Nerve Growth Factor and Neuronal Cell Death

J. Regino Perez-Polo, Perry J. Foreman, George R. Jackson, Din-e Shan, Giulio Taglialatela, Larry W. Thorpe, and Karin Werrbach-Perez*

Department of Human Biological Chemistry and Genetics, and the Marine Biomedical Institute, The University of Texas Medical Branch at Galveston, TX 77550

Contents

*Author to whom all correspondence and reprint requests should be addressed.

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Abstract

The regulation of neuronal cell death by the neuronotrophic factor, nerve growth factor (NGF), has been described during neural development and following injury to the nervous system. Also, reduced NGF activity has been reported for the aged NGF-responsive neurons of the sympathetic nervous system and cholinergic regions of the central nervous system (CNS) in aged rodents and man. Although there is some knowledge of the molecular structure of the NGF and its receptor, less is known as to the mechanism of action of NGF. Here, a possible role for NGF in the regulation of oxidant—antioxidant balance is discussed as part of a molecular explanation for the known effects of NGF on neuronal survival during development, after injury, and in the aged CNS.

Abbreviations: K_d, equilibrium dissociation constant; kD, kilodalton; MC192, monoclonal antibody 192; MC20.4, monoclonal antibody 20.4; NGF, nerve growth factor; NGFR, NGF receptor; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; ALCAR, acetyl-L-carnitine; TM, tunicamycin; WGA, wheat germ agglutinin; RA, retinoic acid.

Index Entries: NGF; NGF receptor; receptor-associated protein; purification; acetyl-L-carnitine; aged; cell death; neurotrophic factor; growth factor; retinoic acid; astrocyte lymphocyte; mitogen.

Cell Death in the Nervous System

There are several developmental and physiological events that have cell death at their core, such as invertebrate metamorphosis, the death of T-lymphocytes in immune reactivity, or neuronal death cell during development. The latter, neuronal cell death during development, is a common occurrence in the nervous system. It can occur as a corollary of the competitive innervation of target tissues at critical stages during development (*see* Fig. 1) as a consequence of traumatic injury, or as one of several manifestations of aging (Cowan, 1973; Hamburger and Oppenheim, 1982; Johnson et al., 1986; Oppenheim, 1985; Perez-Polo and Werrbach-Perez, 1988; Ramon y Cajal, 1928; Thoenen et al., 1981) (Table 1).

Table 1
Neuronal Cell Death

—Competition for target derived trophic factors in development
—Injury-induced secondary cell death
—Aging-associated cell loss and shrinkage of cholinergic CNS neurons

It is known that early during development, there are neurons that express dependence on neuronotrophic factors as target innervation takes place, and that competition for target derived growth factors, which are to be retrogradely transported to the neuronal soma, provides a mechanism for the establishment of appropriate synaptic connections and the culling of those neurons and neurites that do not successfully connect to their appropriate targets. In adulthood, injury to neurons can be lethal in a fashion that depends in part on the distance between the cell soma and the site of axotomy (Ramon y Cajal, 1928). It has been demonstrated that such secondary cell death can be prevented in some measure by increasing levels of neuronotrophic activity *in situ* (Hefti et al., 1984; Nieto-Sampedro et al., 1984; Williams et al., 1986; Whittemore and Seiger, 1987). Finally, there are reports that treatment of the CNS with neuronotrophic factors can spare cholinergic neurons of the CNS from aging associated deficits in rodents (Fischer et al., 1987).

During the development of the nervous system, it has been proposed that, although it is unlikely that in all instances all of the responsible molecular events will be the same, it would appear that, for certain classes of neurons, there may be common regulatory features present during neuronal cell death in development,

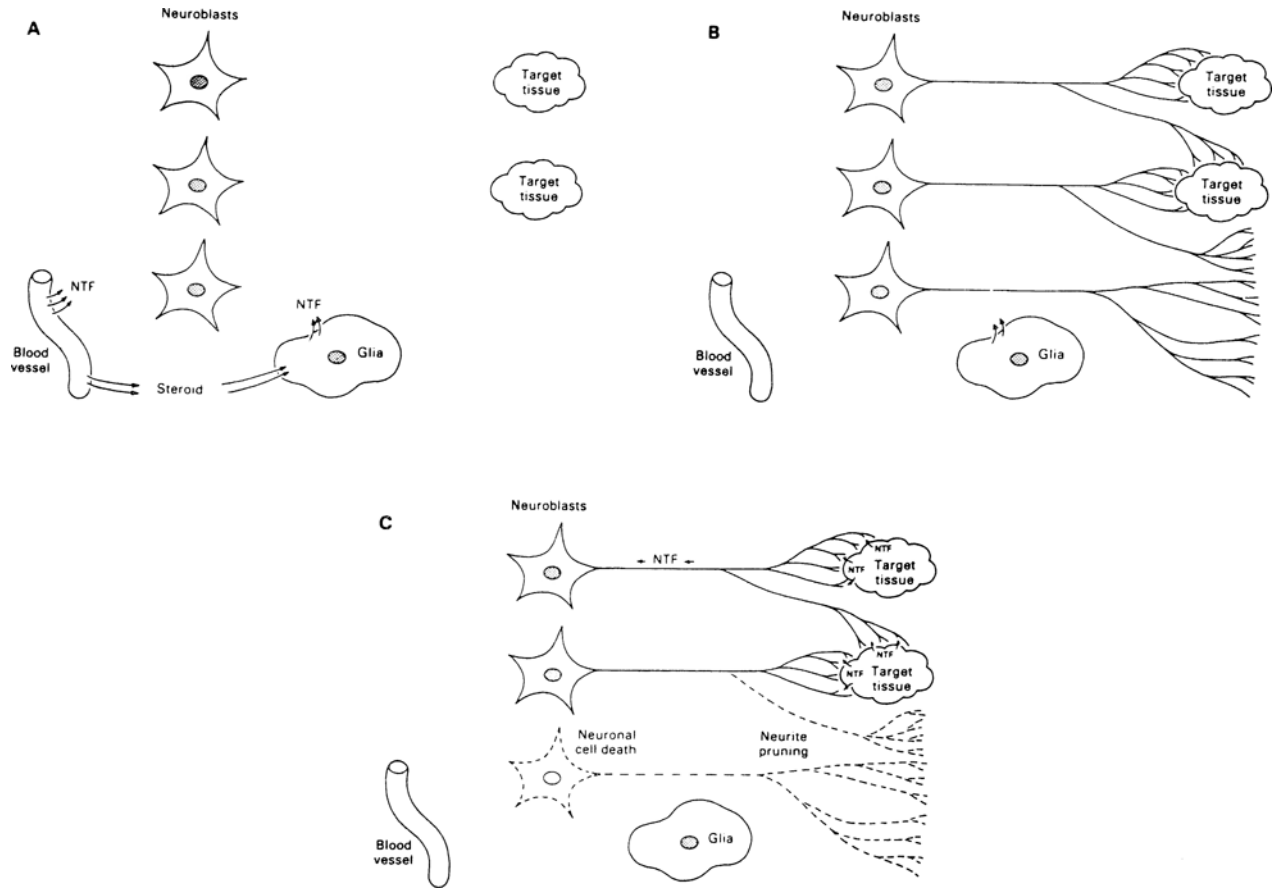

Fig. 1. Proposed mechanism of cell death caused by competition for target derived growth factors during the development of the PNS. **(A)** Increases in the ambient levels of neuronotrophic factors (NTF) stimulate neuroblasts, resulting in maturation to a postmitotic state, stimulation of neurotransmitter metabolism, and production of neurites and growth cones. **(B)** Consequently, some neurons, but not all, are able to synapse on target tissues. **(C)** As NTF activity levels drop in the surroundings of the neuronal cell bodies, only those neurons that can import NTF by retrograde transport from target tissues survive. Thus, only the synapsing neurites remain. Adapted from Perez-Polo and Haber 1984.

following injury or associated with aging in the CNS. For example, the toxicity that is a byproduct of oxidative metabolism may be exacerbated in neurons as a consequence of developmentally driven neuronal cell death, dendritic and axonal injury occurring proximal to the cell soma, or cumulative events accentuated in the aged nervous system. It may also be that certain degenerative neurological disorders, or the even less dramatic manifestations of viral infections, immune challenges, electrical seizures, or chronic stressful stimuli may perturb some neuronotrophic factor mediated events.

Although the number of known or suspected neuronotrophic factors increases on a daily basis, the demonstration of a physiological role for these has been laggard. Certainly, the best characterized neuronotrophic factor is the nerve growth factor protein (NGF). NGF is known to play a crucial role in the regulation of neuronal cell death in the developing sympathetic and embryonic sensory system, and to promote the survival of injured cholinergic neurons of the basal forebrain (Hamburger et al., 1981; Hamburger and Oppenheim, 1982; Korsching, 1986a,b; Levi-Montalcini, 1987; Whittemore and

Seiger, 1987). Although much is known about the regulation of neurite outgrowth and neurotransmitter synthesizing enzymes by NGF (Table 2), less is known about its regulation of neuronal cell death at the molecular level during development, following injury, or as an end point in the aged CNS. Also, given that NGF acts on several target tissues (Table 3), it may be that similar trophic effects are mediated by different molecular mechanisms in different tissues.

Here, we will discuss the role of neuronotrophic activity and, more specifically, of NGF activity in neuronal cell death. It is known that exogenous NGF, or its withdrawal by anti-NGF, has significant and permanent effects on axonal sprouting and the survival of sympathetic, sensory, and striatal neurons (Bostwick et al., 1987; Hulsebosch et al., 1987a,b; Levi-Montalcini, 1987). In particular, we will consider the hypothesis that effects of NGF on oxidant–antioxidant balance might be relevant to the regulation of cell death at critical periods during development, following injury and, more chronically, throughout aging or the course of certain degenerative disorders that are associated with aging. There may be other effects of NGF that are also relevant to cell death and there are also likely to be other factors at play. Although the proposed hypothesis is sweeping, it may be only applicable to some, and not all, of the three cell death associated phenomena discussed here. Furthermore, it may be more relevant for NGF-responsive neurons of peripheral and not central origin, or vice versa. Also, NGF effects on oxidant–antioxidant balance may be one of several effects of NGF that bring about a similar outcome.

