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The regulation of NGF mRNA in the rodent central nervous system

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THE REGULATION OF NGF mRNA IN THE RODENT CENTRAL NERVOUS SYSTEM

By

Ronald P. Hellendall

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

THE REGULATION OF NERVE GROWTH FACTOR mRNA IN THE RODENT CENTRAL NERVOUS SYSTEM

by

Ronald P. Hellendall

Advisor: Dr. Mariann Blum

Recent evidence has indicated a role for nerve growth factor (NGF) in the central nervous system (CNS). The observation that a population of cholinergic cells in the CNS can respond to NGF and that similar cells undergo profound degeneration in Alzheimer's disease has provoked interest in the role and regulation of NGF in the CNS. While evidence supports a neuronal expression of NGF mRNA in the intact adult brain, recent reports demonstrate that other cells found in the CNS have the capacity to express NGF. The finding that Schwann cells in the peripheral nervous system can produce NGF during development and subsequent to injury indicates that the elaboration of trophic factors by glia may play a role in the survival of neurons. Studies were conducted to determine if glia in the CNS can also express this growth factor. High levels of NGF mRNA were found in enriched astrocyte cultures derived from hippocampus, cerebral cortex, hypothalamus, and cerebellum of the neonatal rat brain. A trend toward lower NGF mRNA levels was found when cultures from cerebral cortex were maintained for 25 days *in vitro*. Interleukin-1 (IL-1), a cytokine released by phagocytic cells and shown previously to induce NGF mRNA in the sciatic nerve, significantly increased NGF mRNA in astrocyte cultures from a variety of CNS regions. This induction was not observed in the 25 day cultures nor were NGF mRNA levels changed in whole hippocampus when IL-1 was injected in the lateral ventrical of adult male rats. These data indicate a role for astrocyte

released NGF during specific stages of development of the CNS and suggest a maturational effect where astrocytes no longer provide trophic support for neurons and NGF is primarily expressed in neurons in the adult. The absence of NGF mRNA induction following IL-1 injection in the adult indicates the mature CNS may lack a population cells able to regulate NGF expression in response to trauma. Lack of trophic support may be a component in the inabiltiy of CNS neurons to regrow subsequent to injury.

Acknowledgements

Christine Lackner and Dr. Diana Casper performed the isolation of astrocyte and neuron enriched primary cultures. The generous assistance of Dr. Casper in lending her expertise to the immunocytochemical protocols is appreciated. The hippocampal slice protocol is based upon that used by Drs. Robert Blitzer and Emanuel Landau, Department of Psychiatry, Mount Sinai Medical Center. Dr. Blitzer loaned us a metabolic chamber initially used in these experiments and provided helpful support during the course of these studies. Dr. George Kuchel was an equal participant in the effort to develop the hippocampal slice procedure.

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Introduction

NGF in the peripheral nervous system

Work nearly four decades ago resolved that mouse sarcoma tissue released a factor which could stimulate the growth of embryonic chick sensory and sympathetic ganglia. The substance was termed nerve growth factor (NGF) and has been studied extensively. The most common attribute associated with the biological effects of NGF is its influence on the growth and maintenance of survival of sympathetic chain ganglia and sensory ganglia of neural crest origin (Purves and Lichtman, 1985; Thoenen and Barde, 1980). Dramatic effects have also been consistently observed on adrenal chromaffin cells *in vitro* and on a rat pheochromocytoma cell line (PC12) which has become the basis of much NGF research. Following NGF (or fibroblast growth factor, FGF) exposure PC12 cells undergo biochemical and morphological changes from a round chromaffin-like cell to a phenotype characteristic of differentiated sympathetic neurons (Greene and Tischler, 1982). Although the entire constellation of events accompanying this transformation is not understood, some of the altered parameters include induction of a number enzymes, increased synthesis of proteins which are associated with the early increased activity in cyclic AMP-and Ca^{2+}-phospholipid dependent protein kinases and the rapid induction of the oncogene c-*fos* (see Altin and Bradshaw, 1990; Tiercy and Shooter, 1986). NGF also plays a role in maintaining the differentiated state of cells: removal of NGF from PC12 cells results in the retraction of neurites and the return to normal chromaffin-like biochemical and morphological parameters. In the animal, briefly stated, NGF is involved in the following responses (Thoenen and Barde, 1980): 1) During specific perinatal periods, sympathetic and NGF responsive sensory cells of the neural crest 'compete' for a limited supply of the

