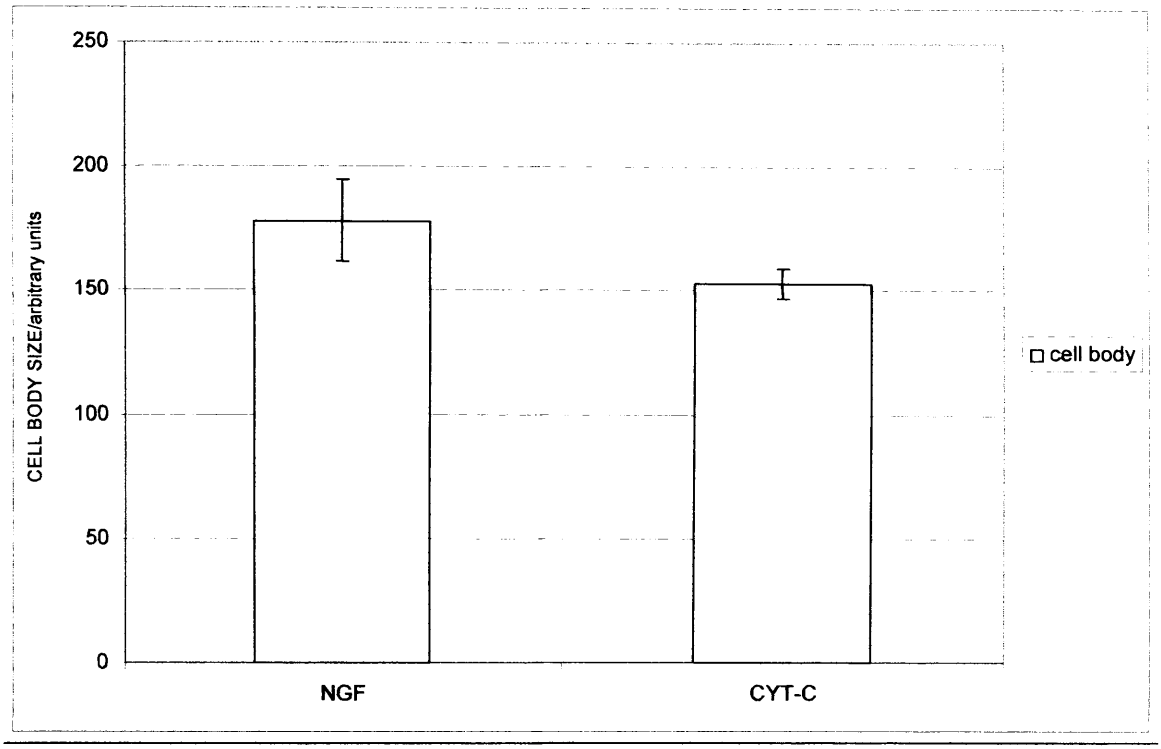

Figure 3.10: Cell body size of 8 months old SCG neurons pre-treated in vivo with NGF or CYT-C. Neurons have been cultured in serum-free medium for 24 hours.

The area covered by neurites in the 8 months old group (see figure 3.11) is significantly increased ($p < 0.05$) by NGF pre-treatment compared to controls (CYT-C).

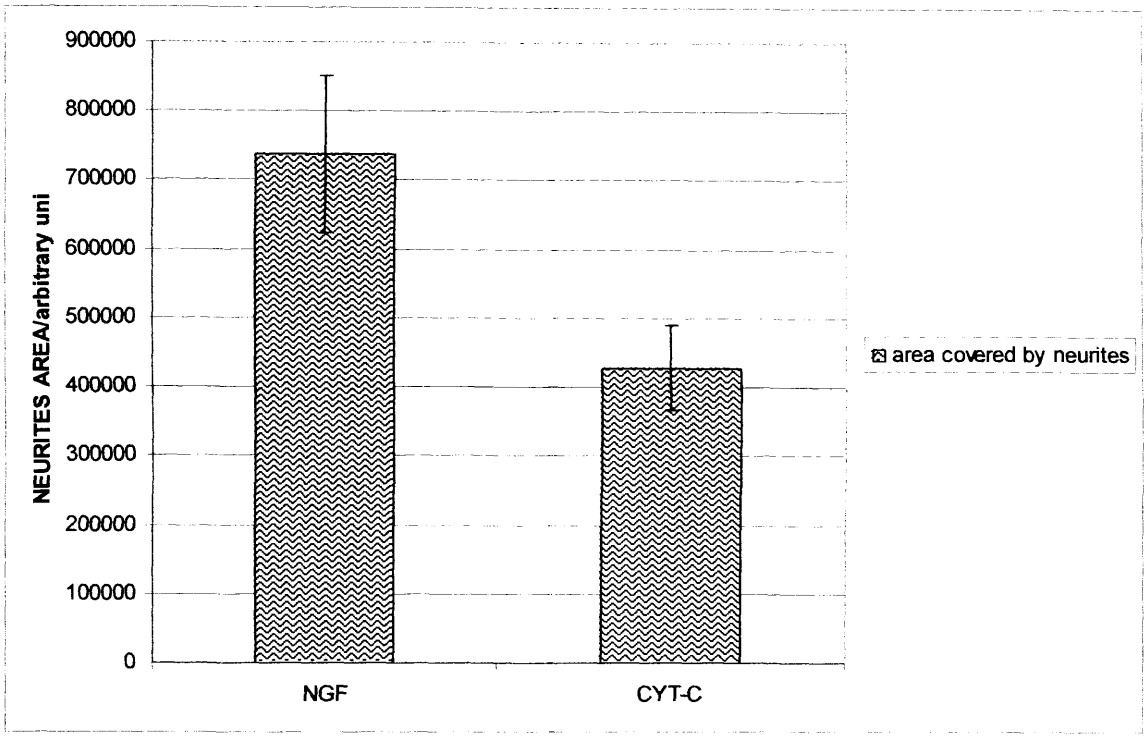

Figure 3.11: Area covered by neurites of 8 months old SCG neurons pre-treated in vivo with NGF or CYT-C. Neurons have been cultured in serum-free medium for 24 hours.

The total neurite length of 8 months old group is also affected by in vivo pre-treatment (see figure 3.12). NGF significantly increases the total neurite length ($p < 0.05$).

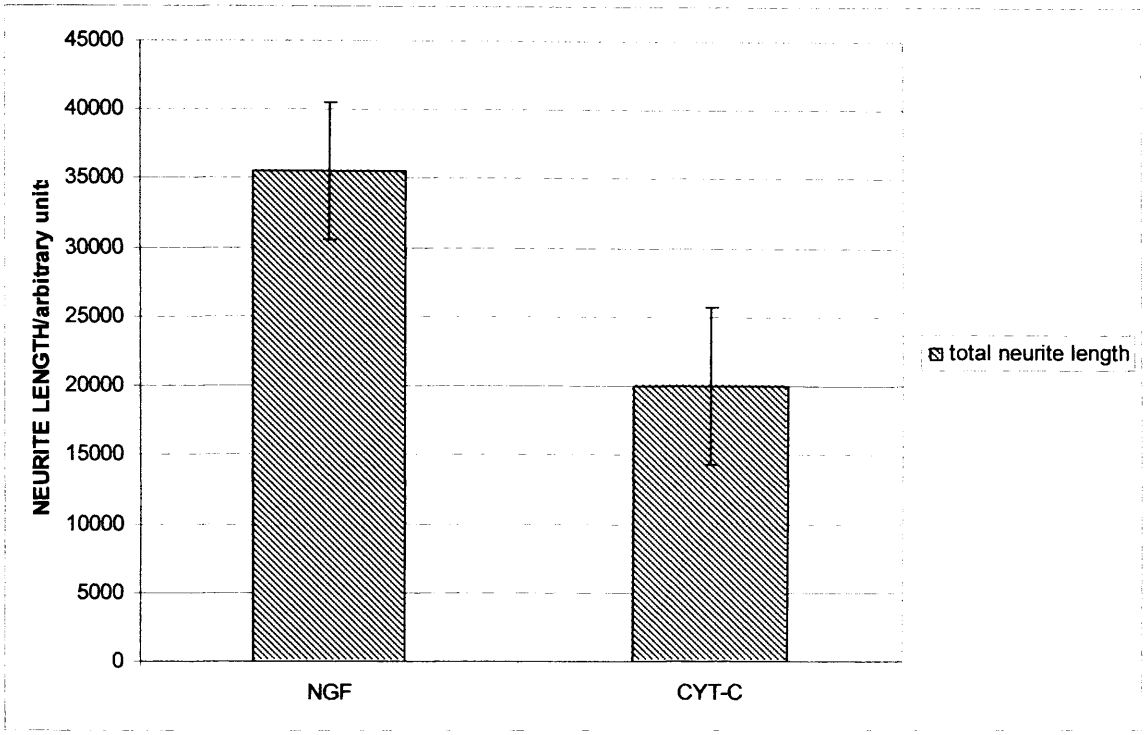

Figure 3.12: Total neurite length of 8 months old SCG neurons pre-treated in vivo with NGF or CYT-C. Neurons have been cultured in serum-free medium for 24 hours.

The number of intercepts of the 8 months old group is affected by pre-treatment in vivo (see figure 3.13). Even though NGF pre-treatment in vivo increased the number of intercepts, the difference is not statistically different.

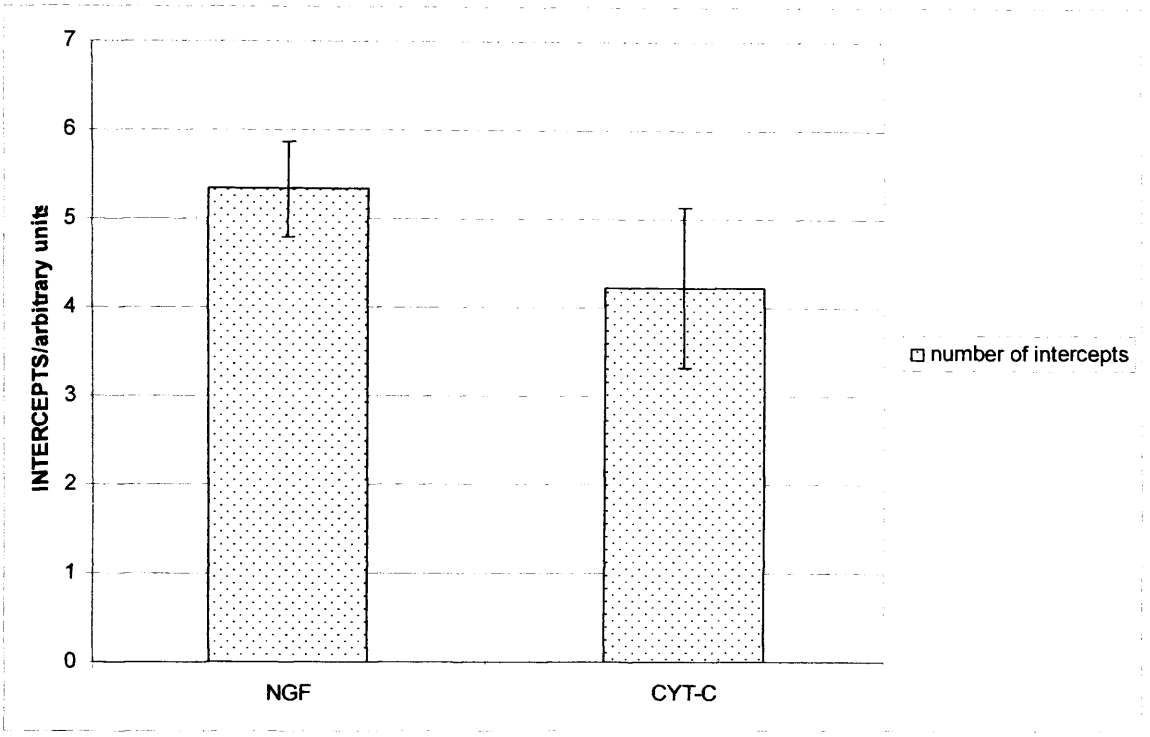

Figure 3.13: Number of intercepts of 8 months old SCG neurons pre-treated in vivo with NGF or CYT-C. Neurons have been cultured in serum-free medium for 24 hours.

3.2.5 Effect of 'early' (age 3-7wks) pre-treatment with NGF, A-NGF or CYT-C treatment on growth of neurons in vitro: 24 MONTH GROUP

The cell body size of neurons pre-treated with NGF in vivo is not affected in 24 months old neurons (see figure 3.14). In contrast, a-NGF seems to be increasing cell body size compared to NGF pre-treated in vivo neurons ($p < 0.05$).

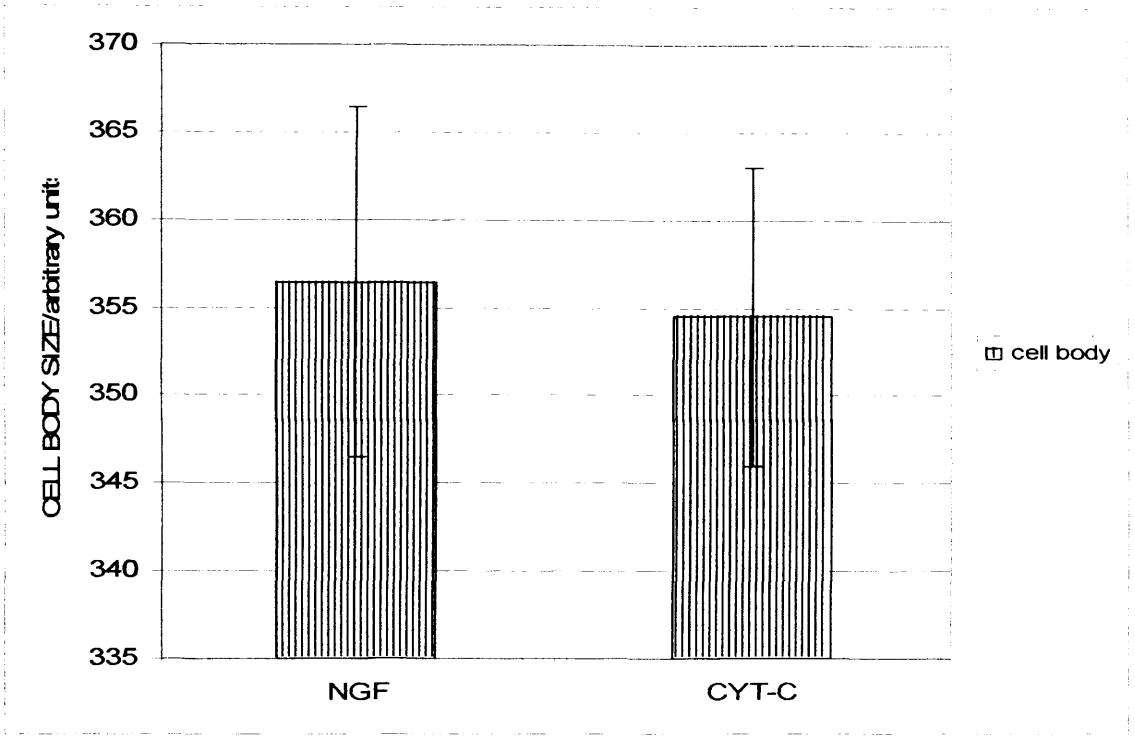

Figure 3.14: Cell body size of 24 months old SCG neurons pre-treated in vivo with NGF, CYT-C or a-NGF (ANGF). Neurons have been cultured in serum-free medium for 24 hours.

A-NGF pre-treatment in vivo caused a reduction of the area covered by neurites ($p < 0.05$) in 24 months old SCG neurons when compared with CYT-C (see figure 3.15).

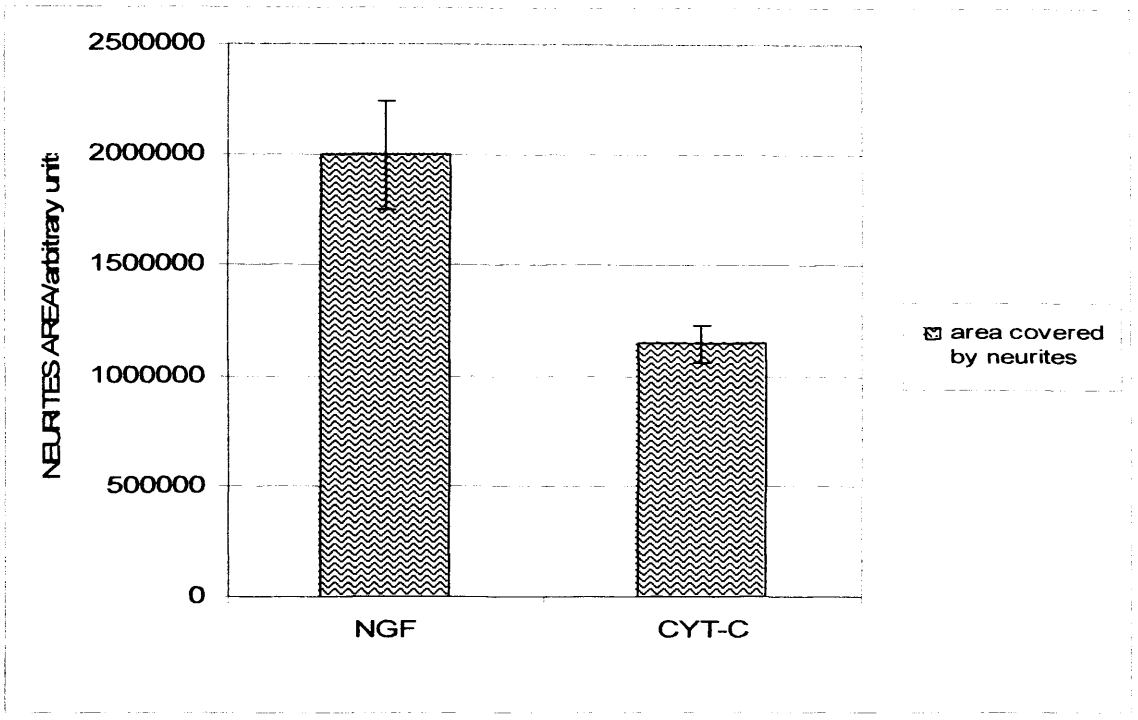

Figure 3.15: Area covered by neurites of 24 months old SCG neurons pre-treated in vivo with NGF, CYT-C or a-NGF (ANGF). Neurons have been cultured in serum-free medium for 24 hours.

The total neurite length results are very similar to those of the area covered by neurites (see figure 3.16); with A-NGF pre-treatment in vivo significantly affecting the length of the neurites compared to the control ($p < 0.05$).

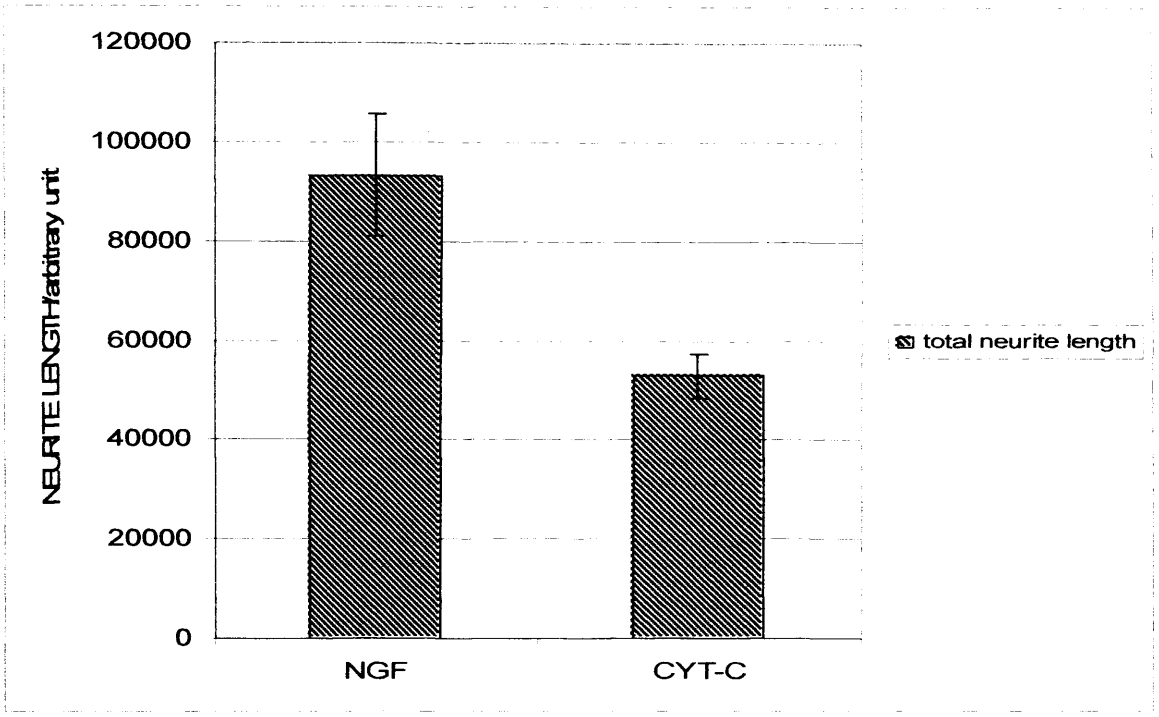

Figure 3.16: Total neurite length of 24 months old SCG neurons pre-treated in vivo with NGF, CYT-C or a-NGF (ANGF). Neurons have been cultured in serum-free medium for 24 hours.

Results obtained calculating the number of intercepts (see figure 3.17) shows that there is a non-significant increase in the number of intercepts when neurons have been treated in vivo with NGF compared to CYT-C , and a significantly ($p < 0.05$) decreased number of intercepts when neurons were pre-treated with a-NGF compared to CYT-C.

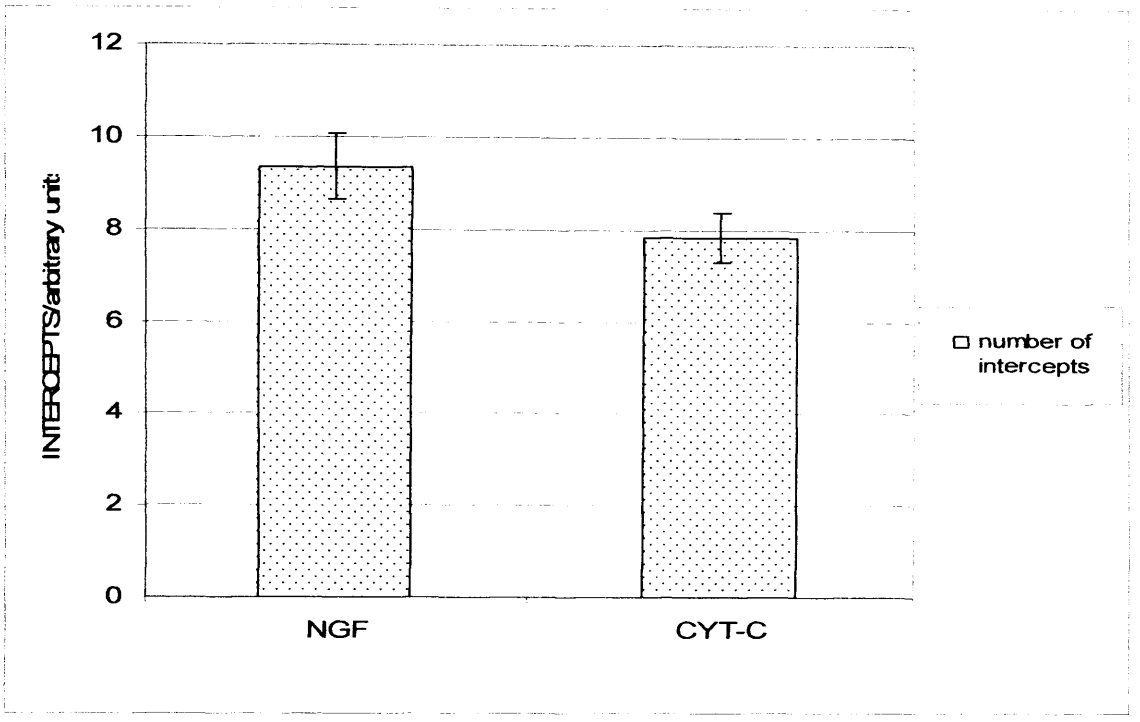

Figure 3.17: Number of intercepts of 24 months old SCG neurons pre-treated in vivo with NGF, CYT-C or a-NGF (ANGF). Neurons have been cultured in serum-free medium for 24 hours.

Results from in vitro cultured and immunostained senescent SCG neurons show that the most sensitive analysis for this group might be the number of intercepts. Results from 24 months group show no significant difference due to NGF pre-treatment.

3.2.6 Effect of 'late' (age 3 months) pre-treatment with NGF or CYT-C treatment on neurons in vitro

Neurons that have been pre-treated in vivo after termination of development (i.e. at 3 months of age) seems to have unaltered cell body size (see figure 3.18). The late treatment group, in contrast to the 24 months group, are neurons that have been examined immediately after termination of the in vivo pre-treatment, while 24 months group have been treated between 3 and 7 weeks of age and examined 22 months after termination of pre-treatment in vivo.

In figure 3.18 are the results of the cell body size of neurons pre-treated in vivo with NGF compared to neurons pre-treated in vivo with CYT-C (control). There is no significant difference between the two groups of pre-treatment in vivo.

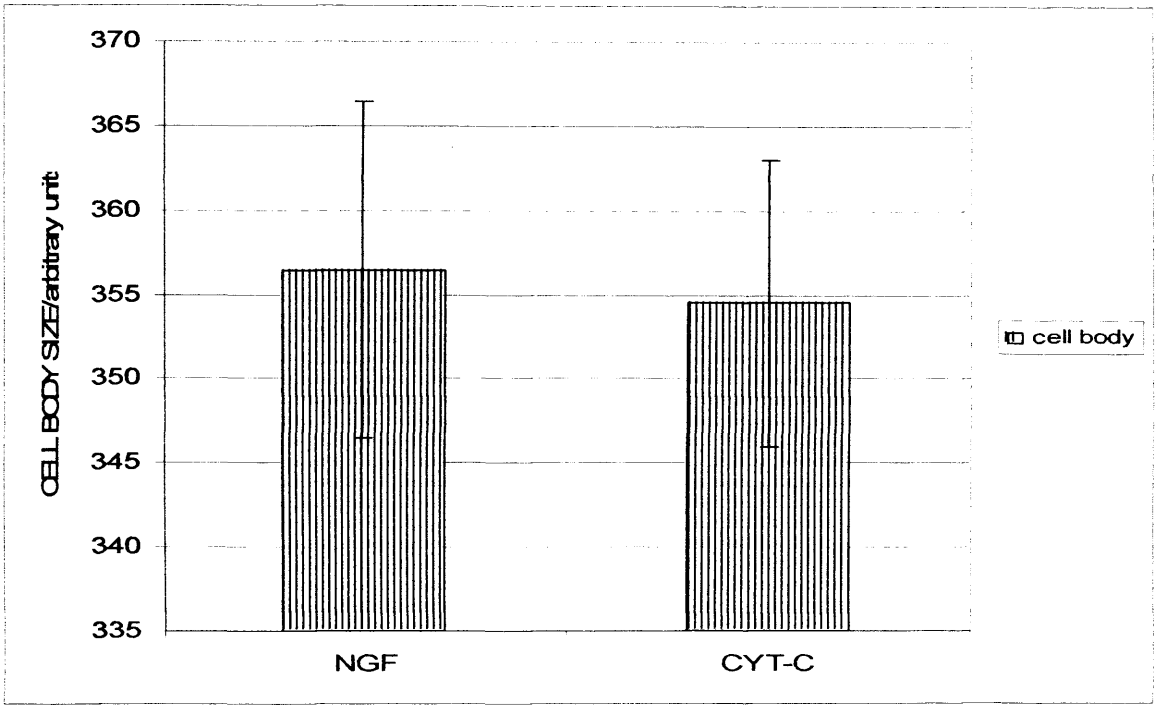

Figure 3.18: Cell body size of late treatment SCG neurons pre-treated in vivo with NGF, or CYT-C. Neurons have been cultured in serum-free medium for 24 hours.

Results from analysis of the area covered by neurites (see figure 3.19) shows a significant difference in neurons pre-treated in vivo with NGF compared to control ($p < 0.05$). NGF pre-treatment in vivo increases significantly the area covered by neurites.

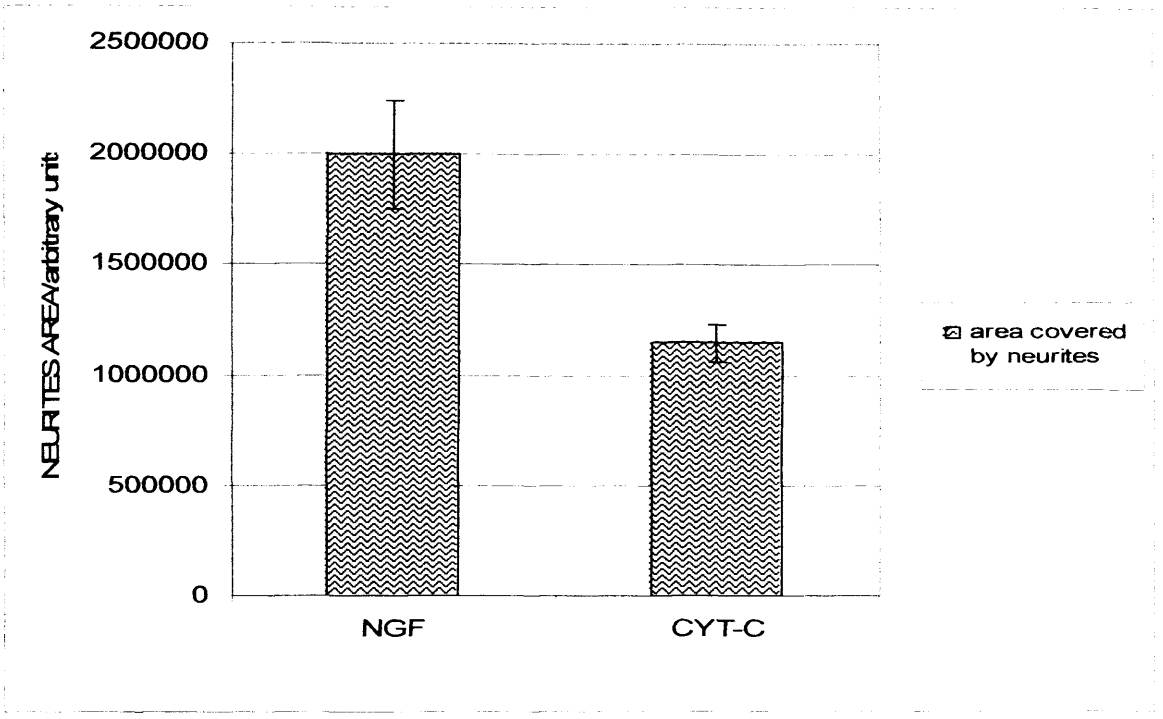

Figure 3.19: Area covered by neurites in late treatment SCG neurons pre-treated in vivo with NGF, or CYT-C. Neurons have been cultured in serum-free medium for 24 hours.

Similarly the total neurite length (see figure 3.20) is significantly different following late pre-treatment in vivo ($p < 0.05$). NGF pre-treatment in vivo significantly increases the length the neurites compared to control.

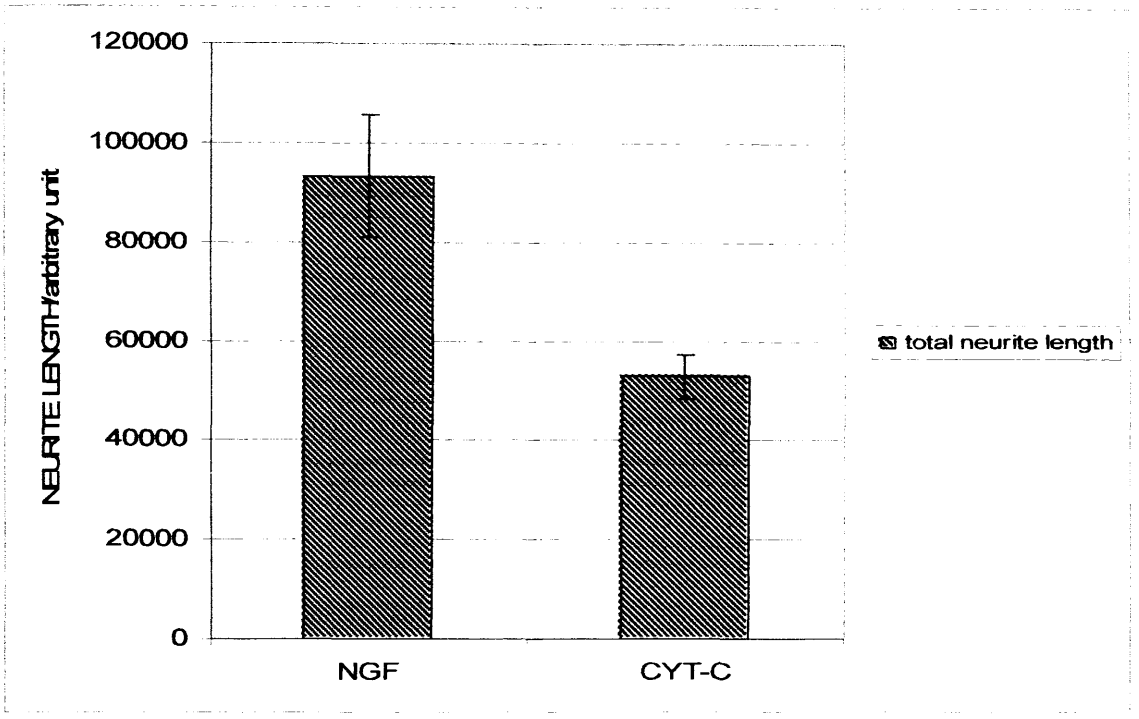

Figure 3.20: Total neurite length in late treatment SCG neurons pre-treated in vivo with NGF, or CYT-C. Neurons have been cultured in serum-free medium for 24 hours.

Number of intercepts has been significantly increased ($p < 0.05$) following pre-treatment in vivo with NGF compared to control (see figure 3.21).

Therefore neurite outgrowth in the late treatment group has been significantly affected by pre-treatment in vivo with NGF compared to control, but not the size of the cell body.