Since neurons have low endogenous levels of antioxidants, the regulation of oxidant–antioxidant balance by neurotrophic factors may control neuronal cell death. Free radicals, such as hydroxyl radicals, can arise as a result of increases in oxygen species and their conversion to hydrogen peroxide by the enzyme superoxide dismutase (Fig. 2). Hydrogen peroxide and ferrous iron, in turn, can yield hydroxyl radicals that initiate self-sustaining lipid peroxidation reactions at the plasma membrane of the neuron. These amplified reactions will spread to the cytoplasm of the cell, where proteins can become inactivated, leading to cell death. Increases in iron species can occur following injury caused by heme release, and also as a concomitant of inflammatory events associated with a compromised blood–brain

Table 2
Cellular Effects of Hydrogen Peroxide

Energy metabolism
HMP shunt activation
Increased oxidized sulfhydryls
Decreased ATP
Decreased NAD
Poly (ADP-ribose) polymerase activation
Decreased glycolytic flux
Damaged molecules
DNA single strand breaks
Increased intracellular free calcium
Plasma membrane "blebbing"
Lipid peroxidation
Cell death

Table 3
Effects of NGF on PC12 Cells

Gene expression
c-*fos*
c-*jun*
c-*myc*
NGFIA
NGFIB
Actin
Ornithine decarboxylase
Second messengers
cAMP (?)
Calcium (influx and release)
Phosphoinositides
Protein kinase C
Protein kinase N
Cytoskeletal proteins
Tubulin
MAP-1/MAP-2/Tau
Vimentin
Neurofilament proteins (68/200 kDa)

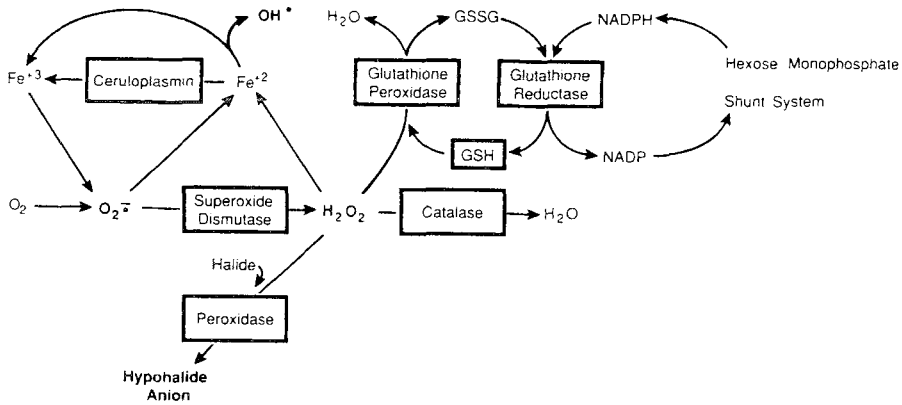

Fig. 2. Some of the principal pathways responsible for oxidant–antioxidant balance and effects on energy metabolism. Here, antioxidant activities are boxed in. Enzymatic activities, such as superoxide dismutase, catalase, and peroxidase, directly act on free radicals or free radical generators. Ceruloplasmic binding of ferrous species influences hydroxyl radical generation. Glutathione metabolism directly affects hydrogen peroxide conversion to water in a series of coupled reactions associated with the hexose monophosphate shunt system. Adapted from Jackson et al., 1990.

barrier. In this instance, cell death has also been called secondary cell death, and may result in part from reoxygenination events that take place after those ischemic events that occur initially following injury. In the aged CNS, it is not clear whether increased free radical damage is a consequence of trophic activity deficits or metabolic responses to ongoing degenerative events resulting in cell death, debris, and diminished antioxidant activities.

There are three principal ways in which hydrogen peroxide can be rendered nontoxic (Fig. 2). Catalase enzymes can convert hydrogen peroxide to water in a very efficient reaction; ceruloplasmin binding to ferrous iron can dramatically shift the equilibrium of the Haber-Weiss equation (1934); and the hexose monophosphate shunt system driven by glutathione peroxidase can generate water from hydrogen peroxide. It is a corollary of this hypothesis that reductions in NGF activity can result in damaging shifts in oxidant–antioxidant balance and energy metabolism (Jackson et al., 1990a,b; Perez-Polo and Haber, 1984; Perez-Polo and Werrbach-Perez, 1985,1987).

There are other sources of toxic molecular species that are affected by intracellular increases in free radical concentrations. The interior of mitochondria contain very high levels of free radical generating molecules and, curiously, have low endogenous levels of antioxidants. Thus, increased peroxidative activity in the cytoplasm will not only disrupt free calcium levels, but will also result in release of calcium from mitochondrial stores or increased influxes of calcium resulting from membrane peroxidation induced damage (Koike et al., 1989). Such an increase in intracellular free available calcium can activate proteases that, in turn, degrade proteins and break nucleic acid strands (Davies, 1987; Richter, 1987; Richter and Frei, 1988). It has been suggested that cumulative free radical insults over time may result in mitochondrial DNA losses to the cytoplasm, and that the cumulative incorporation of this "excised mitochondrial DNA" into the nuclear genome might bring about increased incorrect gene expression, which accounts for aging associated phenomena (Richter, 1988). These explanations for cell death in the nervous system are not mutually exclusive, but rather, may be synergistic.

Another explanation for the NGF cell-sparing effect is that since cycloheximide, a protein synthesis inhibitor, protects cultured sensory neurons from the cell death normally observed following removal of NGF, it may be that NGF

suppresses expression of "suicide genes" (Martin et al., 1988). Thus, there are two possible ways in which trophic factors abrogate the cell death response. The hypothesis that NGF regulates oxidant–antioxidant balance would suggest that NGF sparing of cells marked for death is caused by induction of certain genes, such as genes coding for catalase and glutathione peroxidase. That is, proteins are synthesized that protect the cell from free radical inflicted damage. The suicide gene hypothesis states that neuronal cell death in development is the result of the activation of a cascade of death associated proteins, culminating in so-called "thanatins," or death proteins (Johnson, 1990). Thus, trophic factors act by repressing the synthesis of specific gene products. Evidence for the existence of thanatins in the periphery rests on the use of mRNA transcription and translation inhibitors. There are several candidates for thanatin molecules, but none have been demonstrated to date.

It has not been established whether free radicals and peroxidative damage play a role in development. That is, it is not known whether there are surges of peroxidative damage that result in the death of those neurons that are known to depend on competition for target derived growth factors to prevent their death during development.

The situation is different for secondary cell death caused by injury and aging. It is known that a consequence of neuronal injury or aging is oxidative stress, partially manifested by an increase in peroxidative events that disturb energy metabolism, and damage proteins and membranes (*see* Fig. 2)(Cand and Verdetti, 1989; Hall and Braughler, 1982; Hall 1987; Harman, 1988).

This issue is further complicated because low concentrations of hydrogen peroxide stimulate neuronal metabolism whereas high concentrations of hydrogen peroxide are toxic (Demopoulos et al., 1979; Chan et al., 1984; Laval, 1988). Hydrogen peroxide can have several different effects on energy metabolism in addition to its more direct effects on molecules (Table 2). Thus, it is not surprising that oxidative stress can induce xenobiotic responses, much like exposure of most mammalian cells to heavy metals (Spitz et al., 1987). Thus, the regulation of free radical scavenging mechanisms is important, because free radicals can accumulate and adversely affect the functions of neurons that are highly specialized, have limited regenerative capacity, and a relatively high requirement for oxygen. Such a concentration dependent effect on neuronal survival has also been proposed for NGF effects on calcium intracellular concentrations under the rubric of the "calcium set point" hypothesis of neuronal cell death (Koike et al., 1989).

NGF

NGF is a neurotrophic protein whose structural features have been well-chronicled (Greene and Shooter, 1980; Levi-Montalcini, 1987). NGF has been purified from the submaxillary gland of mice and rats, murine saliva, several snake venoms, the guinea pig prostate, bovine seminal plasma, rodent seminal vesicle, and human term placenta (Perez-Polo, 1985). In some tissues, NGF has been isolated as a subunit containing protein. In all instances, only the β-NGF subunit, henceforth called NGF, has been found to have nerve growth promoting activity (*see* Fig. 3 and Table 4). To date, the role of the quaternary structure of NGF in the mouse submaxillary gland and the nature of the subunits' composition, if any, of NGF in neuronal tissues are not known (*see* Table 5).

The sequence of the α, β, and NGF genes is known, for β-NGF, the gene sequence is known for mouse, rat, bovine, human, and chick NGF, and all are highly conserved (Ebendal et al., 1986; Goedert, 1986; Isackson et al., 1987; Meier et al., 1986; Misko et al., 1987; Schwarz et al., 1989; Scott et al., 1983; Ullrich et al., 1983a,b; Whittemore et al., 1988). The human gene for NGF is on the proximal short arm of chromosome 1 (Francke et al., 1983). Recombinant NGF has been characterized and shown to be biologically active (Bruce and Heinrich, 1989; Edwards et al., 1988). NGF mRNA levels have been determined for brain, superior cervical

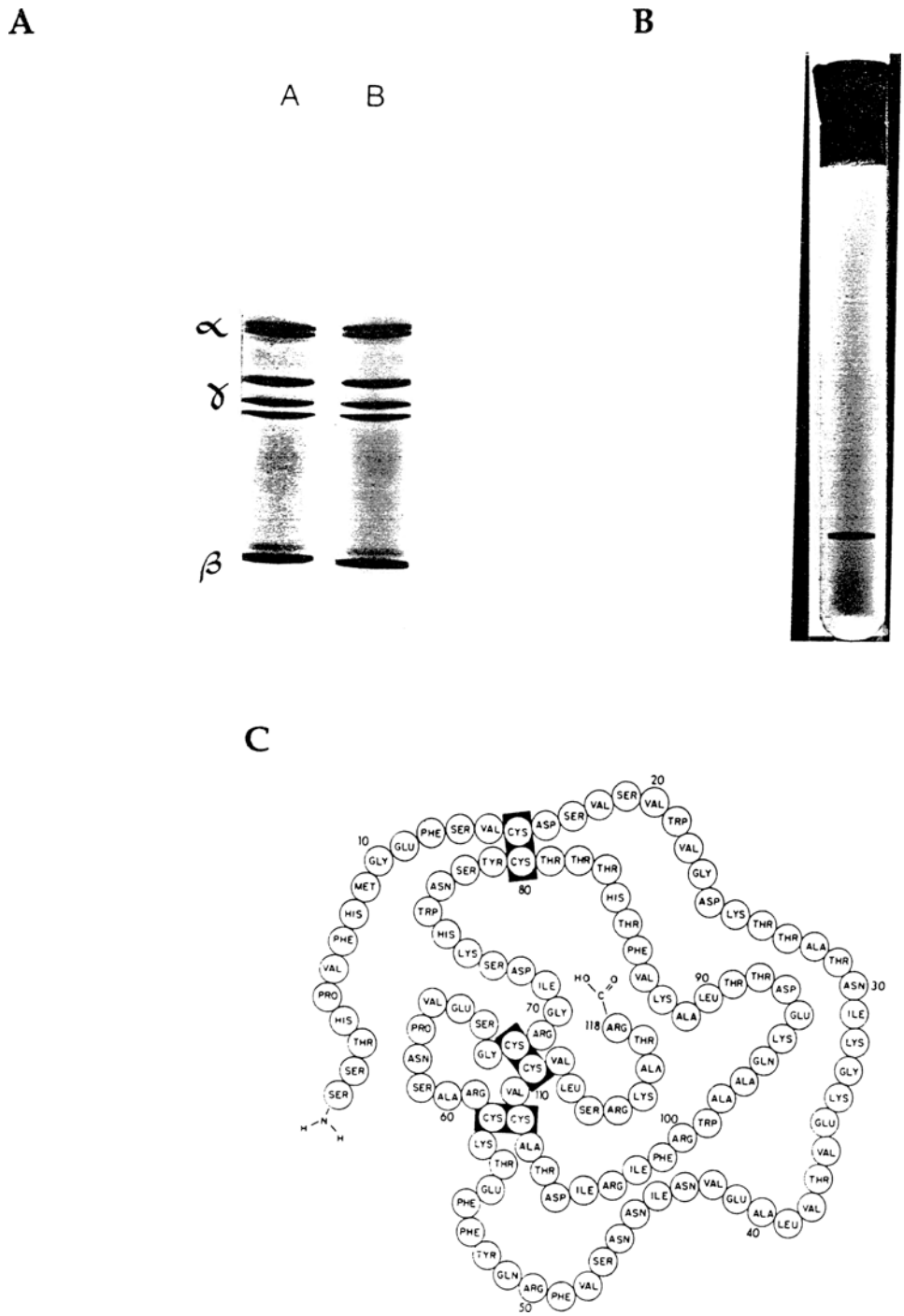

Fig. 3.(A) Separation by analytical isoelectric focusing gel electrophoresis of two samples of murine 7S NGF (from Perez-Polo and Shooter, 1975). Isoelectric focusing on polyacrilamide gels with a 3–10 pH gradiant. (B) Same electrophoretic analysis of murine β-NGF isolated from male submaxillary gland. (C) Amino acid sequence of murine β-NGF (from Hogue-Angeletti and Bradshaw, 1971).