factor produced and secreted from the organ to be innervated. Those cells successful in binding and incorporating NGF and, most likely other factors, will survive while others will not (programmed cell death). Application of NGF or its antibody can dramatically alter the number of surviving cells during this period. In addition, Schwann cells in the developing PNS transiently synthesize NGF and the NGF receptor and both decrease with maturation of the nerve. This has led one group to suggest peripheral glia may provide temporary support for the migrating axons prior to target innervation (Johnson *et. al.*, 1988). 2) Following differentiation, some of these cells appear to require NGF for survival into adulthood while others do not. 3) NGF produces specific biochemical, cytoskeletal and morphological responses in cells and is itself under regulatory control in target organs.

Upon the serendipitous discovery of an extremely rich source of NGF, the male mouse submandibular gland, the amino acid sequence has been elucidated (Angeletti and Bradshaw, 1971) and the gene has been cloned and the sequence is highly homologous across mouse, rat, and human (Scott *et. al.*, 1983; Ullrich *et. al.*, 1983; Whittemore *et. al.*, 1988). A number of excellent reviews describe the subunit structure and processing of NGF to its mature form (Thoenen and Barde, 1980; Greene and Shooter, 1980) and this will not be discussed here. There is evidence, however, that alternate transcripts of NGF mRNA may be produced in different tissues. Using primer extension and S1 nuclease analysis, Rutter's group (Edwards *et. al.*, 1986; Selby *et. al.*, 1987) has shown that four transcripts are produced which predict three closely related precursor proteins in the mouse SMG. While the mature mRNA is not affected, alternate splicing of exon 2 changes the presumptive translation initiation site and therefore displaces the hydrophobic sequences of the signal peptide. The longer transcript, containing the 5' extension inclusive of exon 2, was found to be in ten times greater abundance in the mouse SMG and placenta than the transcript lacking exon 2, while in all other tissues the transcript lacking exon 2 was 5 times in excess (Selby et. al., 1987) The significance of this differential splicing is unclear.

These transcriptional modifications have no effect on the NGF peptide: the final 13KD peptide forms a homodimer upon dissociation from the a 7S complex at low physiological concentrations (Thoenen and Barde, 1980). The dimerized form may play an important role in the induction of internalization subsequent to binding to the receptor and leading to the multiple biological effects of NGF.

As noted by Thoenen and Barde (1980), the most revealing method to analyze the dependence of cells for NGF is to observe the effects of anti-NGF antibodies during various developmental periods and to monitor the requirement of these cells for NGF *in vitro*. Injection of antibodies into newborn rats or mice results in complete loss of sympathetic ganglia after three days with no effect on sensory ganglia (Levi-Montalcini and Booker, 1960; Levi-Montalcini and Angeletti, 1966). Conversely, when NGF is given to 6-day-old rats, the developmentally regulated cell loss does not occur and an actual increase in the number of ganglionic neurons is observed (Hendry and Campbell, 1976; Yanker and Shooter, 1982). Aloe and Montalcini (1979) produced a massive transformation of immature chromaffin cells to sympathetic neurons when rats were injected on day 17 of gestation and on each of the first 10 days of life. Thus a number of different cell phenotypes pass through a sensitive period where their survival and, regarding chromaffin precursors, their final differentiated state is dependent upon NGF. In the adult, sensory ganglia are NGF independent while dependence remains in sympathetic neurons (Otten *et. al.*, 1979; Gorin and Johnson, 1980). Interestingly, the death of cultured neonatal sympathetic neurons by NGF antibody can be completely prevented by inhibiting protein or RNA synthesis suggesting NGF may suppress an ongoing cytotoxic process in cells (Martin *et. al.*, 1988). With the recent development of highly sensitive assays to detect NGF and NGF mRNA, there is now a large body of evidence for the following events underlying its biological actions: 1) NGF is synthesized and released from peripheral target organs of sensory and sympathetic ganglia in direct proportion to (with some notable exceptions) the density of sympathetic innervation (Shelton and Reichardt, 1984;