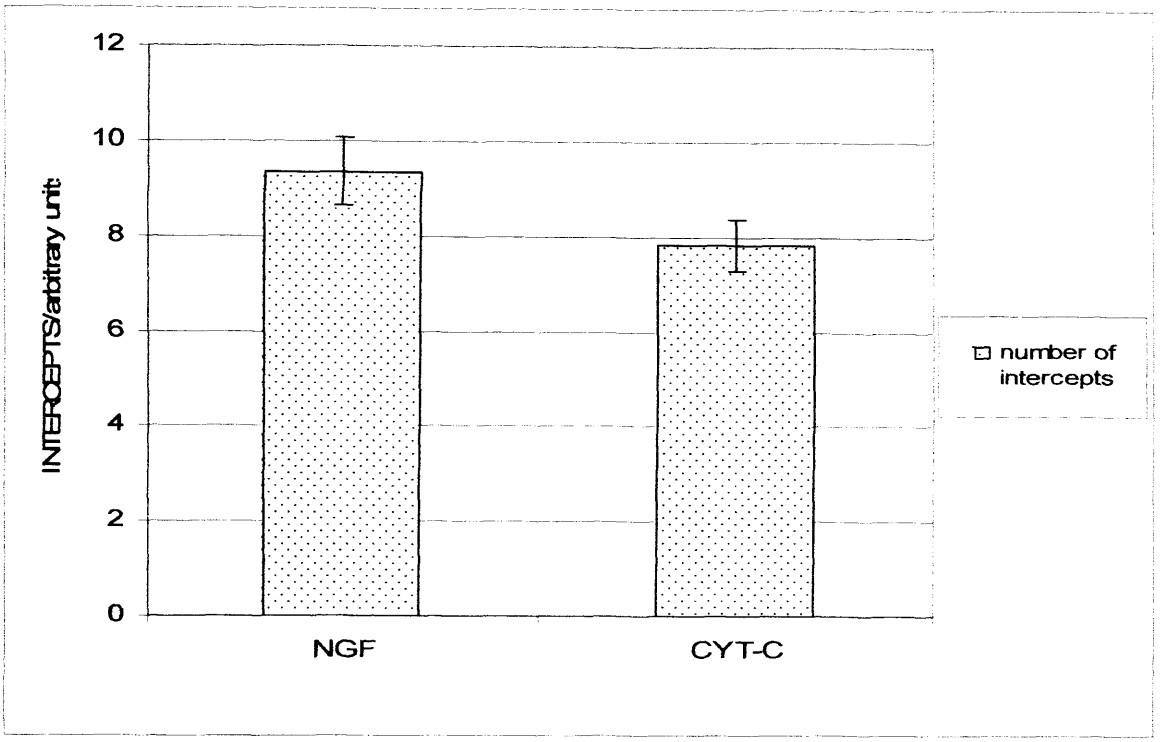

Figure 3.21: Number of intercepts in late treatment SCG neurons pre-treated in vivo with NGF, or CYT-C. Neurons have been cultured in serum-free medium for 24 hours.

Table 3.2 shows the summary of all the measurements from figure 3. to figure 3.21 with a summing up of overall growth.

NEURITES GROWTH	8M	24M	LATE TREATMENT
CELL BODY SIZE	--	-- (or ↓ a-NGF)	--
NEURITES NUMBER	↑	-- (or ↓ a-NGF)	↑
NEURITES LENGTH	↑	-- (or ↓ a-NGF)	↑
NEURITES INTERCEPTS	--	-- (or ↓ a-NGF)	↑
OVERALL GROWTH	↑	-- (or ↓ a-NGF)	↑

Table 3.2: This table shows a summary of the neurites growth in vitro. Arrow pointing up shows increase, pointing down shows decrease and horizontal line shows no increase or decrease.

3.2.7 Effect of 'early' (age 3-7wks) pre-treatment with NGF, A-NGF or CYT-C treatment on innervation of target tissues by MCA and iris projecting neurons: Acute, 8 months, 18 months and late treatment groups; on ex vivo samples

Target tissues are innervated by specific subpopulations of neurons, for simplicity MCA refers to innervation of the MCA target tissue and iris refers to innervation of the iris target tissue. Figure 3.22 shows a significant increase in innervations of acute group MCA following pre-treated in vivo with NGF ($p < 0.05$).

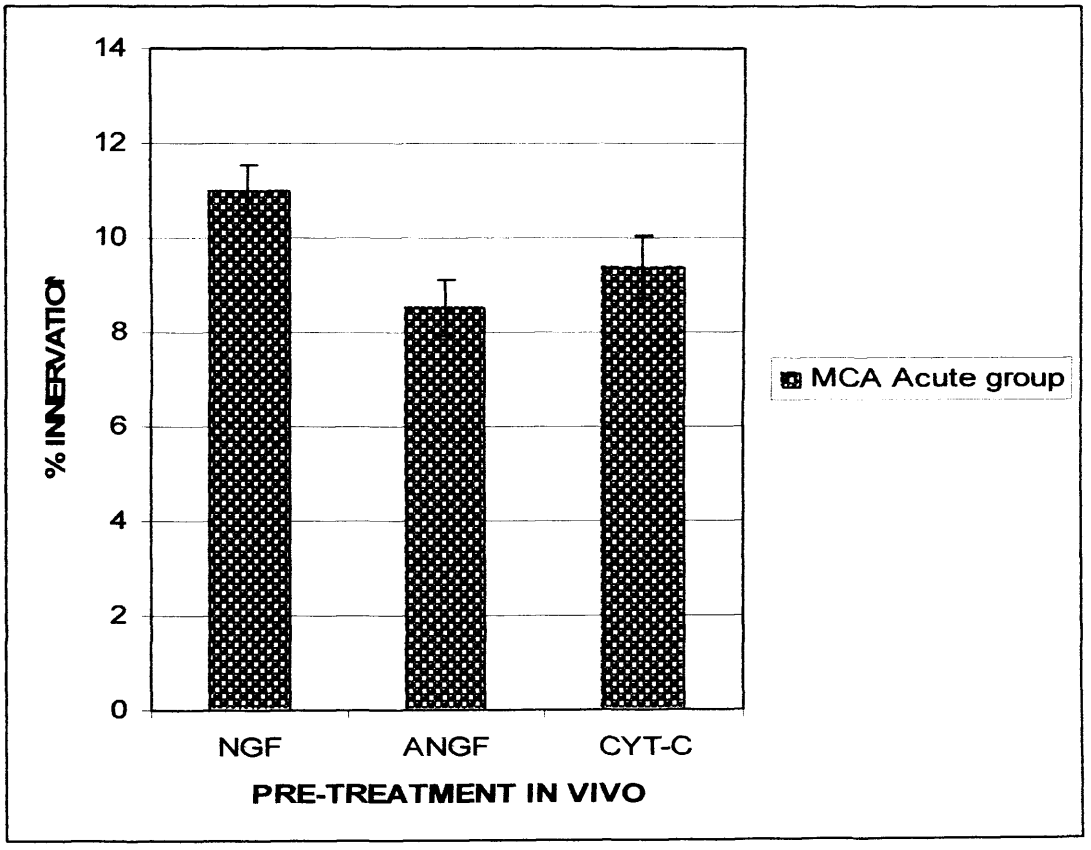

Figure 3.22: Acute group percentage of innervation of the MCA target tissue, following in vivo pre-treatment with either NGF, a-NGF or CYT-C.

Figure 3.23 shows no significant increase in innervations of acute group iris tissue following pre-treated in vivo with NGF.

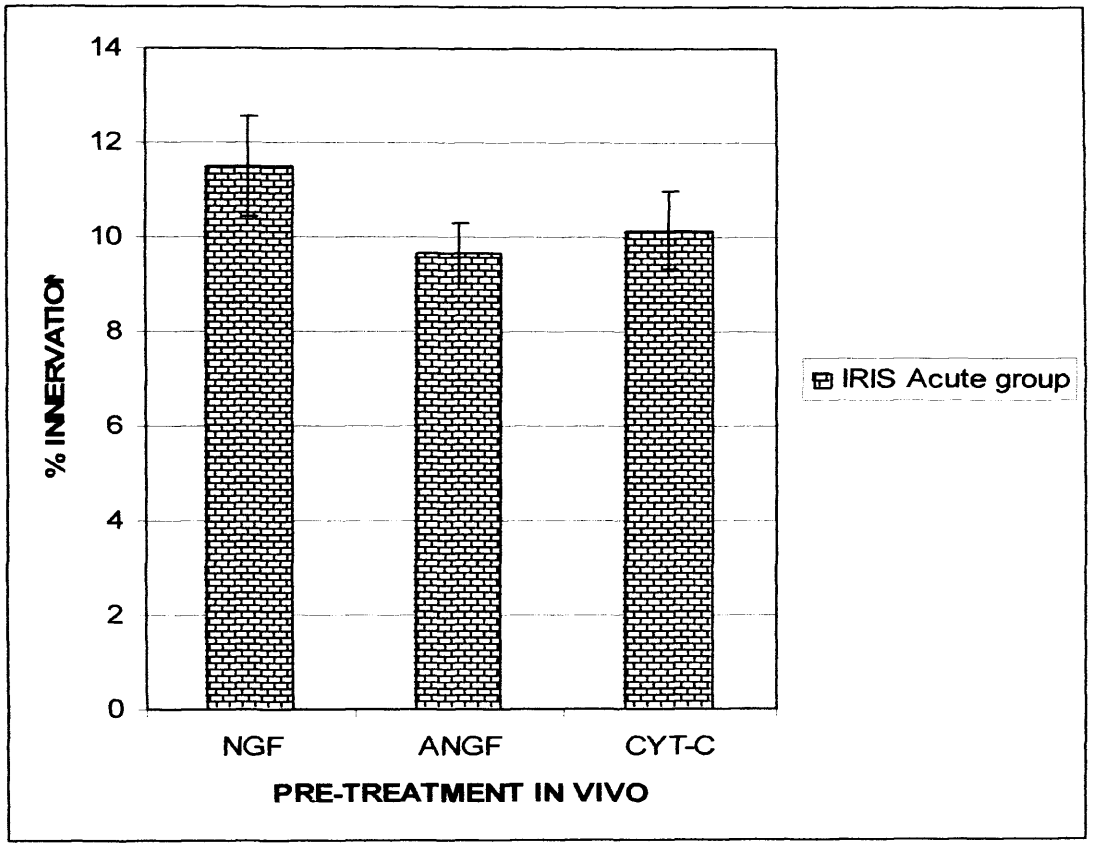

Figure 3.23: Acute group percentage of innervation of the iris target tissue, following in vivo pre-treatment with either NGF, a-NGF or CYT-C.

Figure 3.24 shows the effect of NGF pre-treatment in vivo on the percentage of innervation of the 8 months MCA tissue and figure 3.25 shows the effect on iris, both tissues are not significantly affected by NGF pre-treatment in vivo.

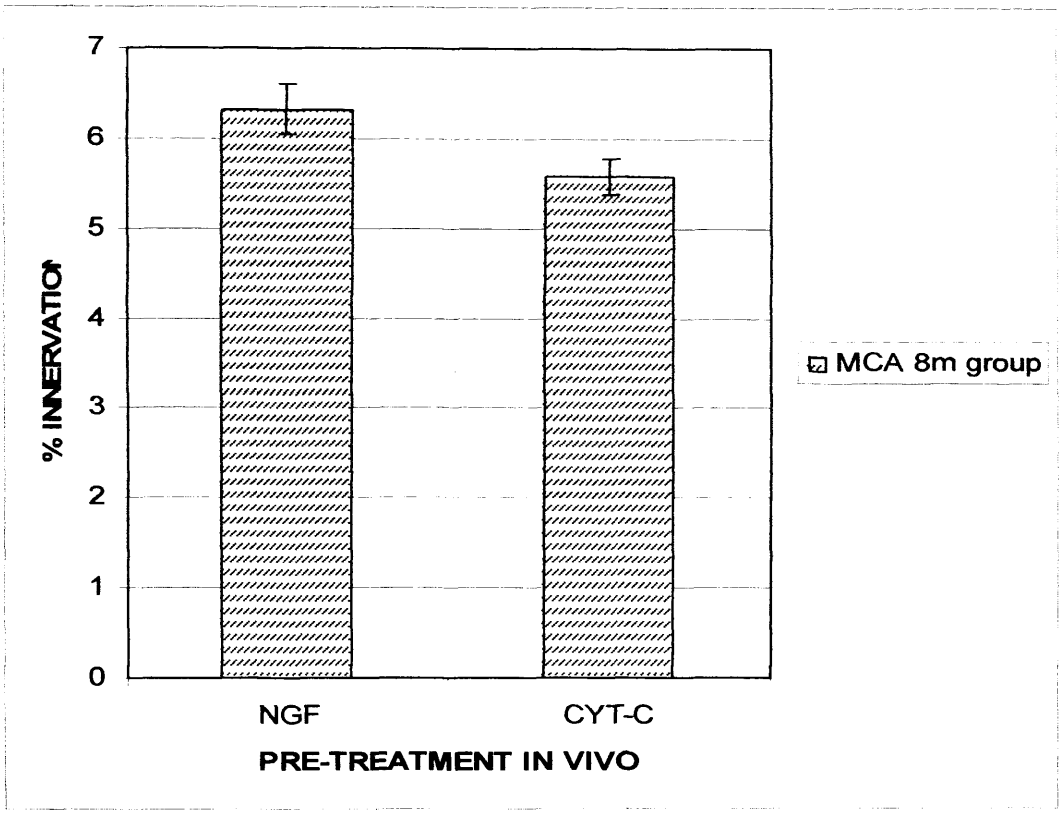

Figure 3.24: 8 months group percentage of innervation of the MCA target tissue, following in vivo pre-treatment with either NGF, or CYT-C.

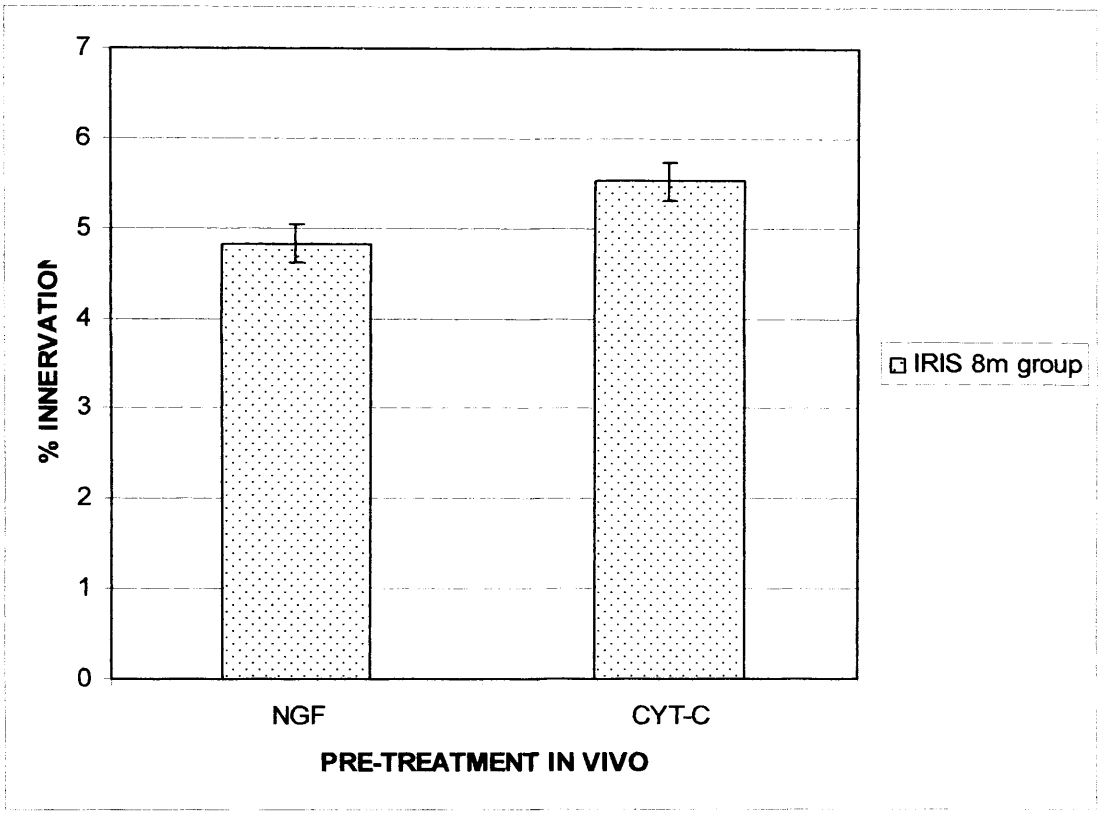

Figure 3.25: 8 months group percentage of innervation of the iris target tissue, following in vivo pre-treatment with either NGF or CYT-C.

Figures 3.26 and 3.27 show the effect on percentage of innervation of NGF pre-treatment in vivo tissue, respectively MCA and iris tissues, both tissues are not significantly affected by treatment when compared to control.

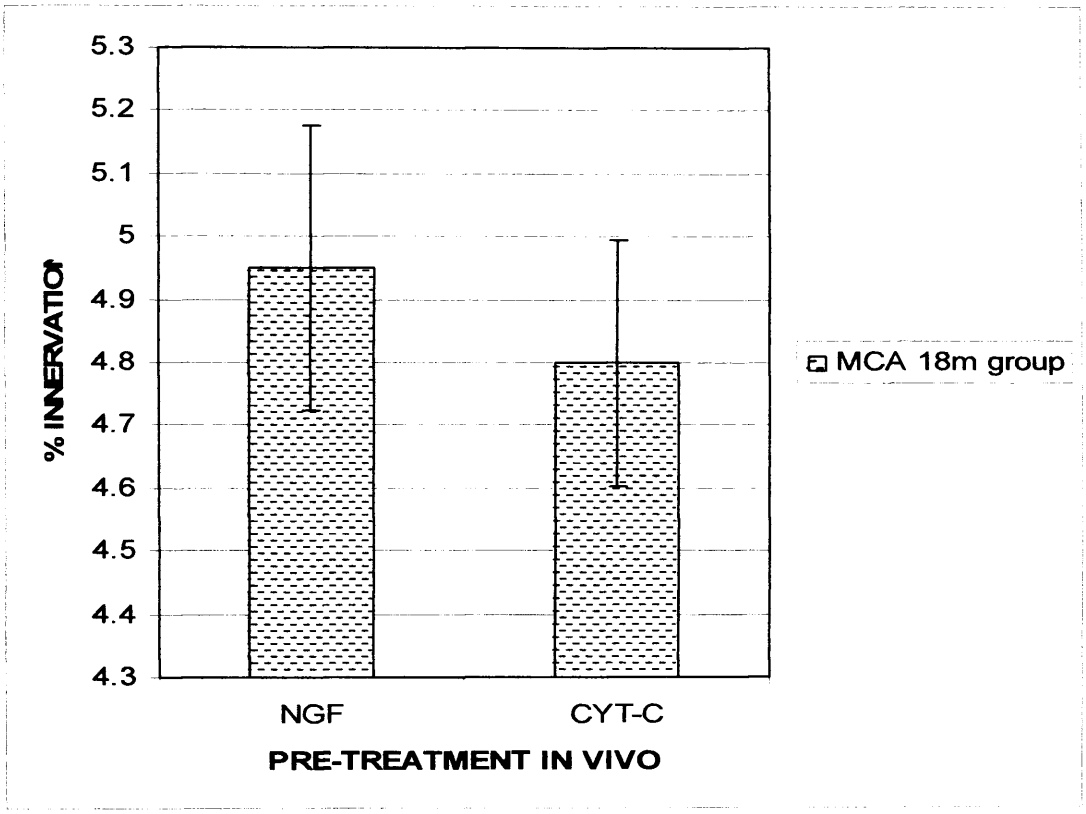

Figure 3.26: 18 months group percentage of innervation of the MCA target tissue, following in vivo pre-treatment with either NGF or CYT-C.

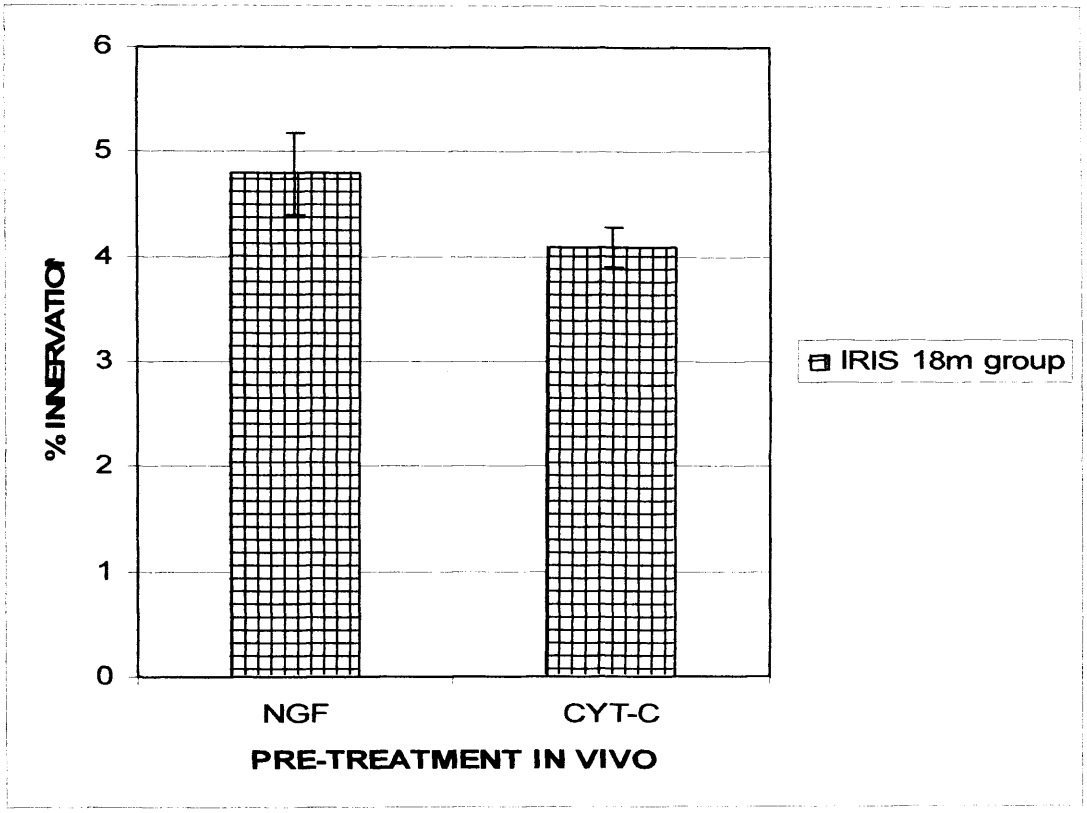

Figure 3.27: 18 months group percentage of innervation of the iris target tissue, following in vivo pre-treatment with either NGF or CYT-C.

Figures 3.28 and 3.29 show the late treatment percentage of innervation of respectively MCA and iris target tissues, following in vivo NGF pre-treatment. MCA tissue shows a significant decrease of innervation following NGF pre-treatment in vivo ($p < 0.05$). No significant effect is present on the iris target tissue.

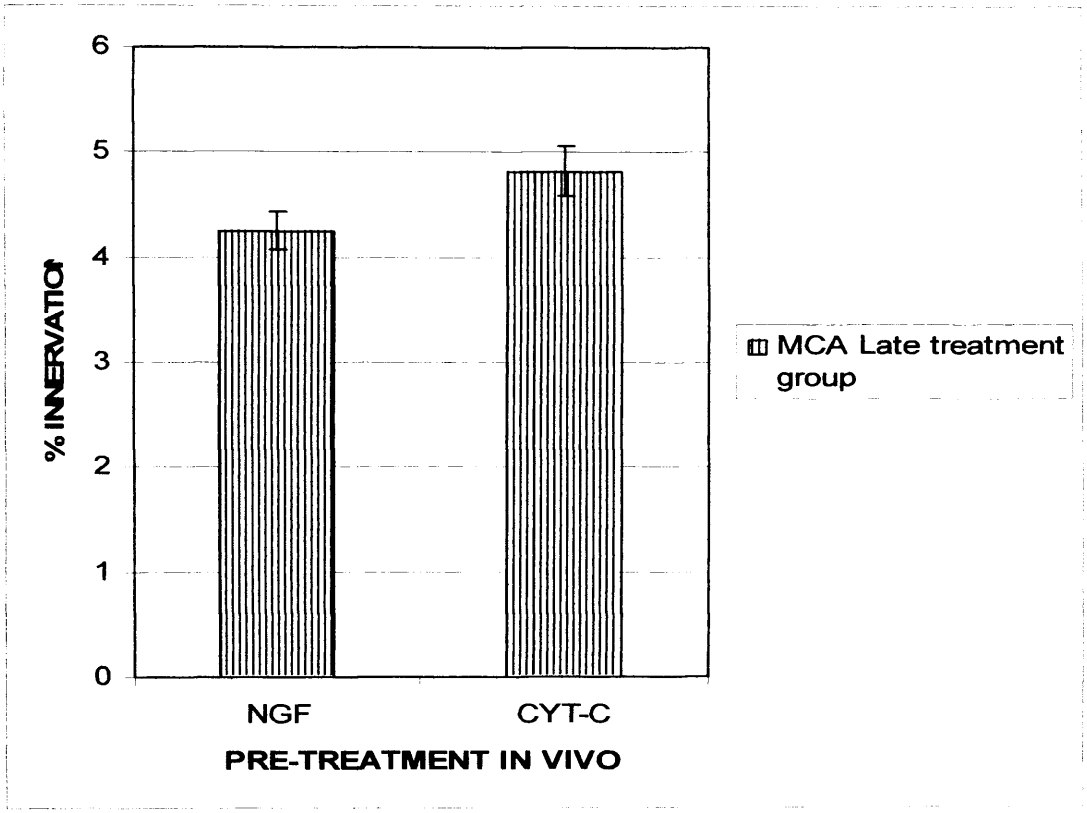

Figure 3.28: Late treatment group percentage of innervation of the MCA target tissue, following in vivo pre-treatment with either NGF or CYT-C.

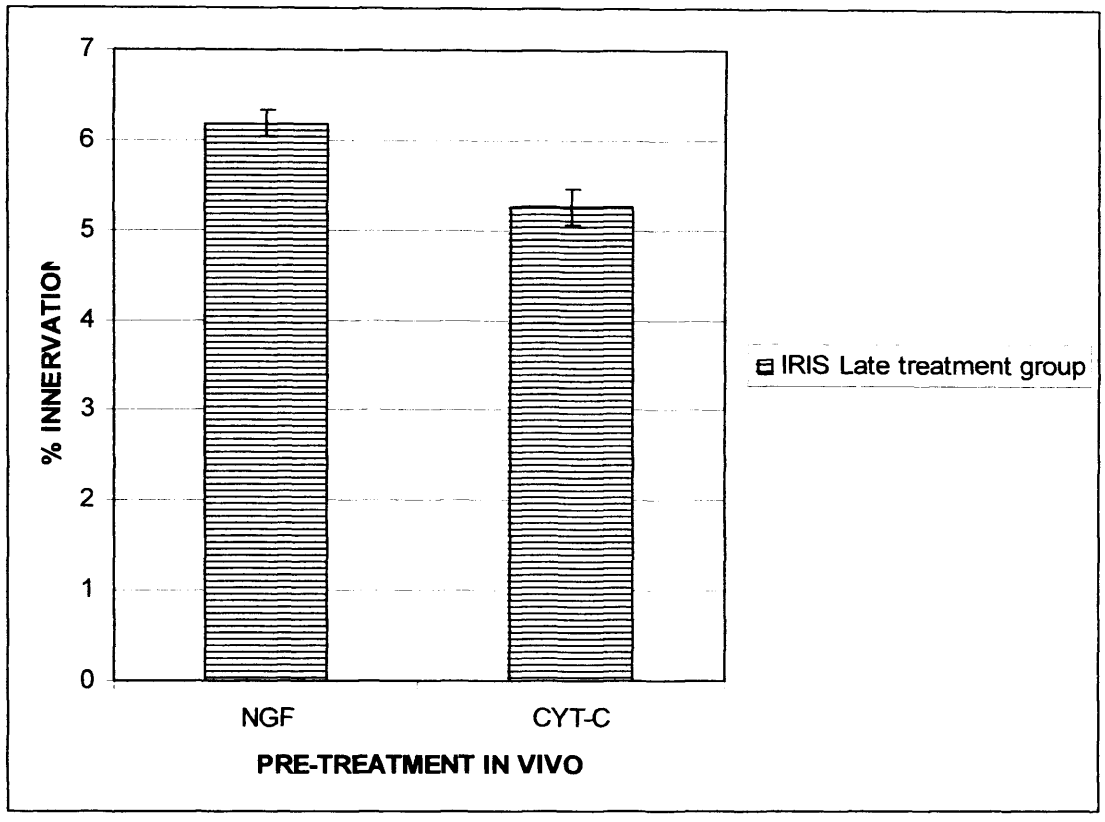

Figure 3.29: Late treatment group percentage of innervation of the iris target tissue, following in vivo pre-treatment with either NGF or CYT-C.

3.3 Discussion

NGF is mandatory for the survival of sympathetic neurons during maturation (Davies, 1996; Levi-Montalcini and Angelletti, 1968; Lewin, 1996); as they mature they lose their absolute dependence on NGF for survival, and NGF becomes involved in selection, specification, and maintenance of differentiated neuronal phenotypes (Lewin, 1996; Lewin *et al.*, 1992; Lindsay, 1996). NGF is also known to cause inflammation (Le Bars and Adam, 2002; Safieh-Garabedian *et* al., 2002; Boucher and McMahon, 2001; Woolf and Salter, 2000; Mannion *et al.*, 1999; McMahon, 1996; Lewin, 1995; Andreev *et al.*, 1995), and pre-treatment in vivo might be causing neuronal toxicity and therefore neuronal death during treatment; resulting in a study carried out on a subset of

neurons (i.e. neurons survived to treatment), rather than the entire population. The results of a simple test carried out with a Promega Elisa kit showed no neuronal death due to NGF pre-treatment in vivo either during the treatment (after 1 week) or immediately after the termination of the treatment regime (after 4 weeks). Even though there is some difference in the amount of cell death between 1 and 4 weeks of pre-treatment in vivo, such difference may be due to normal early development cell death; in fact according to the neurotrophic hypothesis (Purves, 1988) structure and connections of developing and mature neurons are governed by their ongoing interactions with target tissues and therefore target fields requirement and size determines the number of neurons surviving (Davies, 1996). Studies on timing of neuronal death have shown that sympathetic neurons become dependent on the supply of two neurotrophins, NGF and NT3, shortly after they begin to innervate their targets (Crowley *et al.*, 1994; Wyatt *et al.*, 1997; Francis *et al.*, 1999, Orike *et al.*, 2000).

Once the early age developmental mechanisms have been settled according to the neurotrophic hypothesis system, where NGF played a central role in determining the phenotypes of different subpopulations of neurons, the interesting issue is to understand the characteristics of neuronal degeneration in old age. Neuronal degeneration can be assessed by studying the fluctuation in neuronal number with increasing age and the variation between subpopulations of neurons (MCA- and iris-projecting neurons) characterised by differential vulnerability due to the phenotype determined in early age by NGF availability.

Donaldson (1895) already contemplated the importance of neuron number and brain function, and was followed by numerous other scientists, Bok and Van Erp Taalman Kip (1939), Frankhauser *et al.* (1955), Vernon and Butsch (1957), Jerison (1963),

Williams and Herrup (1988). Williams and Rakic (1988) suggested the 'three-dimensional counting' method to estimate number of cells in sectioned material; and in this study we utilised a revised format of the 'three-dimensional counting' method (Williams and Rakic, 1988) to carry out the analysis. Results showed that while a vulnerable subpopulation of neurons (MCA-projecting neurons) decrease in number significantly with age, iris-projecting neurons (resistant neurons) shows only a slight decrease in number with age. These results confirm the hypothesis that neurons receiving a limited amount of NGF in early life are characterised by a phenotype conferring vulnerability in later life.

Rita Levi-Montalcini and Victor Hamburger (1953) described in a seminal paper that the survival of developing neurons is directly related to the availability of their innervating targets, and therefore by pre-treating in vivo neurons early in life we created and environment of overabundance of NGF for all neurons. What is still unknown is if with abundant NGF availability, in early life development, the number of neurons is going to be different.

Research results on the differential numbers of neurons, when cells have been pre-treated in vivo with NGF compared to when they have been pre-treated in vivo with CYT-C (control), showed an overall increased cell number followed treatment. MCA-projecting neurons are more plastic and the availability of NGF caused an increased cell number, which is significant at 8 months ($p < 0.05$); the MCA plasticity is also shown by the results with the A-NGF pre-treatment in vivo where the number of neurons survived are significantly lower compared to control ($p < 0.05$). According to Crowley *et al.* (1994) and Zhou and Rush (1995), lack of NGF caused neuronal death, and similar results are obtained when A-NGF is applied to neurons (De Vries *et* al., 2002;

Shadiack *et al.*, 2001; Tafreshi *et* al., 1998; Luo *et* al., 1997; Qian and Naftel, 1996; Zigmond *et al.*, 1995; Gorin and Johnson, 1979; Bjerre *et al.*, 1975). Furthermore MCA-projecting neurons tend to decrease in number with age and this is in accordance with results from Gatzinsky *et al.* (2004). Iris-projecting neurons don't decrease with age (in accordance with Gatzinsky *et al.*, 2004 results) and increase significantly compared to control at 8 months following NGF pre-treatment in vivo. The above results show that vulnerable neurons (MCA-projecting) can be protected by in vivo NGF pre-treatment and that resistant neurons (iris) dying in old age can be protected as well by in vivo NGF pre-treatment.

The next logical question would be to examine if the NGF pre-treatment in vivo is affecting growth. Crutcher (1989) introduced some important information in understanding the potential of axonal growth within the mature mammalian brain and spinal cord by looking at axonal regeneration stating that differential axonal growth may be attributed to absence of growth-promoting substances, or the presence of growth-inhibiting substances.

During ageing basal forebrain cholinergic neurons are prone to degeneration for unknown reasons. De Lacalle *et al.* (1996) studied the reduced retrograde labelling with fluorescent tracer and found neuronal atrophy of basal forebrain cholinergic neurons in aged rats.

Results from the 8 months group shows that while there is no difference in the cell body size of neurons, neurites number and length are significantly increased by NGF pre-treatment in vivo ($p < 0.05$). The 24 months group shows no increase in either cell body size or neurites following NGF pre-treatment in vivo but a significant decrease following A-NGF pre-treatment in vivo ($p < 0.05$). The above results clearly show that NGF availability increases neuronal growth at 8 months but not at 24, while lack of

NGF (A-NGF in vivo pre-treatment) causes the neurons to retract neurites and eventually die in old age; death of neurons following neurite retraction was also found by studies carried out by Mearow and Kril (1995).

The late treatment group (neurons pre-treated in vivo after termination of development) results are very similar to the 8 months results; where the cell body size is maintained while the neurites are increased following NGF pre-treatment in vivo compared to control. The above results show that NGF pre-treatment has also an effect on growth after the termination of development. According to Gorin and Johnson (1980), and Orike *et al.* (2001), neurons are dependent on NGF for survival in early development but only for growth after termination of development.