Table 4
Quaternary Structure of NGF

Species: Tissue	Subunits present		
	α	β	γ
Mouse: submaxillary gland	+	+	+
Rat: submaxillary gland	+	+	–
Snake: venom	–	+	+
Guinea pig: prostate	–	+	–
Human: term placenta	+	+	–
Bovine: seminal plasma	?	+	–
Bovine: seminal vesicle	?	+	–

ganglia, and spinal cord, and correlated with NGF protein levels as a function of development, innervation, and response to injury (Auberger et al., 1987; Ayer-LeLievre et al., 1988; Goedert et al., 1986; Heumann et al., 1984, 1987; Korsching et al., 1985, 1986; Large et al., 1986; Lu et al., 1989; Rennert and Heindrich, 1986; Shelton and Reichardt, 1984). Although there is only one mature form of NGF expressed in the nervous system, there are two different precursor forms caused by differential RNA splicing (Edwards et al., 1986).

Levels of NGF mRNA and protein in the PNS and CNS correlate with the density of sympathetic innervation (Korsching et al., 1985; Shelton and Reichardt, 1984). NGF mRNA and protein are widely distributed in CNS. The highest levels are in cortex and hippocampus, which are terminal regions for projections from basal forebrain cholinergic neurons. This is where NGF effects on ChAT induction and cell sparing, following lesions, have been best documented (Gnahn et al., 1983; Hefti et al., 1984; Mobley et al., 1986). It should be emphasized that, at early developmental stages, NGF and NGF receptor mRNA levels are highest in noncholinergic CNS structures, such as the cerebellum and outside the nervous system in the immune system (Buck et al., 1987,1988; Ebendal et al., 1986; Ernsfors et al., 1988; Large et al., 1986). Here, it is not known what the role of NGF is during development, and these noncholinergic neurons do not remain NGF-responsive into adulthood (Dreyfus, 1989; Whittemore and Seiger, 1987).

Table 5
Studies on NGF

Biological variables studied
- Neurite outgrowth
- Cell hypertrophy
- Cellular proliferation
- Synaptogenesis
- Cell survival
- Cell death
- Neurotransmitter expression
- Neuro-immune-endocrine activation

Biological phenomena of interest
- Development
- Regeneration after spinal and head trauma
- Chronic degenerative neurological dysfunction
- Aging associated phenomena
- Behavioral disorders

The Nerve Growth Factor Receptor

The first step of NGF action is the binding to specific membrane receptors (Banerjee et al., 1973; Frazier et al., 1973; Herrup and Shooter, 1973). In the PNS, two distinct NGF receptor (NGFR) sites have been found (Godfrey and Shooter, 1986). In the CNS, where NGF binding has not been as extensively characterized, preliminary reports would suggest that NGF binding activity has similar kinetic properties to its PNS counterparts (*see* Figs. 4 and 5) (Angelucci et al., 1988a; Bernd et al., 1988; Cohen-Cory et al., 1989; Raivich and Kreutzberg, 1987; Taglialatela et al., 1990). It should be emphasized that, although the NGF receptor expressed by PC12 cells and some peripheral neurons has been partially characterized, less is known about the structural properties of NGFR expression in CNS and nonneural tissues. In part, this is as a result of the use of NGFR genetic probes based on the cDNA coding for only one of the NGF binding sites, the low affinity receptor (Radeke et al., 1987).

There are different ways to characterize NGF receptor activity. One method is to use receptor binding assays. Two NGF binding activities have been demonstrated for most neuronal

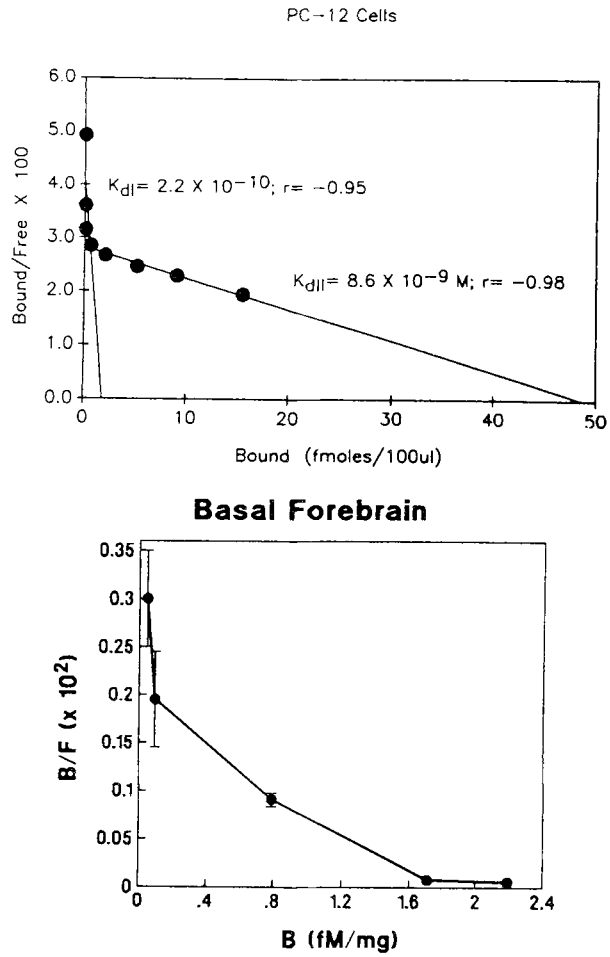

Fig. 4. Scatchard analysis of equilibrium binding of ^{125}I-NGF to solubilized samples. **(A)** PC12 cells; **(B)** rodent basal forebrain cells or homogenized tissues were solubilized in 0.5% NP40 and assayed for NGF binding activity, as described in Angelucci et al., 1988.

tissues with equilibrium dissociation constants (K_d) of around 10^{-11} and $10^{-9}M$ (Stach and Perez-Polo, 1987; Sutter et al., 1979). The former represents a high affinity, low capacity binding site (NGFR-I) that has a slow dissociation rate constant for ligand; the latter represents a low affinity, high capacity binding site (NGFR-II) that has a fast dissociation rate constant. It is generally believed that the NGFR-I is the physiologically relevant receptor present in neuronal populations (Green et al., 1986; Sonnenfeld and Ishii, 1985). Unfortunately, most demonstrations of high affinity NGF binding in the CNS

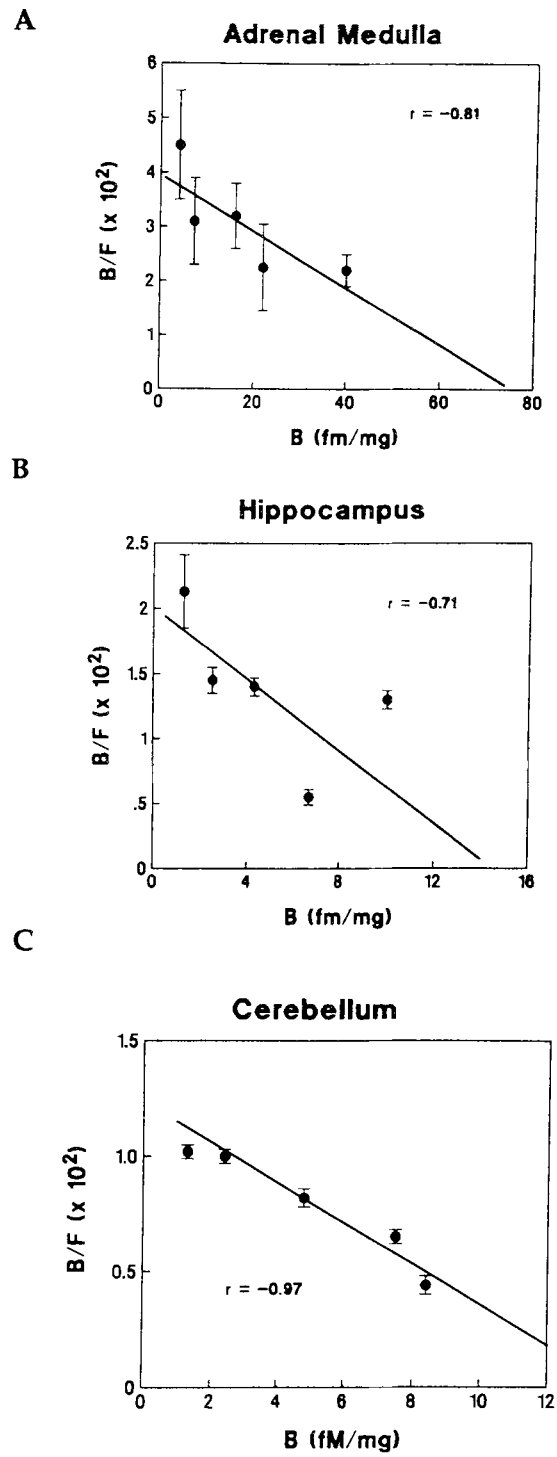

Fig. 5. Scatchard analysis of equilibrium binding of ^{125}I-NGF to solubilized samples of **(A)** adrenal medulla; **(B)** hippocampus; and **(C)** cerebellum. Adapted from Angelucci et al., 1988.

have been indirect, and have relied on autoradiographic analysis of tissue sections exposed to ^{125}I-NGF, and not on Scatchard analysis of specific, saturable binding of an NGF ligand (Cohen-Cory et al., 1989; Riopelle et al., 1987 a,b; Yip and Johnson, 1987). The one exception would suggest that the proportion of low affinity sites to high affinity sites is greater outside the PNS making such an analysis difficult (*see* Figs. 4 and 5)(Angelucci et al., 1988a; Taglialatela et al., 1990). It has been proposed that binding of NGF to the low affinity receptor converts it to its high affinity counterpart (Landreth and Shooter, 1980). There is also evidence that molecular species other than NGF can increase the proportion of high affinity receptors to NGF in isolated fractions of NGFR practically devoid of high affinity binding at the expense of the low affinity sites present there almost exclusively (Marchetti and Perez-Polo, 1987). The NGF–NGFR complex is internalized via high affinity binding mechanisms, a step thought to be necessary for NGF action, although there is no direct evidence for this (Bernd and Greene, 1984; Green et al., 1986; Hosang and Shooter, 1987).

A second approach that has been useful for the study of NGFR structure is sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS–PAGE) of ^{125}I-NGF, which has been covalently crosslinked to NGFR. SDS–PAGE analysis has also been carried out on immunoprecipitated NGF–NGFR complexes, iodinated surface proteins of NGF responsive cells, and partially isolated NGFR enriched fractions relying on immunoaffinity chromatography, preparative isoelectric focusing, and reverse-phase high-performance liquid chromatography, RP-HPLC (*see* Fig. 6)(Beck et al., 1989; Buxser et al., 1985; Green and Greene, 1986; Grob et al., 1985; Hosang and Shooter, 1985; Kouchalakos and Bradshaw, 1986; Marano et al., 1987; Marchetti and Perez-Polo, 1987; Massague et al., 1982; Puma et al., 1983). Most descriptions of rodent NGFR are consistent with a 70–80 kD protein as the prevalent NGFR species, whereas, as shown in Fig. 6 and also Puma et al., 1983 and Riopelle et al., 1987b, in human NGFR bearing cells, NGFR proteins of 92.5 kD have been reported. In both instances, there are higher mol wt species of NGFR recognized. Kouchalakos and Bradshaw (1986) have analyzed the various reports for NGFR, and described a set of four different species of NGFR: class A (70–81 kD), class B (87–105 kD), class C (120–145 kD), and class D (190–300 kD). The low affinity NGFR-II in PC12 cells, in human neuroblastoma LA-N-1 cells, and in human melanoma A875 cells was assigned to classes A and B. The larger M_r NGFR in the class D category could be dimers of the class B since their peptide maps are similar and there is some evidence for interconversion of the D to the B class under reducing conditions (Buxser et al., 1985; Grob et al., 1985; Marchetti and Perez-Polo, 1987). It is likely that B and C forms are part of one spectrum of biologically active NGFR, and A and D, respectively, represent truncated or aggregated forms (DiStefano and Johnson, 1988a,b). Ambiguities as to reported sizes may be the result of differences in the glycosylation or phosphorylation of NGFR, as well as of the many existing disulfide linkages present (Grob et al., 1983, 1985; Ross et al., 1984). Also, there is indirect evidence for a receptor associated protein with structural and functional effects on NGF binding (Hosang and Shooter, 1985; Marchetti and Perez-Polo, 1987). One difficulty here is that data from different tissues and species using different techniques are difficult to compare. It would appear that in most cases NGFR can be identified as two or more different mol wt species.