Heumann *et. al.*, 1984). The current evidence does not support the view that NGF acts as a chemotropic agent during development but rather provides the substrate for survival subsequent to innervation (Davies *et. al.*, 1987). 2) The factor binds to a cell surface receptor on the presynaptic membrane, is rapidly endocytosed and retrogradely transported to the soma in a membrane bound vesicle. Demonstration that retrograde transport is crucial for many of the effects of NGF was shown when disruption of axonal transport had similar consequences as the addition of NGF antibodies. 3) NGF induces both transcription dependent and independent changes in responsive cells.

While other diffusible factors unquestionably play a role in the development and maintenance of neural crest derived cells, glucocorticoids are especially important having apparently antagonistic effects to NGF on developing chromaffin cells (Landis and Patterson, 1981). Whereas dissociated chromaffin cells from neonatal rats display neurites within 3 days of exposure to NGF, dexamethasone (10^{-5} M, DEX) completely abolishes this effect (Unsicker et. al., 1978). Greatly reduced fiber outgrowth was found by this group when cultured neonatal sympathetic ganglia were cultured with DEX. Tyrosine hydroxylase (TH) activity was unaffected in the presence of DEX alone though when DEX and NGF were combined, TH induction was greater than with NGF alone. In perinatal chromaffin cells DEX blocks the NGF induced increase in choline acetyltransferase (ChAT) activity and stimulates TH activity (Edgar and Thoenen, 1978) by increasing TH mRNA levels (Baetge et. al., 1981). This opposing effect by DEX is not observed in PC12 cells Thus glucocorticoids, diffusing from the developing adrenal cortex may directly influence the phenotypic fate of a subpopulation of neural crest derived cells (Landis and Patterson, 1981, Smith and Fauquet, 1984).

In a separate context, glucocorticoids also appear to regulate NGF expression: exposure of L-929 murine fibroblast cells to 10^{-7}M cortisone for 3 days abolished the normal constitutive release of NGF into the conditioned medium (Siminoski et. al., 1986). This reduction is reflected in a parallel 90% down regulation of NGF mRNA (Siminoski et. al.,

1987). Similarly, when L cells are exposed to 10^{-6}M DEX for 4 hrs, NGF mRNA levels are reduced to 10% of controls (Wion et. al., 1986). These findings contrast with the mouse submandibular gland where 5 or 10 day application of corticosterone gives a 200% increase in NGF in females (Walker et. al., 1981). Thus glucocorticoids may exert tissue specific regulation on NGF synthesis.

A number of recent studies in the PNS have begun to reveal mechanisms which may underlie the regulation of expression of NGF and NGF-R during development and following injury in the adult. Thoenen's group (Davies et. al., 1987) has studied the sensory innervation of the whisker pad by the trigeminal ganglia in the embryonic mouse as a prototype for the induction and regulation of NGF and its receptor during development. Following exposure of explants of embryonic trigeminal nerve to ^{125}I-NGF (to display NGF receptors), emerging sensory fibers remained unlabeled until a developmental stage where they would be reaching their target. This implies that the initiation and direction of growth of developing neurites is independent of NGF. In addition, apparent support for this view comes from the same study showing lack of NGF mRNA at the target site until the arrival of the trigeminal fibers. However, there is evidence that a diverse population of outgrowing peripheral nerve fibers may be responsive to NGF during development and that NGF is produced in the peripheral nerve at a similar stage. A series of recent reports have shown that NGF receptor is transiently expressed in a large variety of peripheral ganglia during development at a period corresponding to post mitotic differentiation and target innervation (Buck *et. al.*, 1987; Yan and Johnson, 1988; Enfors *et. al.*, 1989; Katz *et. al.*, 1990a). In addition, at birth, in the rat sciatic nerve, NGF mRNA and NGF-R mRNA are 120- and 10-fold higher respectively than the adult and this expression appears to be localized to Schwann cells (Heumann et. al., 1987a). All Schwann cells, not just those encapsulating the sympathetic and sensory fibers, appear to participate in this induction (Bandlow et. al., 1987). Thus a variety of developing peripheral neurons are responsive to