Is NGF pre-treatment in vivo affecting innervation of target tissues? This research results shows that, in accordance with the subpopulations' neurons number results, the MCA target tissue tend to be mainly affected. MCA subpopulations of neurons are more plastic compare to iris and therefore respond more readily to and increase or decrease of NGF availability. If MCA neurons were not plastic the a-NGF treatment on acute iris neurons would have caused a decrease in neuron number, as for resulted for the MCA-projecting neurons. Furthermore increase in iris acute neuron number, following NGF pre-treatment, does not occur maybe because the level of iris neurons surviving at this age is already at its upper limit.

These results are in accordance with review of Bleys and Cowen (2001) where they extensively describe the characteristics of innervation and plasticity MCA tissue with particular reference to target associated factors, for example NGF.

CHAPTER IV

IN VITRO NEURONAL SURVIVAL OR MORTALITY AND SELECTIVE VULNERABILITY FOLLOWING PRE-TREATMENT IN VIVO WITH NGF OR NGF ANTIBODIES

4.1 Introduction

Neurons are initially dependent on NGF for survival but, during postnatal development, sympathetic neurons lose their dependence on NGF for survival but continue to require it for growth and for development of mature neurotransmitter phenotype (Gorin and Johnson, 1980, Orike *et al.*, 2001), similarly happens in sensory neurons (Diamond *et al.*, 1992).

The purpose is to investigate the possibility that the level of exposure of developing neurons to NGF early in life determines the capacity to survive the stresses and strain of ageing. Such investigation is carried out in two ways: one ex-vivo (as explained in chapter III) and one in vitro with primary culture of SCG neurons pre-treated in vivo with NGF, NGF antibodies or CYT-C (control).

Furthermore this study investigates selective vulnerability of subpopulations of SCG neurons. According to Purves (1980), neurons compete for a limited supply of neurotrophic factors, and therefore targets and neurotrophic availability regulate the population of innervating neurons (Korsching, 1993). Suggesting that the differential behaviour and the selective vulnerability of the MCA and the iris projecting neurons could be associated not only with the type of innervation but also with the neurotrophic availability from target tissues.

PI3-K mediates neurotrophin-induced survival as well as regulating vesicular trafficking of various kinds (Brunet *et al.*, 2001; Cantley, 2002). According to Furman *et al.*

(1998) there are multiple enzymes phosphorylating the D3 position of inositol lipids; neurotrophins activates the heterodimeric class Ia enzyme consisting of a regulatory subunit (p85) and a catalytic subunit (p110); with 3 catalytic isoforms (α, β, and γ) and 4 regulatory isoforms (p85α and β, p55α and γ). Few differences among isoforms are just beginning to be defined (Bartlett *et al.*, 1999; Shin *et al.*, 1998; Tolias and Cantley, 1999). Andjelkovic *et al.* (1998) and Bartlett *et al.* (1997) have defined the isoforms important in neurotrophin signalling. Trk activation by NGF pre-treatment leads to stimulation of PI3-K and binding of the regulatory subunit to the linker proteins Grb-2 and Gab1/2; according to Downward (1998) the catalytic subunit is also stimulated by binding directly to the activated Ras proteins. MacInnis and Campenot (2002) suggest that following NGF stimulation there is an abnormal increase in PI3-K activity. Class I PI3-K generates lipids products, such as phosphatidylinositol3,4-P2 (PtdIns3,4-P2), phosphatidylinositol3,4,5-P3 (PtdIns3,4,5-P3), and phosphatidylinositol-3-P (PtdIns3-P), that binds to PH domains on target proteins. Akt/PKB binds to PtdIns3,4,5-P3, becomes phosphorylated and mediates neuronal survival. Alessi *et al.* (1997) found that PtdIns3,4,5-P3 also binds to the PH-domain containing kinase PDK1, which is an enzymes that phosphorylates and associates with Akt. Brunet *et al.* (2001) and Dudek *et al.* (1997) studied the complex array of survival-promoting pathways activated by Akt kinase. The three main survival pathways activated by Akt are explained as follows:

1) the proapoptotic protein Bad is inactivated by Akt-dependent phosphorylation, allowing bcl-2-dependent survival (Datta *et al.*, 1997; and del Peso *et al.*, 1997);
2) Akt phosphorylates transcription factors of the Forkhead family and reduces expression of several antiapoptotic genes (Biggs *et al.*, 1999; Brunet *et al.*, 1999; and Kops *et al.*, 1999);

3) Akt phosphorylates and inactivates glycogen synthase kinase3 (GSK3).

Growth factor such as: Brain-derived neurotrophic factor (BDNF), insulin, glial derived neurotrophic factor (GDNF), and insulin-like growth factor-1 (IGF-1) seems to have a remarkable effect on neuronal survival. BDNF belongs to the same group of factor as NGF, and they are identified by their capacity to suppress neuronal apoptosis in many populations of neurons. BDNF provided survival through PI3-K in spinal cord motor neurons (Dolcet *et al.*, 1999), in retinal ganglial cells (Meyer-Franke *et al.*, 1998), cerebellar granule cells (Skaper *et al.*, 1998), cortical neurons (Takei *et al.*, 1999), as well as many other groups of neurons. Both Skaper *et al.* (1998) and Takei *et al.* (1999) found that the protection by BDNF/PI3-K was also partially dependent on ERK1/2, suggesting the possibility that both pathways are involved in neuronal survival.

Members of the GDNF family do play essential early roles in development of SCG. The requirement for GDNF family members precedes the dependence of SCG neurons on NT-3, NGF, and TrkA (Huang and Reichardt, 2001). According to Durbec *et al.*, (1996) mice lacking c-ret (i.e. the tyrosine kinase activated by GDNF) lack all neurons in the SCG without any obvious phenotype in the sympathetic chain at the trunk level, with a loss of neural crest-derived precursors. Nishino *et al.* (1999) reported that mutation of the GFRα3 binding subunit has a similar effect. GFRα3 mediates activation of c-ret by artemin, a protein closely related to GDNF.

In mammals, insulin and IGFs are key hormones for the regulation of metabolism, growth, and differentiation. In *Caenorhabditis elegans* (*C. elegans*) the gene that encodes the protein named DAF-2 is orthologous to the mammalian insulin receptor, and is involved in longevity as well as entry into the growth-arrested dauer larva, a kind of diapause, of the nematode (Hsin and Kenyon, 1999). Kirkwood (2001) suggested the

hypothesis that survival mechanisms in postmitotic neurons and organisms are linked. This suggests that growth arrest, ageing and metabolism should be closely related to one another in the animal kingdom, and therefore *C. elegans* has been widely used as model for studying and insulin/IGF-signaling pathway in several aspects (Kawano *et al.*, 2000).

Insulin has a dual effect, while attenuating neuronal apoptosis in mouse cortical culture it induces neuronal necrosis with 48 hours of exposure (Noh *et al.* 1999). Exposure to insulin led to tyrosine phosphorylation of the insulin receptor and activation of protein kinase C (PKC) and PI3-K; inhibitors of tyrosine kinase and PKC, but not PI3-K, attenuated the insulin neurotoxicity. Conversely, inhibition of PI3-K, but not PKC, caused an apoptotic effect of insulin. Cao *et al.* (2000) suggests that gene activity-dependent emergence of excitotoxicity contributed to insulin neurotoxicity, macromolecule synthesis inhibitors and N-methyl-D-aspartate (NMDA) antagonists blocked it. NMDAs are critically involved in synaptic plasticity in the visual cortex of the rat. Exposure to insulin increased the level of the NR2A subunit of the NMDA receptor without altering NR1 or NR2B levels. NR1, NR2A, and NR2B changes in expression may underlie developmental changes. Therefore insulin can be both neuroprotective and neurotoxic in the same cell system but by way of different signaling cascades.

4.1.1 Aims

This chapter aims at studying the effect of pre-treatment in vivo with NGF, anti-NGF or CYT-C (control) across ages (acute, 8 months, 18 months, 24 months) by looking at neuronal behaviour in vitro.

A further group, eight months old, 'the subpopulations group' have been examined in order to understand if there is any distinction in survival/mortality pattern of vulnerable (characterised by multi-unit innervation, i.e. MCA) versus resistant (single unit innervation i.e. iris) populations of neurons.

The 'late treatment' is a group of animals that have been treated with the same regime as all the other groups with the exception that it started at three months of age, instead of seven weeks, and animals have been sacrificed 6 months after termination of treatment. Three months has been determined as the age at which development has been terminated and therefore adulthood starts. These experiments aim to test if the NGF pre-treatment has the same effect whether it has been started late (i.e. adulthood) or early (i.e. during the developmental stage).

4.1.2 Hypothesis

Cowen *et al.* (2003) described extensively how sympathetic neurons undergo age-related neurodegeneration with reduced plasticity of neurotrophin receptor expression and innervate tissues with low levels of neurotrophin.

Therefore we hypothesize that cells pre-treated in vivo with NGF will be permanently affected and their survival pattern will be significantly changed.

The appropriate technique to test such hypothesis is cell culture which allows to study the effect as well as the specific time and mode of in vivo pre-treatment. While chapter III reported the effect of pre-treatment on ex vivo tissue, the in vitro results from this chapter gives us an opportunity to investigate the capacity of adult and ageing neurons to survive under in vitro conditions which includes absence of neurotrophic support. Orike *et al.* (2001a) were the first to establish a method for dissociation of SCGs

eliminating the confounding influences of serum and non-neuronal cells. Furthermore Orike *et al.* (2001) discovered that neuron survival is unaffected by treatment with ant-NGF antibodies in vitro and duration of neuronal survival in culture increases significantly with age.

4.2 Results

4.2.1 Effect of 'early' (age 3-7wks) pre-treatment with NGF, anti-NGF or CYT-C treatment on in vitro neuron survival and mortality: ACUTE GROUP

Results show interesting variation between different regimes of in vivo pre-treatment, as well as discrepancy at different ages. These results are analysed by looking at the different temporal phases as separate life stages and consequently the differences between the percentage of neuronal survival and the mortality rate become very relevant (Tatar M., personal communication). The effect of pre-treatment changes between age groups, and therefore it is important to analyse these effects both as single results as well as comparing them across ages. Intra-age-groups statistical analysis is carried out using analysis of variance (ANOVA) followed by the Tukey HSD post hoc test.

In vivo pre-treatment of SCG neurons cultured in the absence of NGF or anti-NGF are shown in figure 4.1

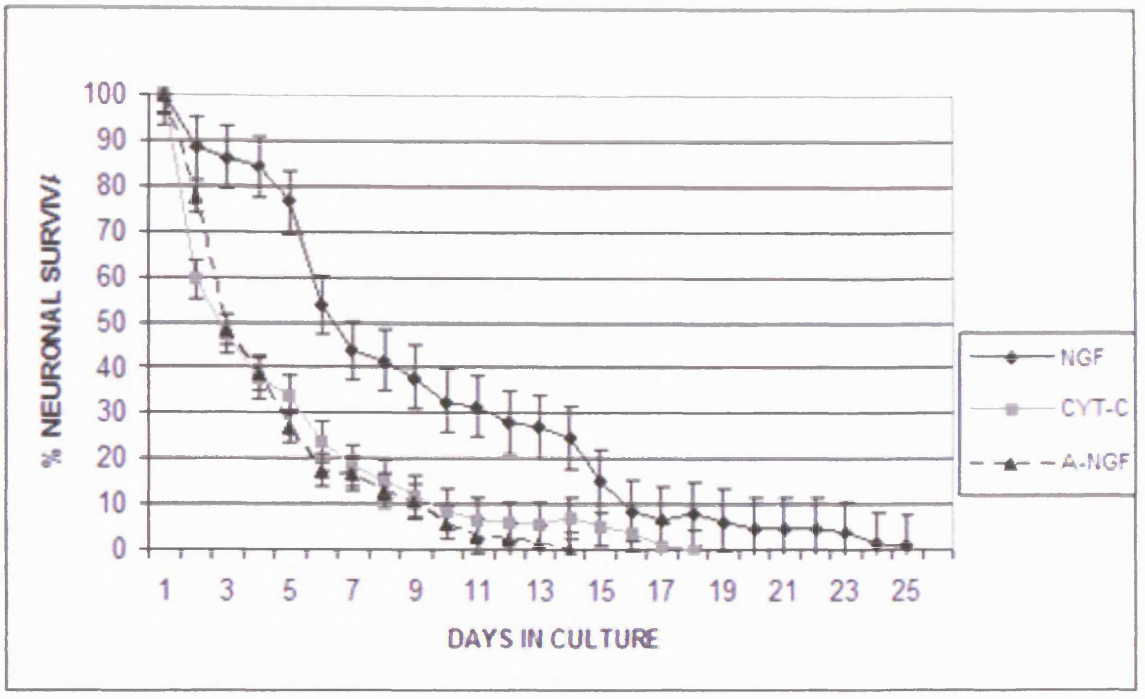

Figure 4.1: In vitro survival of acute group SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF (diamond, black solid line), NGF antibodies (i.e. A-NGF; triangle, black segmented line), or CYT-C as a control (i.e. CYT-C; square, grey solid line).

Generally, in neurons taken from animals soon after the cessation of treatment (acute group), a higher number of neurons from animals pre-treated in vivo with NGF tend to survive when compared to the number of neurons that have been pre-treated with either cytochrome-c or anti-NGF antibodies. The survival curve shown in figure 4.1 may be considered as divided into 3 phases: phase 1, days 1 to 3-5; phase 2, days 3-5 to day 15; phase 3, days 15 to 25 (or more) (see Methods in chapter II). In phase 1, the three groups behave similarly, indicating a common recovery response to the in vitro manipulations. However, already from day 3, the NGF pre-treated group begin to survive significantly better than the other two groups. Throughout phase 2, the NGF group survive significantly better than the control or anti-NGF pre-treated groups. Thus there are significant differences between NGF and CYT-C and between A-NGF and NGF (both $p<0.01$); while the difference between CYT-C and A-NGF is not significant. Survival analysis provides formal terms with which to describe cell survival in culture

(see Appendix – Glossary). A-NGF shows lower l_t (i.e. cumulative proportion of surviving cells) as well as a lower t to t + 1 (i.e. survival over whole period in culture) compared to both CYT-C and NGF. In contrast NGF presents the highest t to t + 1 as well as the highest l_t. Phase 3 seems to start at day 11 in this group. All A-NGF cells are dead by phase 3, while the CYT-C and NGF curves adopt a similar behaviour, with the only difference that NGF cells have a higher t to t+1 compared to CYT-C.

Mortality analysis provides further proof of the priming effect of NGF pre-treatment in the acute group by showing that NGF pre-treated cells exhibit a higher percentage survival (l_t) despite the high mortality rate in phase three compared to the control (figure 4.2).

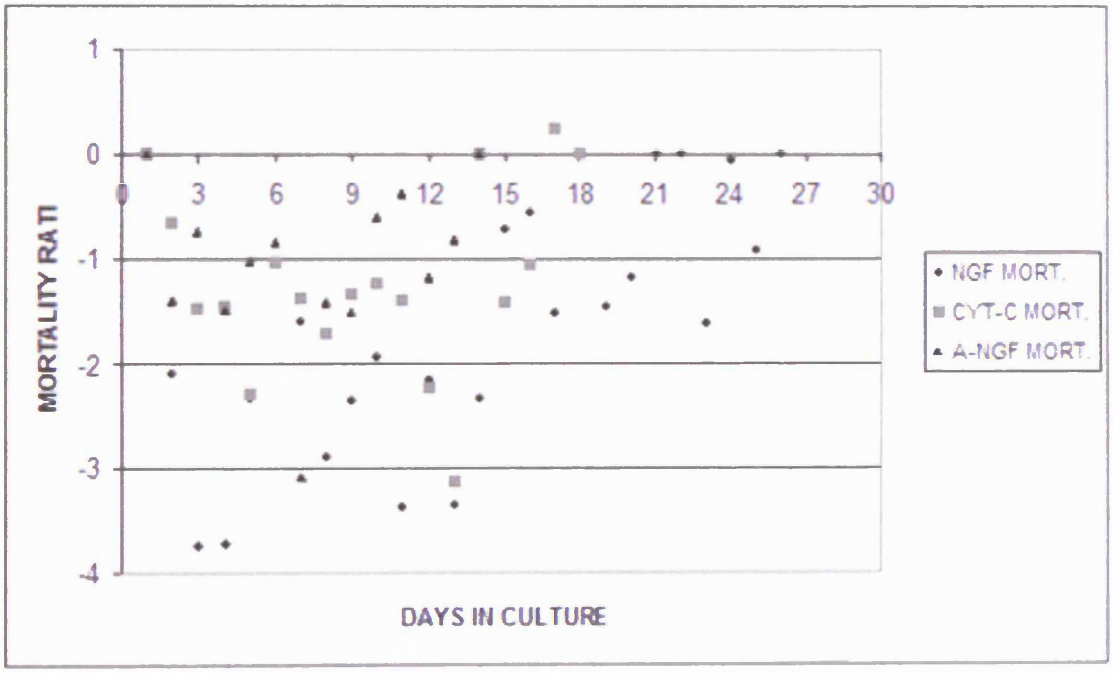

Figure 4.2: log plot of the acute group mortality rate in vitro of SCG neurons treated in vivo at 7 weeks old with NGF (black diamond), NGF antibodies (i.e. A-NGF; black triangle) or cytochrome-C (i.e. CYT-C; grey square). The lower values in the y axis (i.e. -3) indicate the lower mortality while higher values (i.e. -1) represent higher mortality.

NGF pre-treatment therefore delays the onset of cell death in vitro compared to anti-NGF and control pre-treatments, causing not only an increase but also a change in the survival pattern.

4.2.2 Effect of 'early' (age 3-7wks) pre-treatment with NGF, or CYT-C on in vitro neuron survival and mortality: 8 MONTH GROUP

SCG neurons were taken 6 months after pre-treatment at 3-7wk, i.e. at 8 months of age, with NGF or CYT-C (see chapter II). Survival of these neurons was examined as before (see 4.1). No animals were treated with anti-NGF for this group. Eight months of age in the Sprague Dawley rat represents adulthood, including achievement of maximum crown-rump length (T Cowen, personal observations). NGF and CYT-C pre-treated neurons show a similar steep decline in survival over Phase 1 (Days 1-5; Fig. 4.3). In Phase 2, however, NGF cells exhibit significantly ($p < 0.01$) enhanced survival (maintained l_t) compared with CYT-C neurons (steep decrease in l_t) (Fig. 4.3) indicating that increased availability of NGF in early postnatal life has significant and prolonged effects on the capacity of neurons to survive in vitro in the absence of NGF or serum. In contrast to the acute group, few neurons from 8 month old animals in either treatment group survive beyond Phase 2. Therefore phase 1 is characterised by evidence of early cell loss in both groups, while phase 2 shows the main differences between the treatments at 8 months. Phase 1 is characterised by a higher but not significant l_t for CYT-C; when phase 2 starts NGF shows the characteristic plateau, and the CYT-C shows a steep decrease in l_t (see figure 4.3). Phase 3 shows a rapid decline in l_t for both treatments, with a faster decline in CYT-C compared to NGF, even though t to t+1 is longer in CYT-C compared to NGF.

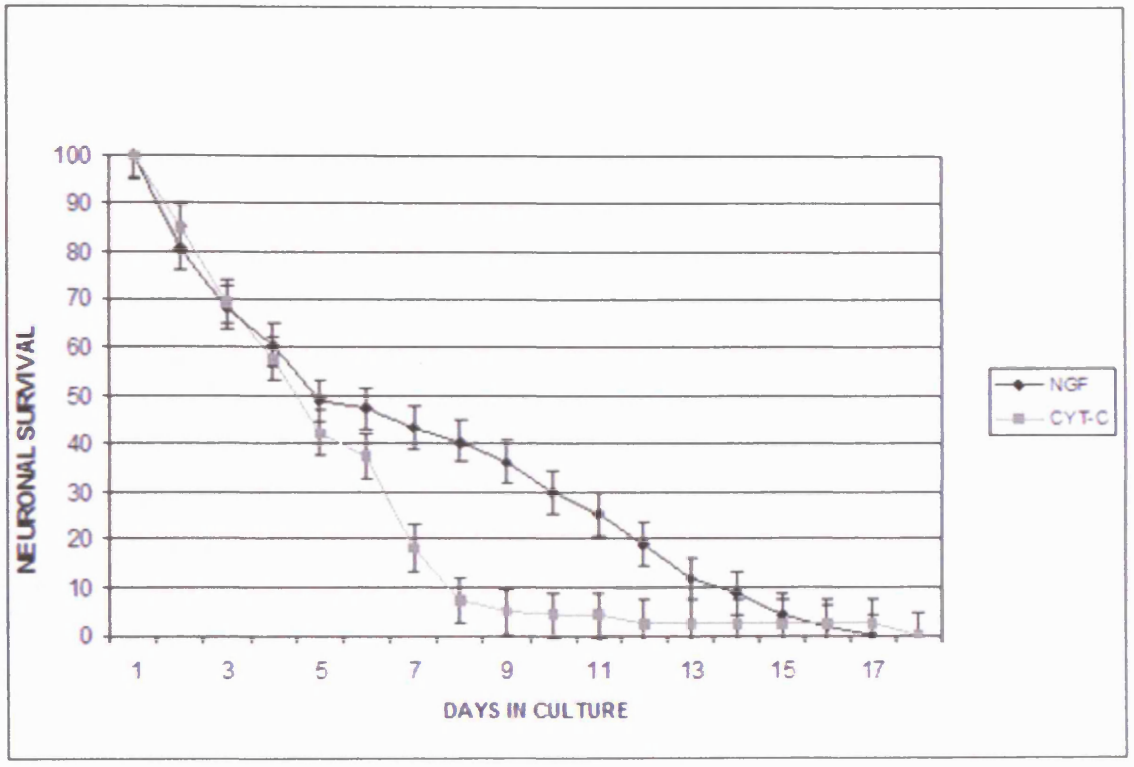

Figure 4.3: In vitro survival of 8 months group of SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF (diamond, black solid line), or CYT-C as a control (square, grey solid line).

The mortality rate results (see figure 4.4) show a phase one with slightly but not significant higher mortality of NGF pre-treated neurons compared to CYT-C, followed by phase two where NGF pre-treated neurons have a substantially higher l_t (therefore a low mortality) compared to CYT-C.

Phase three is distinguished by a low mortality of NGF pre-treated neurons. These behaviours may be due to the fact that NGF pre-treated neurons once plated in vitro tend to have a worst reaction than CYT-C at the beginning (i.e. phase one), but a lower mortality rate in phase three, which may be attributed to the in vivo NGF pre-treatment positive effect.

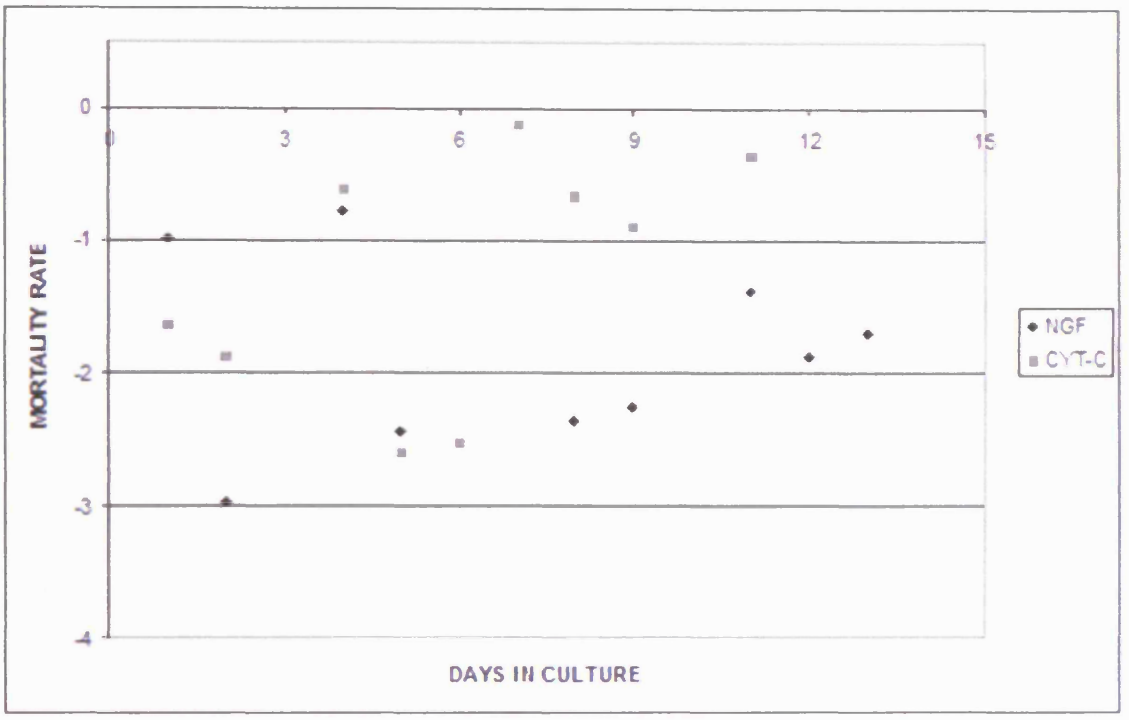

Figure 4.4: log plot of the 8 months group mortality rate in vitro of SCG neurons treated in vivo at 7 weeks old with NGF (black diamond) or CYT-C (grey square). The lower values in the y axis (i.e. -3) indicate the lower mortality while higher values (i.e. -1) represent higher mortality

4.2.3 Effect of 'early' (age 3-7wks) pre-treatment with NGF, anti-NGF or CYT-C on in vitro neuron survival and mortality: 18 MONTH GROUP

In this group, SCG neurons were taken 16m after pre-treatment with NGF, anti-NGF or CYT-C, i.e. at 18 months of age. Eighteen months represents an age intermediate between senescence (24m), when approximately 50% of the cohort has died, and 8 months, representing the achievement of full adulthood. Eighteen months is also the age around which vulnerable neurons appear to be lost due to ageing SCG (Gatzinsky *et al.*, in press). Over the large majority of the survival period in vitro, the NGF pre-treated neurons exhibit significantly enhanced survival compared with CYT-C pre-treated cells ($p < 0.01$). A-NGF neurons, in contrast, exhibit significantly lower levels of survival than either of the other pre-treatment groups (both $p < 0.01$; see figure 4.5). In the early part of phase 1, NGF and CYT-C cells show a similar decline in survival, while A-NGF

neurons already in phase 1 survive less well than either of the other two groups. However, from day 3, NGF neurons show a significant higher l_t compared to the control and A-NGF. A-NGF has the shortest t to t+1, and all neurons were lost by day 7 NGF neurons had the longest t to t+1. Phase three is characterised by a significantly higher NGF l_t compared to control. The results at eighteen months are similar to results from earlier groups, in that NGF pre-treatment significantly increases both l_t and t to t+1 compared with CYT-C. However, in this group, A-NGF has a more rapid effect than NGF and a far more deleterious effect on survival overall compared to the acute group. In addition, the maximum survival time for all treatments was shorter than in previous groups.

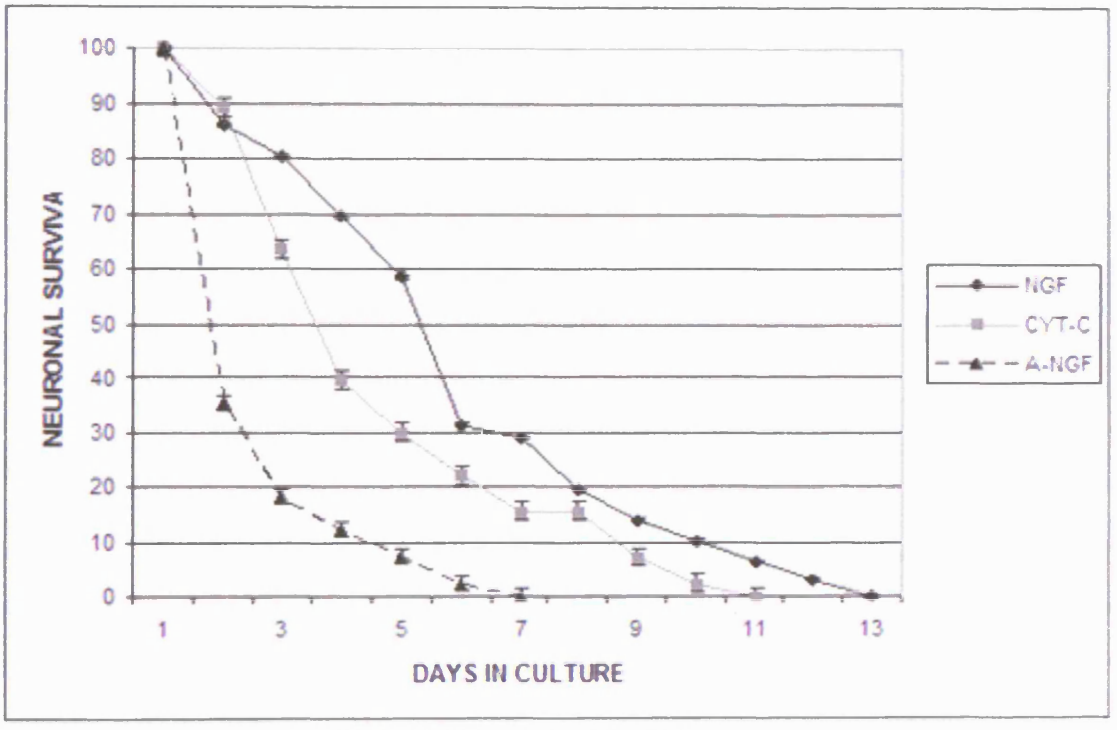

Figure 4.5: In vitro survival of 18 months group of SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF (diamond, black solid line), NGF antibodies (i.e. A-NGF; triangle, black segmented line), or CYT-C as a control (i.e. CYT-C; square, grey solid line).

In phase 1, the mortality rate for 18m A-NGF neurons is very high, while that for NGF neurons is low (figure 4.6). The mortality of the latter tends to increase slightly in phase two, but it is still characterised by a longer t to t+1, showing once more the positive effect of 'primed cells' due to the in vivo pre-treatment with NGF and the resulting delayed cell death in vitro. CYT-C neurons show mortality values intermediate between those of NGF and A-NGF.

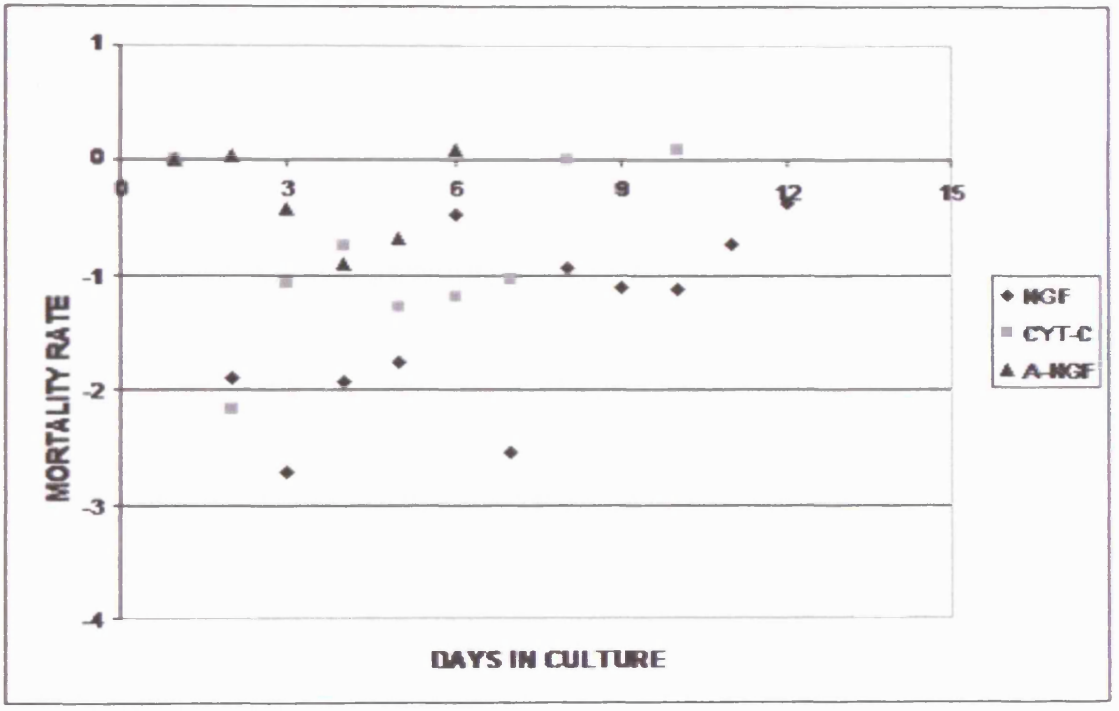

Figure 4.6: log plot of the 18 months group mortality rate in vitro of SCG neurons treated in vivo at 7 weeks old with NGF (black diamond), NGF antibodies (i.e. A-NGF; black triangle) or cytochrome-C (i.e. CYT-C; grey square). The lower values in the y axis (i.e. -3) indicate the lower mortality while higher values (i.e. -1) represent higher mortality

4.2.4 Effect of 'early' (age 3-7wks) pre-treatment with NGF, anti-NGF or CYT-C treatment on in vitro neuron survival and mortality: 24 MONTH GROUP

24 months of age in Sprague Dawley rats from the RFUCMS colony represents senescence, i.e. the age by which approximately half of the cohort has died.

Results from the twenty-four months groups are very different from previous results (see figure 4.7). Even though there are some significant differences when comparing the three different groups ($p = 0.02$) and between the A-NGF and the CYT-C ($p = 0.003$), there is no significant difference between the NGF and the CYT-C, or between the NGF and A-NGF groups. The l_t is very similar between treatments although A-NGF has the longest t to t+1 value. Compared to the 18m neurons, those at 24m on average survive longer, reinforcing the suggestion that vulnerable neurons have already died.