The third approach to the characterization of NGFR relies on recombinant DNA technology. Both rat and human NGFR are the product of a single gene that does not appear to undergo differential splicing (Johnson et al., 1986; Large et al., 1989; Radeke et al., 1987). Based on the known NGFR DNA sequence, it has been determined that the human NGFR is synthesized as a precursor molecule with 427 amino acids (Johnson et al., 1986). The rat NGFR is highly homologous, and is a synthesized 425 amino

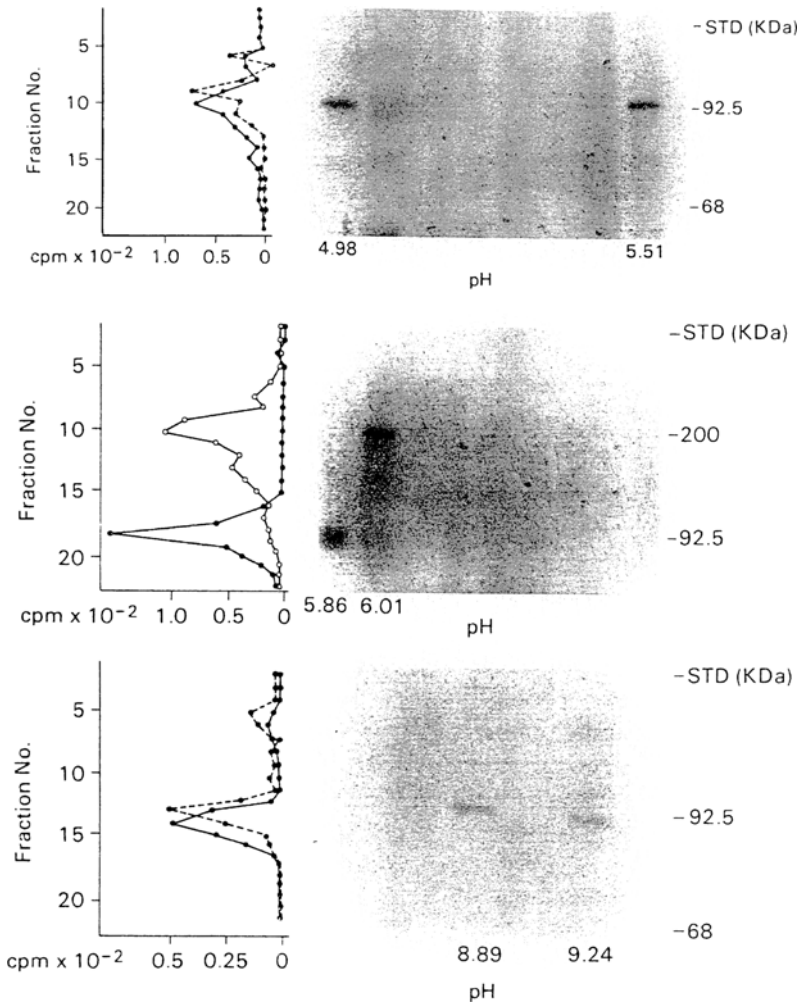

Fig. 6. SDS-PAGE analysis of immunoprecipitated iodinated NGFR samples that were partially isolated by lentil lectin chromatography and preparative electrofocusing on granulated gel, PEGG. (A) through (C) are different PEGG fractions. From Marchetti and Perez-Polo, 1987.

acid precursor protein (Radeke et al., 1987). After removal of the *N*-terminal signal peptide, the protein core consists of 399 (human) or 396 (rat) amino acids, with an estimated mol wt of 42 or 49 kD. The protein core is subsequently glycosylated to yield a 75–80 kD NGFR (Grob et al., 1985; Johnson et al., 1986). However, the identification of only a single gene for NGFR does not explain the existence of two different NGF binding activities, or of the different molecular species of NGFR. The gene for NGFR has been localized on human chromosome 17 (Huebner et al., 1986; Rettig et al., 1986).

Receptors for NGF are present on cells derived from all three germ layers, consistent with the hypothesis that NGF is not exclusively a neuronotrophic factor (Perez-Polo and Werrbach-Perez, 1987; Thomson et al., 1988; Thorpe et al., 1987a,b,1989; Thorpe and Perez-Polo, 1987). For the CNS and PNS, NGFR and NGF mRNA expression appear to be coordinated and related to density of innervation (Buck et al., 1987; Korsching, 1986a,b; Whittemore and Seiger, 1987). There is NGF mRNA present in the hippocampus, cortex, thalamus/hypothalamus, brainstem, striatum, cerebellum, and

spinal cord, in decreasing order. In some CNS regions, such as cerebellum, there is NGF and NGFR mRNA early in development not associated with cholinergic neurons (Whittemore and Seiger, 1987). The distribution of NGFR protein in the CNS is most evident in the hippocampus, frontal cortex, basal forebrain, and cerebellum (Angelucci et al., 1988a; Taniuchi et al., 1986a).

NGFRs are synthesized predominantly in the cell bodies of cholinergic neurons and subsequently transported via anterograde transport to the axon terminals (Buck et al., 1987). At the terminals, NGFR binds NGF, and the NGF–NGFR complex is internalized and retrogradely transported to neurons of the basal forebrain nuclei (Johnson et al., 1987; Seiler and Schwab, 1984). The continuous flux of NGF and NGFR, as well as, of the NGF–NGFR complex, may have regulatory significance on target tissues innervated, innervating neurons, or both (Hefti, 1986). However, NGF binding activity, NGFR protein, and NGFR mRNA in all regions of brain and lymphoid tissues, during some stages of development, have been reported for both the chick and the rat (Buck et al., 1988; Ernfors et al., 1988).

Model Systems

There are obstacles to the study of the mechanism of action of NGF in vivo. It is difficult to have homogenous cellular populations, to observe isolated cellular events, and to quantitatively manipulate the cellular environment (Perez-Polo, 1987). Cell lines have distinctive advantages because of their homogeneity and relative ease of production. Although caution must be exercised before extrapolating conclusions gleaned from studies with transformed cells, these in vitro paradigms are useful when used in conjunction with the more complex in vivo paradigms.

Three neuronal cell lines that have proven useful in the study of NGF are the PC12 rat pheochromocytoma line, the SK-N-SH-SY5Y (SY5Y), and the LA-N-1 human neuroblastoma lines. The rat pheochromocytoma cell line PC12 is the most extensively studied NGF responsive cell line (Greene and Tischler, 1976). NGF has several major effects on PC12 cells that have been classified temporally and based on their RNA transcription dependence Table 3, (Greene, 1984; Levi et al., 1988):

1. In common with other growth factors, NGF elicits rapid cell-surface ruffling, stimulated ion fluxes across the cell membrane, and internalization of the NGF ligand (Connolly et al., 1979);
2. NGF induces short-term transcription-independent phosphorylation of several cytoplasmic proteins (Halegoua and Patrick, 1980; Romano et al., 1987); and transcription of some proto-oncogenes, such as c-*myc*, c-*fos*, and c-*jun* (Milbrandt, 1986, 1988; Wu et al., 1989);
3. NGF induces short-term synthesis of ornithine decarboxylase (Greene and McGuire, 1978);
4. NGF induces long-term transcription-dependent synthesis of those cytoskeletal proteins and cell adhesion molecules that are required for normal neurite growth; and
5. NGF can induce mitotic arrest under some conditions for PC12 cells.

This broad spectrum of responses is not unique to NGF, but rather, represents the response of the PC12 cell; other classes of NGF responsive cells may display a different spectrum of responses (Thorpe et al., 1989). Also, even for one cell line, such as the PC12 line, the different cell responses may not be coupled, and represent different segments of physiologically distinct outcomes. For some cell types like astrocytes and Schwann cells, ambient conditions can drastically affect the NGF response (Bothwell et al., 1980; Burstein and Greene, 1978,1982; Green et al., 1986; Levi et al., 1988).

Human neuroblastoma cell lines are another model for studying the structure of NGFR and the effects of NGF (Perez-Polo and Werrbach-Perez, 1985, 1987). These cell lines are genetically stable, dependent on NGF for cell survival when grown in the absence of serum, and reversibly responsive to NGF. Similar to the findings in PC12 cells, treatment of neuroblastoma cells with NGF induces neurite outgrowth and hypertrophy (*see* Figs. 7–9)(Perez-Polo et

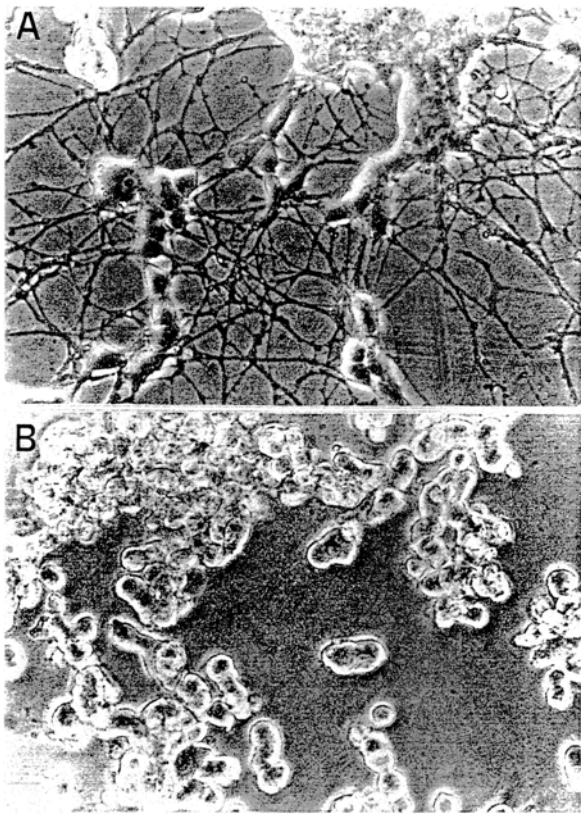

Fig. 7. PC12 cells culture in **(A)** the presence of 1 μg/mL NGF for 6 d and **(B)** control (250×). Culture conditions as described in Jackson et al., 1990.

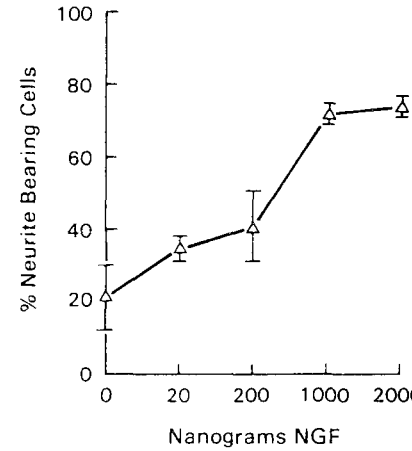

Fig. 8. Dose–response curve of 4 d of 1 μg/mL NGF treatment on neurite outgrowth on human neuroblastona cell line SK-N-SH as described in Reynolds and Perez-Polo, 1987.

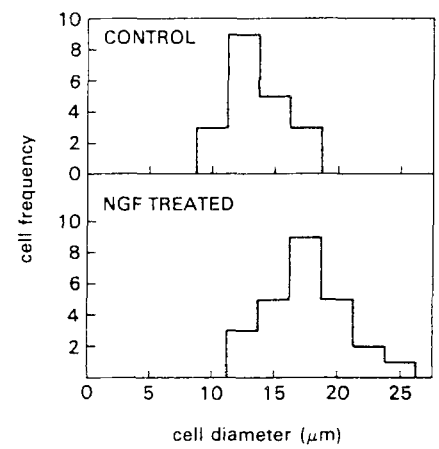

Fig. 9. Effect of 1 μg/mL NGF on SY5Y cell diameter in culture, as described by Perez-Polo et al., 1982a.

al., 1979; Sonnenfeld and Ishii, 1982), increases protein synthesis (Perez-Polo et al., 1982; Sonnenfeld and Ishii, 1982), and induces electrical excitability (Kuramoto et al., 1981). The study of NGF effects on neuroblastoma cells offers unique opportunities since the cells possess properties not present in PC12 cells.