NGF during the period of neurite outgrowth into an environment containing nonneuronal cells expressing NGF.

An important consideration for studies localizing and analyzing expression of the NGF receptor, and a possible explanation of the apparent discrepancy with the Thoenen studies, is that the overwhelming majority of this work uses probes and techniques which solely detect the low affinity receptor. As noted, this receptor *per se* does not mediate the effects of NGF and its relationship to the high affinity, physiologically relevant receptor remains controversial. While this topic is beyond the scope of this report, Johnson (see Yan and Johnson, 1988) has reported a difference in total NGF-R protein found in the developing rat superior cervical ganglion when detected by anti-NGF-R immunohistochemistry alone (low affinity only) and when measured by a two site immunoprecipitation assay. The latter is thought to measure the high affinity as well as low affinity receptor. These considerations may not pertain to the trigeminal ganglion study since direct binding was used; it is possible that low affinity sites were not saturated or that the trigeminal pattern of innervation differs from other systems in the developing PNS.

In addition to the apparent widespread role during development, a similar function is suggested for NGF following trauma to the PNS. Subsequent to a transection of the sciatic nerve in the adult, there is a dramatic, though topographically regulated, induction of NGF and NGF-R in Schwann cells near and distal (from the cell soma) to the site of the cut (Schwab and Thoenen, 1985; Heumann et. al. 1987a, b; Taniuchi et al., 1988). Thoenen's group (Heumann et. al., 1987, Lindholm et. al., 1987) has made an important finding using this paradigm: the NGF induction is biphasic with the second increase corresponding temporally with the macrophage invasion of the injury site. Interleukin-1ß, a cytokine released by activated macrophages and monocytes, was found to produce a similar induction of NGF mRNA in explanted rat sciatic nerve (Lindholm *et. al.*, 1987). An hypothesis, derived from work with the sciatic nerve and proposed by Johnson and colleagues (Taniuchi *et. al.*, 1986; Taniuchi *et. al.*, 1988; Johnson *et. al.*, 1988) states that

axonal contact is a major determinant in Schwann cell NGF and NGF-R expression. Thus, axonal degeneration subsequent to lesion induces NGF and NGF-R expression in Schwann cells devoid of axolema contact. NGF is released by these glia where it plays a role in supporting regrowth of the nerve. The Johnson model accounts for the observations that Schwann cells only express the low affinity receptor and that NGF has no physiological role for Schwann cells themselves: the Schwann cell derived NGF binds to the receptors on its surface where it is recognized and removed by high affinity receptors that are expressed on the regrowing nerve. Reexpression in the mature animal of the NGF receptor is found in hypoglossal (Wood *et. al.*, 1990) and NGF -R mRNA in spinal cord (Enfors *et. al.*, 1989) motor neurons ~7d after crush injury to their axons and returns to (very low) adult levels following reinnervation of the target. Thus when the regenerating axon reaches the target, neuronal NGF-R expression and Schwann cell expression of NGF/NGF-R is down regulated and trophic support is once again supplied by the innervated organ. The authors speculate that this paradigm may also explain the sequence of events observed with respect to NGF and NGF receptor expression during development. That is, during injury the peripheral nervous system may recapitulate a developmental program with respect to NGF: the cells along the path of the regenerating nerve reexpress NGF and NGF receptor to lay down a permissive pathway for nerve growth. The hypothesis that axonal contact and agents released by phagocytic cells regulates nonneuronal expression of a molecule known to have trophic properties for neurons of the central nervous system has provoked interest in the ability of CNS glia to respond in a similar fashion.