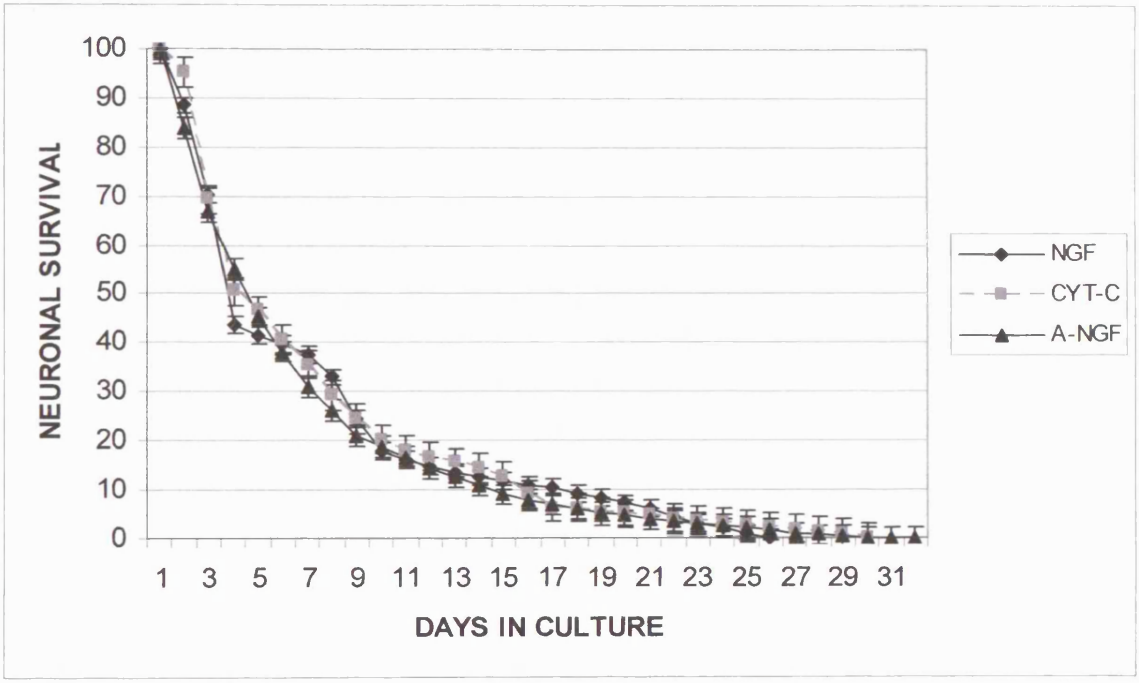

Figure 4.7: In vitro survival of 24 months group of SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF (diamond, black solid line), NGF antibodies (i.e. A-NGF; triangle, black segmented line), or CYT-C as a control (i.e. CYT-C; square, grey solid line).

Results form the mortality rate gives us more information on the behaviour of this particular group (see figure 4.8). In actual fact there is a longest t to t+1 for the A-NGF compared to the other two pre-treatment in vivo; but if we observe the mortality rate across the three different phase we may notice that during phase one the highest

mortality is recorded for NGF and the lowest for CYT-C. The end of phase two shows a very low mortality for NGF, again this may indicate the real survival behaviour after a phase one of settlement. Phase three shows the lowest mortality for CYT-C.

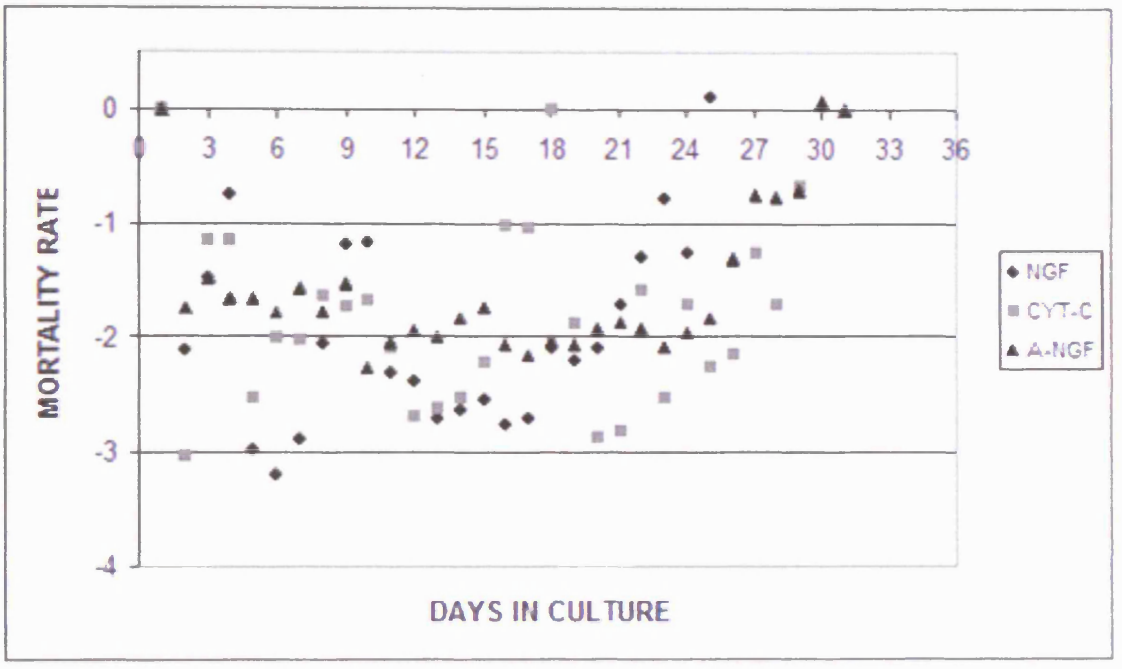

Figure 4.8: log plot of the 24 months group mortality rate in vitro of SCG neurons treated in vivo at 7 weeks old with NGF (black diamond), NGF antibodies (i.e. A-NGF; black triangle) or cytochrome-C (i.e. CYT-C; grey square). The lower values in the y axis (i.e. -3) indicate the lower mortality while higher values (i.e. -1) represent higher mortality

4.2.5 Effect of 'early' (age 3-7wks) pre-treatment with NGF, anti-NGF or CYT-C treatment on in vitro neuron survival and mortality: ACROSS AGE COMPARISONS

During phase one of the CYT-C pre-treated groups across ages, there are no particular differences between the age groups. However we can notice a difference in l_t, starting in phase two and continuing in phase three. The 24 month group has t to t+1 and l_t which are significantly higher compared to the acute ($p < 0.01$), to the 8 months ($p < 0.01$), and the 18 months ($p < 0.01$). The shortest t to t+1 is in the 18 months group. It seems that

survival of control (CYT-C pre-treated) neurons is unexpectedly better in the most aged group compared to the younger stages, at least over the longer periods in culture.

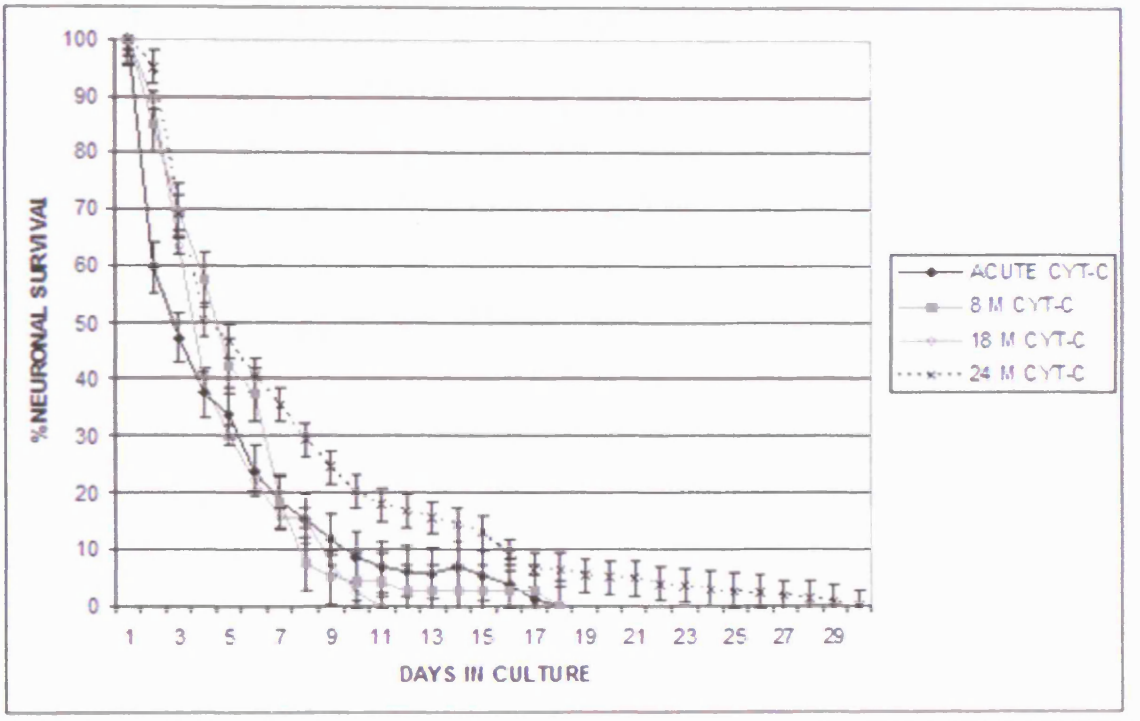

Figure 4.9: Comparison of in vitro survival of SCG neurons pre-treated in vivo with CYT-C across ages. Acute group (diamond, black solid line); 8 months group (grey square, solid line); 18 months group (grey empty diamond, solid line); 24 months group (black cross, dotted line).

Comparisons across ages of neurons pre-treated in vivo with NGF are shown in figure 4.10. The acute group seems to have an lt significantly higher than the 8 months ($p = 0.01$), the 18 months ($p = 0.0007$), and the 24 months ($p = 0.002$) groups, i.e. NGF pre-treatment enhances survival most at the earliest stage. The eighteen month group has the lowest response to NGF and exhibits the shortest overall duration of survival in vitro, differing substantially from the other groups; again suggesting that the 18m is the age at which the most vulnerable neurons die.

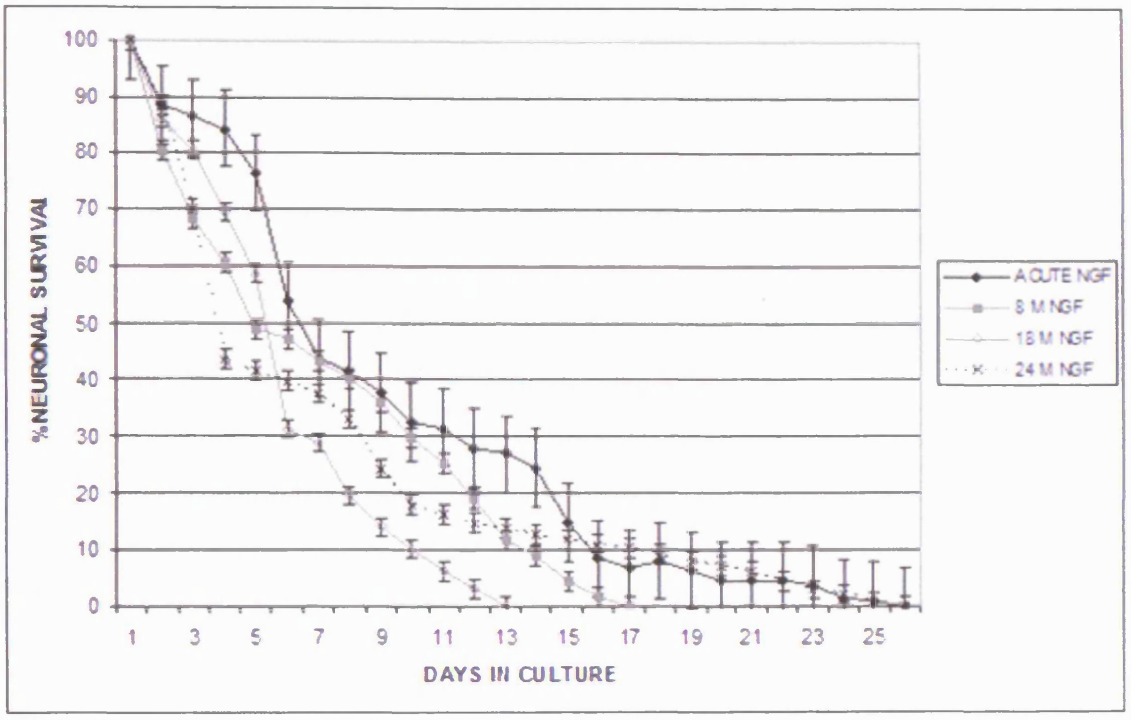

Figure 4.10: Comparison of in vitro survival of SCG neurons pre-treated in vivo with NGF across ages. Acute group (diamond, black solid line); 8 months group (grey square, solid line); 18 months group (grey empty diamond, solid line); 24 months group (black cross, dotted line).

Comparisons across ages of neurons pre-treated in vivo with A-NGF are shown in figure 4.11. The 24 month group is relatively unaffected by A-NGF pre-treatment in vivo as the l_t is the highest of all (see figure 4.11). The l_t of the acute, the 18 months and the 24 months are significantly different ($p < 0.01$); with the eighteen months being the lowest of the three, showing again that this is the age at which the most vulnerable neurons die. The t to t+1 of the 18 months is the lowest, while the 24 months is the highest. This comparison underlines once again the lack of an apparent pre-treatment effect on the 24 month group, suggesting that only 'super' neurons are left at this age.

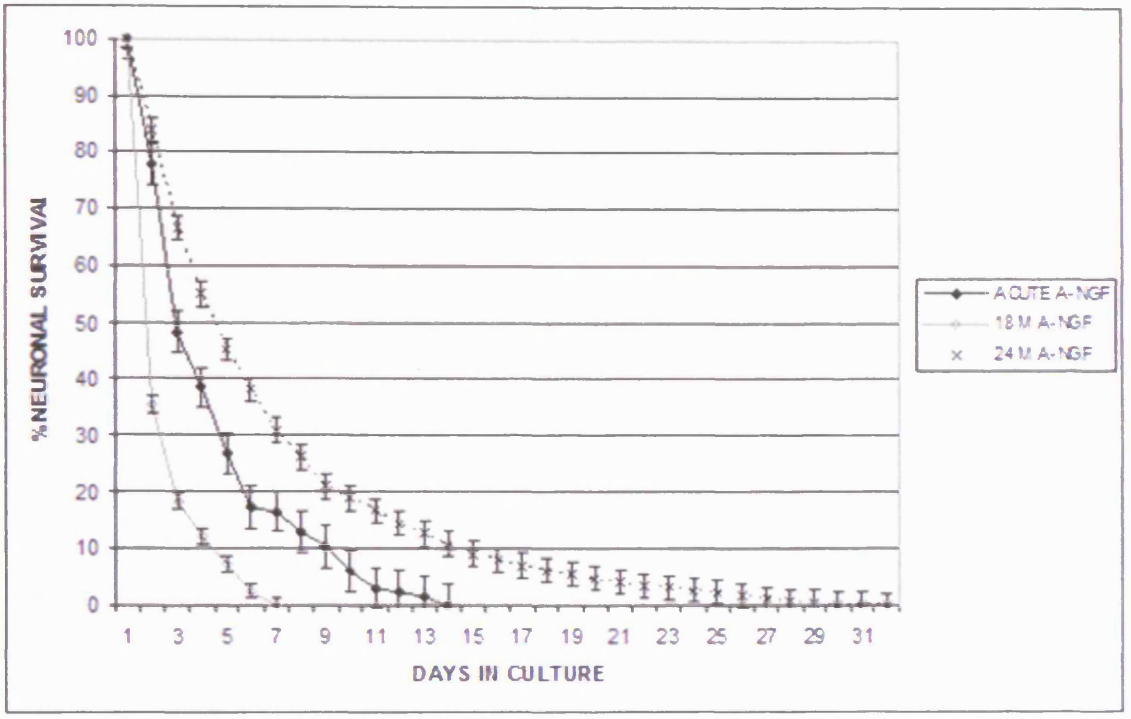

Figure 4.11: Comparison of in vitro survival of SCG neurons pre-treated in vivo with A-NGF across ages. Acute group (diamond, black solid line); 8 months group (grey square, solid line); 18 months group (grey empty diamond, solid line); 24 months group (black cross, dotted line).

4.2.6 Effect of 'late' (age 3 months) pre-treatment with NGF or CYT-C treatment on in vitro neuron survival and mortality

The effect of the pre-treatment administered at later period of postnatal development, i.e. at 3 months as opposed to 3-7 weeks as in the previous experiments, demonstrates a completely different effect. NGF pre-treatment at this later stage fails to provide any protection of neuron survival in vitro at any stage of the culture period. While there is no difference in survival during phase 1, there appears to be reduced survival of the NGF pre-treated group compared to CYT-C in phase 2. In addition, CYT-C pre-treated cells appear to survive farther into phase 3 than their NGF-pre-treated counterparts. Thus postnatal age has a major effect on the capacity of neurons to alter their in vitro survival response to NGF pre-treatment., in fact, as shown in figure 4.12, SCG neurons

pre-treated in vivo with NGF don't manifest a higher l_t, with the exception of phase one. Phase two shows a significantly lower l_t for NGF pre-treated neurons compared to the control ($p < 0.01$); t to t+1 is also lower for NGF compared to CYT-C. NGF pre-treatment in vivo seems to have a negative effect in vitro SCG.s neuronal survival.

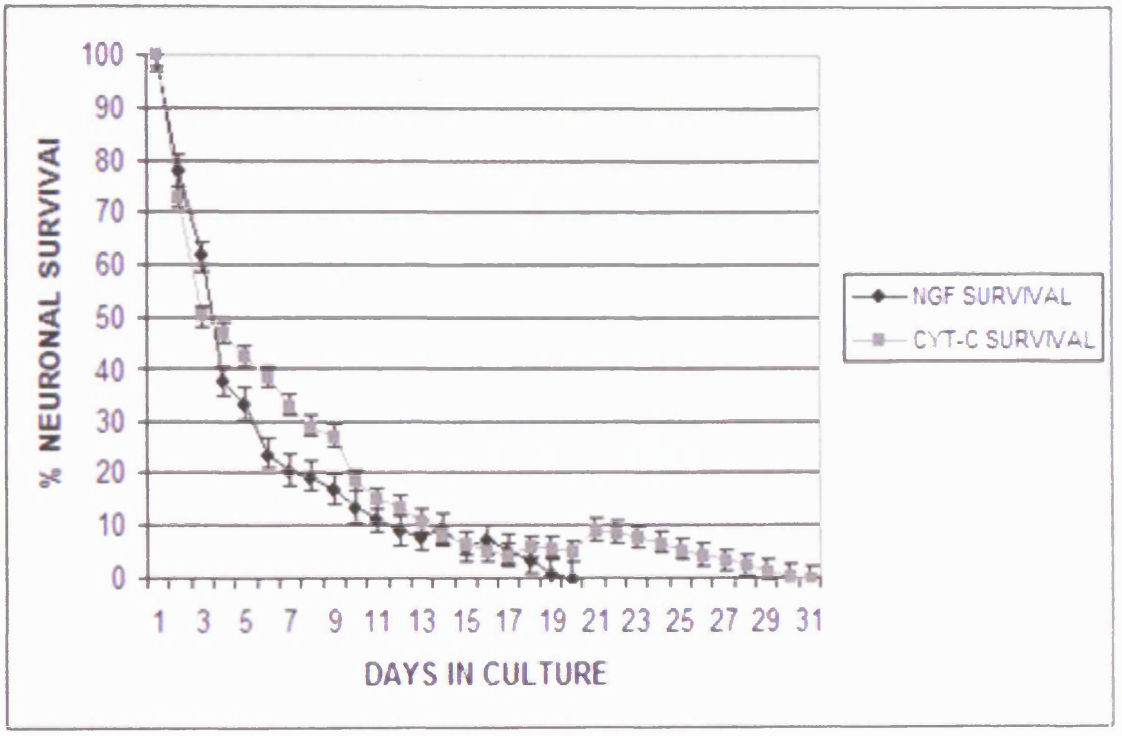

Figure 4.12: In vitro survival of late treatment group of SCG neurons from animals pre-treated in vivo at 3 months old with NGF (diamond, black solid line), or CYT-C (square, grey solid line).

The mortality rate (figure 4.13) shows some more details on the behaviour of the cells with different pre-treatment. CYT-C pre-treated cells shows a high mortality in phase one contrasted by a very low one in phase two. NGF pre treated neurons present the highest mortality in phase three.

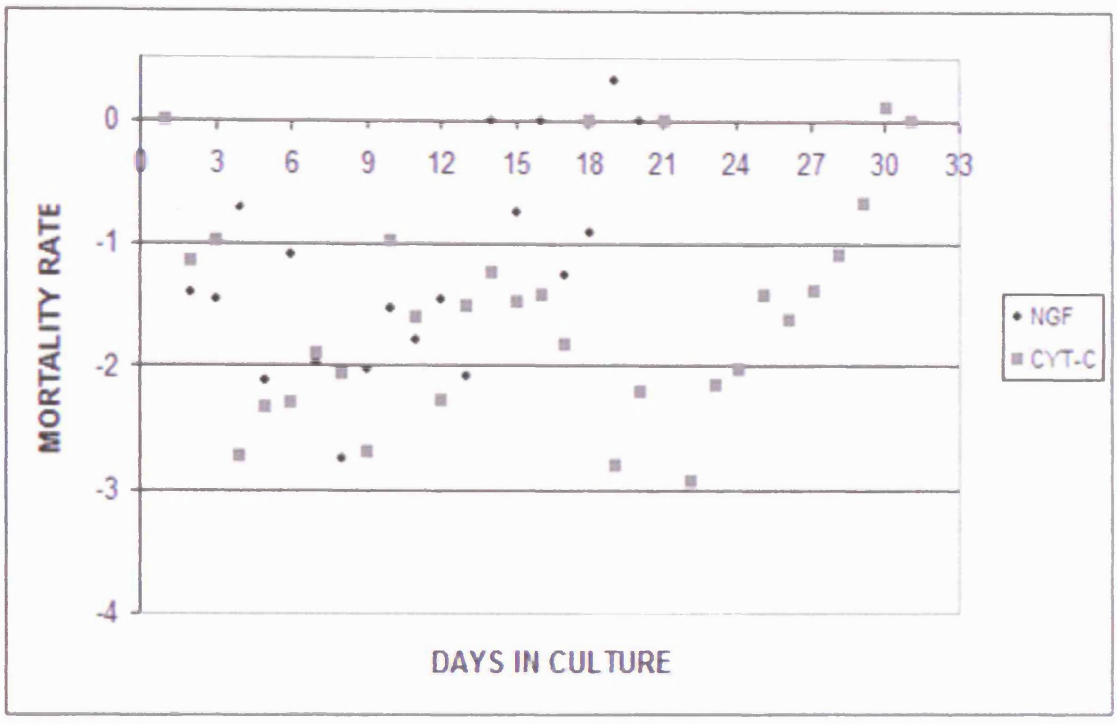

Figure 4.13: log plot of the late treatment group mortality rate in vitro of SCG neurons treated in vivo at 3 months old with NGF (black diamond) or cytochrome-C (i.e. CYT-C; grey square). The lower values in the y axis (i.e. -3) indicate the lower mortality while higher values (i.e. -1) represent higher mortality.

The l_t of the 8 months group is significantly higher than the late treatment group ($p < 0.01$). Therefore, from comparison of the early and late treatment groups at 8m it seems that the 'early' in vivo pre-treatment has a significant effect in increasing neuronal survival across ages, while late treatment doesn't have the same effect.

4.2.7 Effect of 'early' (age 3-7wks) pre-treatment with NGF, or CYT-C treatment on in vitro neuron survival and mortality: SUB-POPULATIONS OF NEURONS PROJECTING TO THE IRIS AND MCA

In these experiments, the subpopulations of neurons projecting to iris and MCA were retrogradely traced some days before the ganglia were removed for tissue culture. Pre-treatment with NGF or CYT-C was carried out as before and the animals were killed (6m after termination of treatment), i.e. at 8 months of age. The results in figure 4.14

show how the two different subpopulations of SCG neurons respond in vitro after the different in vivo pre-treatments. Phase 1 exhibited similar survival responses for all groups. Phase 2, however, saw the lowest survival in the CYT-C pre-treated, MCA-projecting neurons, while CYT-C-treated iris-projecting neurons survived somewhat better (although there is no significant difference, $p = 0.08$), i.e. control MCA neurons seems to be more vulnerable than iris neurons under in vitro conditions, just as they are during ageing. Also during phase 2, NGF pre-treatment significantly enhanced survival of MCA neurons ($p < 0.01$), indicating plasticity of phenotype of this subpopulation during early postnatal life. However, NGF pre-treatment had some significant effect on survival of iris-projecting neurons ($p < 0.01$) compared to control, as well as between MCA and iris ($p < 0.01$), both showing a very small standard error. Only iris neurons (NGF or CYT-C pre-treated) and NGF pre-treated MCA neurons survived significantly into phase 3. Phase two and phase three describe an interesting behaviour; in phase two l_t of the MCA subpopulation pre-treated with NGF, is the highest, showing a large effect of the pre-treatment on a subpopulation characterised by a single unit innervation. Phase three shows a significant lower l_t of the MCA subpopulation pre-treated with CYT-C, while the NGF pre-treatment seems to have pushed the survival curve up to the iris level.

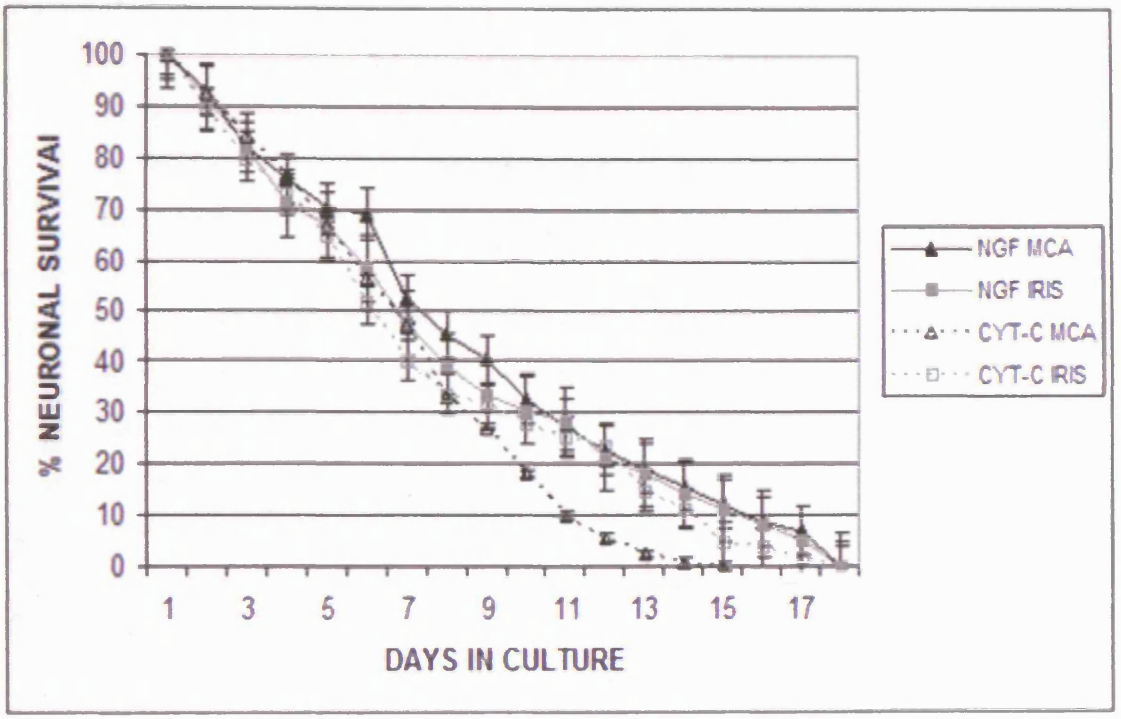

Figure 4.14: Comparison of in vitro survival of subpopulations projecting either to the iris or to the MCA target tissues of SCG.s neurons pre-treated in vivo with NGF or CYT-C. MCA pre-treated with NGF (black triangle, solid line); iris pre-treated with NGF (grey square, solid line); MCA pre-treated with CYT-C (black triangle, dotted line); iris pre-treated with CYT-C (grey square, dotted line).

In figure 4.15 the results have been separated, and it shows MCA pre-treated either with NGF or CYT-C. L_t of MCA pre-treated in vivo with NGF is significantly higher ($p < 0.01$); therefore the survival behaviour of vulnerable subpopulation of neurons, such as MCA, can be enhanced with pre-treatment in vivo with NGF during maturation.

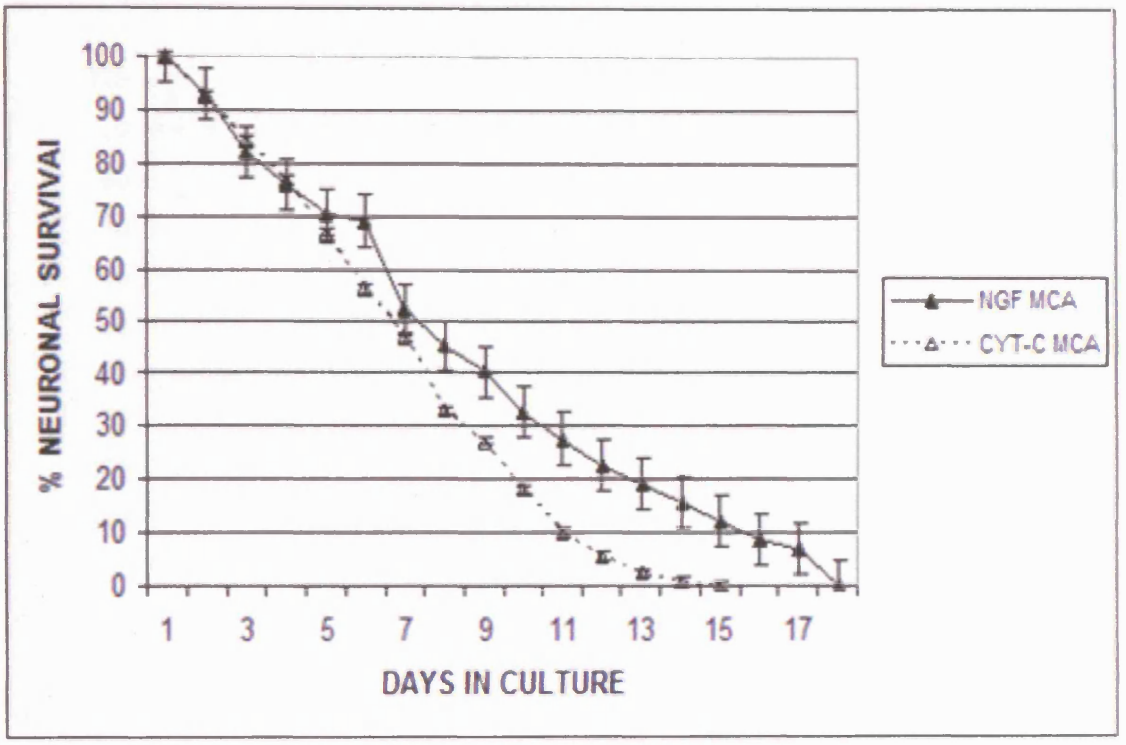

Figure 4.15: Comparison of in vitro survival of subpopulations of SCG neurons projecting to MCA in animals pre-treated in vivo with NGF or CYT-C. MCA pre-treated with NGF (black triangle, solid line); MCA pre-treated with CYT-C (black triangle, dotted line).

Results of the iris projecting neurons pre-treated in vivo with either NGF or CYT-C are shown in figure 4.16. Even though there is significance difference between the two curves ($p < 0.01$), in every phase there seems to be a similar behaviour. Despite the fact that NGF pre-treatment in vivo seems to have elevated significantly the l_t of these neurons, if we compare these results with those from the MCA, we can observe that the increase in survival due to pre-treatment is much lower for the iris compared to the MCA.

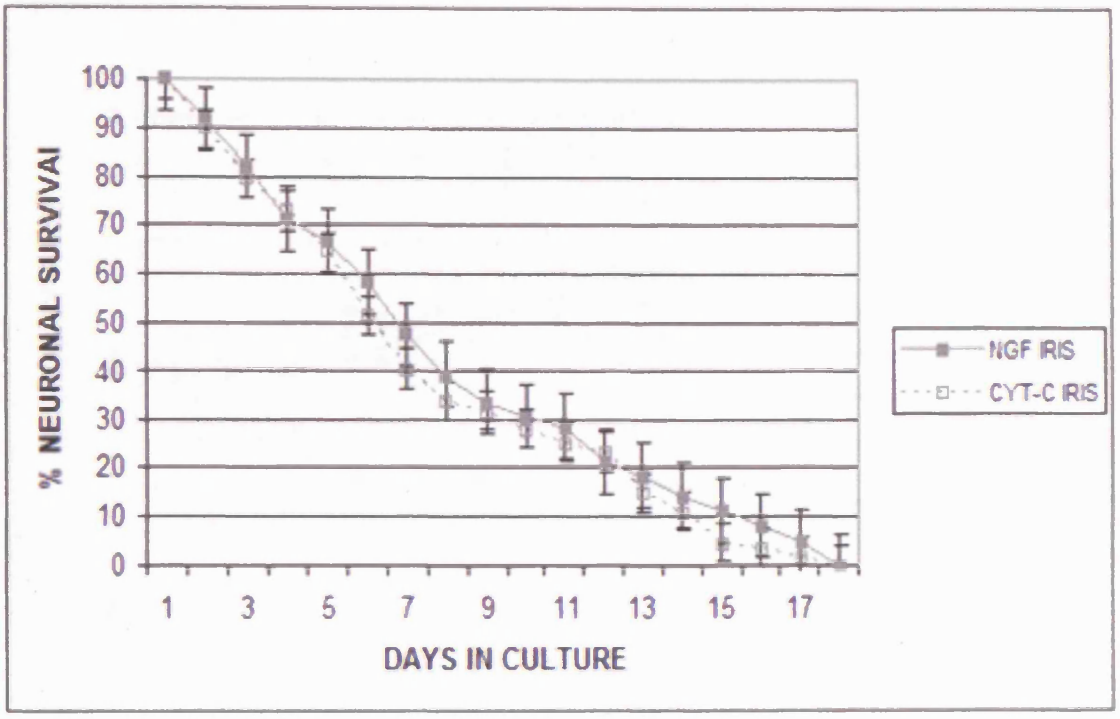

Figure 4.16: Comparison of in vitro survival of subpopulations of SCG neurons projecting to the iris in animals pre-treated in vivo with NGF or CYT-C. Iris pre-treated with NGF (grey square, solid line); iris pre-treated with CYT-C (grey square, dotted line).

The mortality rate graph gives us interesting information as well (see figure 4.17). There is very low mortality for all groups during phase one, which starts to increase only during the first part of phase three. The subpopulations group is therefore characterised more by phase three rather than phase two or one like previous groups. Phase three is showing a higher mortality rate for both iris and MCA pre-treated in vivo with CYT-C; meaning that the effect of NGF pre-treatment has elevated l_t, in MCA subpopulation more than in the iris one.