First, only the high affinity NGFR-I type binding has been detected in neuroblastoma SY5Y cells (Sonnenfeld and Ishii, 1982, 1985). Second, SY5Y cells are reported to have NGFR mRNA of the same size as that reported for the low affinity NGF receptor and, when the SY5Y NGFR gene is transfected to mouse fibroblast-like L cells, the receptor expressed is also the NGFR-II type (Chao et al, 1986; Hempstead et al., 1989). This implies that, in SY5Y cells, there may exist a specific cellular environment that is responsible for the expression of the NGFR gene as an NGFR protein expressing high affinity binding.

NGFR in Model Systems

Two binding sites for NGF have been described for LA-N-1 and PC12 cells, using equilibrium binding assays. They are the high affinity binding site (NGFR-I) and the low affinity binding site (NGFR-II) identical to that described for sensory and sympathetic neurons

(Sutter et al., 1979; Stach and Perez-Polo, 1987). It is believed that the physiologically relevant binding site for neurons is the high affinity site, and that the low affinity site may act as a "reservoir" of NGF binding sites because the equilibrium dissociation constant (K_d) for NGFR-I binding is approximately equal to the half-maximal effective concentration of NGF *in situ*; and in the absence of NGFR-II, such as in neuroblastoma SY5Y cells, cells remain responsive to NGF (Sonnenfeld and Ishii, 1985). Also, there are PC12 cell variants that possess NGFR-II but not NGFR-I, and do not respond to or internalize NGF (Green et al., 1986). It has also been proposed that internalization of the NGF–NGFR complex is necessary for NGF action (Bernd and Greene, 1984; Green et al., 1986; Hosang and Shooter, 1987). That conversion of NGFR II to NGFR I may be a physiologically relevant event is suggested by the fact that tunicamycin (TM) and wheat germ agglutinin (WGA) increase the affinity of NGFR to NGF (Baribault and Neet, 1985; Grob and Bothwell, 1983; Landreth et al., 1985). When TM and WGA treatment deplete the number of NGFR-II on PC12 cells, the PC12 neurites will no longer respond to NGF by turning in the direction of increasing concentrations of NGF. Furthermore, at the NGF concentration required for the chemotactic response of neurite turning or increased tyrosine hydroxylase activity, roughly half the low affinity binding sites are occupied. This finding is compatible with the idea that these particular effects of NGF are mediated through NGFR-II (Gundersen and Barrett, 1980; Hefti et al., 1982). Also, NGF induces ornithine decarboxylase activity in cultured neural crest cells as well as increased thymidine uptake in rodent lymphocytes, and both of these cell types express only NGFR-II (Bernd, 1986; End et al., 1983; Thorpe et al., 1987a,b,c, 1989). It may be that NGFR-I and NGFR-II are responsible for a different spectrum of cellular responses to NGF.

Alternatively, the presence of high affinity NGF binding on a cell does not necessarily mean that there are NGF responses. There are high affinity NGF binding sites on the A875 cell line that do not respond to NGF (Buxser et al., 1985; Rubenstein et al., 1985). Also, WGA treatment increases high affinity binding of NGF to PC12 cells when it blocks the NGF response (Grob and Bothwell, 1983; Hashimoto et al., 1985; Landreth et al., 1985).

The fact that there are several mol wt values reported for NGFR and that all of these appear to be recognized by the two species specific monoclonal antibodies to NGFR, MC192 to the rodent species and MC20.4 to the human species, would suggest that the NGFR identified share an epitope and/or are modified forms of NGFR derived from a core protein (Green and Greene, 1986; Hosang and Shooter, 1985). One such modification, the binding of a receptor-associated protein to the NGF binding subunit may be required for NGF action. Such binding may facilitate hydrophobic interactions between NGFR and other proteins, such as an adaptor complex, or may provide a hydrophobic environment for the regulation of protein kinase C (Snoek et al., 1988).

Based on the molecular species of putative NGFR reported for PC12 cells, one can propose a tentative model that accounts for the relationships among the different molecular species of NGFR. The basic assumption in this model is that all the high molecular weight species of NGFR are derived from the interactions of a core protein, with other proteins (Green and Greene, 1986; Marchetti and Perez-Polo, 1987). There is evidence that the NGF receptor-associated protein is not encoded by the NGFR gene that has been cloned and sequenced (Hempstead et al., 1989; Radeke et al., 1987). An understanding of whether the different forms of NGFR play a role in the different aspects of NGF activity is not at hand.

Effects of Retinoic Acid on NGF Action

Retinoic acid (RA) is a vitamin A metabolite that is known to affect cell differentiation (Brockes, 1989; Giguere et al., 1989). It can also

modify glycoprotein synthesis, and has been shown to stimulate neurite extension and inhibit cellular proliferation of human LA-N-1 neuroblastoma cells (Sidell, 1982; Sidell and Horn, 1985). Although RA increases NGF binding in LA-N-1 and PC12 cells (Haskell et al., 1987; Jackson et al., 1990a,b), the effects are not mediated solely through its effects on NGFR expression.

Differentiation can be induced by RA in a variety of NGF-unresponsive cell types (Pohl et al., 1988). RA is also capable of inducing expression of the NGF gene in mouse L cells (Wion et al., 1987). Even for some NGF-responsive cells, RA does not elicit the same responses as NGF. For example, RA has no effect on basal or NGF-stimulated levels of ChAT in rat septal cultures (Knusel and Hefti, 1988), but it does cause SK-H-SH-SY5Y to differentiate into cholinergic neurons (Sidel and Horn, 1985). It inhibits expression of c-*myc* in neuroblastoma cells as compared to NGF stimulation of c-*myc* expression in PC12 (Greenberg et al., 1985; Thiele et al., 1985). RA also has opposite effects from NGF on prostaglandin E-stimulated accumulation of cyclic AMP in neuroblastoma cells (Yu et al., 1988). On PC12 cells, RA has no effect on morphology, although it does protect PC12 cells from hydrogen peroxide treatment in a similar way to the protection conferred by NGF (Jackson et al., in press). Thus, NGFR expression and hence NGF activity are modulated by substances, such as RA, that can in part have similar effects on such cells as NGF, and can also augment the NGF effect.

Known Effects of NGF

Nerve growth factor (NGF) regulates neuronal cell death, neurite extension, and synapse formation during the development of sensory and sympathetic ganglia, and is also trophic to some neurons in the central nervous system (Levi-Montalcini, 1987; Thoenen and Barde, 1980; Whittemore and Seiger, 1987). The role played by NGF in the PNS has been well established and extensively reviewed (Greene and Shooter, 1980; Levi-Montalcini, 1987). During neuronal development, increased ambient levels of NGF in the region of the developing neurons provide guidance to outgrowing neuronal fibers in a process that may involve increased synthesis of NGF and NGFR by Schwann cells (Assouline and Pantazis, 1989; DiStefano and Johnson, 1988b). Once target tissues are innervated, Schwann cell synthesis, secretion of NGF and NGFR is curtailed, and target derived NGF is taken up at nerve terminals and retrogradely transported to the soma to maintain the differentiated state of the neuron (*see* Fig. 1 and Tables 5 and 6) (Hamburger and Oppenheim, 1982; Levi-Montalcini, 1987).

Although less is known about the role of NGF in the development of the CNS, there is evidence that it provides trophic support to basal forebrain cholinergic neurons (Gnahn et al., 1983; Hefti et al., 1984; Whittemore and Seiger, 1987; Williams et al., 1986). For example, NGF is synthesized in hippocampus and frontal cortex, and released in the proximity of nerve terminals of the basal forebrain where it is bound by NGFR, internalized, and retrogradely transported to mostly, but not only, the cholinergic neurons of the basal forebrain nuclei (Johnson et al., 1987). After fimbria-fornix transection, a lesion that interrupts the NGF–NGFR flux between the hippocampus and the basal forebrain, the cholinergic neurons of the diagonal band of Broca and septum undergo rapid cell death or severe cell shrinkage. Exogenous administration of NGF prevents this phenomenon in rats, when the fimbria-fornix has been severed or aspirated, thus demonstrating that the cell death exhibited by the cholinergic neurons of the basal forebrain under these conditions is likely caused by the lack of retrogradely transported NGF of hippocampal origin (Hefti, 1986; Will and Hefti, 1985; Williams et al., 1986). In aged humans and rats, there are deficits in NGF and NGFR (Angelucci et al., 1988b; Gomez-Pinilla et al., 1989; Hefti and Mash, 1989; Koh and Loy, 1988; Larkfors et al., 1987; Mufson et al., 1989a,b). The intraventricular infusion of NGF into aged rats rescues septal cholinergic neurons and im-

proves behavioral performances in a spatial orientation task (Fischer et al., 1987).

It must be remembered that NGFR is also expressed in noncholinergic areas of the brain and spinal cord, where it may play different roles in development, such as the regulation of cell migration (Schatteman et al., 1988) and neurite outgrowth (Collins and Dawson, 1983). Also, not all NGF-responsive tissues are in the nervous system. NGF has been shown to act as a mitogen on cultured chromaffin cells (Aloe and Levi-Montalcini, 1979; Lillien and Claude, 1985) and some classes of hemopoietic cells (Matsuda et al., 1988; Thorpe and Perez-Polo, 1987). Thus, different target cells respond to NGF in different fashions.

NGF Responses and Receptors in Glia

NGF does not induce proliferation in astrocytes cultured in serum containing media (Yong et al., 1988a,b). However, astrocytes are capable of supporting the survival of NGF-dependent neurons in vitro (Lindsay, 1979), and can synthesize and secrete NGF (Furukawa et al., 1986; Tarris et al., 1986) in vitro and in vivo after hypoxic injury (Lorez et al., 1989). It has been proposed that low affinity NGF receptors can stabilize neuron–glia interactions (Zimmermann and Sutter, 1983). There, type II, low affinity NGFR may be required for directed axonal growth along Schwann cell surfaces that are decorated with type II NGFR with bound NGF that is released to the high affinity type I NGFR on the advancing growth cone surface during early development or regeneration in PNS (DiStefano and Johnson, 1988b; Taniuchi et al., 1986b). The inability to identify NGFR in adult astrocytes in vivo is consistent with the proposed role for NGFR during early stages of development of nonneuronal cells.

The mol wt of the NGFR present on astrocytes has not been reported, whereas the mol wt of the NGFR–NGF complex present on Schwann cells is similar to the 92 kD NGF–

Table 6
Studies on NGF

Target tissues: In vitro studies
Explanted and dissociated sensory and sympathetic ganglia
Adrenal chromaffin cells
Rat pheochromocytoma (PC 12)
Human neuroblastoma (LAN and SKN-SH subclones)
Explanted CNS tissues
Lymphocytes, thymocytes, and macrophages
Schwann cells, astrocytes, and aligrodendrocytes
Others
Target tissues: In vivo studies
Peripheral nervous system
Central nervous system
Immune system
Adrenal hypothalamic pituitary axis
Reproductive system

NGFR complex present in PC12 cells (DiStefano and Johnson, 1986b; Taniuchi et al., 1986a). When soluble receptor binding assays for NGF are carried out on purified cultured rat astrocytes, there is a low affinity NGF binding site present, with a equilibrium dissociation constant (K_d) of around 2.7 n*M* consistent with the presence of type II or low affinity NGFR (*see* Fig. 10)(Marchetti et al., 1987). Curiously, immunohistochemical staining, using the MC192 monoclonal antibody to rodent NGFR, does not demonstrate the presence of NGFR on nonneuronal cells in adult rat brain, except for Muller glia in retina (Kiss et al., 1988; Schatteman et al., 1988; Foreman et al., unpublished observations) or in cultures of purified astrocytes (DiStefano and Johnson, 1988a; Perez-Polo et al., unpublished observations). It is possible that the loss of neuronal contacts associated with tissue culture may induce the expression of NGFR mRNA and NGFR protein in astrocytes under some culture conditions but not others, in a manner that is similar to that reported for Schwann cells after axotomy (Heumann et al., 1987; Taniuchi et al., 1986a). Thus, the species of NGFR expressed on some NGF responsive cells may depend on their cellular environment.