NGF in the central nervous system

In a manner analogous to the support provided by the peripheral target tissue by NGF for sympathetic and sensory ganglia, NGF protein and NGF mRNA are found in the rodent brain with highest levels in areas corresponding to the terminal fields of cholinergic

neurons of the basal forebrain (Korsching *et. al.*, 1985; Shelton and Reichardt, 1986; Whittemore *et. al.*, 1986; Ayer-LeLievre *et. al.*, 1988; Gall *et. al.*, 1991). ^{125}I-NGF injected into the cortex or hippocampus of the adult rat is retrogradely transported to large perikarya of cells in the nucleus basalis and medial septum (Schwab *et. al.*, 1979; Seiler and Schwab, 1984). In contrast to the NGF sensitivity of peripheral adrenergic neurons, there was no transport nor effect of NGF antibodies on the noradrenergic neurons of the locus coeruleus. NGF receptor immunohistochemistry and NGF-receptor mRNA *in situ* hybridization studies have indicated a colocalization of the receptor with cells of the basal forebrain stained for acetylcholinesterase (AChE) or choline acetyltransferase (ChAT), markers for cholinergic cells (Hefti *et. al.*, 1986b; Raivich and Kreutzberg, 1987; Springer *et. al.*, 1987; Batchelor *et. al.*, 1989). These studies indicate that cholinergic cells along the basal forebrain are sensitive to NGF produced in their target fields. The dependency of these neurons for trophic support supplied by NGF has been demonstrated in a number of experiments where axons of septal neurons have been transected by lesioning the fornix: the cholinergic cells of the medial septum will degenerate under this paradigm (Gage *et. al.*, 1986; Armstrong *et. al.*, 1987). When NGF is infused into the hippocampus of the lesioned adult animals, there is a significant increase of survival of these cells accompanied by an increase in hippocampal ChAT activity (Hefti *et. al.*, 1984, 1986a; Williams *et. al.*, 1986; Kromer *et. al.*, 1987; Gage *et. al.*, 1988b). NGF can also influence basal forebrain neurons during development. Embryonic septal cholinergic cells *in vitro* respond to NGF by induction of ChAT activity (Hefti *et. al.*, 1985; Hatanaka and Tsukui, 1986; Martinez *et. al.*, 1987) and can stimulate survival and fiber outgrowth when cultured at low density (Hartikka and Hefti, 1988a). Administration of NGF into the intact neonatal animal also results in a significant increase in septal, hippocampal and striatal ChAT activity (Gnahn *et. al.*, 1983; Mobley *et. al.*, 1985; Mobley *et. al.*, 1986; Johnston *et. al.*, 1987). Similar studies in the adult have produced a less robust (but significant) induction of ChAT. However, it appears a longer exposure is necessary and the striatal response is significantly

diminished (Gnahn *et. al.*, 1983; Whittemore and Singer, 1987; Johnston *et. al.*, 1987; Fusco *et. al.*, 1989; but see Gage *et. al.*, 1989). These effects on ChAT activity appear to occur through the NGF mediated synthesis of new enzyme molecules in the basal forebrain and their subsequent anterograde transport to the target site (Fusco *et. al.*, 1989).

While NGF appears to play a role in the development and maintenance of CNS cholinergic neurons, a number of studies indicate that in the adult animal the NGF/cholinergic system may not be amenable to adaptive responses. Transection of the fimbria/fornix in the neonatal rat increases the concentration of NGF protein and NGF mRNA in the hippocampus. In contrast, only the protein levels are elevated in the adult hippocampus (Goedert *et. al.*, 1986; Whittemore *et. al.*, 1986, 1987; Korsching *et. al.*, 1986; Larkfors *et. al.*, 1987). The increased levels of NGF protein in the adult have been attributed to the accumulation of NGF as a result of the loss of retrograde transport by the basal forebrain neurons. This apparent lack of transcriptional regulation in the mature animal may reflect a loss of plasticity in neuronal and nonneuronal cell populations and may indicate an altered role and availability of NGF during ontogeny. A number of reports indicate a more widespread expression of NGF receptor during embryogenesis than is found in the mature brain (Yan and Johnson, 1988; Schatteman *et. al.*, 1988) and a colocalization of the receptor with low levels of NGF mRNA expression as early as embryonic day 16 (Lu *et. al.*, 1989). Thus, in parallel with the peripheral nervous system, NGF may initially be involved in the trophic support of a number of neuronal populations through highly localized interactions. During development this evolves into the restricted, target derived pattern of the adult. This switch in expression profile may involve a change in the cell type expressing NGF.