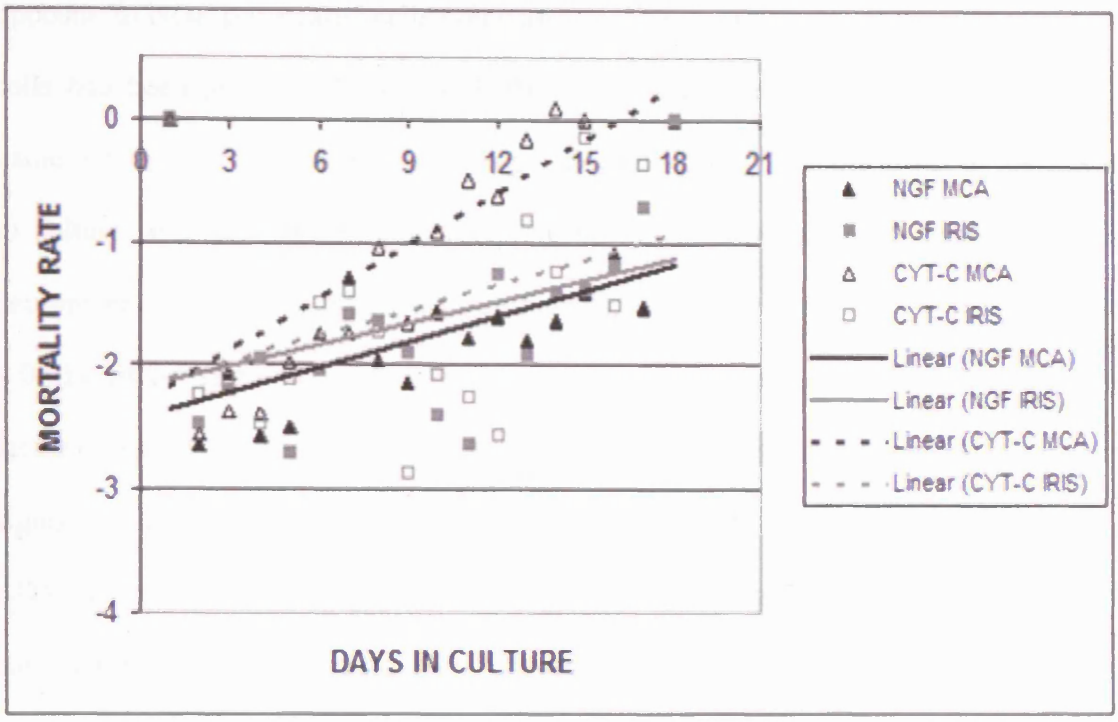

Figure 4.17: log plot of the subpopulation groups of SCG neurons projecting either to the iris or to the MCA target tissues eight pre-treated in vivo at 3-7 weeks old. MCA pre-treated with NGF (black triangle, solid line); iris pre-treated with NGF (grey square, solid line); MCA pre-treated with CYT-C (black triangle, dotted line); iris pre-treated with CYT-C (grey square, dotted line). The lower values in the y axis (i.e. -3) indicate the lower mortality while higher values (i.e. -1) represent higher mortality; trendlines are purely illustrative, the results are based on p-values.

4.2.8 Effect of in vitro treatment with growth factors (insulin, IGF, BDNF and GDNF) on survival and mortality of SCG neurons, pre-treated at 3-7wk of age, with NGF or CYT-C: ACUTE GROUP

It is interesting to know whether the in vitro responsiveness of acute SCG neurons to neurotrophic and growth factors was altered by in vivo pre-treatment during early postnatal life with NGF (figure 4.18) or CYT-C (figure 4.19). The effects obtained by adding growth factors in culture to pre-treated neurons were remarkable. NGF, insulin, IGF-1, BDNF and GDNF all had similar effects in vitro. However, their effects were

opposite in NGF pre-treated cells compared to CYT-C pre-treated cells: thus where the cells had been pre-treated with NGF the effect of all the factors was to lower the l_t value; while where the cells have been pre-treated with CYT-C the effect of the factors in culture was the opposite and therefore served to increase the l_t level. The l_t for neurons pre-treated with NGF was significantly lowered by insulin ($p < 0.01$), IGF-1 ($p < 0.01$), GDNF ($p = 0.01$), and BDNF ($p < 0.01$). Comparisons of the effect of the same factor on neurons subject to different pre-treatments in vivo, insulin and IGF-1 showed significant differences ($p < 0.05$, $p = 0.01$, respectively), but neither GDNF ($p = 0.06$) or BDNF ($p = 0.5$) showed significant differences, suggesting that these factors supported comparable level of neuron survival, irrespective of the pre-treatment.

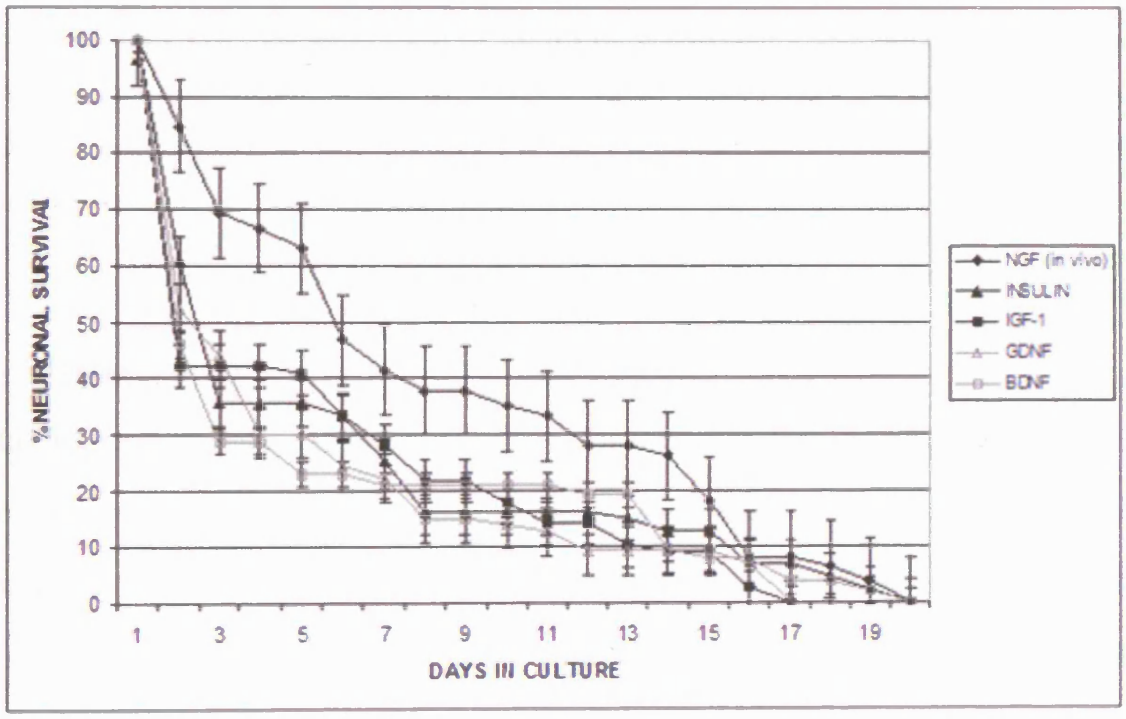

Figure 4.18: Effects of in vitro growth factor treatment on survival acute SCG neurons pre-treated in vivo at 3-7 weeks with NGF. The legend reads: NGF (NGF applied in vivo; black diamond); INSULIN (i.e. insulin applied in vitro, NGF applied in vivo; black triangle); IGF-1 (i.e. IGF-1 in vitro, NGF in vivo; black square); GDNF (i.e. GDNF in vitro, NGF in vivo; grey triangle); BDNF (i.e. BDNF in vitro, NGF in vivo; grey square).

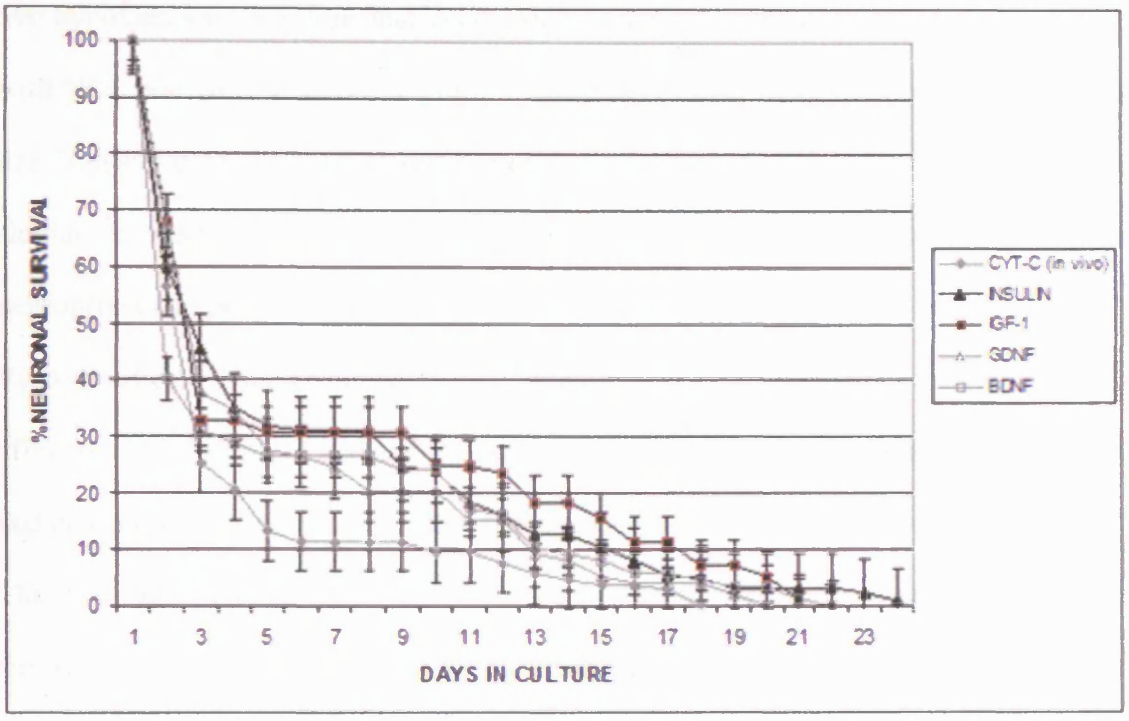

Figure 4.19: Effects of in vitro growth factor treatment on survival acute SCG neurons pre-treated in vivo at 3-7 weeks with CYT-C. The legend reads: CYT-C (CYT-C applied in vivo; grey diamond); INSULIN (i.e. insulin applied in vitro, CYT-C applied in vivo; black triangle); IGF-1 (i.e. IGF-1 in vitro, CYT-C in vivo; black square); GDNF (i.e. GDNF in vitro, CYT-C in vivo; grey triangle); BDNF (i.e. BDNF in vitro, CYT-C in vivo; grey square).

4.3 Discussion

In Chapter I, as well as in the introduction of this chapter three main assumptions have been stated: firstly adult neurons become partly independent of neurotrophic factors for their survival in vitro (Diamond *et al.*, 1992; Orike *et al.*, 2001); secondly there is no evidence for age-changes in level of expression of neurotrophic factors which might explain altered patterns of survival (Kuchel *et al.*, 1999; Cowen, 2002; Gatzinsky et al., 2004); and thirdly evidence that vulnerability is associated with early-life (and perhaps life-long) exposure to different levels of neurotrophic factors (Cowen *et al.*, 2003).

My hypothesis is therefore that pre-treatment during early postnatal life (maturation) with NGF and NGF antibodies would permanently affect SCG neuron survival in later life. If this hypothesis is proved, can altered availability of NGF in later (adult) life have similar effects? Are we looking at a maturational phenomenon, or at a lifetime neuroprotective programme?

Results indicate that altering NGF availability during postnatal life has a significant effect on the capacity of sympathetic neurons to survive in vitro in the absence of serum and neurotrophic factors and without supporting glial cells.

The first question regarding my results is whether early life 're-setting' of neuron survival is equally effective with different pre-treatments and whether this effect changes with age?

My studies of neuron survival in vitro have shown three major phases. In all groups studied, phase one is characterised by an initial high mortality, probably due to a settling time in the new in vitro environment. Once the mortality rate reaches a plateau, normally between day 3 and 5, the second phase starts with a lower mortality, followed by the third phase of high mortality, which may be attributed to a mutation accumulation effect. The end of the third phase is characterised by low mortality and the prolonged survival of a very few 'super neurons' which may be comparable to human centenarians.

Acute group: Comparison of the acute effects of in vivo NGF pre-treatment in the present study with in vitro NGF treatment (Orike et al., 2001) reveals different effects on survival: NGF pre-treatment in vivo enhances neuronal survival in vitro (see above), while NGF treatment in vitro does not. Results, from the acute group, show that the NGF effect, from day 3 in vitro, enhances and extends survival, right through phase

three. According to Tsui-Pierchala and Ginty (1999), the half life of the signaling NGF-phospho-TrkA complex in adult sympathetic neurons is about 2 days, indicating that even at this early stage, active NGF signaling is unlikely to be retained long enough to cause an altered survival response and for that reason we can assume that the effect in vitro is a 'conditioning' response to NGF and not just an ongoing direct effect of the treatment.

In contrast, anti-NGF shows no effect at this stage; this might be due to a temporary inflammatory effect caused by the anti-NGF pre-treatment in vivo which in turn initiated endogenous NGF production (Mcmahon, 1996; Safieh-Garabedi *et al.*, 2002). This endogenous upregulation of NGF might have rescued neurons and hence masked the effect of the anti-NGF pre-treatment.

Another possible explanation is that anti-NGF pre-treatment has killed neurons in vivo and therefore we are looking at a normal survival pattern of the remaining cells in vitro. However, the neuron counts that were made on ganglia pre-treated in the same way in vivo (see Chap. III) do not demonstrate any significant cell loss as a result of pre-treatment with anti-NGF, therefore the latter possibility is effectively ruled out.

Thirdly, I suggest a combination of neuronal depletion (due to NGF deprivation) in some areas and rescue (by upregulation of endogenous NGF as a result of inflammatory responses) in other areas such as skin, ears and other target tissues.

Accordingly from the NGF pre-treatment results we may deduce that SCG neuronal cells become primed by the NGF pre-treatment in vivo. However the following question arises: do neurons retain such characteristics throughout life?

8 months group: This question is answered by the NGF effect 6 months after termination of treatment, where neurons survival pattern is still high compared to

control, particularly in phase two, and therefore neurons have been primed by the in vivo pre-treatment and what we were looking at in the acute group is not only a temporary effect.

NGF has a number of roles in the development of neurons, as is widely known; during development NGF promotes survival and later supports maturation of sympathetic neurons through growth, enhancement of tyrosine hydroxylase synthesis and other aspects of mature neuronal phenotype (Conover and Yancopoulos, 1997; Johnson *et al.*, 1986; Snider, 1994). Once mature, SCG neurons lose their absolute dependence on NGF for survival; NGF signaling shifts to regulation of neuronal phenotype and function (Johnson and Deckwerth, 1993; Sofroniew *et al.*, 1990, 1993; Svendsen *et al.*, 1994).

The 8 month's NGF effect, from day 5, enhances the proportion of neurons surviving, principally in phase 2, but no extension of survival is detected. Results were most exciting, because they almost mirrored the acute group results with the exception of phase one, in fact NGF pre-treatment had no significant effect on neuronal survival pattern until day 5 in culture. Phase two, changes in which are characteristic of every group, revealed a clear significant increase in survival showing that NGF pre-treated cells are primed compared to CYT-C (control).

The natural next question is: how would the primed cells react to pre-treatment during ageing? Understanding of changes with increased age has been attempted by several scientists. According to Cowen *et al.* (2003), ageing shows a decrease in neurotrophin uptake.

18 months group: Therefore 16 months after termination of pre-treatment in vivo we looked at the results in 18 months old SCG neurons. The 18 month NGF pre-treated

group shows enhanced survival from day 2, but the shortest t to t+1 of all groups. NGF pre-treatment shows a small extension of survival, but this doesn't reach phase 3. The A-NGF effect is stronger, compared with acute, affecting neurons from day 1 and reducing maximum survival time to 7 days (shortest of whole study). Ageing neurons in vitro had a very similar result to the acute group in terms of phase pattern, with two exceptions; primarily t to t+1 is half as long, and therefore as neurons age lifespan in vitro is reduced, and secondly the NGF antibody pre-treatment has a marked effect by reducing neuronal survival. The anti-NGF effect may also support the idea that neuronal vulnerability is increasing at 18 months (Gatzinsky *et al.*, 2004) i.e. neurons may be close to a threshold affecting their capacity to survive in vitro and therefore both NGF and anti-NGF pre-treatment in vivo have a marked effect on survival of neurons in vitro.

24 months group: There is an unexpected absence of difference between the three pre-treatments in vivo; these results may be explained by the fact that the majority of the neurons (vulnerable neurons) have already died (around 18 months of age) and the remaining cells are the 'super' neurons, a subpopulation which for reason we do not presently understand are capable of surviving perhaps beyond the mean lifespan of the organism. This subpopulation may include the protected iris-projecting neurons which survive successfully to 24 months (Gatzinsky *et al.* 2004)

Across age comparisons show another interesting result, when pre-treated with anti-NGF or NGF, the 24 months group have in general a better survival curve in vitro compared to the 18 month group; except for phase one with NGF pre-treatment when the 24 months group has lower survival. The anti-NGF and phase three of the NGF pre-

treated cells (corresponding to senescence in culture) have a positive effect on the 24 months group.

Across-ages comparison is very important: the results show that NGF pre-treatment in vivo rescues successfully in acute, 8m and 18m groups, but not in 24m, while anti-NGF gives adverse effects only at 18m, and not in acute (possibly because of inflammatory response) or at 24m (because all vulnerable neurons have died earlier). No anti-NGF pre-treatment have been done at 8 months.

In vivo pre-treatment has therefore been shown to prime cells such that their in vitro survival is altered when administered before the end of development (3 to 7 weeks old), but are cells primed as well if treatment is begun after the end of maturation in early adulthood, starting at 3 months old?

The late treatment group show that this is apparently not the case. In contrast to treatment at 3-7wk, NGF pre-treatment at 3 months doesn't enhance the survival curve but on the contrary it reduces survival. Therefore, we are looking at a developmental or maturational phenomenon, rather than a lifetime regulatory mechanism of neuronal survivability. We predict therefore that the association between target levels of NTFs and neuronal vulnerability established in Gatzinsky *et al.* (2004) is based on the exposure of neurons to differing levels of NTFs during development and maturation only and that later life alterations will not affect this aspect of phenotype which we can therefore designate as a 'survivability programme' which operates during late development (maturation).

Subpopulations group: How vulnerable subpopulations react to the pre-treatment is the next issue; results confirmed observations from Gatzinsky *et al.* (2004), where it was shown that age-related neurodegeneration and neuronal cell death, in subpopulations of SCG neurons, are associated with the level of NGF availability from the target, which was high for the iris and low for cerebral vessels (CV); NGF uptake was correspondingly low for the vulnerable CV projecting neurons and higher for the protected iris-projecting neurons. I have shown that the survival pattern of vulnerable neurons (CV-projecting) pre-treated in vivo with NGF is changed and enhanced to reach the level of protected subpopulations of neurons (iris). However the survival curve of protected iris projecting neurons is not enhanced by pre-treatment with NGF. An hypothesis could be that iris neurons are already programmed (by high target levels of NGF) to survive maximally, while the MCA neurons are not, and only in the latter group can their later life survivability be re-set by altering levels of NGF availability during maturation.

The difference may lie in the phenotype and functional adaptations that differentiate the two populations: single-unit innervation by large complex neurons characterises the iris-projecting population, while multi-unit innervation by smaller, simpler neurons characterises the CV-projecting group (Andrews *et al.*, 1996; Burnstock, 1993). When both subpopulations receive high levels of NGF during maturation, the survival levels of both populations at later stages in vitro become very similar. Thus, NGF pre-treatment enhances survival of MCA neurons (both l_t and t to t+1, extending into phase 3) but not in iris neurons (either proportion or duration of survival). Therefore the level of NGF supplied by the target tissues seems to determine the phenotype of the cells as well as their vulnerability (Cowen *et al.*, 2003; Andrews *et al.*, 1996; Cowen, 1984). Furthermore there is a maximum potential level of survival for SCG neurons which

corresponds to the level reached by the iris-projecting neurons. This helps to understand the 24 months group results. These 'super' neurons resemble, and include, iris-projecting neurons which do not exhibit age-related cell death (Gatzynsky *et al.* 2004). They appear to be resistant to pre-treatment with both anti-NGF and NGF, hence either their survival is regulated by different mechanisms, or their 'survival threshold' is set at such a level that they are unaffected by pre-treatment.

The regulation of apoptosis by survival factors is critical for normal development and proper functioning of the nervous system. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) belong to the first identified group of factors suppressing neuronal apoptosis (Levi-Montalcini and Booker, 1960; Barde, 1989).

Cowen *et al.* (1996) failed to find an association between age-related neuronal vulnerability and reduced NGF availability. Katoh-Semba, Semba *et al.* (1998) studied the brain of senescence-accelerated (SAM) mice, and also, despite evidence of neurodegeneration, found no reduced levels of neurotrophin expression compared with wild-type animals. My results, however, show that when NGF levels are varied before the end of development in vivo neurons become primed (results for the acute group) and retain such characteristic later on in life (8 and 18 months group). This emphasises the above conclusion that early life conditions, including the level of availability of target-derived factors, in some way set the subsequent capacity of neurons to survive into old age.

In vitro treatment with growth factors suggests that altered exposure to NGF changes the pattern of acute responsiveness of SCG neurons to other NTFs and growth factors.

The addition in culture of exogenous growth factors such as, insulin, IGF-1, GDNF, and BDNF caused a very interesting effect; all factors caused decreased survival of neurons pre-treated in vivo with NGF, but enhanced survival when cells had been pre-treated with CYT-C.

Are external factors triggering a receptor mediated response? According to Segal (2003) various tissue culture and animal experiments indicate that the nature of the receptor ligand specifies the signaling pathways activated. Furthermore in vivo studies (Fan *et al.*, 2000) demonstrate clearly that biological responses elicited by for example TrkB (BDNF receptor) signaling are modulated by the identity of the activating ligand.

Or are external factors converging on common pathways which result in stimulating phosphorylation of CREB? Ginty *et al.* (1994) suggested that CREB might be involved in growth factor-stimulated gene expression. Bonni *et al.* (1995) revealed that CREB plays a critical role in mediating growth factor induction of c-fos gene transcription, and showed that induction occurs in a Ser 133-dependent manner, and activation of CREB-dependent transcription. Finkbeiner *et al.* (1997) found that BDNF stimulates both Ca^{2+}/calmodulin-dependent kinase IV and the Ras-MAPK cascade, leading to CREB phosphorylation at Ser 133. Nakajima *et al.* (1996) suggested that certain signals initiated by growth factor stimulation interfere with the association of CREB with CREB-binding protein (CBP), for example NGF stimulation of Ras-MAPK pathway in PC12 cells induces a CBP-RSK (RSK is the ribosomal protein SG Kinase, a substrate of ERKs) interaction, thereby sequestering CBP and inhibiting c-AMP responsive element- (CRE) and CREB- mediated transcription. Insulin also represses cAMP-induced activation of CREB, and both NGF and insulin represses c-AMP-induced activation of CREB (Yeagley *et al.*, 1998).

In conclusion, my results show:

1. That NGF by itself increased survival during later life, and that anti-NGF decreased it. Both effects were most marked at 8-18 months.

2. The lack of effect of late treatment effect suggests that only those neurons pre-treated before the end of development are affected.

3. Subpopulations of neurons are affected differently according to the level of NGF they receive, but pre-treatment affects CV-projecting neurons more than iris-projecting neurons possibly due to the fact that iris-projecting neurons are already programmed to reach a maximum threshold, while CV-projecting neurons survivability can be re-set by altering levels of NGF availability before the end of development; possibly due to the difference in phenotypes between the two subpopulations of neurons.

4. A range of growth and neurotrophic factors by themselves also increased survival; however, pre-treatment in vivo with NGF together with the treatment in vitro with other factors appears to repress survival.

We have shown a programme that can be manipulated during maturation but not in adulthood, which has effect in late life neuron survival in vitro. How should we understand this apparent contradiction? We suggest that early life conditions which determine trophic availability, phenotype and function, have a 'knock on' or pleiotropic effect on late life survival. Furthermore, we hypothesize that there are adaptations i.e. increasing survival of vulnerable neurons does not enhance function during maturation or in adult life. Alternatively I hypothesize an adaptive outcome of this programme, namely functional adaptation of neuronal phenotype to the particular needs of different targets (as previously explained in the general introduction and the chapter introduction). If NGF pre-treatment in vivo confers additional protection by inducing vulnerable neurons to survive than we might suggest that target receive additional

innervation but which may not contribute to function. Alternatively NGF pre-treatment in vivo protects neurons that survive postnatal competition but remain vulnerable to age-related degeneration.

The NGF pre-treatment in vivo caused an increased in survival of SCG.s neurons, with changes that are likely to involve the PI3-K/Akt pathway with possible activation of PTEN, p53, and GSK-3β, and/or ERK pathway.

CHAPTER V

SIGNALING PATHWAYS INVOLVED IN ALTERED SURVIVAL OF ADULT AND AGEING NEURONS INDUCED BY NGF PRE-TREATMENT IN VIVO

5.1 Introduction

The nature and mechanisms of activation of anti-apoptotic signaling pathways triggered by neurotrophins have been extensively described by Segal and Greenberg (1996), Kaplan (1998), Friedman and Greene (1999), and Klesse and Parada (1999). The Trk neurotrophin receptors trigger activation of the signaling kinases PI3-K (Franke *et al.*, 1997; Hemmings, 1997), and ERK1/2 (Lewis *et al.*, 1998; Cobb, 1999; Grewal *et al.*, 1999) which play a key role in the survival of developing sympathetic and other neurons. Yao and Cooper (1995), and Xia *et al.* (1995) first studied the role of PI3-K and ERK1/2 in suppressing apoptosis in PC12 cells. NGF mediated survival was shown to be transduced by PI3-K in SCG neurons as well as in DRG and PC12 cells (Crowder and Freeman, 1998; Klesse and Parada, 1998, Meyer-Franke *et al.*, 1999).

Following TrkA phosphorylation, PI3-K catalyzes the production of 3-phosphoinositides, including PI-3,4,5-P_3, which bind to and activate PDK1 which in turn associates with and phosphorylates the serine-threonine kinase Akt. Akt phosphorylates Bad inducing its association with the 14-3-3 protein, and sequestering it from heterodimerization with Bcl-X_L. At this point Bcl-X_L is able to heterodimerize with Bax, preventing Bax homodimerization. Homodimerized Bax is a key element in apoptotic signaling, via altering mitochondrial membrane potential. The balance of Bax: Bax homodimers versus Bax: Bcl-X_L heterodimers may determine whether the cell lives or dies (Allsopp *et al.*, 1993) . The anti-apoptotic targets for PI3-K/Akt pathway are

negative regulation of GSK-3β, caspase 9, FKHRL1 and Bad and positive regulation of L-Ca, IKKα, and eNOS.

Akt (also known as PKB) has two mechanisms of regulation: binding of the lipid to the PH domain, and activation by autophosphorylation (Burgering and Coffer, 1995). The exact mechanism of this phospho-regulation became apparent when Alessi *et al.* (1996) demonstrated that Akt could be activated by treating cells with insulin or IGF-1, and this activation was accompanied by phosphorylation on residues Thr308 and Ser473. These phosphorylations were completely blocked by wortmannin (Arcaro and Wymann, 1993) and LY294002 (Vlahos *et al.*, 1994). Kohn (1995 and 1996) demonstrating the role of Akt in glucose uptake and metabolism. Cross *et al.* (1995) first described GSK-3β as a substrate for Akt. Akt phosphorylates GSK-3β on S^9 to inactivate it, and neurons in which this pathway is activated become resistant to apoptotic stimuli. The second regulatory site on GSK-3β is Y^{216}, phosphorylation of which is necessary for functional activity and increased response to apoptotic signals. GSK-3β inhibits the microtubule-associated protein (MAP)1B and hyperphosphorylates the microtubule-associated protein τ (i.e. tau, which form paired helical filaments in Alzheimer's disease). As described by Müller *et al.* (1994), MAP1A and B are members of the same family of high molecular weight microtubule-binding phosphoproteins. MAP1B is found in neurons particularly during development, and several phosphorylated isoforms are present only in axons. Gordon-Weeks (1997) described the importance of MAP1B in regulation of axon outgrowth by influencing the organisation and dynamics of microtubules in growing and regenerating axons and growth cones (Gordon-Weeks and Fischer, 2000).

The second survival pathway downstream of TrkA is the Ras-MAP kinase cascade. Following phosphorylation of tyrosine 490 on TrkA, the adaptor protein Shc is recruited

to the receptor; Shc activates Ras which in turn recruits and activates Raf. Raf is a serine-threonine kinase that phosphorylates the MAP kinase kinase MEK on 2 serines initiating the activity of a dual specificity kinase which activates the MAP kinase Erk1/2 via phosphorylation of threonine 202 and tyrosine 204. At this point phosphorylated Erk1/2 may either translocate into the nucleus, where they phosphorylate the transcription factor Elk-1, or they may phosphorylate the kinase Rsk. Phosphorylation of Elk-1 allows interaction with the accessory transcription factor SRF which binds to the serum response element (SRE) within the c-fos promoter region and contributes to initiation of transcription. On the other hand phosphorylation of Rsk causes its nuclear translocation and phosphorylation of CREB on serine 133. CREB is bound to the coactivator protein CPB, which binds to SRF-Elk complex that leads to c-fos transcription. A simplified diagram is shown in figure 5.1.

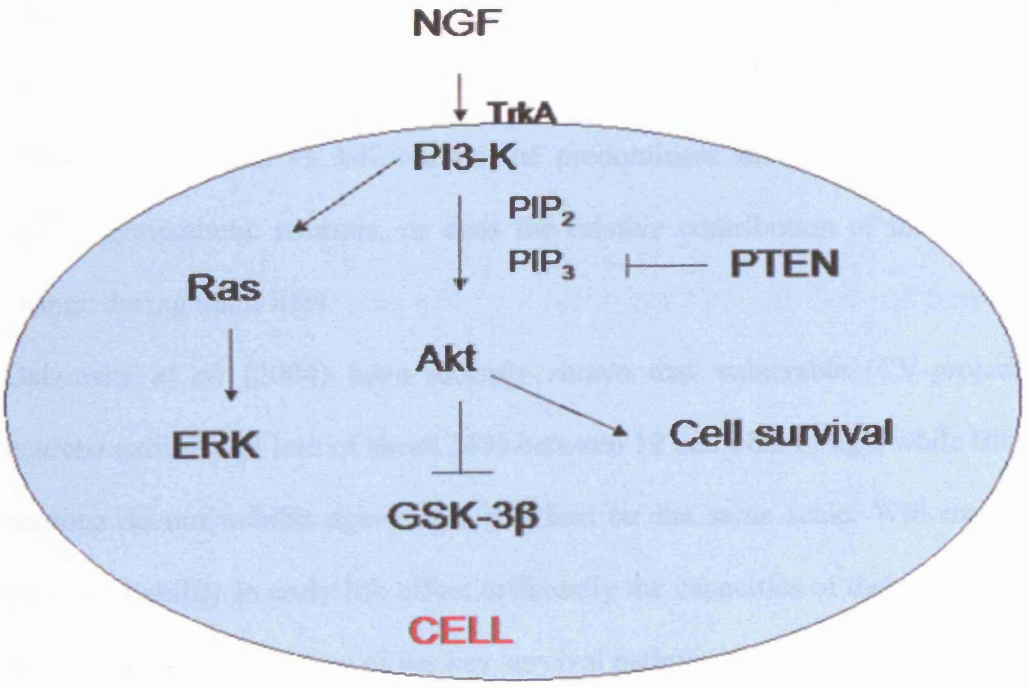

Figure 5.1: Schematic representation of the relevant signaling pathways in SCG neurons. Arrows indicate stimulation and line indicates inhibition.

Orike *et al.* (2001) and others have shown that sympathetic and sensory neurons become at least partially independent of NGF and of NGF induced Trk signalling for their survival during maturation. Presumably because adult neurons are no longer acutely dependent on NGF availability, they are better able to resist injury and possible separation from their target tissues. Do these 'NGF-independent' neurons therefore continue to require a longer term supply NGF for their survival?

NGF-independent adult sympathetic neurons continue to require activation of the PI3-K pathway for their survival, despite their loss of responsiveness to Trk activation (Orike *et al.*, 2001). The question is therefore raised: does NGF-independence depend on an intrinsic and ongoing upregulation of PI3-kinase signalling in adult sympathetic neurons? and do different levels of PI3-K activity distinguish those neurons which are, or are not, vulnerable to age-related cell death?

Furthermore according to Orike *et al.* (2001) there is a greater sensitivity of in vitro survival of adult sympathetic neurons to PI 3-K inhibition compared to inhibition of ERK/MAP-K. Does PI 3-K remain the predominant survival pathway for adult and ageing sympathetic neurons, or does the relative contribution of these two pathways change during adult life?

Gatzinsky *et al.* (2004) have recently shown that vulnerable (CV-projecting) SCG neurons exhibit cell loss of about 50% between 12 and 18m of age, while iris projecting neurons do not exhibit age-related cell loss on the same scale. Will manipulation of NGF availability in early life affect differently the capacities of these subpopulations of neurons to resist inhibition of the key survival pathways?

5.1.1 Aims

This chapter aims at studying the effect of pre-treatment in vivo with NGF, A-NGF, or CYT-C (control) across ages (acute, 8 months, 18 months, 24 months) on survival pathways (PI3-K and ERK1/2) by looking at neuronal behaviour in vitro. In a further experiment, the 'late treatment' group of animals were treated with the same regime as all the other groups with the exception that treatment started at 3 months of age (instead of 7 weeks) and animals were sacrificed 6 months after termination of treatment (i.e. at a time interval comparable to the 8 months group). By three months of age male Sprague Dawley rats are fully grown and sexually mature; this age has therefore been chosen as representing the start of adulthood.

These experiments aim to test if the NGF pre-treatment has an effect on survival pathways in adult and ageing neurons and whether the effect is the same when pre-treatment has been started late (i.e. adulthood) or early (i.e. during early postnatal development).

5.1.2 Hypothesis

Orike *et al.* (2001) have described how PI3-K inhibition in vitro affects survival of 12 wks old SCG neurons, and Crowder and Freeman (1998) showed that prenatal neurons are responsive to PI3-K in the presence of NGF. Therefore my hypothesis is that in vivo manipulations of NGF levels during postnatal maturation will affect permanently one or both of the PI3-K and ERK survival pathways and that NGF pre-treatment will protect neurons from inhibition of those pathways. Furthermore, the differential effects of NGF pre-treatment on subpopulations of neurons may be the result of different degrees of alteration in the activity of key survival pathways. We predict that different age groups will respond differently to either pre-treatment in vivo and/or to treatment in vitro, with

the 8m and the 18m groups being most vulnerable to inhibition of survival pathways as well as to reduced availability of NGF in early life.

This hypothesis is tested in ex vivo tissues (Chap. III) and in cell culture (Chap. IV and here) which allows us to study the effect of different in vivo pre-treatment regimes as well as in vitro treatment with pathway inhibitors.

5.2 Results

5.2.1 Pharmacological inhibition of PI3-kinase and ERK signaling pathways, and regulation of survival induced by NGF pre-treatment in vivo: Acute group

The acute group (SCG neurons taken for dissociated cell culture immediately after the cessation of treatment) results of SCG neurons pre-treated in vivo with NGF are shown in figure 5.2.