This may have important significance for differences in neuronal responses, such as cell death during development, as compared to following injury since cellular environments and involvement of the immune system resulting from inflammatory events are likely to be very different in these two circumstances.

NGF and the Immune System

The role for NGF in inflammation and the immune system is not well understood. It has been suggested that NGF may affect the immune response indirectly by increasing sympathetic innervation in immunocompetent organs and, thus, increase catecholamine induced lymphoblast transformation (Abramchik et al., 1988). On the other hand, direct demonstration of NGF binding, NGFR protein, and NGFR mRNA on isolated lymphocytes (*see* Figs. 10, 11, and 12 [Ernfors et al., 1988; Morgan et al., 1989; Thorpe et al., 1987ab]) would suggest that NGF may have direct effects on lymphocytes. In comparison, soluble receptor binding assays for NGF, using lymphocytes, show a single low affinity binding site, with a K_d of around 2.5 n*M* (Morgan et al., 1989; Thorpe et al., 1987a,b). NGF not only increases the proliferation of lymphocytes, but also potentiates the lymphoproliferative responses to several T-cell and B-cell mitogens (Thorpe and Perez-Polo, 1987). NGF also increases the expression of interleukin-2 receptors on lymphocytes (Thorpe et al., 1987b). Unlike astrocytes, the expression of NGFR persists in lymphocytes of adult rats and humans (Ernfors et al., 1988), suggesting that some, but not all, lymphocytes are under the constant influence of NGF. However, it should be remembered that around 85–90% of spleen cells do not stain for NGFR (Thorpe et al., 1987a). Whether these cells reflect the presence of lymphocytes in a quiescent stage, in a way similar to the astrocytes in adult brain, is not clear. Macrophages can respond to injury by synthesizing IL-1, which in turn can stimulate Schwann cells to synthesize and secrete NGF

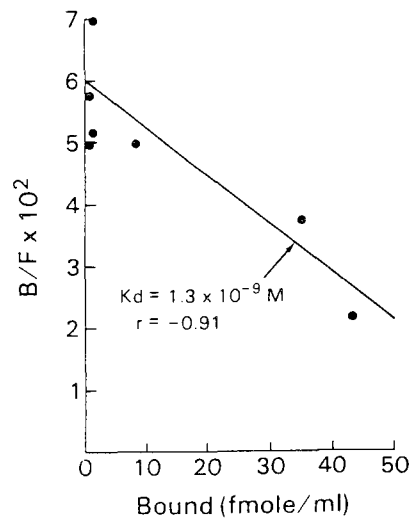

Fig. 10. Scatchard analysis of NGF equilibrium binding to solubilized rat astrocytes in vitro, as described in Marchetti et al., 1987.

(Heumann et al., 1987). More direct stimulatory actions of NGF on most cells have been demonstrated in vivo and in vitro (Aloe, 1990). The effects of NGF on glial and lymphoid cells may be restricted to their early development, or to consequences of injury associated with inflammation in the latter case.

Outcome of Neuronal Injury

It has been established that, although neurons of the adult mammalian PNS are able to regenerate, the opposite is true for most of the CNS, in which abortive sprouting is more common (Ramon y Cajal, 1928). In those instances in the periphery, where it has been established that regeneration takes place, it has been demonstrated that ambient conditions under the control of Schwann and satellite cells are permissive for axonal sprouting, growth, and synaptogenesis. In the periphery, neurons that are isolated from target tissues, for example as a consequence of injury, exhibit a more rigorous dependence on the appropriate survival factors. Also, a procession of metabolic changes takes place in the Schwann and satellite cells, such as expression of NGF and NGFR mRNA,

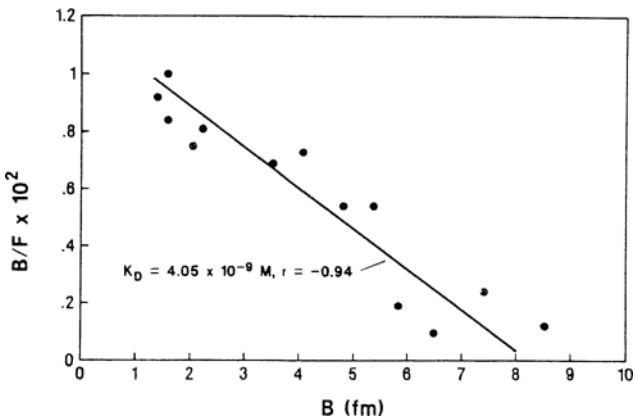

Fig. 11. Scatchard analysis of equilibrium NGF binding to human peripheral blood monocytes, as described in Morgan et al., 1989.

among others, that may account in part for the success of regeneration in the periphery, as compared to the CNS. The time sequelae involved in these injury induced changes in the nonneuronal cells of the periphery may be one of the important factors that differentiate the PNS from the CNS with respect to regeneration. Less is known about the molecular signals that act on glial and mast cells as part of the inflammation, gliosis, and scarring associated with neuronal injury. Thus, manipulations of ambient levels of neuronotrophic substances, and of other time-dependent events involved in the neuronal response to injury may answer the question of whether external manipulation of the organism can overcome the inability of the CNS to recover functionally from certain traumatic injuries.

The Effect of Exogenous NGF on the Outcome of Neuronal Injury

The human neuroblastoma cell line SK-N-SH-SY5Y (SY5Y) is a nearly diploid NGF responsive cell line that in the presence of NGF extends neurites, and undergoes cell hypertrophy. In vitro NGF treatment of SY5Y cells protects them against 6-hydroxydopamine (6-OHDA), a known generator of H_2O_2 (Tiffany-

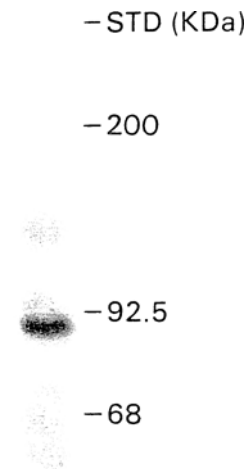

Fig. 12. SDS-PAGE analysis of immunoprecipitated rodent splenocyte NGFR from Thorpe et al., 1987.

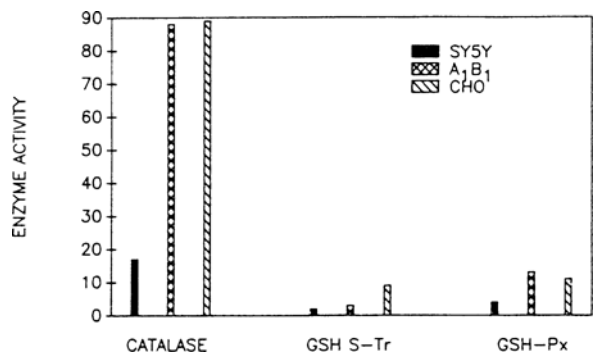

Fig. 13. Antioxident enzyme levels of SY5Y, human glioma A_1B_1, and Chinese hamster ovary CHO cells. Enzyme levels expressed as units of activity per 10_6 cells. Adapted from Tiffany-Castiglioni et al., 1982.

Castiglioni and Perez-Polo, 1981). The SY5Y line has low endogenous levels of catalase, glutathione transferase, and glutathione peroxidase activity as compared to A1B1 human glioma cells and CHO Chinese ovarian hamster fibroblast cells (Fig. 13)(Tiffany-Castiglioni et al., 1982). The protection of the SY5Y was shown to be caused by NGF stimulation of metabolic events, and not as a result of dopamine uptake, morphological, or extracellular substrata associated events (Tiffany-Castiglioni et al., 1979, 1982; Tiffany-Castiglioni and Perez-

Polo, 1980, 1981; Perez-Polo and Werrbach-Perez, 1985, 1987). Thus, when SY5Y uptake of labeled dopamine was measured in the presence of 6-OHDA, there was a marked inhibition of dopamine uptake, suggesting that the 6-OHDA, a dopamine analog, could compete for the labeled dopamine. This would suggest that, if dopamine, and hence 6-OHDA uptake, were to be blocked, there would be a reduction in toxicity resulting from the intracellular 6-OHDA. Since cocaine, a dopamine blocker, does not significantly alter 6-OHDA toxicity, 6-OHDA effects are independent of dopaminergic transport systems (Perez-Polo et al., 1982b). Similarly, treatment of SY5Y with dibutyryl cyclic AMP, which elicits neurites but not other NGF effects, did not protect cells from 6-OHDA, but rather exacerbated toxicity (Tiffany-Castiglioni and Perez-Polo, 1980; 1981; Perez-Polo and Werrbach-Perez, 1985, 1987). Rather, 6-OHDA generated free radicals appear to be the principal toxic agent, and catalase, but not superoxide dismutase, SOD, affords almost total protection from 6-OHDA insults in culture (Tiffany-Castiglioni et al., 1982; Perez-Polo and Werrbach-Perez, 1985, 1987).

Since 6-OHDA toxicity relies on the generation of hydrogen peroxide, the application of hydrogen peroxide itself can be used as a model of free radical damage in the CNS (Kovachich and Mishra, 1980; Pellmar, 1986, 1987). Hydrogen peroxide reacts with transition metals, such as copper and iron, to produce the very reactive hydroxyl free radical through the Fenton reaction (Haber and Weiss, 1934). The hydroxyl radical is a strong oxidant and, as such, can initiate lipid peroxidation.

Chain reactions in the lipid environment of the plasma membrane result in an amplification of cytoplasmic hydroxyl radical levels, which in turn inactivate proteins, cleave DNA, and disrupt energy metabolism (Kim et al., 1985; Hyslop et al., 1988; Imlay et al., 1988). Treatment of both PC12 and SY5Y with NGF increases catalase and glutathione peroxidase activity levels, but has no effect on SOD activity (Fig. 14). Dose–response curves for H_2O_2 toxicity, and for the protection afforded by catalase and NGF to PC12 and SY5Y, are similar (Figs. 15 and 16). In both instances, addition of exogenous SOD to cultures offers no protection. This is not surprising, since for fixed low levels of catalase activity, addition of SOD will further dismute those oxyl radicals present under culture conditions and further elevate the concentration of hydrogen peroxide (Fig. 2). Since PC12 cells aggregate in the presence of NGF, making dye exclusion tests for viability difficult to evaluate, ^{35}S-met incorporation into protein can be used as a viability test under certain conditions, as described in Tiffany-Castiglioni and Perez-Polo (1980). If PC12 cells are protected from H_2O_2 by NGF in the presence or absence of aminotriazole, AZ, a small mol wt inhibitor of catalase that can cross the plasma membrane, NGF protection is significantly reduced (Fig. 17). It is interesting that, for cells not treated with NGF, there is a significant loss in viability in those cultures exposed to H_2O_2 and AZ when compared to those deprived of AZ, suggesting that AZ is acting here to reduce endogenous levels of cellular catalase activity. Taken together, these results suggest that neuronal cells in vitro are more susceptible to free radical damage compared to nonneuronal cells, and that NGF confers partial protection to PC12 and SY5Y cells against H_2O_2 by virtue of its stimulation of catalase, but not superoxide dismutase activity.