The data outlined above would indicate an influence of NGF on cholinergic cells, the mechanism of the effect and the ongoing source of NGF in the developing and the intact or injured adult animal is yet to be resolved. Studies applying *in situ* hybridization against NGF mRNA have shown signal in the pyramidal cell layer in all fields of the hippocampus

proper and in the granule cell layer of the dentate gyrus (Ayer-LeLievre *et. al.*, 1988; Whittemore *et. al.*, 1988; Gall *et. al.*, 1991). In addition to this neuronal expression of NGF, there may be periods during development and in response to injury when expression of NGF (and its receptor) may reside in additional (or alternate) cell phenotypes. As noted above, in the peripheral nervous system, the expression of NGF is induced in numerous cells types and NGF-R in Schwann cells in the area surrounding the degenerating axon of the lesioned sciatic nerve; there is evidence the pattern of this reexpression may recapitulate the distribution of NGF and NGF-R during development. The expression of NGF in nonneuronal cells in the central nervous system is less well known. While NGF mRNA is found in neurons in the hippocampus, there is now a body of work indicating that, at least *in vitro*, astrocytes can express NGF and that various factors and conditions can regulate this expression. Enriched astrocyte cultures can express the mRNA for NGF and can produce the biologically active protein (Lindsay *et. al.*, 1979; Norrgren *et. al.*, 1980; Furikama *et. al.*, 1986, 1987; Assouline *et. al.*, 1987; Yamakuni *et. al.*, 1987; Houlgatte *et. al.*, 1989 Gadient *et. al.*, 1990; Schwartz *et. al.*, 1990; Spranger *et. al.*, 1990; Yoshida *et. al.*, 1991). There is also evidence that isolated peripheral macrophages can synthesize (Otten *et. al.*, 1987) and release NGF (Otten *et. al.*, 1987; Mallet *et. al.*, 1989) and macrophages isolated from brain can secrete NGF in response to lipopolysaccharide (LPS, a bacterial cell wall endotoxin) stimulation (Mallet *et. al.*, 1989). Furikama *et. al.* (1986, 1987) measured levels of NGF in cultured astrocyte following various periods in culture: in short term cultures, long before reaching confluence, astrocytes produce high levels of NGF (per mg protein). As the cultures expanded and approached confluence the relative levels of NGF in the cultures decreased until significantly lower levels of NGF are present in the confluent plates. When the cells were split and replated at a lower density NGF levels returned to non-confluent levels. These studies may indicate that the proliferative state of astrocytes (and possibly other glia) plays an important role in the expression and release of NGF. Thus, in a paradigm analogous to the observed expression following

lesion in the PNS, physical injury, trauma or disease in the CNS may lead to astrocytic proliferation and the reexpression of NGF (and other growth factors).

The role of glia in the central nervous system

Nonneuronal cells in the CNS have become the recent focus of heightened research as the perceived scope of their function has expanded to encompass an active participation in the development, positioning, and maintenance of the integrity of neurons. Part of the evolution of this perception is the concept that this role is partially mediated by the release of growth factors. The work described in this report has studied agents which control the regulation of NGF mRNA levels in astrocytes. The objective was to determine if astrocytes, in a fashion similar to Schwann cells in the periphery, express NGF during transient periods of ontogeny and subsequent to brain trauma in a partial recapitulation of developmental events. The following sections provide a brief background on astrocyte derived growth factor involvement in development and injury and a more extensive description of the involvement of phagocytic cells and their released factors in the development and response to injury in the CNS.