NGF pre-treatment resulted in enhanced survival in vitro compared to neurons from animals receiving CytC ($p < 0.05$). The enhanced survival with NGF was effectively blocked by inhibition of PI3-kinase, which was however ineffective in the Cyt-C pretreated group. Inhibition of ERK, in contrast, was effective in both groups. These results suggest that altered NGF availability in early postnatal life enhances neuron survival by upregulating PI3-kinase, but not ERK/MAP-kinase. The contrasting results of inhibiting PI3-kinase and ERK in the control group suggest that at this early postnatal stage, and in the absence of altered NGF availability, ERK/MAP-kinase is the more important survival pathway.

There is a significant higher l_t (i.e. larger overall number of neurons alive at the time of counting) as well as t to t+1 (i.e. individual neurons surviving for longer) for untreated neurons in vitro compared to LY (i.e. LY294002; PI3-K inhibitor) or UO (i.e. UO126; ERK inhibitor) ($p = 0.01$; $p < 0.01$ respectively), meaning that both PI3-K and ERK

inhibitors have two causes: reductions in number of neurons and reduction in survival time. The survival pathways are clearly inhibited and ERK inhibition has at this stage a stronger effect on survival compared to PI3-K inhibition ($p = 0.05$). It is important to note that both LY and UO exert a rapid inhibitory effect during phase 1 – i.e. during the initial settling phase in culture (days 1to day 3/5). There is a clear separation between phase one, with a sharp decline with LY and UO; while phase 2 in all the in vitro treatment shows a similar rate of decline in neuron survival, resulting in shortened maximal survival for the inhibited cultures compared to the control (untreated) cultures. (NB no phase 3 for treated cultures).

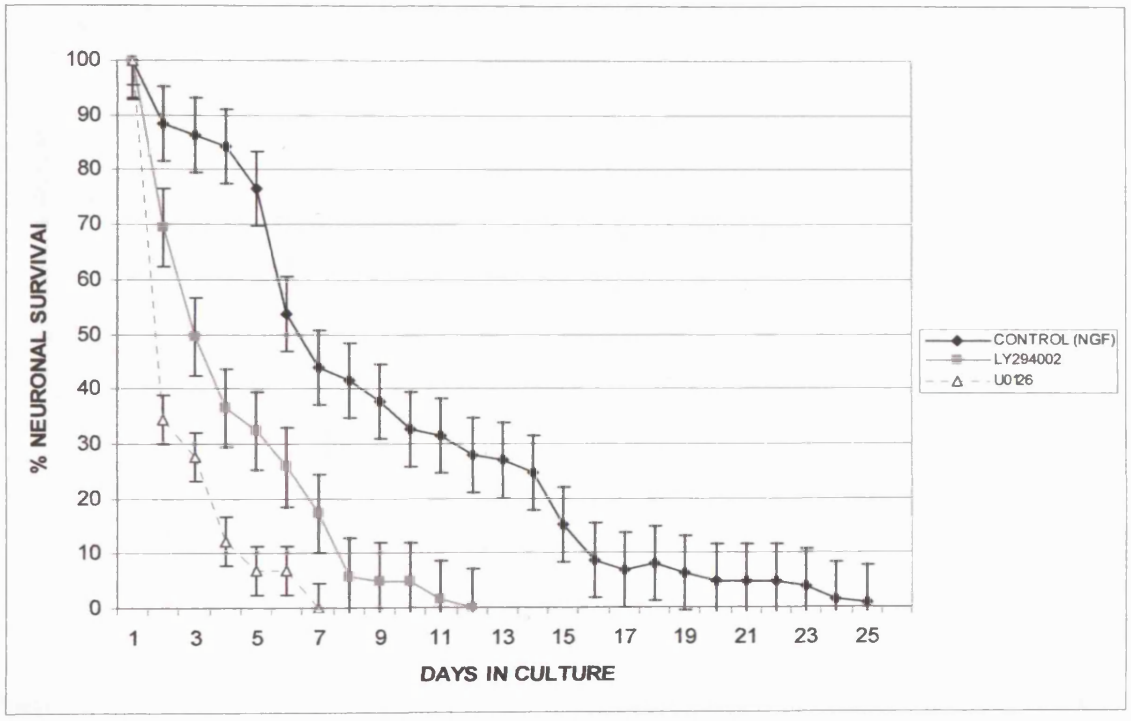

Figure 5.2: In vitro survival of acute group SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF (diamond, black solid line); pre-treated in vivo with NGF and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with NGF and treated in vitro with U0126 an ERK inhibitor (white triangle, dotted line).

Figure 5.3 shows SCG neurons pre-treated in vivo with CYT-C and inhibited in vitro with either LY294002 or UO126. LY doesn't have a significant effect on survival

compared to the untreated cultures, while UO has a significant effect compared to untreated ($p < 0.01$). This may show that at this stage of development ERK plays a predominant role in regulating cell survival in untreated neurons, although this contradicts the findings of a previous study (Orike *et al.*, 2001) where 12 weeks old SCG neurons have been studied.

Thus, NGF pre-treated neurons tend to be more vulnerable to PI3-K inhibition compared to the CYT-C controls suggesting that NGF pre-treatment in vivo is acting mainly on PI3-K rather than on ERK-mediated survival pathways.

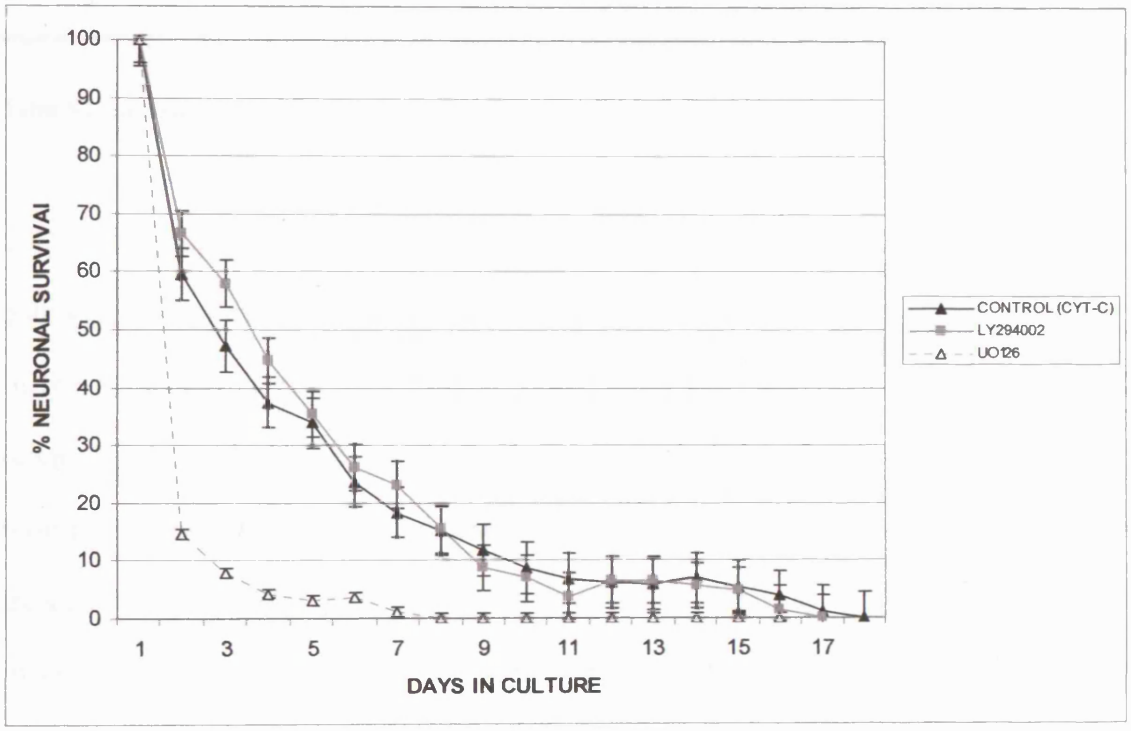

Figure 5.3: In vitro survival of acute group SCG neurons from animals pre-treated in vivo at 3-7 weeks old with CYT-C (triangle, black solid line); pre-treated in vivo with CYT-C and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with CYT-C and treated in vitro with U0126 an ERK inhibitor (white triangle, dotted line).

Table 5.1 summarises the ANOVA p-values results, with no significant difference in l_t when comparing CYT-C with LY, and no significance between treatments when UO is added in vitro.

p-VALUES ACUTE GROUP	NGF	CYT-C
UNTREATED vs. LY294002	0.00115	Not significant (0.2)
UNTREATED vs. UO126	0.00972	0.000362
LY294002 vs. UO126	0.00578	0.00112
LY294002 (NGF) vs. LY294002 (CYT-C)	0.000241	
UO126 (NGF) vs. UO126 (CYT-C)	Not significant (0.06)	

Table 5.1: Summary of the p-value results for the acute group

5.2.2 The 8 months group: pharmacological inhibition of PI3-kinase and ERK signaling pathway, and regulation of survival induced by NGF pre-treatment in vivo

In the 8 months group neurons have been pre-treated in vivo with NGF or CYT-C (control) as in the acute group but observation are carried out six months after termination of the in vivo pre-treatment in order to detect whether the effect of treatment is still effective. Results show significant increase in survival following NGF in vivo pre-treatment compared to control ($p < 0.05$). Both PI3-K and ERK inhibitor have significant effect in causing decreased survival in vitro following NGF in vivo pre-treatment (both $p < 0.05$). See figure 5.4. Both inhibitor have significant effect on CYT-C in vivo pre-treated neurons (both $p < 0.05$), but compared to the acute group, where ERK inhibition was stronger, the 8 months group seems to be principally affected by PI3-K inhibition ($p < 0.05$). These latest results are suggesting that there is a switch in survival pathway with age, specifically between 7 weeks and 8 months of age.

NGF in vivo pre-treated neurons shows that LY and UO both depress survival significantly in vitro with a lower l_t compared to control (both $p < 0.01$). See table 5.2. Comparing LY with UO effect on NGF in vivo pre-treated neurons, there is no significant difference at 8m which is different from the acute group where UO was significantly more potent than LY, suggesting that the effects of NGF pre-treatment on PI3-K-mediated survival have increased with time.

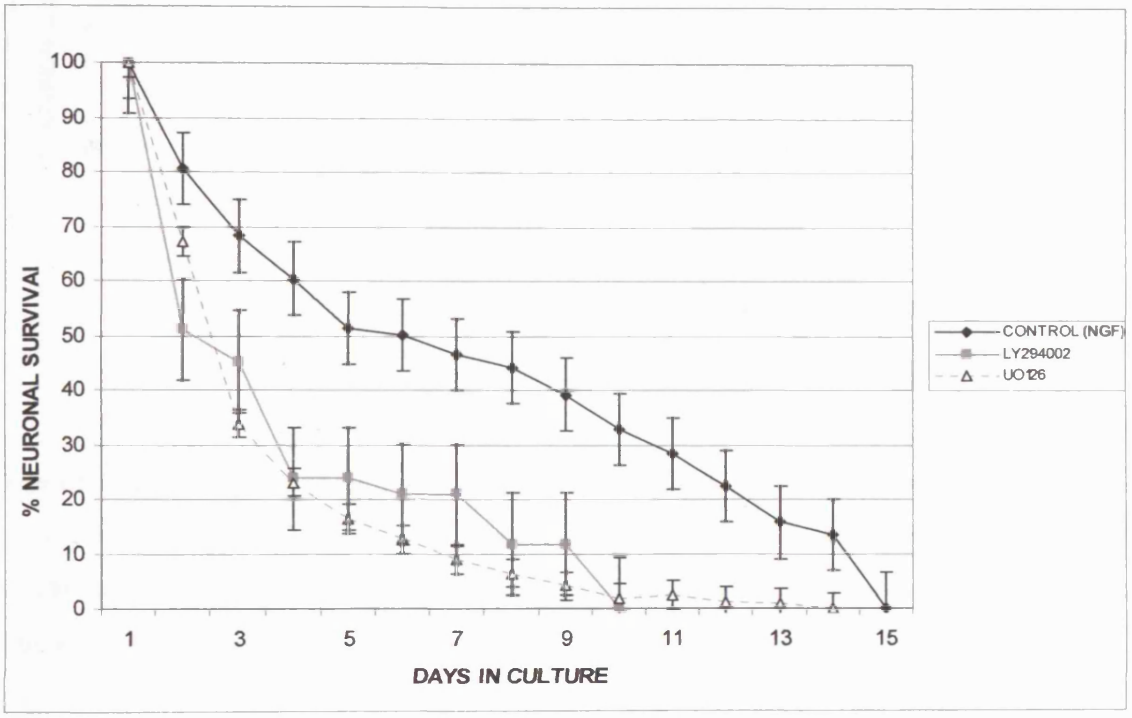

Figure 5.4: In vitro survival of the 8 months old group SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF (diamond, black solid line); pre-treated in vivo with NGF and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with NGF and treated in vitro with U0126 an Erk inhibitor (white triangle, dotted line).

Results from figure 5.5 provide strong confirmation that NGF pre-treatment affects neuronal survival by upregulating PI3-kinase. Thus, with NGF and CYT-C pre-treatments, the effects of UO are similar ($p = 0.03$). However, following NGF pre-treatment, resistance to LY is significantly increased compared to UO ($p<0.002$), compared to the NGF pre-treatment results in figure 5.3. There is a significant

difference between LY and UO ($p < 0.01$) as well as between the two LY pre-treated in vivo with NGF or CYT-C and the two UO (respectively $p < 0.01$, and $p = 0.01$).

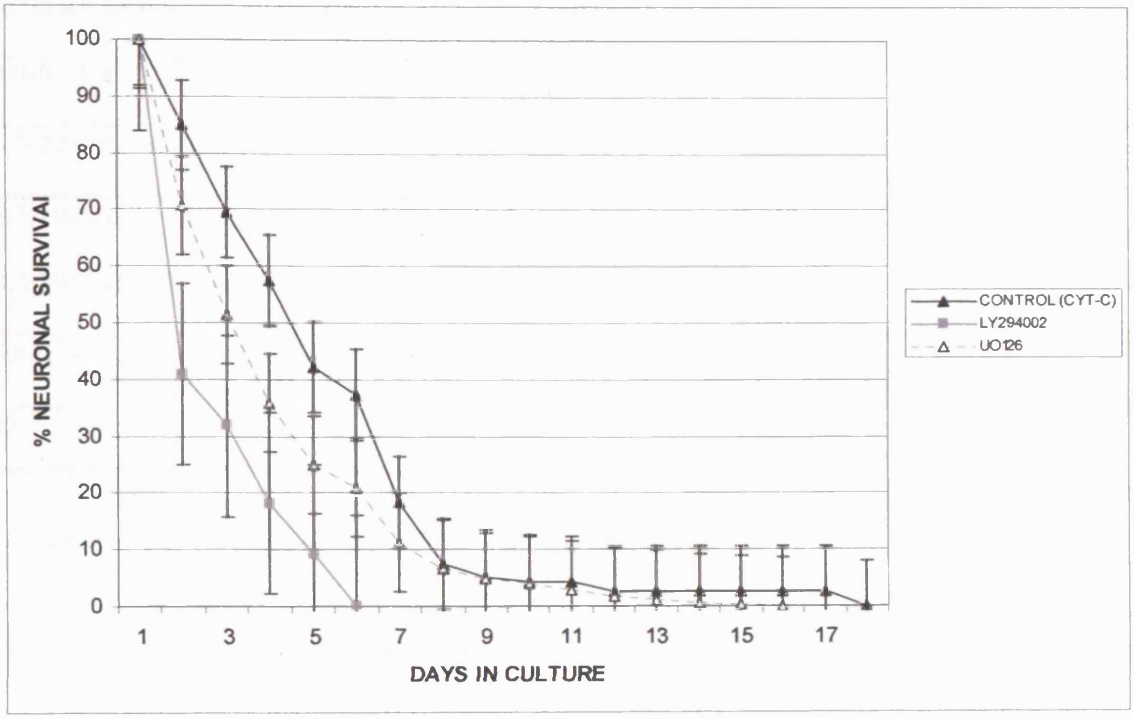

Figure 5.5: In vitro survival of the 8 months old group SCG neurons from animals pre-treated in vivo at 3-7 weeks old with CYT-C (triangle, black solid line); pre-treated in vivo with CYT-C and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with CYT-C and treated in vitro with U0126 an Erk inhibitor (white triangle, dotted line).

Table 5.2 summarises the p-value results for the 8 months group.

p-VALUES 8 MONTHS OLD GROUP	NGF	CYT-C
UNTREATED vs. LY294002	0.00141	0.002
UNTREATED vs. UO126	0.00543	0.003
LY294002 vs. UO126	Not significant (0.3)	0.002
LY294002 (NGF) vs. LY294002 (CYT-C)	0.001	
UO126 (NGF) vs. UO126 (CYT-C)	0.01	

Table 5.2: Summary of the p-value results for the 8 months old group

5.2.3 The 18 months group: pharmacological inhibition of PI3-kinase and ERK signaling pathway, and regulation of survival induced by NGF pre-treatment in vivo

In the 18 months group the NGF in vivo pre-treatment significantly enhance survival compared to control (CYT-C), $p < 0.05$.

However, the effect is less than in the 8m group and there are no significant differences between the inhibitory effects of LY and UO. A possible explanation for this result is that vulnerable neurons which were rescued by NGF in vivo pre-treatment (as demonstrated in Chap. III) have been lost between 15 and 18m, confirming results from a recent study (Gatzinsky et al., 2004).

Results from figure 5.6 shows that NGF pre-treatment in vivo effect is still effective where $p < 0.01$ for the untreated group compared to LY and to UO. The interesting effect is that in phase one and two l_t seems to be higher for UO compared to LY, something that was not seen in previous age groups. It is possible that during ageing the survival pathways switch such that neurons that were pre-treated with NGF availability are more affected by inhibition of PI3-K compared to inhibition of Erk.

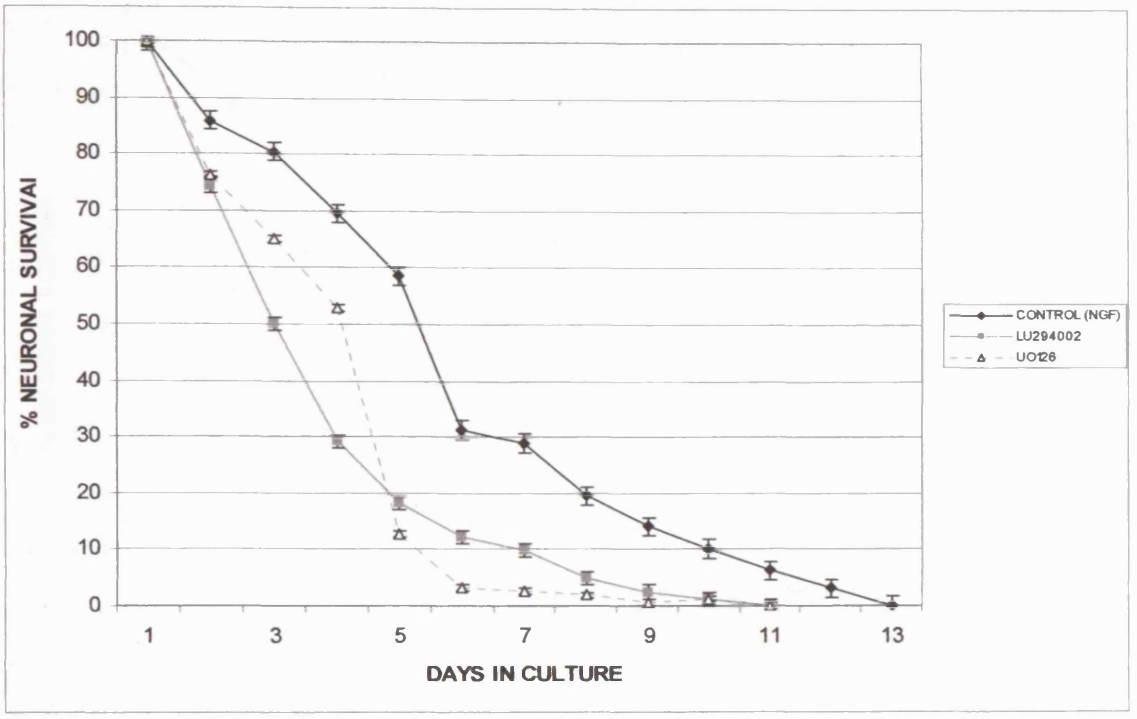

Figure 5.6: In vitro survival of the 18 months old group SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF (diamond, black solid line); pre-treated in vivo with NGF and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with NGF and treated in vitro with U0126 an Erk inhibitor (white triangle, dotted line).

The results seems to be slightly different when neurons have been pre-treated in vivo with CYT-C, both survival pathways inhibited in vitro seems to have the same effect on neuronal survival. See figure 5.7.

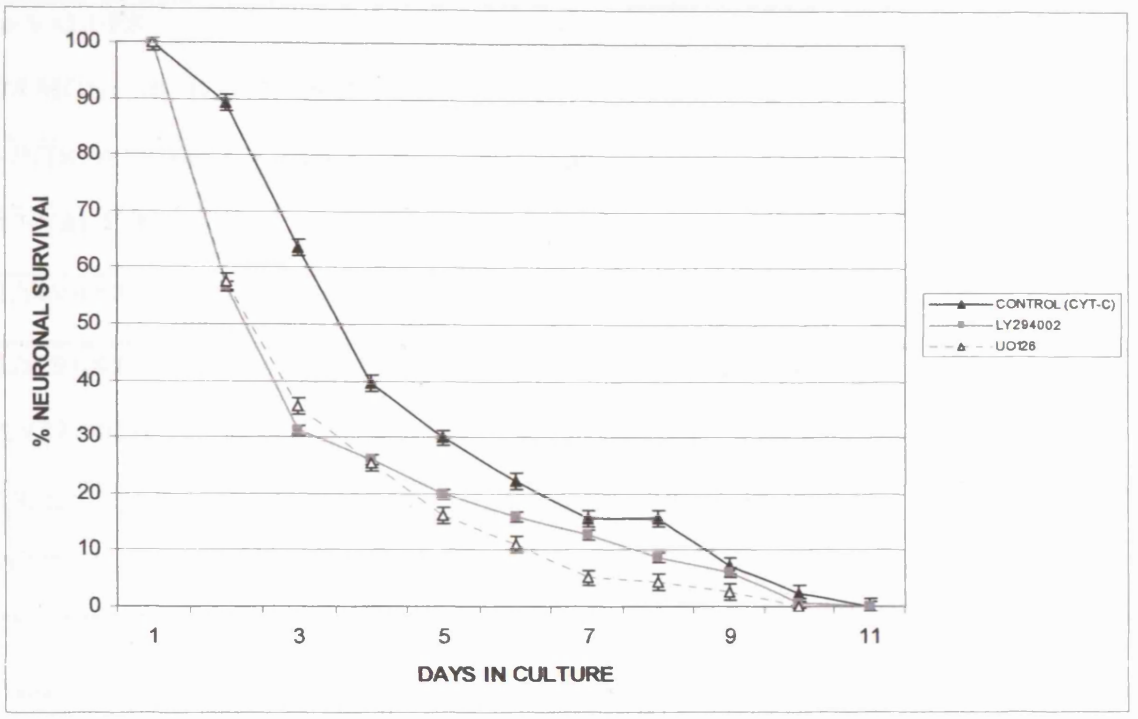

Figure 5.7: In vitro survival of the 18 months old group SCG neurons from animals pre-treated in vivo at 3-7 weeks old with CYT-C (diamond, black solid line); pre-treated in vivo with CYT-C and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with CYT-C and treated in vitro with U0126 an Erk inhibitor (white triangle, dotted line).

The untreated neurons l_t is significant both compared to LY ($p < 0.05$) and compared to UO ($p < 0.01$); but there is no significant difference between LY and UO inhibition in vitro or the comparison of these inhibition between in vivo pre-treatment (see table 5.3).

p-VALUES **18 MONTHS OLD GROUP**	NGF	CYT-C
UNTREATED vs. LY294002	0.001	0.02
UNTREATED vs. UO126	0.001	0.004
LY294002 vs. UO126	Not significant (0.7)	Not significant (0.09)
LY294002 (NGF) vs. LY294002 (CYT-C)	Not significant (0.4)	
UO126 (NGF) vs. UO126 (CYT-C)	Not significant (0.2)	

Table 5.3: Summary of the p-value results for the 18 months old group

The 18 months group is particularly interesting as pathways may change significantly due to old age

5.2.4 The 24 month group: pharmacological inhibition of PI3-kinase and ERK signaling pathway, and regulation of survival induced by NGF or A-NGF pre-treatment in vivo

At 24 months post-treatment, NGF has a small, but non-significant, effect on neuron survival in vitro. Equally, inhibiting both pathways has similar effects in the NGF and CYT-C in vivo pre-treated groups. A-NGF in vivo pre-treatment significantly decrease neuronal survival compared to control ($p < 0.05$) and both, PI3-K and ERK, inhibitor significantly reduce neuronal survival in vitro when neurons have been pre-treated with A-NGF in vivo ($p < 0.05$). Furthermore there is significant decrease in neuronal survival between PI3-K and ERK inhibition in vitro of neurons pre-treated in vivo with A-NGF compared to CYT-C ($p < 0.05$)

The NGF and the CYT-C pre-treatment in vivo results in general confirm the interpretation of the 18m data, namely that most of the vulnerable neurons in the SCG have been lost by 18m, leaving a residue of 'super-survivors'.

The 24 month group results from SCG pre-treated in vivo with NGF show similar effects of inhibiting both pathways with similar l_t (see figure 5.8), although t to t+1 is different, with LY longer than UO which may account for the significant difference between the 2 treatments ($p < 0.05$). Cultures treated with LY or UO are significantly different from the untreated group (both $p < 0.01$).

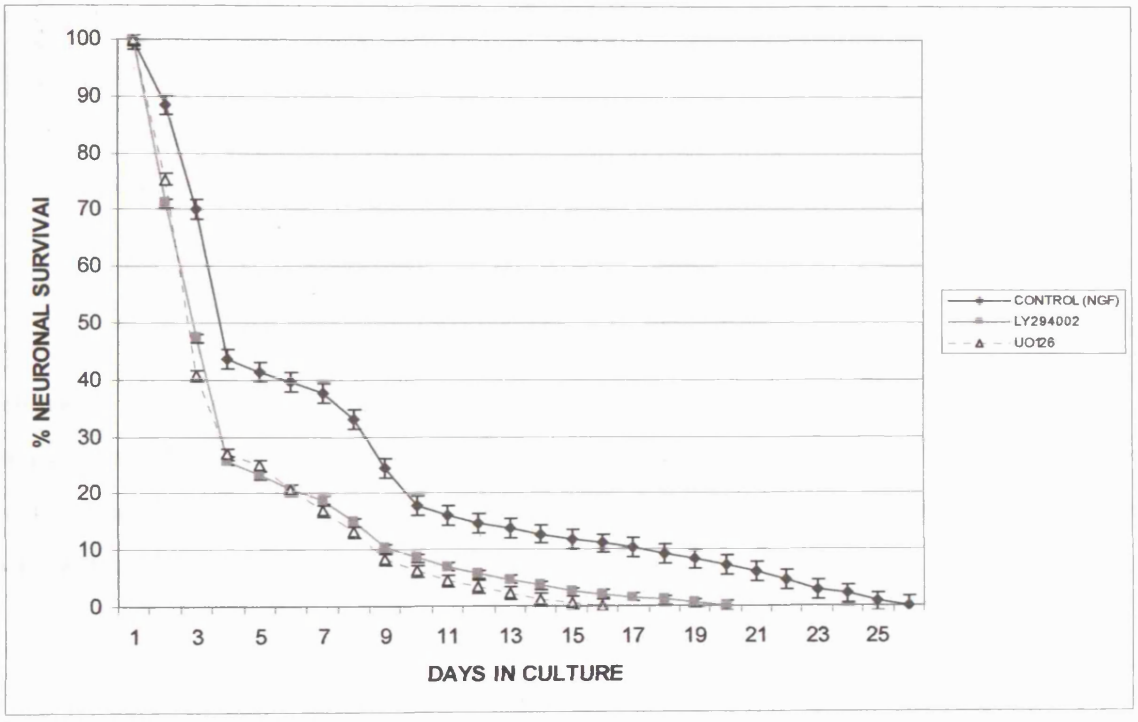

Figure 5.8: In vitro survival of the 24 months old group SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF (diamond, black solid line); pre-treated in vivo with NGF and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with NGF and treated in vitro with U0126 an Erk inhibitor (white triangle, dotted line).

In figure 5.9 results show that inhibition of the two pathways have similar effects on l_t with some difference in the t to t+1 ($p < 0.01$). Inhibition with LY or UO is significantly different from the untreated group (both, $p < 0.01$).

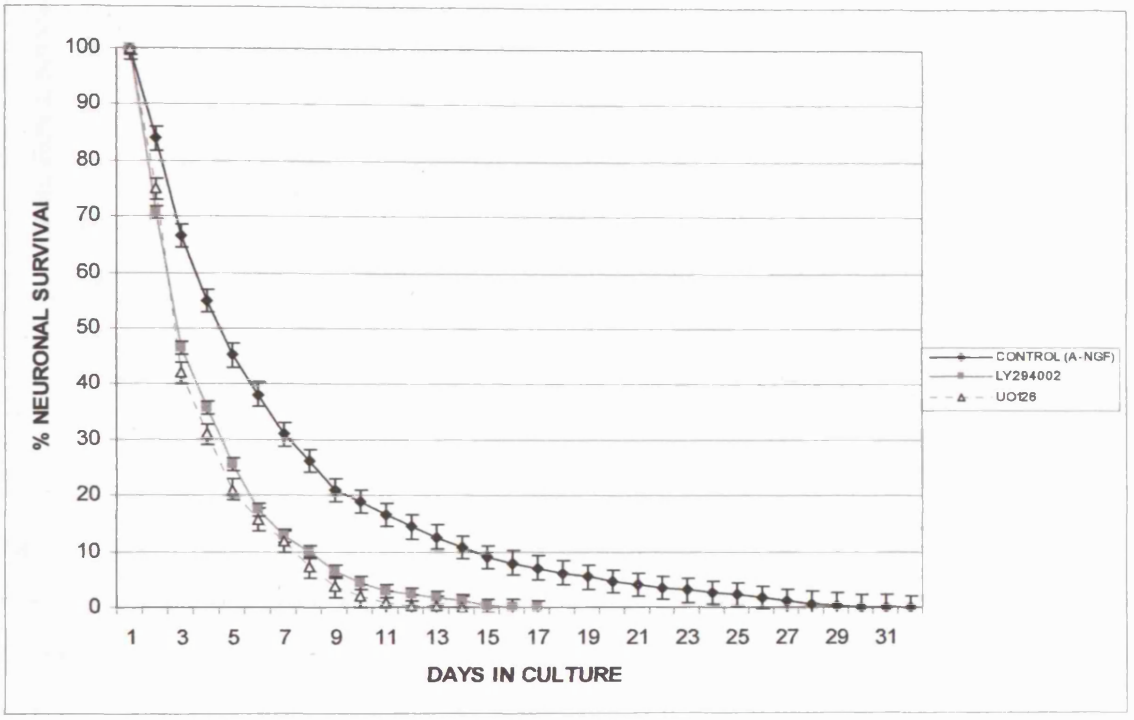

Figure 5.9: In vitro survival of the 24 months old group SCG neurons from animals pre-treated in vivo at 3-7 weeks old with anti-NGF (diamond, black solid line); pre-treated in vivo with anti-NGF and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with anti-NGF and treated in vitro with U0126 an Erk inhibitor (white triangle, dotted line).

In figure 5.10 SCG neurons have been pre-treated in vivo with CYT-C. The results show that the results of in vitro inhibition of both survival pathways are similar with no significant difference between them. The treated groups are significantly different from the untreated group (both $p < 0.01$).

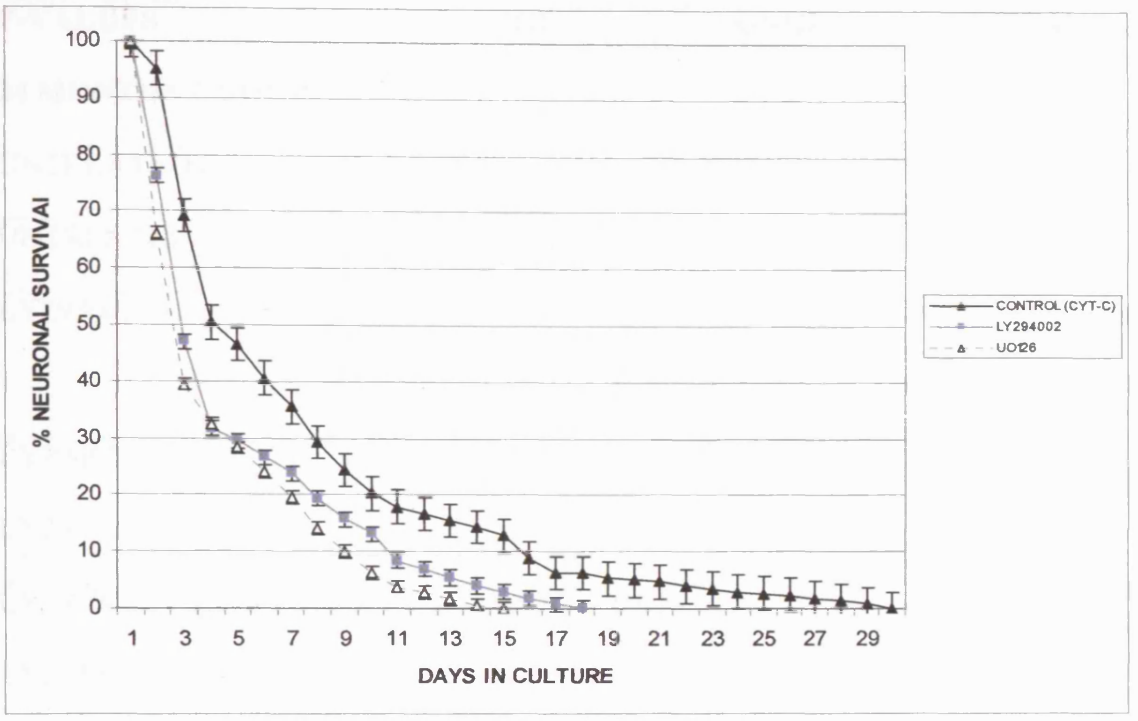

Figure 5.10: In vitro survival of the 24 months old group SCG neurons from animals pre-treated in vivo at 3-7 weeks old with CYT-C (diamond, black solid line); pre-treated in vivo with CYT-C and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with CYT-C and treated in vitro with *U0126 an Erk inhibitor (white triangle, dotted line).*

Table 5.4 show the p-values for the 24 months group; from the exact values results there is no significant difference in the UO pathways of both pre-treatment in vivo compared to the control, while there is significance between the two pre-treatment in vivo ($p < 0.01$). The LY pathways results show significance between all pre-treatment in vivo ($p < 0.01$).