The Conditioning Lesion Paradigm

If a neuron suffers a second injury in close temporal proximity to a first injury, the rate at which regeneration takes place is significantly accelerated (Gutmann, 1942). This effect on regeneration by a prior injury is called the "conditioning lesion" paradigm. Conditioning lesion effects have been described for the CNS (Grafstein and McQuarrie, 1978; McQuarrie and Grafstein, 1982; Nieto-Sampedro and Cotman, 1985; Nieto-Sampedro et al., 1987) and the PNS (Edstrom and Kanje, 1988; Jenq et al., 1988; Perry et al., 1987). One proposed mechanism of ac-

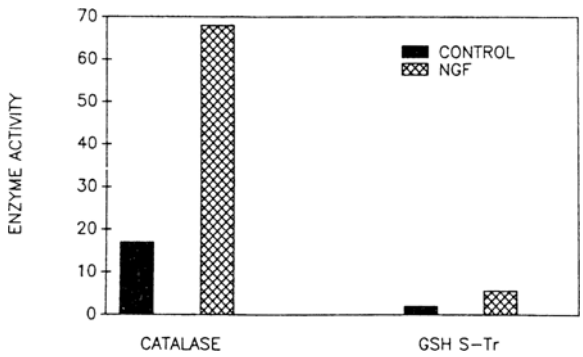

Fig. 14. Effect of NGF (1 μg/mL) treatment on the antioxidant enzymes catalase and glutathione transferase in SY5Y cells after 5 d in culture. Enzyme activity expressed in Units 1 10_6 cells, as described in Tiffany-Castiglioni et al., 1982. Similar results were obtained on PC12 cells (data not shown). In all instances, there were no effects on SOD activity levels by NGF treatment, irrespective of whether activity was determined on a per 10^6 cells or per mg of protein basis (data not shown).

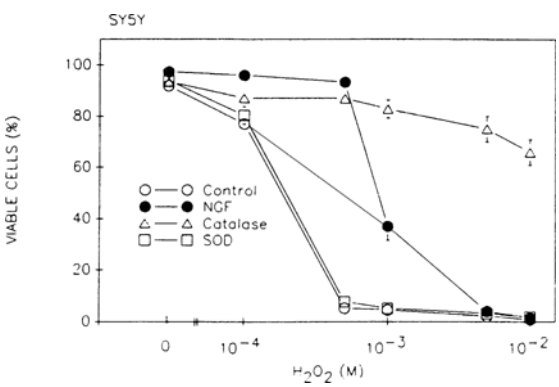

Fig. 15. Dose response of H_2O_2 effect on SY5Y cell viability determined by dye exclusion following treatment with (50 μg/mL) superoxide dismutase, SOD (50 μg/mL), or NGF (1 μg/mL) for 5 d, as described in Jackson et al., 1990a.

tion is that injury induces the synthesis and secretion of neuronotrophic and neurite promoting factors. Thus, at the time of the second lesion, cells are primed in the sense that critical mRNA species would be available for translation independent of transcription. Also since the increase in trophic activities takes 6–12 d, an effect of a prior lesion is to augment significantly ambient levels of neuronotrophic growth

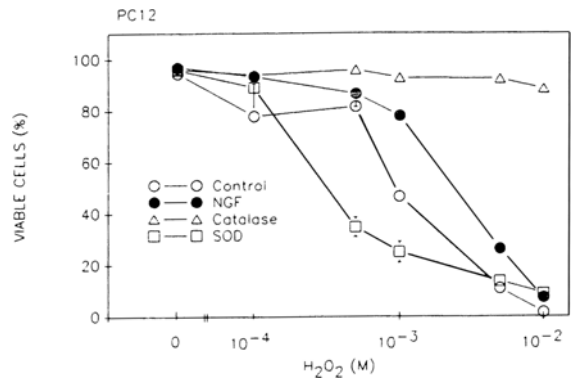

Fig. 16. Dose response of H_2O_2 effect on PC12 cell viability determined by dye exclusion, following treatment with as described in Morgan et al., 1989.

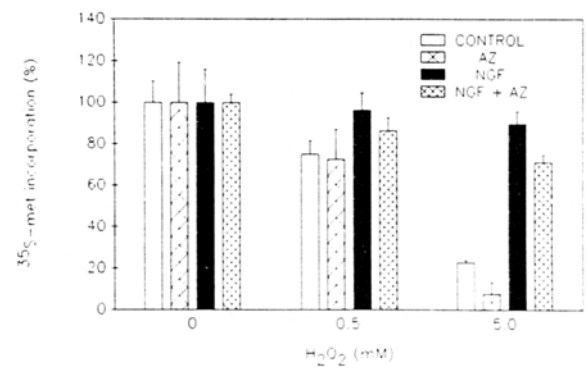

Fig. 17. Effect of aminotriazole on NGF protection of PC12 from H_2O_2. Viability determined by ^{35}S-met incorporation into protein, as described in Jackson et al., 1990a.

factors to beneficial levels at a time when, as a consequence of axotomy, there is a disruption of the retrograde transport of growth factors from nerve terminals to the soma of the neuron. Increased availability of growth factors then can reduce secondary neuronal cell death, and can have the greatest biological impact and facilitate the acceleration of recovery from further injury (Manthorpe et al., 1983; Needels et al., 1985, 1986; Nieto-Sampedro et al., 1983, 1984, 1987; Whittemore et al., 1985).

Since many traumatic or ischemic injuries to the nervous system generate free oxygen radical species (Halliwell and Gutteridge, 1985; McCord, 1985), and NGF treatment in vitro imparts resistance to H_2O_2 (Jackson et al., 1990a;

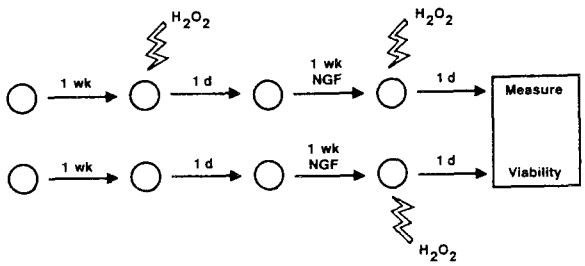

Fig. 18. Conditioning lesion paradigm. PC12 cells are grown in log phase at equal cell densities. Cells are exposed, or not, to hydrogen peroxide at three concentrations (0, 0.5, and 5 m*M*) of H_2O_2. Survivors are harvested 1 d later and grown for 1 wk exposed, or not (not shown), to NGF for 1 wk, at which time, *at equal cell densities*, cells are exposed to varying concentrations of H_2O_2 and viability measurements are carried out 1 d later, as described in Jackson et al., 1990a,b.

Tiffany-Castiglioni and Perez-Polo, 1981; Tiffany-Castiglioni et al., 1982), it is of interest to know the extent to which conditioning lesions enhance regeneration by virtue of induction of catalase, stimulated NGF autocrine effects, or in other ways (Jackson et al., 1990b). In focusing on catalase, it is clear that only one component of antioxidant cellular defenses is being addressed, and that the mechanism by which NGF stimulates catalase activity may not be similar to effects on other aspects of oxidant–antioxidant balance and energy metabolism.

Trophic Effects of Conditioning Lesions

When PC12 cells are exposed to a conditioning lesion consisting of either 0.5 m*M* ("low-dose") or 5.0 m*M* ("high dose") H_2O_2 for 30 min, followed by a recovery period in the presence or absence of NGF (1 μg/mL), cells display an enhanced ability to survive a second H_2O_2 treatment (Figs. 18, 19, 20). In the absence of NGF, the effect of a low-dose conditioning lesion is an apparent upward displacement in the dose response to H_2O_2 for lesioned survivors. This is not what is observed in the presence of NGF, in which the effect of the conditioning lesion on the response of PC12 to further injury is enhanced by NGF.

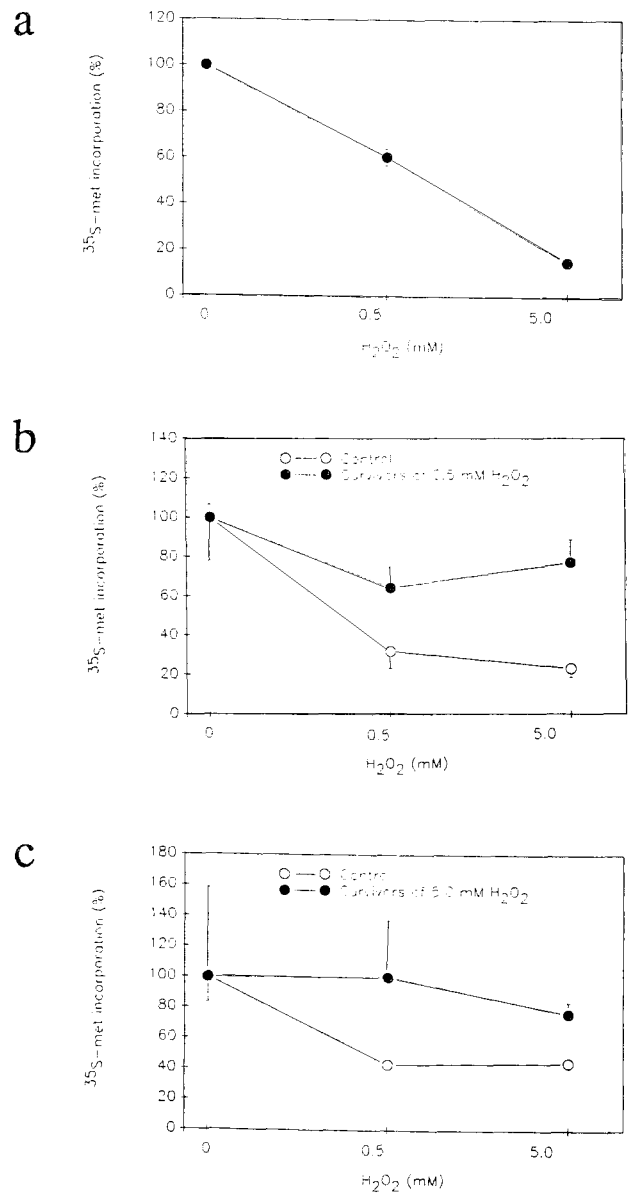

Fig. 19. PC12 cells were grown and exposed to H_2O_2 conditioning lesions as described in Fig. 19 and in Jackson et al., 1990a,b. (**A**) Survivors of conditioning lesion. (**B**) Effect of 0.5 m*M* H_2O_2 conditioning lesion on subsequent H_2O_2 injury. (**C**) Effect of 5 m*M* H_2O_2 conditioning lesion on subsequent H_2O_2 injury.