P-VALUES 24 MONTHS GROUP	NGF	A-NGF	CYT-C
UNTREATEDvs.LY294002	0.00004	0.0002	0.000006
UNTREATED vs.UO126	0.00003	0.0006	0.00002
LY294002 vs. UO126	0.02	0.007	Not significant (0.07)
LY294002(NGF) vs. LY294002(CYT-C)	0.001		
LY294002 (NGF) vs. LY294002 (A-NGF)	0.004		
LY294002 (A-NGF) vs. LY294002 (CYT-C)	0.0005		
UO126 (NGF) vs. UO126 (CYT-C)	Not significant (0.7)		
UO126 (NGF) vs. UO126 (A-NGF)	0.007		
UO126 (A-NGF) vs. UO126 (CYT-C)		Not significant (0.2)	

Table 5.4: Summary of the p-value results for the 24 months old group

5.2.5 The late treatment group pharmacological inhibition of PI3-Kinase and ERK signaling pathway, and regulation of survival induced by NGF pre-treatment in vivo

The late treatment group shows a significant decrease in survival following NGF pre-treatment in vivo compared to CYT-C ($p < 0.05$). PI3-K and ERK inhibitors seems to

affect significantly both NGF and CYT-C in vivo pre-treated neurons ($p < 0.05$); but while neurons pre-treated in vivo with NGF tend to be affected by both inhibitors equally in vitro, control neurons are principally affected by ERK inhibition ($p < 0.05$). The late treatment group pre-treated in vivo with NGF (see figure 5.11) when treated with survival pathways inhibitor seems to show a significant decrease in l_t (for both PI3-K and ERK inhibition $p < 0.01$). There is no significant difference between the two inhibitory survival pathways.

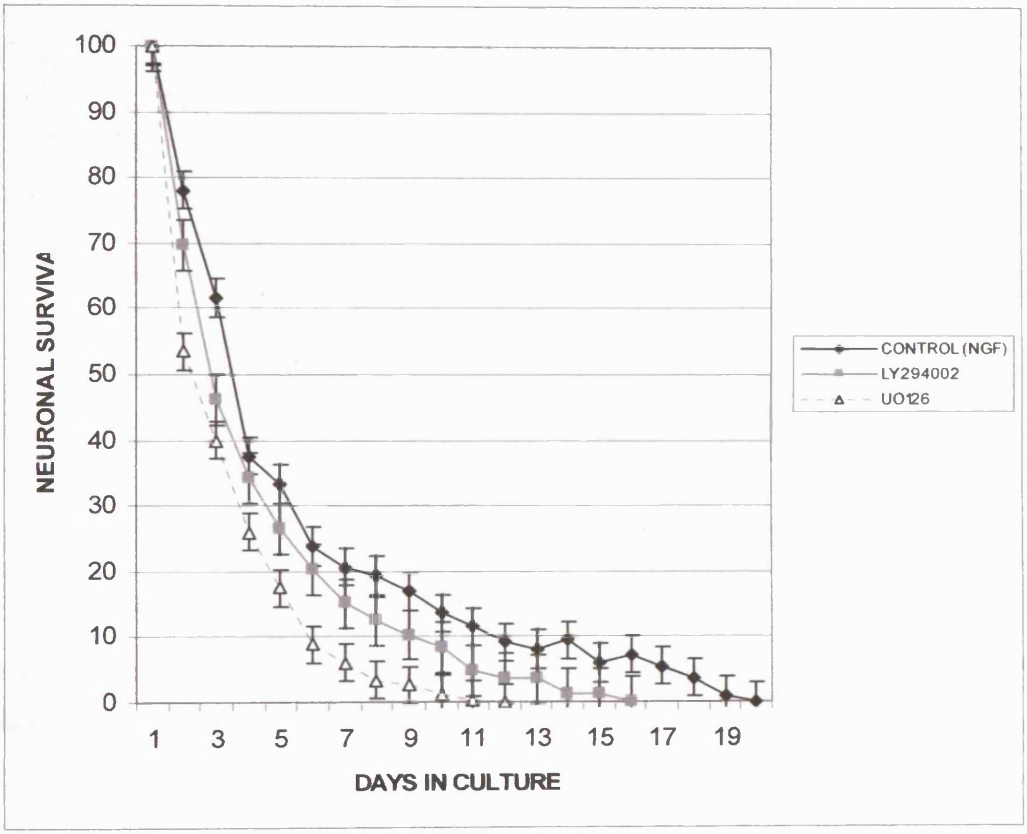

Figure 5.11: In vitro survival of the late treatment group SCG neurons from animals pre-treated in vivo at 3 months old with NGF (diamond, black solid line); pre-treated in vivo with NGF and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with NGF and treated in vitro with U0126 an Erk inhibitor (white triangle, dotted line).

Neurons pre-treated in vivo with CYT-C are significantly different from those where one of the two survival pathways had been inhibited in vitro (both $p < 0.01$). See figure

5.12. The difference between NGF and CYT-C pre-treatment in vivo is that the ERK inhibition is significantly more effective than PI3-K inhibition ($p < 0.05$) when pre-treated in vivo with CYT-C; while the NGF pre-treatment in vivo significantly affect both, ERK and PI3-K inhibition ($p < 0.05$ and $p < 0.01$ respectively).

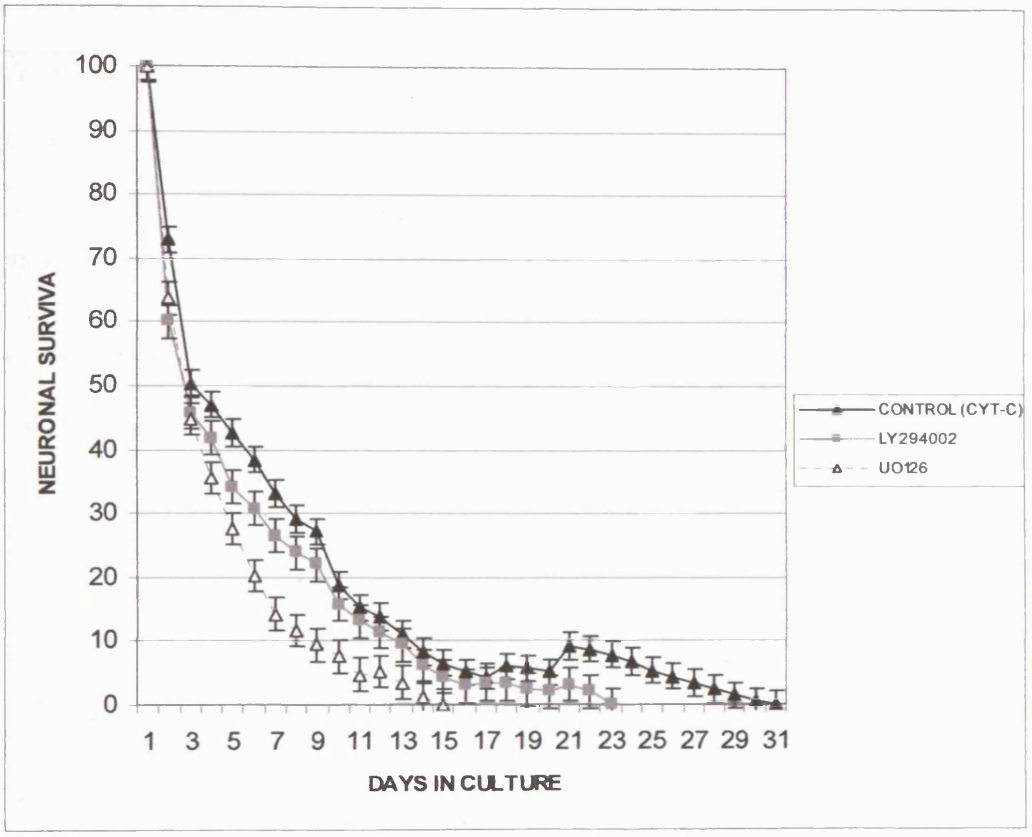

Figure 5.12: In vitro survival of the late treatment group SCG neurons from animals pre-treated in vivo at 3 months old with CYT-C (triangle, black solid line); pre-treated in vivo with CYT-C and treated in vitro with LY294002 a PI3-K inhibitor (square, grey solid line); pre-treated in vivo with CYT-C and treated in vitro with U0126 an Erk inhibitor (white triangle, dotted line).

Table 5.5 summarises the level of significance resulted from inhibition of either ERK or PI3-K pathway on the late treatment group of neurons.

p-VALUES **LATE TREATMENT GROUP**	NGF	CYT-C
UNTREATED vs. LY294002	0.0009	0.0000008
UNTREATED vs. UO126	0.001	0.000004
LY294002 vs. UO126	Not significant (0.06)	0.01
LY294002 (NGF) vs. LY294002 (CYT-C)	0.0001	
UO126 (NGF) vs.UO126 (CYT-C)	0.0212	
NGF vs. CYT-C	0.0002	

Table 5.5: Summary of the p-value results for the late treatment group

5.2.6 Across ages pharmacological inhibition of PI3-Kinase and ERK signaling pathway, and regulation of the survival induced by NGF or A-NGF pre-treatment in vivo

Results of pharmacological inhibition of PI3-K (LY294002), on SCG neurons, pre-treated with NGF is shown in figure 5.13. There is significant differences between groups ($p < 0.05$) the 18 months group has the lowest l_t, with the exception of the 8 months group during phase one. The acute group seems to be the one less affected by the pharmacological inhibition, excluding the 24 months group in phase three.

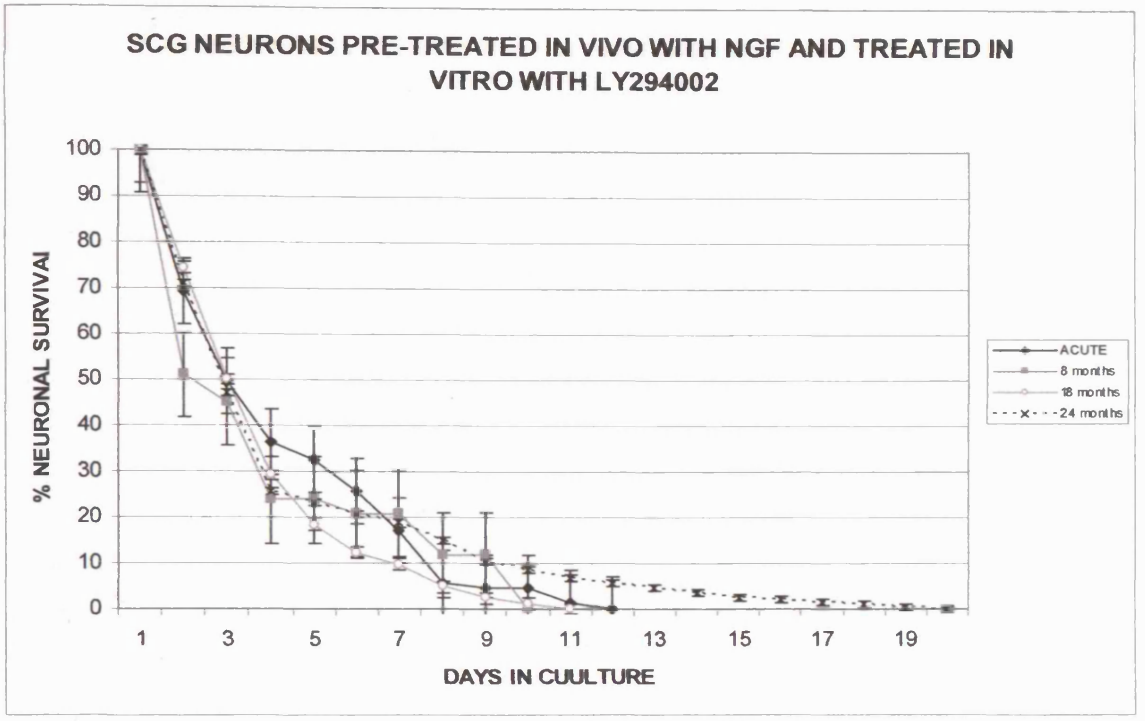

Figure 5.13: In vitro survival across ages of SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF and treated in vitro with LY294002 a PI3-K inhibitor; acute group (black diamond); 8 months old group (grey square); 18 months old group (white triangle); 24 months old group (black dotted line).

Figure 5.14 shows the effect of the ERK inhibitor, UO126, in vitro when neurons have been pre-treated in vivo with NGF. There is significant difference between groups ($p < 0.01$) even though ERK inhibitor seems to have a reverse effect compared to PI3-K inhibitor. The acute group seems to be the one most negatively affected while the 8 months is intermediate. Eighteen months seems to be affected negatively only during phase three.

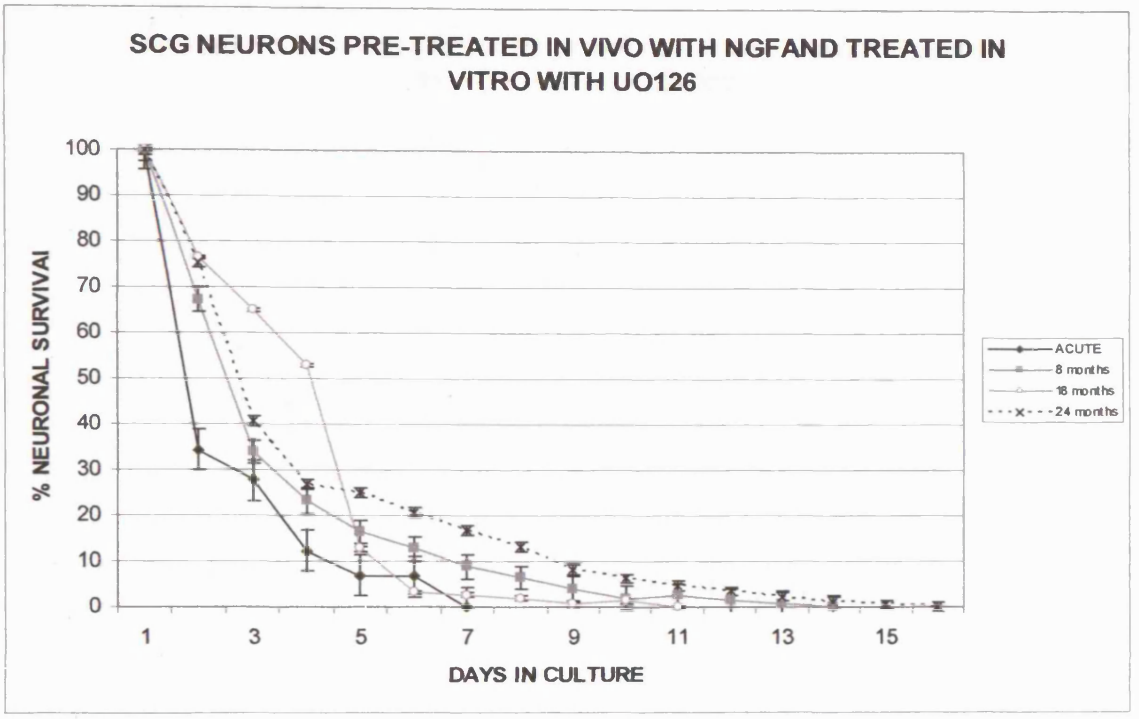

Figure 5.14: In vitro survival across ages of SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF and treated in vitro with UO126 an ERK inhibitor; acute group (black diamond); 8 months old group (grey square); 18 months old group (white triangle); 24 months old group (black dotted line).

Figure 5.15 shows results of neurons treated in vitro with LY294002 and pre-treated in vivo with CYT-C. There is significant difference between groups ($p < 0.01$) and results show behaviour similar to NGF pre-treatment with the exception that the effect is more drastic. PI3-K inhibition affects mainly the 8 months group as for the NGF pre-treated groups but the negative effect continues in phase 2.

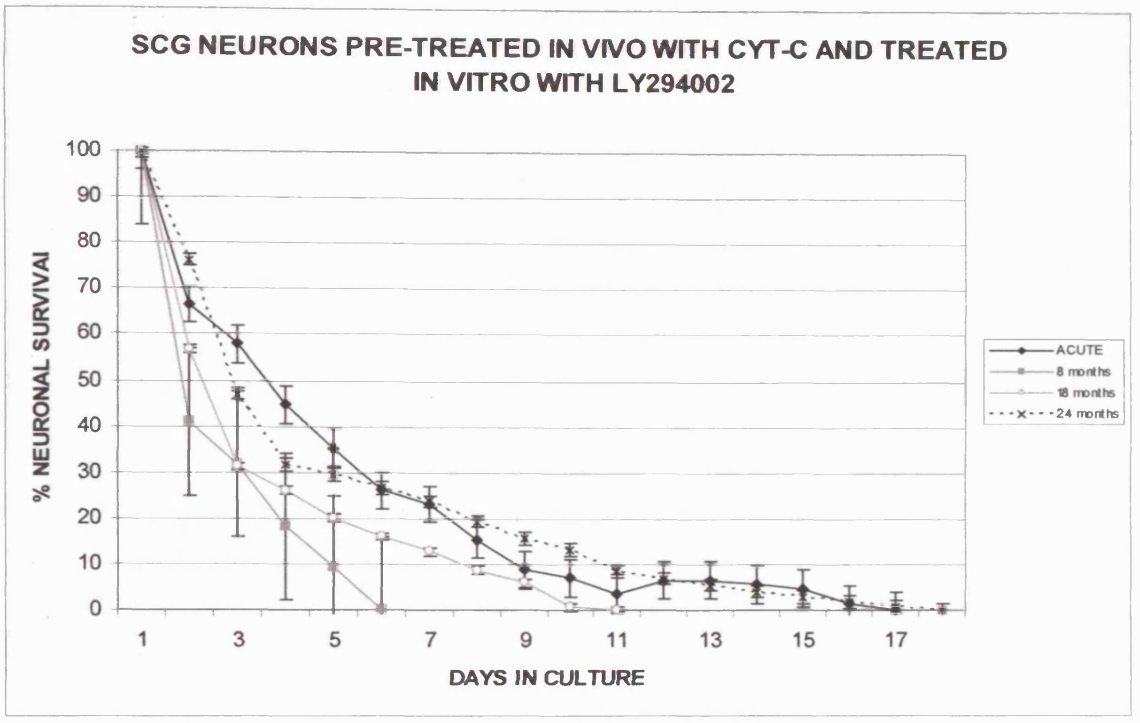

Figure 5.15: In vitro survival across ages of SCG neurons from animals pre-treated in vivo at 3-7 weeks old with CYT-C and treated in vitro with LY294002 a PI3-K inhibitor; acute group (black diamond); 8 months old group (grey square); 18 months old group (white triangle); 24 months old group (black dotted line).

Figure 5.16 shows results of neurons pre-treated in vivo with CYT-C and in vitro with ERK inhibitor. There is significant difference between groups ($p < 0.01$), and, as resulted from NGF pre-treated neurons, the ERK inhibition causes a reverse effect compared to PI3-K inhibition. The acute group is negatively affected throughout each phase.

Table 5.6 shows the ANOVA exact p-value results comparing groups between in vivo pre-treatment and in vitro treatment.

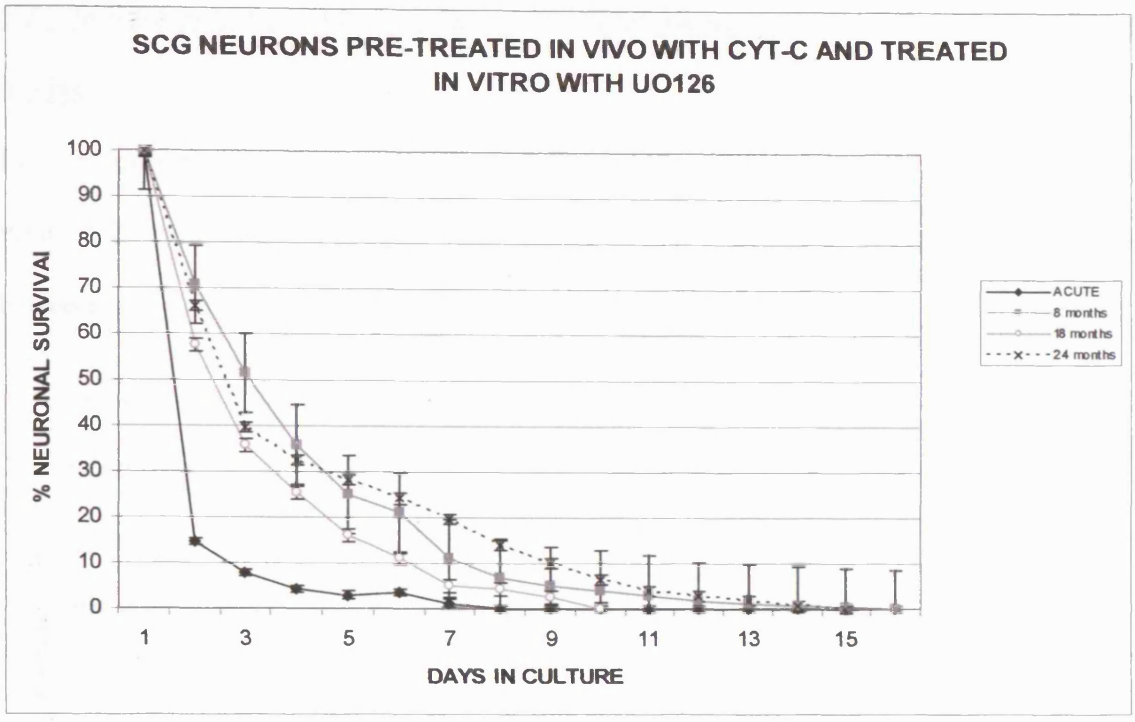

Figure 5.16: In vitro survival across ages of SCG neurons from animals pre-treated in vivo at 3-7 weeks old with CYT-C and treated in vitro with UO126 an ERK inhibitor; acute group (black diamond); 8 months old group (grey square); 18 months old group (white triangle); 24 months old group (black dotted line).

P-VALUE ACROSS AGES	NGF	CYT-C
LY294002	0.02	0.00008
UO126	0.005	0.004

Table 5.6: Summary of the p-value results across ages

5.2.7 In vitro p-Akt and GSK-3β upregulation following 'early' and 'late' pre-treatment in vivo

In vitro phospho-Akt regulation: p-Akt upregulation following early pre-treatment in vivo of 24 months old SCG neurons (figure 5.17). There is no significant difference between early pre-treatments in vivo.

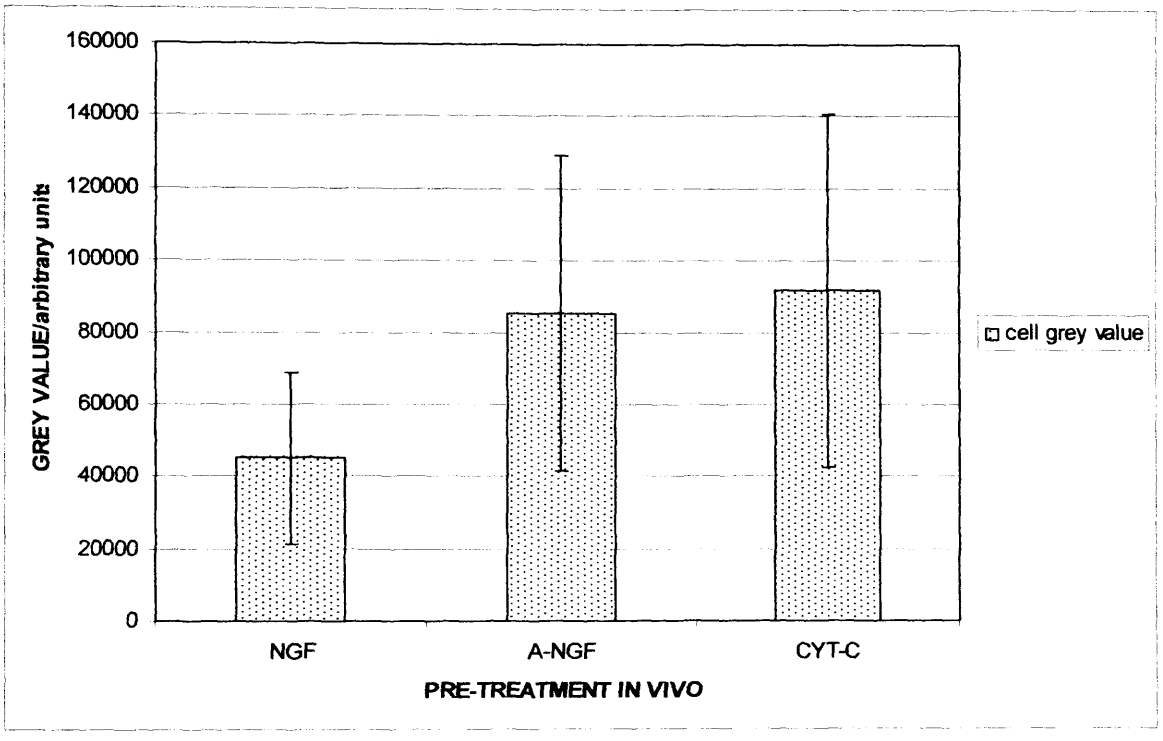

Figure 5.17: Phospho-Akt regulation of 24 months old group of SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF, A-NGF or CYT-C (control); results on immunostained sections of SCG.

Results for the 24 month group are consistent with the results of chapter 4 and previous results in this chapter, showing that vulnerable neurons have died by this age and we are not expecting any significant difference between in vivo pre-treatments.

Figure 5.18 shows that NGF late pre-treatment in vivo down regulates p-Akt. There is significant difference between grey values of neurons pre-treated with NGF compared with the control ($p < 0.05$).

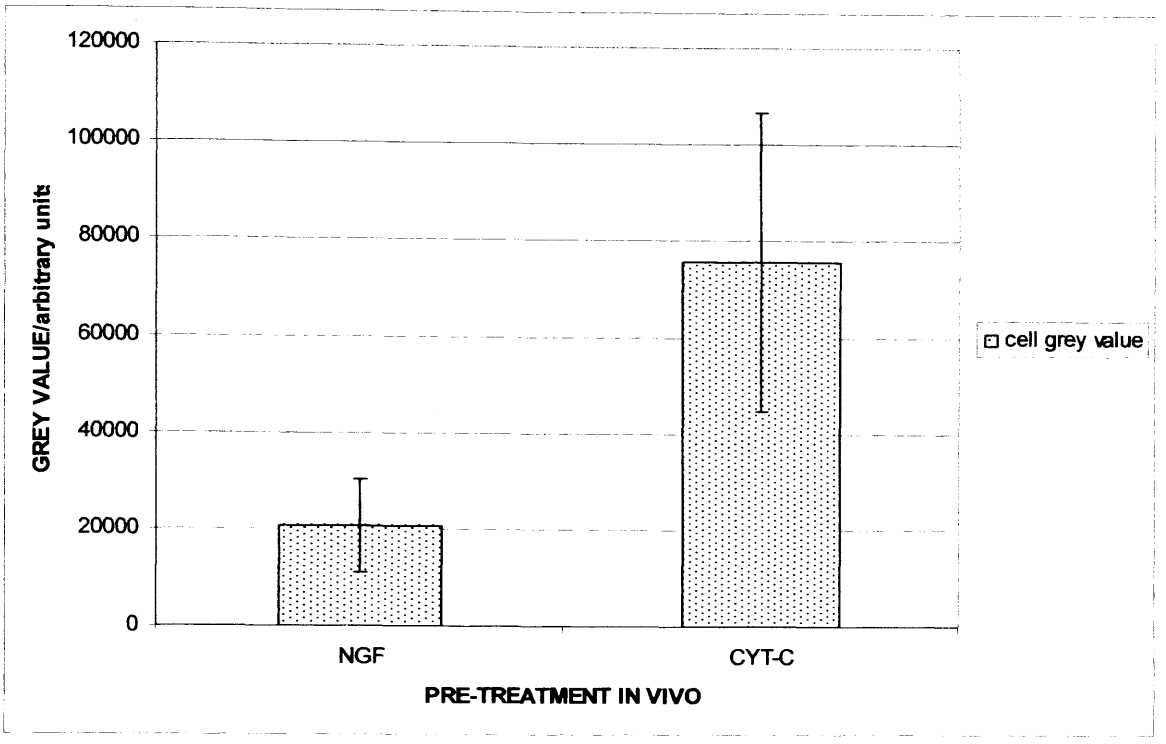

Figure 5.18: Phospho-Akt regulation of the late treatment group of SCG neurons from animals pre-treated in vivo at 3 months old with NGF or CYT-C (control); results on immunostained sections of SCG.

In vitro GSK-3β regulation:

In the 24 months group GSK-3β immunoreactivity there is no significance when it is down-regulated as a consequence of pre-treatment with NGF, compared with CytC (see figure 5.19). There are no significant differences in GSK-3b immunoreactivity between the anti-NGF and CYT-C or between the NGF and anti-NGF pre-treated groups. This result is expected because it indicates a that vulnerable neurons have already died, by 18 months according to previous data, and there is no significant effect of NGF pre-treatment on SCG neurons.

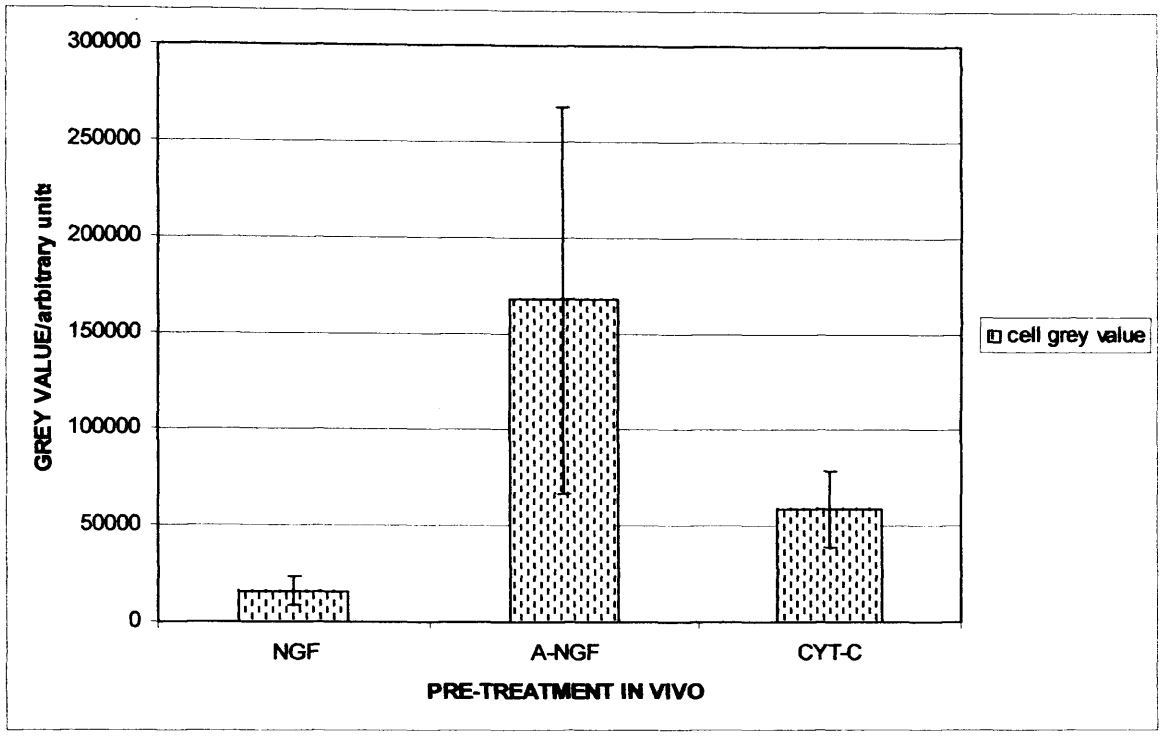

Figure 5.19: GSK-3β regulation of the 24 months group of SCG neurons from animals pre-treated in vivo at 3-7 weeks old with NGF, A-NGF or CYT-C (control). Measurements of grey values obtained from MAP1B immunostaining with BUGS.

In the late treatment group GSK-3β is significantly down regulated as an effect of NGF treatment, see figure 5.20 ($p < 0.05$). These results show comparable results with previous survival pathways experiments, where the late treatment group showed significant decrease in survival following NGF pre-treatment in vivo.

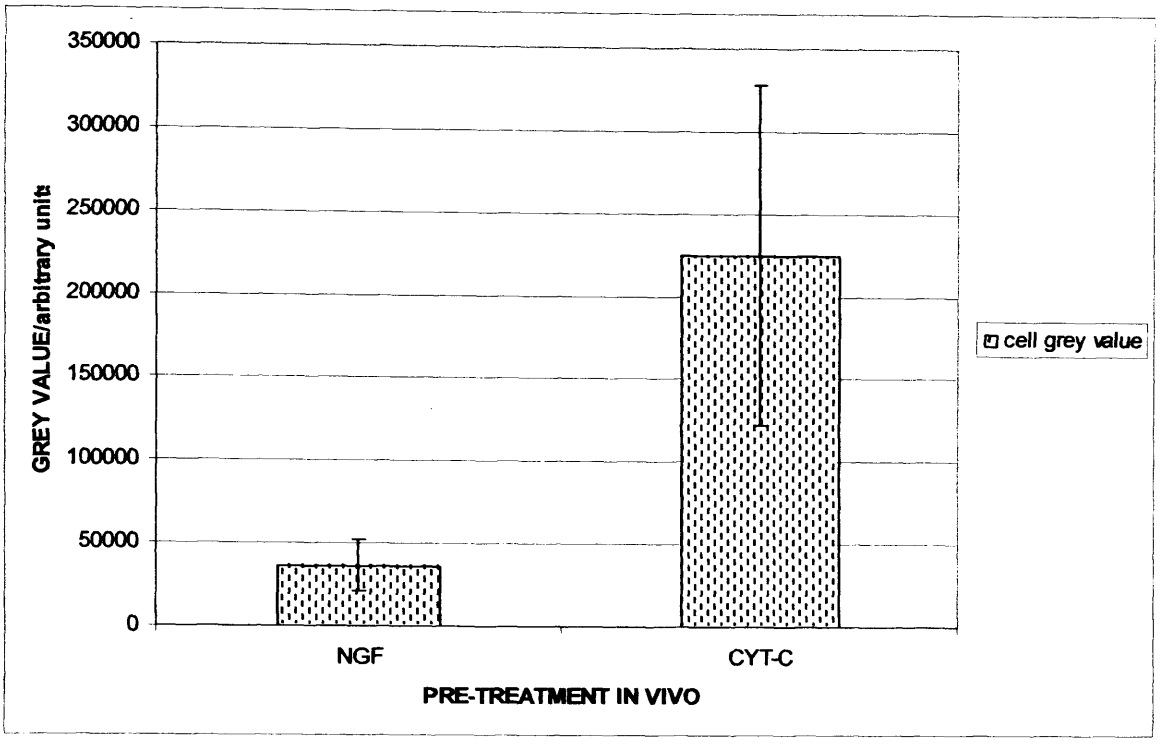

Figure 5.20: GSK-3β regulation of the late treatment group of SCG neurons from animals pre-treated in vivo at 3 months old with NGF or CYT-C (control). Measurements of grey values obtained from MAP1B immunostaining with BUGS.

Table 5.7 shows a summery of the results of section 5.7 in this chapter, comparing NGF in vivo pre-treatment to control, as well as 24 months control to late treatment control.

P-VALUE **P-Akt and GSK-3β**	**p-Akt**	**GSK-3β**
24 m	Not significant	Not significant
Late treatment	$P < 0.05$	$P < 0.05$
24 m vs. late treatment	Not significant	Not significant

Table 5.7: Summery of cell grey value significance results of p-Akt and GSK-3β in 24 m and late treatment neurons.

5.3 Discussion

Extracellular stimuli, such as NGF, are transmitted intracellularly by signaling cascades that involve the interaction of macromolecules and the generation of second messenger molecules that pass signals between the origin and the target of the signal. The principal pathways involved in survival of sympathetic and other neurons are described in the Introduction, but to summarise, one class of second messenger is generated by phosphorylation of PI3-K , while ERK/MAP-kinase are the principal mediators of a second survival pathway.

My hypothesis is that pre-treatment during early postnatal life with NGF or A-NGF would permanently affect survival pathways of SCG neurons in later life by upregulating one or both of these pathways. Mutually the general introduction and the chapter introduction describe the hypothesis that the pre-treatment in vivo is affecting both survival pathway causing differential results.

If this hypothesis is proved, can altered availability of NGF in later (adult) life have similar effects?

The questions are very similar to those raised in chapter IV: Are we looking at a maturational phenomenon, or at a lifetime neuroprotective programme? And, is early life're-setting' of neuron survival equally effective with different pre-treatments, and do the effects change with age?

Inhibition of PI3-kinase and ERK survival pathways in vitro had very different effects in the different age and treatment groups (acute, 8m, 18m, 24m and late treatment). As described in Chap. IV, effects of NGF in vivo pre-treatment resulted in a marked enhancement of neuron survival in vitro in the acute and 8m groups, with smaller effects at 18m and no effect at 24m. I attributed the age-related decline in the effect of

NGF to the death of vulnerable neurons around the 18m stage (Gatzinsky et al. 2004). The results of inhibition of PI3-kinase and ERK appear to demonstrate that long-term NGF-induced upregulation of PI3-kinase is the principal mechanism underlying the enhanced survival that I have shown. This effect is most clearly demonstrated in the acute and 8m groups and has largely disappeared by 18m and 24m. According to the results in this chapter Erk inhibitor seems to affect neurons differently compared to PI3-K inhibitor. ERK seems to kill neurons quicker (shorter survival time t to t+1) suggesting not only that PI3-K and ERK have different developmental timetables (PI3-K acting more on 8, 18, and 24 months; ERK acting on the acute and late treatment mainly), but that they might act on different populations of neurons. A preliminary experiment was carried out to see if adding both inhibitors together had an additive effect, this resulted in a total death before the counting time, suggesting that further more articulated research needs to be done in order to answer this question.

Acute group: in the acute group, the determinant phase, in showing neurons' responses to pathway inhibition, is phase one (in fact in vitro survival pathways inhibition act within few hours of application). In this age-group, when neurons are pre-treated with CYT-C, ERK1/2 inhibition (achieved by application of UO126) has a major effect on cell survival, particularly during this early phase of the cultures compared to PI3-K inhibition (achieved by application of LY294002). Furthermore, when neurons are pre-treated with NGF in vivo, PI3-K inhibition is ineffective; it is only in phase 1 that substantial reduction in neuron survival shows that PI3-K inhibition becomes effective, again. ERK inhibition remains overall significantly more effective than PI3-K inhibition and its effects are not altered by the NGF pre-treatment regime. Orike *et al.* (2001) shows that LY is effective in inhibiting in vitro survival of SCG neurons at 12 weeks,

therefore my results from studies of neurons at 7wks following CYT-C in vivo pre-treatment is evidence that PI3-K is upregulated during the postnatal period, between 7 and 12 weeks of age. Furthermore Crowder and Freeman (1998) showed that perinatal neurons are responsive to PI3-K in the presence of NGF, this may be due to the fact that the half life of the signaling NGF-Phospho-TrkA complex in adult sympathetic neurons is about 2 days, indicating that in my experiments even in the early age, the effect of NGF pre-treatment on subsequent survival in vitrois a 'conditioning' response to NGF and not just an ongoing direct effect of the NGF treatment (Tsui-Pierchala and Ginty, 1999).

Comparison of the LY and UO curves shows that both have similar curve in NGF and CYT-C graphs although survival level is different, statistical analysis shows some difference between LY for NGF and LY for CYT-C, indicating that NGF pre-treatment in vivo upregulates the PI3-K survival pathway but not the ERK1/2 survival pathway in the acute group. This suggests either a survival threshold below which neuron survival is controlled by ERK and above which is controlled by PI3-K, or alternatively, different subpopulations of neurons may be preferentially responsive to the two pathways.

8 months group: particularly interesting is the switch in the dependence of the 8 months group on PI3-K. In contrast to the acute group, the control (Cyt-C) group now shows PI3-K as the major survival pathway. This is in agreement with Orike *et al.* (2001) suggesting that there is a maturational increase in the role of PI3-K in neuron survival in the absence of NGF.

NGF pre-treatment in vivo markedly enhances survival in vitro (in agreement with the results of chapter IV). Both LY and UO seem to inhibit survival in NGF in vivo pre-treated neurons to similar extents. However, LY inhibition of PI3-K depresses survival

significantly less in the NGF pre-treated group compared to CYT-C, while the effect of UO is unchanged in the two groups. Again this suggests that the rescue is the result of an intrinsic upregulation of PI3-K signaling since it occurs in the absence of ongoing NGF signaling. Furthermore at this particular age UO inhibition seems to be unaffected by NGF pre-treatment in vivo.

18 months group: 16 months after termination of treatment neurons are old and seem to show both pathways, PI3-K and ERK1/2, to be equally inhibited with PI3-K creating more vulnerability for neurons when inhibited in phase one and two; despite the rescuing effect of NGF pre-treatment. These results suggest the idea that at this stage there is an 'attrition mechanism', on one side there is the ageing effect and on the other side the intrinsic elevation of PI3-K which keeps the NGF responsive neurons alive has been reduced to a point where LY and UO effects are no longer different. In turn, this suggests that the vulnerable neurons are now close to their survival threshold, in fact according to Gatzinsky *et al.* (2004) by 18 months many of the sympathetic neurons of the rat SCG which are vulnerable to ageing have already died.

The 24 months group: this shows a similar effect on survival when either of the pathways are inhibited. With age both pathways react similarly to inhibition therefore we might be looking at an additive effect of age and survival pathway inhibition. As expected, the responses of this group are closely similar to the 18 months group, there is no rescue effect of NGF, or depressive effect of A-NGF (as seen in chapter IV) and no difference between the inhibitory effects of LY and UO. Once again, this indicates that those neurons lifted above a 'survival threshold' by NGF pre-treatment in early life have already been lost.

Late treatment group: results are similar to those found in chapter IV; these latest results show that inhibitors in the late treatment group have a similar effect to the acute group (of this chapter) as far as the inhibitory pathways are concerned but an opposite effect with the NGF in vivo pre-treatment. In fact NGF in vivo pre-treatment is significantly decreasing survival ($p < 0.05$) possibly due to a dependence effect. Neurons treated at 3 months of age, after termination of development, may be dependent on extra exogenous NGF supplied by in vivo pre-treatment and possibly neurons become more vulnerable to external insults, which in this case are the pathway inhibitors. Late pre-treatment in vivo with NGF had significant effect on ERK survival compared with CYT-C. While in neurons pre-treated in vivo with CYT-C, ERK inhibition was principally affecting survival, neurons pre-treated in vivo with NGF seems to be equally affected by ERK inhibition and PI3-K inhibition. We may assume that we are intervening in a maturational programme with a limited time window of sensitivity, which acts through a long-lasting intrinsic upregulation of PI3-K activity.

It is also interesting to observe the effect of inhibition across ages as there seems to be a different effect according to the age stage considered. Neurons pre-treated in vivo with NGF show marked inhibition of survival in vitro with LY at 8 and 18 months, and a switching of survival pathways for the acute group and late treatment (i.e. UO inhibition affecting survival significantly more compared to LY inhibition); while UO is most effective on the acute group. For all groups NGF pre-treatment in vivo increased survival.

These data suggest, as I have argued previously, that PI3-K becomes the principal survival pathway in adult sympathetic neurons, while ERK1/2 is more important during earlier stages of development. In addition, altering availability of NGF during early

postnatal life appears to enhance survival of adult sympathetic neurons through an intrinsic upregulation of PI3-kinase activity which can be maintained in the adult neuron in the absence of NGF. The significance of this observation will be discussed below in chapter VI.

PI3-K is a key enzyme for regulating neuronal cell survival; the downstream signalling partner of PI3-K is Akt. However, there is still some debate about the role of Akt following NGF stimulations. Yano *et al.* (1998) and Virdee *et al.* (1999) reported that Akt can promote survival independently of PI3-K activity, and this may be driven by anti-apoptotic second messengers such as calcium or cAMP (Filippa *et al.*, 1999).
Philpott *et al.* (1997) reports questioned the role of Akt in NGF mediated survival of SCG neurons. My results shows that PI3-K mediates survival of SCG neurons in response to NGF and some preliminary results (see chapter VI) show that Akt is upregulated following in vivo NGF pre-treatment. We therefore deduce that both Akt and PI3-K are upregulated following NGF in vivo pre-treatment.
My results show that for both the 24 months group and the late treatment group there is no significant difference in the p-Akt regulation.
There is a possibility that the survival pathway may change according to the age of the neurons, in fact my signalling pathway experiments showed that the two survival pathways (PI3-K and ERK1/2) shifted in relative importance according to the age group of the neurons; in addition to the effect of cell loss due to ageing.

Neurotrophins were originally identified as survival factors only, but recent studies reveals how they elicit many biological effects, including proliferation, synaptic modulation, and axonal pathfinding; specifically the nature of the signaling cascade and

the biological responses that is elicited are specified by the ligand, the temporal pattern and the special location of the stimulation. Hetman and Xia review (2000) extensively how both ERK and PI3-K can mediate anti-apoptotic signaling of neurotrophins; suggesting that in most cases of trophic deprivation PI3-K is the main player, whereas ERK dominates as the major neuroprotective mechanism in damaged cells.

The strength of this experiment is to examine the relation of a treatment effect applied in vivo (i.e. manipulation of NGF availability) to the inhibition of specific survival pathways in vitro; therefore we look at an effect in the animal and we observe the changes at different developmental stages in vivo but we consider specific pathways in vitro.

According to Franke *et al.* (1997) although multiple responses are not probably explained by the action of a single downstream target, the signaling pathway from PI3-K to Akt may mediate some cellular responses of PI3-K (Burgering and Coffer, 1995; Cross *et al.*, 1995; Franke *et al.*, 1995; Kohn *et al.*, 1995) such as protection from apoptosis (Dudek *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997; Khwaja *et al.*, 1997; Kulik *et al.*, 1997).

Downstream of PI3-K there are both Akt and GSK-3β, both produced some interesting preliminary results in this study. Results form the 24 months group showed, as expected, no significant difference when pre-treated in vivo with NGF, according previous data, the vulnerable neurons have probably already died by this age and we are dealing with a subset of 'super neurons' that are masking the effect of NGF. Particularly interesting are the results for the late treatment group, where the effect of NGF pre-treatment in vivo significantly downregulates p-Akt and GSK-3β. These results show that PI3-K provoked a 'dependence effect' where neurons that have been

receiving NGF in vivo after termination of development die once exogenous NGF supply stops. GSK-3β resuction in phosphorylayion causes a decrease in apoptosis, possibly as an effect of the secondary survival pathway, ERK. Dozza *et al.* (2004) showed how both PI3-K and ERK are causing down regulation of GSK-3β in neuroblastoma cells, it can be hypothesized that a similar mechanisms is affecting SCG neurons. Bhat *et al.* (2000) studied the effect of NGF withdrawal from differentiated PC12 cells which lead to phosphorylation at Y^{216}, a GSK-3β regulatory site, leading to GSK-3β activity and cell death. Crowder and Freeman (2000) pointed out how in sympathetic neurons PI3-K and Akt are important mediators in cell survival and how GSK-3β is inhibited upon phosphorylation by Akt. According to Wagner *et al.* (1996) and Lovestone *et al.* (1994), GSK-3β in addition to a role in apoptosis it hyperphosphorylates the microtubule-associated protein τ, a mechanism implicated in paired helical filament formation in Alzheimer's disease. The link with Alzheimer's disease rises interest for future research.

CHAPTER VI

GENERAL DISCUSSION

6.1 Brief discussion of results

This thesis shows how pre-treatment in vivo with NGF at a specific point during development affects SCG neurons (see section 6.2). In summary, results show that following pre-treatment in vivo there is an effect on neuronal number, with differential effect on different subpopulations of neurons (MCA versus iris projecting neurons). MCA projecting neurons (a vulnerable subpopulation of SCG neurons) increase in number (acute group), growth and innervation of specific target tissues following NGF pre-treatment in vivo, showing a maintained plasticity after termination of development and therefore a potential target site for future therapeutics.

NGF pre-treatment in vivo also increases neuronal survival time throughout life in vitro, showing that the limited supply of NGF, in real life, primes neurons to a reduced potential.

The results on survival also show a difference in the mode of action between the two major survival pathways (PI3-K and ERK), with PI3-K being the predominant in adult life and ERK acting mainly in early life. This shows a double survival mechanism which is both plastic and capable of shifting predominance according to factors such as NGF stimuli and/or ageing.

Furthermore if the NGF pre-treatment in vivo is applied after termination of development, neurons show plasticity by developing an 'addiction'/dependance to NGF; pre-treatment termination results in death of the neurons.

Preliminary results show upregulation of Akt which is downstream of PI3-K (see figure 5.1 in chapter V), and is activated in NGF-dependent survival of SCG neurons (Pierchala *et al.*, 2004). Biological consequences of Akt activation are survival, increase in cell number and growth, which are all characteristics relevant to cancer-cell growth. In fact Hanahan and Weinberg (2000) observations of human cancers and animal models indicate that tumour development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human or animal cells into cancer cells.

Further preliminary results show reduction of the GSK-3β phosphorylation (see figure 5.1 in chapter V), which inhibits and is determinant for cytoskeletal rearrangement, glucose metabolism and cell survival; regulation of GSK-3β has been widely studied in relation to Alzheimer's disease. Alzheimer's disease is associated, among other histological changes in the brain, with an intracellular deposition of neurofibrillary tangles that are composed of the microtubule-associated protein *Tau*; GSK-3β phosphorylates *Tau* and is therefore implicated in the progression of Alzheimer's disease (Teter and Ashford, 2002).

6.2 Detailed discussion

6.2.1 Chapter III: neuron numbers and growth

Results from chapter III showed an increase in SCG neuronal number (ex vivo) due to NGF pre-treatment in vivo, as opposed to control where neurons which have not been pre-treated in vivo with NGF showed a decrease in neuronal number with age ex vivo (significant for the MCA-projecting neurons and less significant for the iris-projecting neurons). Increased survival in SCG neurons by NGF pre-treatment in vivo is thought to have been achieved by rescuing neurons that would otherwise have undergone atrophy due to loss of axon branches and dendrites and neuronal death would have caused neighbouring neurons to die

too (Thrasivoulou and Cowen, 1995; Andrews *et al.*, 1996). Understanding the effect of altered neurotrophin on the ageing of the nervous system will have important implications in understanding age-related neurodegenerative diseases. According to Kudwa *et al.* (2002), accumulation of exogenous NGF decreases with age in target tissues of SCG neurons following intracerebroventricular infusions of NGF.

A further interesting result is the plasticity of young MCA-projecting neurons compared to iris-projecting neurons: results showed that MCA-projecting neurons are very responsive to the availability of NGF, and with NGF antibodies there was a significant decrease in survival of MCA-projecting neurons in vitro.

Additionally, growth of axons and dendrites has been shown as a consequence of exogenous NGF supply on NGF neurons both in vivo and in vitro (Gavazzi and Cowen, 1993; Andrews and Cowen, 1994; Thrasivoulou and Cowen, 1995; Isaacson and Crutcher, 1998; Orike *et al.*, 2001). Previous studies have not investigated the permanent effect of NGF on SCG neurons across ages, while this research examined such effect at several age points and it is important to remember that there are high levels of NGF only during the treatment but it returns to normal levels shortly after termination of the treatment. Chapter III results showed an increase in neurite numbers and lengths following NGF pre-treatment in vivo. It is clear in this case that NGF was stimulating growth of neurons in early age, but not at 24 months when the effect of NGF was not stimulating growth anymore, while anti-NGF was clearly decreasing growth. These results show plasticity in young age which disappear some time before 18 months of age. Further results of chapter III show an increase of innervation in specific target tissues, following pre-treatment in vivo with NGF. MCA target tissue shows an increased innervation compared to control, in accordance with previous results showing an increased number in MCA-projecting neurons. This supports

the idea that the limited amount of NGF available in early development causes MCA-projecting neurons to be more plastic compared to iris-projecting ones, in order to survive. Iris neurons may be already maximally stimulated and therefore have no further capacity for plasticity. Kuchel *et al.* (1997) showed that MCA-projecting neurons are more vulnerable to atrophy specifically with age causing a decrease of innervation of the specific target tissue.

The late treatment group showed a decrease in innervation which also reflects results in chapter IV, where an addiction effect is hypothesized: a large availability of NGF supplied by the pre-treatment in vivo and a sudden lack of extra NGF caused neurons to have an increased vulnerability and die and therefore a decreased innervation of the target tissues.

As we now understand the extent of the effect of in vivo pre-treatment with NGF, future research should investigate the mode of action (maybe) by looking at receptors.

Miller *et al.* (1991, 1994), Wyatt and Davies (1995) studied NGF receptors (trkA, p75) and specifically trkA and showed that trkA receptors of SCG neurons of rats and mice before or after birth do not respond to NGF treatment. However, according to Horton *et al.* (1997), p75 receptors do increase after NGF treatment. More specifically, the ratio of trkA and p75 determines the SCG response to exogenous NGF. In this research, observing the differential effect that pre-treatment with NGF in vivo has on survival compared to growth of SCG neurons, it would be interesting, for future research, to investigate how exactly TrkA and p75 are regulated in response to NGF pre-treatment in vivo.

6.2.2 Chapter IV: survival and mortality in vitro and external factors

Orike *et al.* (2001) showed how 12 weeks old SCG neurons in culture survive in the absence of neurotrophins, while retaining their growth dependency on these neurotrophins. Furthermore if NGF is administered in vivo, as it is done in this PhD research, rather than

in vitro (Orike *et al.*,2001; Price *et al.*, 2005), and at a crucial time before termination of development, there is a permanent upregulation of PI3-K/Akt pathway which increases survival and is detected up to 15 months after termination of treatment.

Results in chapter IV showed that the effect of NGF pre-treatment in vivo on neuronal survival in vitro when, at the time of culturing, there were no abnormal levels of NGF. Chapter IV results reported a significant increase in survival and a decrease in mortality which were not only a result of the acute group but were clearly present in the 8 months group as well. More precisely, the acute group results delayed the onset of cell death in vitro causing also a change in the survival pattern, while the 8 months group, despite the NGF pre-treatment in vivo, caused an increased number of neurons to survive, but for a shorter time. Possibly the treatment was causing precocious ageing of the neurons: results from the mortality assays revealed an unexpectedly higher vulnerability of NGF pre-treated neurons compared to controls. Early ageing in the 8 months group was possibly due to a variation of the activity of the tumour suppressor p53 which can upset the balance of neuronal turnover by affecting the rate of cell proliferation. Medrano and Scrable (2005) showed in genetically engineered mice in which p53 activity was increased, that premature loss of neurogenic capacity is linked to accelerate organismal ageing.

Results from chapter IV showed that while at 18 months NGF in vivo pre-treated neurons tended to retain the positive effect of NGF and were affected negatively by anti-NGF, at 24 months the effect of the pre-treatment in vivo was no longer distinguishable from the effect of ageing cells. At 24m neurons pre-treated with anti-NGF or NGF had either different survival mechanisms or their 'survival threshold' was set at a different level so that they were unaffected by pre-treatment.

The increased survival phenomenon appears to resemble the numerous examples of a retained memory, a kind of conditioning stimulus in neural, immune and other cells indicating a long term NGF effect (Dutton *et al.*, 1998; Kamimura and Murakami, 2005). Results from chapter IV, on neurons from the late treatment group, showed that if treatment is applied late, after termination of development, neurons were not primed, showing again that priming may be related to a long-term memory of the cells by resetting the survival pathway through treatment.

There is a developmental programme involving local differences in availability of neurotrophins which has 'knock-on' or pleiotropic effects on survivability in vitro, and perhaps in vivo. What is the purpose of this programme? Clearly, if it were only to regulate neuronal survival in adult life, selective pressure would ensure that all neurons were exposed to maximum levels of NGF. According to the results on subpopulations of chapter IV, iris neurons already survive maximally, while MCA neuron survival can still be boosted as a result of different levels of neurotrophins. Low levels of NGF are 'adaptive' for MCA-projecting neurons (related perhaps to the small size, relative simple axonal and dendritic arbors, Andrews *et al.*, 1996) and the pleiotropy is that these adaptive feature become a hazard in ageing. These results are in accordance with findings by Gatzinsky *et al.* (2004), and therefore propose another link to the hypothesis that the capacity of NGF uptake in later life is linked to the age-related vulnerability of specific neurons.

NGF is not the only neurotrophin affecting survival of SCG neurons; therefore, the preliminary results of chapter IV on the effect of external factors (insulin, IGF, BDNF and GDNF) on survival and mortality are particularly interesting. Insulin and IGF-1 are growth promoting peptides (LeRoith and Roberts, 1993); in the nervous system IGF-1 appears to regulate tissue growth, supporting differentiation of fatal neurons in culture, stimulating

protein synthesis and promoting neurite outgrowth (Recio-Pinto *et al.*, 1984; Ishii and Recio-Pinto, 1987; Werner *et al.*, 1989; 1994; Mozell and McMorris, 1991; Heidenreich, 1993), while insulin regulates and restores IGF-1 levels in rats (Taylor *et al.*, 1987).

Results from chapter IV showed that neurons which were pre-treated with NGF in vivo were significantly affected by in vitro addition of insulin or IGF-1, by increasing neuronal survival in controls and decreasing survival with NGF pre-treatment, suggesting there may be an optimal survival threshold which neurotrophic factors such as insulin and IGF-1 tend to re-establish when altered by NGF pre-treatment in vivo. Therefore it can be hypothesized that neurotrophic factors are the result of a homeostatic mechanism controlling survival (and perhaps growth) which prevents a synergistic effect of growth factors which could cause either too many neurons to survive (during development), or those neurons that do survive to grow too large (during adulthood) and hence force the neuron out of its 'proper' phenotypic shape. Kimpinski and Mearow (2001) showed that IGF-1 can elicit extensive neurite extension in adult sensory neurons and this can potentiate NGF-induced neuritogenesis.

According to Foukas *et al.* (2004) insulin has an important effect in NT signalling; insulin has been shown to stimulate PI3-K (Rondinone *et al.*, 2000).

Therefore, while IGF-1 is related to Akt activity, insulin affects the PI3-K/Akt signaling pathway through phosphorylation of the insulin receptor substrate-1 (IRS-1), (Foukas *et al.*, 2004).

6.2.3 Chapter V: neuronal survival pathways in vitro

Results from chapter V revealed some interesting information on the main survival pathways in SCG neurons: PI3-K/Akt which were recently extensively reviewed by

Sofroniew *et al.* (2001) and Vivanco and Sawyers (2002) and the ERK pathway recently reviewed by Segal (2003). Altering availability of NGF during early postnatal life appeared to enhance survival of adult sympathetic neurons through an intrinsic upregulation of PI3-kinase activity which was maintained in the adult neuron in the absence of NGF. In fact, PI3-K activates phosphatidylinositol (3, 4, 5) in a wide range of tumours which in turn activates Akt, triggering responses that drive tumour progression such as cell growth, proliferation, survival and mortality. Akt results are preliminary and may be used as base for future research possibly using Western blot to quantify the qualitative Akt results in this thesis.

It has never been reported before that survival signaling pathways (PI3-K and ERK) in SCG neurons, pre-treated with NGF in vivo before the end of development, work together, shifting importance with age: ERK being the major survival pathway before the end of development and PI3-K in adulthood and during ageing. As expected, late treatment (after termination of treatment) did not affect significantly PI3-K survival pathway (results form chapter V). What causes the neurons to die? It appears that at a certain stage in development, about the time that neuronal axons reach their targets, neurons begin to shift to an apoptotic state, and unless something happens, they die. This suggest that they receive a 'signal' that counteracts the apoptotic self-destruction programme, and that signal comes from trophic factors binding to trophic factor receptors in the neurons' plasma membrane, activating various internal signaling pathways involving protein kinases.

Earlier studies showed ERK, as the main survival pathway regulating cell proliferation and survival (Xia *et al.,* 1995; Mazzoni *et al.*, 1999). Nevertheless, in recent years new discoveries regarded PI3-K as an important regulator in mammalian cells (Hlobilkova *et* al.,

2003; Pierchala *et al.*, 2004). On the contrary, Lentzsch *et al.* (2004) reported that some survival pathway, including PI3-K/Akt and MAPK/ERK, are redundantly stimulated to independently promote proliferation and survival of multiple myeloma cells. The present research shows that both survival pathways might be working together to promote survival, shifting predominance with age, possibly due to a redundancy of survival pathways.

Results from chapter V showed interesting effects of 'NGF dependence' in the late treatment group, where neurons pre-treated in vivo with NGF tended to be negatively affected by lack of NGF in culture and therefore showed a decreased Akt activity. This is possibly due to the fact that adult neurons supplied with large amounts of NGF might be developing dependence to NGF. Eichler *et al.* (1992) described the dependence between intracellular calcium and NGF in dorsal root ganglion (DRG) in culture, explaining that a variation in calcium channels activation may affect survival in adult neurons.

Results from chapter V showed a decreased activation of GSK-3β following pre-treatment in vivo with NGF in all groups (young and ageing neurons as well as in the late treatment group). One downstream target of Akt is GSK-3β which, among other functions, associates with presenilin and phosphorylates the microtubule-associated protein tau, implicating GSK-3 in the progression of Alzheimer's disease (Harwood, 2001).

Active Akt phosphorylates and inactivates GSK-3β as well as mediating cell survival. According to Bhat *et al.* (2000) GSK-3β increases in neurons susceptible to degeneration but its pathway exerts a pro-apoptotic effect independently in response to NGF deprivation. GSK-3β is also a downstream target of insulin stimulation and regulates glycogen synthase, the protein translation initiation factor eIF2B, and the transcription factor C/EBPα (Cohen,

1999). GSK-3β is involved in the Wnt signaling pathway, influencing pattern formation during embryonic development and regulation of cell proliferation (Dale, 1998).

Cordes and Beuningen (2004) provided molecular insight into the importance of extracellular-matrix (ECM)-dependent cell survival; where they suggested a novel pathway that makes direct phosphorylation of GSK-3β by the integrin-linked kinase (ILK), showing that ILK, Akt and GSK-3β are involved in modulation of the cell cycle. Since integrin and growth factor receptors are colocalised at the source of integrin signalling, which are called focal adhesions, convergence and mutual modification between these networks are highly likely to occur. This crosstalk could result in differentiated regulative schemes of cellular functions such as cell growth and survival.

Therefore results in chapter V of down regulation of GSK-3β in all groups might be due to an upregulation of Akt (see also figure 5.1 chapter V). Differently in the late treatment group the decreased activation of GSK-3β could be hypothesized as an effect of ERK (Dozza *et al.*, 2004) which acts in the same way as PI3-K in blocking GSK-3β and therefore increasing survival.

6.3 Future research and alternative treatments

The results of Ruit *et al.* (1990) showed that sympathetic ganglion cells remain dependant on NGF for survival and maintenance of dendritic geometry even in maturity and old age. According to my initial results, neurons are NGF independent but benefit from high level of NGF availability by changing their response during survival. It seems that NGF availability early in life results in more independency from NGF later; with the exception of neurons which have been pre-treated with NGF 'later in life' and develop dependence.

NGF might have caused intrinsic altered regulation not dependent on PI3-K receptors so that NGF acts on survival, through down regulation of PTEN.

Ruit and Snider (1991) repeated a similar treatment in the 2-3 postnatal weeks showing a marked effect on survival, size and arborisation 6 months after completion of treatment, when number, size and branching of cells changed considerably in SCG neurons. Ruit and Snider (1991) suggest number, size and branching of cells remain malleable throughout postnatal life, and they also sustained the idea that primary dendrites, a fundamental determinant of organization within sympathetic ganglia, were permanently altered. A further hypothesis for future research is that NGF treatments might have influenced integrins, which affect survival and growth, interacting with laminin and also with PI3-K. According to my results, on 7 weeks old rats, no permanent alteration on neurites in vitro was visible yet. This might also be due to the difference in the injected quantities of neurotrophic factor and antibodies, which were ten times lower in my experiment compared to Ruit *et al.,* the emerging idea was that NGF influences primary dendrites only during the critical development period, while in maturity NGF acts as 'maintenance' factor, but neurons lose the capacity to respond with wholesale rearrangements of dendritic architecture.

According to Orike *et al* (2001) SCG neurons acquire NGF-independent survival by week 12, but they remain still dependent on neurotrophins for growth. On the other hand my results show survival independence already at week 7 of age with NGF treatment increasing both time as well as number of surviving cells, suggesting that NGF treated cells might have retained higher survivability.

The present research is a comprehensive investigation on the effect of NGF pre-treatment in vivo which raises numerous questions on potential future research which explores mechanisms of survival into details. For example PTEN, a major tumour suppressor

protein, causes dephosphorylation of PIP_3 (a second intracellular messenger produced by PI3-K) and therefore deactivation of PI3-K (Leslie and Downes, 2002). PTEN is one of the most common targets of mutation in human cancer; its cellular functions have been reported using different experimental model organisms; regulation of cell division, cell survival, apoptosis and cell migration in human and mouse cells, involvement in dauer formation in *Caenorhabditis elegans*, and regulation of cell size in Drosphila eye (Huang *et al.*, 1999).

The pre-treatment in vivo, used in this research, is not resulting in deactivation of PI3-K, as Akt resulted in being upregulated following the treatment in vivo; consequently, as an investigator should always raise questions for future research, the natural question for future studies is: how is the antagonistic effect of PTEN acting on PI3-K signaling?

Graff (2002) suggested that functional loss of PTEN and subsequent activation of the Akt pathway have been implicated in the progression of prostatic adenocarcinoma; the Akt pathway can suppress the apoptotic response, undermine cell cycle control and enhance production of key growth and survival factors.

Akt and Erk regulate caspase-mediated apoptosis in prostate cancer cells via p53 (Hu *et al.*, 2005); p53 is a proapoptotic pathway which is suppressed by activation of PI3-K, therefore future research might concentrate in understanding how p53 causes cells to arrest or die in case of DNA damage, hypoxia, oxidative stress, excessive mitogenic stimuli, or telomerase shortening (reviewed in Hahn and Weinberg, 2002). At the same time excess p53 activity causes ageing (Sharpless and DePinho, 2002) in response to increased metabolism, ROS and DNA damage.

At the moment there are no studies linking autophagy and diet restriction to PI3-K activation. Autophagy, meaning 'self-eating', is activated during gross developmental changes and times of nutrient deprivation and wanes with age, it is a key mechanisms in modulation of insulin signaling and caloric restriction, promoting longevity. Insulin, binding its receptor on the plasma membrane, activates PI3-K. Genetic interaction studies by Rusten *et al.* (2004) on Drosophila showed that PI3-K signaling represses programmed autophagy, and that there is a link between hormonal induction of autophagy and regulatory function of the PI3-K signaling pathway in vivo.

In order to target specific groups of cells precisely, a variation in treatment regimes may be appropriate as these may give more specific results: a change of regime of treatment where synthetic NGF instead of NGF 2.5 S Murine may be used. The synthetic NGF having a smaller molecular weight will be easily absorbed through the blood-brain barrier and therefore less likely to create inflammation.

Xie and Longo (2000) studied neurotrophic mimetic strategies. They identified specific neurotrophin protein domains likely to modulate receptor interaction, guiding synthesis of neurotrophin small-molecule peptidomimetics corresponding to individual domains and functioning via selected receptors to trigger neurotrophic-like signal transduction. They also suggested the possibility to design neurotrophin antagonists that would inhibit neurotrophin action in the context of neurotrophin-induced cell death, aberrant sprouting, and growth in general. Mahoney and Saltzman (1999) studied an alternative effective local delivery of NGF by implanting small polymer pellets that slowly release NGF; illustrating

pharmacotectonics a drug-delivery system arranged spatially in tissues to create a concentration field which enhance the action of potent agents.

6.4 Summary

The main points summarizing the effect of NGF pre-treatment in vivo on SCG neurons across ages, as established in chapter III to V are as follows:

- Increased number of neurons in the ganglia;
- Vulnerable subpopulation of neurons increased in numbers;
- Increased neurite growth both in early as well as late treatment;
- Increased innervation in target tissues;
- Increased survival time of single neurons and decreased mortality;
- Constitutively activated survival pathways.

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APPENDIX

GLOSSARY

- **A-NGF**: Anti NGF antibody;
- **BDNF**: Brain derived neurotrophic factor;
- **CYT-C**: Cytochrome-c used as a control for its similarity to NGF in structure and molecular weight;
- **GDNF**: Glial derived neurotrophic factor;
- **GSK-3β**: glycogen synthase kinase-3β;
- **IGF**: Insulin-like growth factor;
- **MCA**: Middle cerebral artery;
- **NGF**: Nerve Growth Factor first discovered by Rita Levi-Montalicini;
- **PI3-K**: phosphatidylinositol 3-kinase;
- **SCG**: Superior cervical ganglia;
- **Terms from survival analysis for describing survival of cells in culture:**
  - $\mathbf{N_t}$: Number of live cells;
  - **t to t+1**: Total period in culture;
  - $\mathbf{d_t}$: Number of dead cells;
  - $\mathbf{l_t}$: Cumulative proportion of surviving cells;
  - $\mathbf{q_t}$: Specific mortality per unit time (period) in culture;
  - $\mathbf{p_t}$: Period-specific survival ($p_t = 1 - q_t$);
  - $\mathbf{u_t}$: Mortality rate (per period);