The conditioning lesion enhancement of NGF protection from H_2O_2 cannot be accomplished by increasing NGF concentration (Jackson et al., 1990b). Also, the conditioning lesion by itself does not dramatically alter the catalase-

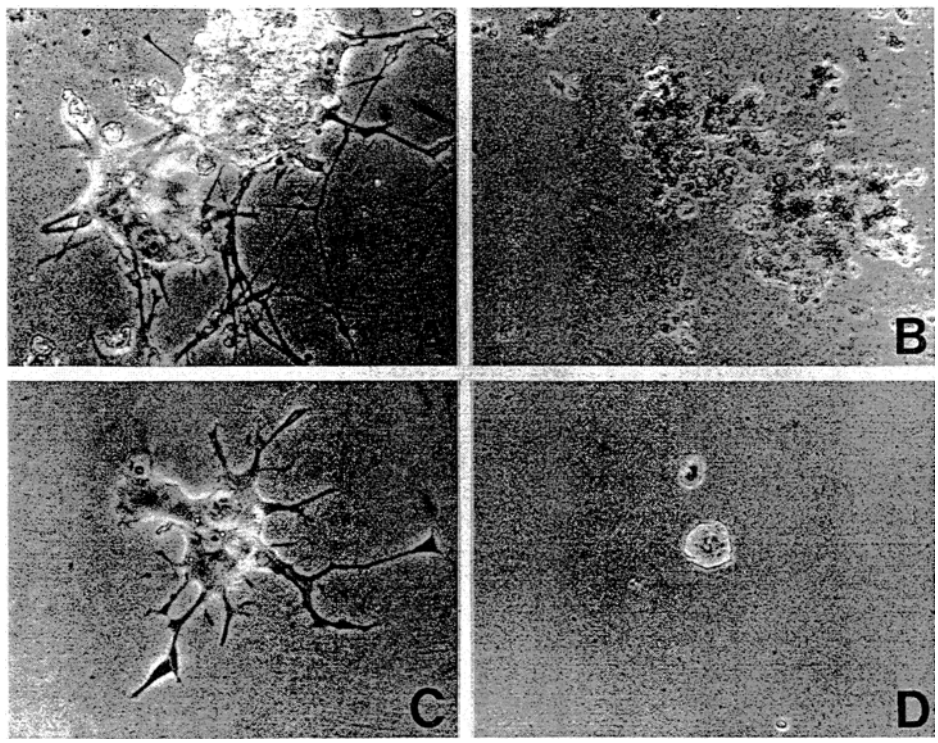

Fig. 20. Morphology of PC12 cells conditioned at 0.5 m*M* H_2O_2 and controls: (A) conditioned at 0.5 m*M* H_2O_2 and treated with NGF; (B) conditioned at 0.5 m*M* H_2O_2 without NGF; (C) control PC12 cells treated with NGF alone; (D) control PC12 cells treated with neither H_2O_2 nor NGF. Phase contrast. x250. From Jackson et al., 1990b.

specific activity measured in lesioned survivors. This would suggest that NGF protection and conditioning lesion protection mechanisms may have overlapping components, but are different with regard to effects on the regulation of catalase activity. The net effect of the high dose conditioning lesion is primarily cytotoxic, and NGF is incapable of eliciting neurite extension among these surviving PC12 cells. Furthermore, the survivors of the low-dose conditioning lesion display an accelerated rate of neurite elongation, following NGF exposure, which is analogous to the enhanced rate of axonal outgrowth reported for conditioning lesions in vivo (data not shown). The degree of neurite extension observed in lesion survivors after 24 h is comparable to that seen only after 3–4 d of NGF treatment in control cells. This would suggest that the conditioning lesion does not merely select cells, but rather has a stimulatory effect on cells, although stimulation of catalase activity is not a major component of this stimulus as opposed to the NGF effect.

Antioxidant Effects of a Conditioning Lesion

Two explanations of the conditioning lesion phenomenon may be proposed: first, PC12 cells in culture express a spectrum of antioxidant capabilities. The conditioning lesion selects for those cells with more robust enzymatic defenses. Second, the conditioning lesion itself acts to induce free radical detoxifying activities. The second explanation proposes that sublethal doses of H_2O_2 are capable of inducing a xenobiotic response that is similar to the heavy metal induction of metallothionein proteins or of DNA repair enzymes by ionizing radiation (Durnham and Palmiter, 1981, 1984; Kyse and

Tyrrel, 1987). There are reports of induction of hydrogen peroxide resistance by pretreatment with low concentrations of H_2O_2 (Laval, 1988; Spitz et al., 1987).

If the first explanation is correct, i.e., the conditioning lesion acts via a selective, rather than an inductive, mechanism, one would expect that a stronger selective stimulus would result in a more pronounced resistance to injury. This clearly is not the case, as evidenced by the lack of significant resistance imparted by pretreatment with 5.0 m*M* H_2O_2. Thus, the available evidence seems to favor the second explanation that sublethal H_2O_2 treatment stimulates antioxidant activities in PC12. It should be noted, however, that the two hypotheses are not mutually exclusive and that, for different concentrations of H_2O_2 exposure, different mechanisms may be involved. The results discussed here would be in agreement with there being a stimulation of antioxidant activities by lesions at sublethal levels. Since NGF treatment of PC12 cells does induce small, but significant, increases in the activity of catalase (Jackson et al., 1990a), one possible explanation is that pretreatment with a sublethal dose of H_2O_2 increases resistance to further insults via an enhancement of catalase activity. To test this hypothesis, one can compare the levels of catalase activity in naive PC12 cells to the survivors of low-dose H_2O_2 treatment, both in the presence and absence of NGF. In all instances, H_2O_2 treatment resulted in lower levels of catalase-specific activity in surviving PC12, irrespective of whether NGF treatment was involved or not. Thus, PC12 survivors of a conditioning lesion had lower levels of catalase-specific activity as compared to unlesioned PC12 cells, and NGF-treated PC12 survivors of a conditioning lesion had lower levels of catalase-specific activity as compared to unlesioned NGF-treated PC12 cells. Clearly these results speak against selection of PC12 cells within the population that have high endogenous levels of catalase activity by NGF or conditioning lesions. Also, this result would suggest that, although the mechanism of action of protection from H_2O_2 by NGF and the conditioning lesions may share certain elements, the stimulation of catalase activity is not one of them (Jackson et al., 1990a,b).

These data are supportive of the hypothesis that one of the effects of the conditioning lesion is to shift cellular oxidant–antioxidant balance to a more resistant state, and to accelerate NGF-induced neurite outgrowth. It is not known whether such a mechanism is present following neuronal injury in vivo, where injury-induced neuronotrophic factors may act in a paracrine fashion, minimizing a secondary wave of neuronal cell death. Such an interpretation is consistent with the results obtained in vitro, and provides a mechanistic model for the study of conditioning lesions and the design of therapeutic strategies for neuronal injury. This would suggest that cell survival and cell death are highly regulated events and that both oxidative stress, as evidenced in a conditioning lesion, and neuronotrophic factors have direct and somewhat different regulatory roles through induction of catalase activity in the case of NGF, and of other antioxidant mechanisms in the case of conditioning lesions. This is particularly interesting because these specific conditioning lesion effects speak to autocrine-like responses that are independent of externally provided growth factors in a possible paracrine fashion. The nature of such "lesion-dependent" messenger molecules is not known.

NGF and the Aged CNS

It would appear that there may be a correlation between cognitive deficits expressed in the aged and the levels of trophic activity in cholinergic areas of the CNS, as measured by the functional levels of critical growth factors, such as NGF and their receptors (Cortes et al., 1989; Eldridge et al., 1989 a,b; Flood and Coleman, 1988; Gage et al., 1988; Gomez-Pinilla et al., 1989; Hefti and Mash, 1989; Koh and Loy, 1988; Lahtinen, 1989; Larkfors et al., 1987, 1988; Mufson et al., 1989a,b; Pezzoli et al., 1988). Since there is a reduction in trophic and principally

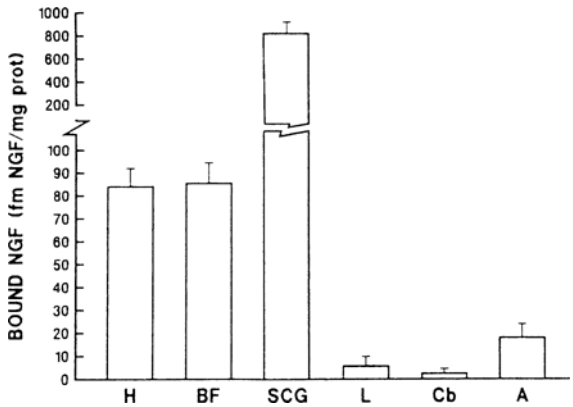

Fig. 21. NGF binding in rodent brain. B_{max} obtained by Scatchard analysis of ^{125}I-β-NGF binding to NP-40-solubilized tissues of rat brain. H: hippocampus, BF: basal forebrain, as compared to SCG: superior cervical ganglion, L: liver, Cb: cerebellum, and A: adrenal. From Angelucci et al., 1988.

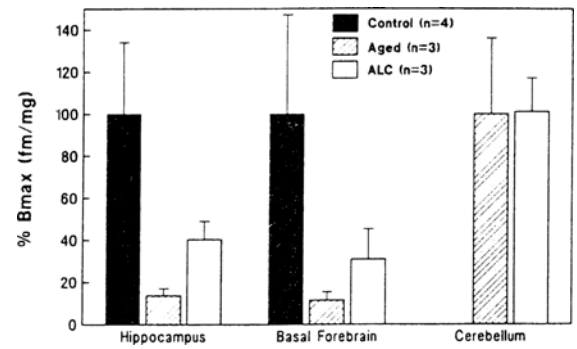

Fig. 22. Effect of aging and acetyl carnitine treatment of aged rodents on NGF binding in rodent brain. Relative B_{max} obtained by Scatchard analysis of ^{125}I-β-NGF binding to NP-40-solubilized brain tissues of young, aged, or ALC-treated aged rats expressed as percent of values obtained for 4-mo-old rats. From Angelucci et al., 1988.

NGF associated activity in those cholinergic regions that display age-associated pathology (Gomez-Pinilla et al., 1989; Hefti and Mash, 1989; Koh and Loy, 1988; Kudo et al., 1989; Larkfors et al, 1988; Mufson et al., 1989a,b), it is not surprising that there is a reduction in the NGF and NGF binding capacity in the neurons of the aged rodent basal forebrain and hippocampus in the CNS, as well as in sympathetic neurons in the PNS (*see* Figs. 21 and 22) (Angelucci et al., 1988a; Uchida and Tonionega, 1987). Similar deficits in NGF and NGFR protein and mRNA have also been demonstrated in the CNS although, at the present time, it is not known if these deficits are a consequence of neuronal atrophy and cell loss there, or are a cause of such cell loss and atrophy (Flood and Coleman, 1988). It has been suggested that addition of exogenous NGF may reverse some cognitive deficits in the aged (Phelps et al., 1989).

It is not known if the mechanism by which NGF rescues basal forebrain cholinergic neurons following deafferentation lesions in the adult is the same as that by which NGF has sparing effects on aged rat cholinergic neurons of the CNS. It is difficult to speculate as to differences in possible mechanisms of cell death, such as death resulting from neuronal injury as discussed here and cell death among NGF responsive neurons in the aged CNS. It is encouraging that acetyl-L-carnitine, a substance that ameliorates some age-associated cognitive deficits in aged rodents (Angelucci et al., 1986; Angelucci and Ramacci, 1986) and that appears to prevent age-associated decreases in NGF binding in hippocampus and basal forebrain (Angelucci et al., 1988), can also stimulate NGF binding activity in PC12 cells (Taglialatela et al., 1990a,b). Although the precise mechanism by which acetyl-L-carnitine stimulates NGFR expression is not known, it is likely to be a general stimulation of trophic activity in the CNS acting by appropriate increased receptor expression.

Effects of Cold Stress on NGF Binding Activity

It is intriguing that, during late aging events in the CNS, there is a similar loss of corticosteroid and NGF receptors associated with cholinergic neuronal shrinking and cell death in the hippocampus (Angelucci et al., 1988a,b). One explanation is that age-associated reductions in NGF activity might be the result of a disinhibition of the hypothalamic–pituitary–adrenocortical–axis (HPAA), and the resultant

prolonged exposure of the CNS to increased corticosterone in the rat, as aging progresses. In this context, many of the age-associated deficits would result more directly from the disinhibition of feedback mechanisms in HPAA. Two obvious consequences would be a lowering of immunoreactivity and of the stress response setpoint, and the feedback mechanisms responsible for the return of corticosteroid plasma levels to basal values after a stressful event. Thus, stressful events might have been expected to have an effect on NGF binding capacity in hippocampus. When adult rats are exposed to 1 h of cold stress for 5 d, there is a reduction in the measured B_{max} for ^{125}I-NGF in the hippocampus and basal forebrain, in agreement with the hypothesis that corticosteroid and NGF binding activity are related. The nature of this relationship is not simple. There are also reports of other behavioral variables that have effects on NGF activity in vivo (Alleva et al., 1986, 1987; Aloe et al., 1986; Lakshmanan, 1986, 1987).

Understanding the role of NGF action in aged dysfunction in CNS will require a knowledge of the molecular mechanisms by which NGF and corticosteroid affect neuronal cell death in the hippocampus. Whether these aspects of cell death share common features with cell death regulation after injury or during development remains to be determined. The use of common NGF responsive neuronal tissues in the paradigms described here may prove useful to the understanding of neuronal cell death mechanisms.

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