Astrocytes

There exists a large body of evidence that astrocytes interact with neurons during development and subsequent to injury in the central nervous system. The role of astrocytes in the maturation and support of neurons has begun to be appreciated as the number of cell surface, extracellular matrix components and soluble factors produced by astrocytes and found to influence neurons has become evident (reviewed in Vernadakis, 1988; Hatten, 1989). Astrocytes can enhance neuronal survival and neurite elongation (Banker, 1980; Noble *et. al.*, 1984). Conversely, neurons can diminish the proliferation and enhance the differentiation of astrocytes (Hatten, 1985; 1987). Migration of many postmitotic neurons to their final position occurs along the processes of radial glia in the developing nervous

system (Levitt and Rakic, 1980; Hatten and Liem, 1981; Hatten, 1990). Developing astrocytes express a variety of cell surface proteins (N-CAM, G4/L1) and elaborate extracellular matrix molecules (laminin, fibronectin) which are important for neuronal-glial interactions (Manthorpe *et. al.*, 1983; Liesi *et. al.*, 1983; Prochiantz and Mallat, 1988). These studies indicate astrocytes play an important role in axonal guidance and neuronal migration in development and these properties are mediated through cell surface properties or released agents (Manthorpe *et. al.*, 1986).

Raff and colleagues have conducted extensive studies of the developing optic nerve (reviewed in Raff, 1989) to unravel the process of the development of glial diversification in the CNS. Using immunohistochemical markers, this group has identified two types of astrocytes (type I and type II) in the rat optic nerve and in rat brain which are distinguished by characteristic morphology, antigenic phenotype and response to trophic agents. Type 1 astrocytes first appear in the rat optic nerve at E16 (and in the brain at E15-16), oligodendrocytes on the day of birth and type II astrocytes at P7. These three classes of glia represent two separate cell lineages: the type II astrocyte and oligodendrocyte are derived from a common precursor, the O-2A progenitor while the type I astrocyte arises from a separate lineage. These studies also indicate that the proliferation of the progenitors and their differentiation into astrocytes and oligodendrocytes are influenced by the presence of factors in the immediate extracellular environment and at least some of these factors are produced by the developing astrocyte population.

Experimental lesion paradigms have provided evidence that astrocytes are involved in the events following injury to the CNS. A well studied paradigm that is relevant to the current work is lesion of the perforant path or the entorhinal cortex which removes a major extrinsic input to the dentate gyrus. These inputs innervate the outer two-thirds of the molecular layer (ML) where they terminate on dendrites of dentate granule cells. The inner one third includes innervation from contralateral hippocampal pyramidal cells and from cholinergic cells in the medial septum (Rose *et. al.*, 1976). Using a gold chloride stain,

Rose *et. al.* (1976) found astrocytes either evenly distributed in the ML or aligned in a row corresponding to the border of the entorhinal input in the intact rodent brain. Following lesion in the adult rat brain, the terminals in the outer part of the ML degenerate and sprouting is observed from the inner zone to partially reinnervate the vacated sites in the outer zone. Concomitant with this sprouting an astrocytic hypertrophic response is observed where astrocyte processes multiply and thicken while displaying a distinct process polarity towards the deafferented area. There is also an apparent shift in distribution of cells from the inner ML towards the outer ML although the histological procedure could not distinguish between astrocyte proliferation or an alteration in staining characteristics of individual astrocytes. More recent evidence using an antibody against glial fibrillary acidic protein (GFAP), an intermediate filament protein specific to astrocytes, indicates a sequence of events in various injury paradigms where an initial response of phagocytic cells is followed by alterations in the morphology and distribution of astrocytes near the lesion site (Gage *et. al.*, 1988; Giulian *et. al.*, 1989; Fagan *et. al.*, 1990). The temporal association of injury to a reactive process in astrocytes suggests a glial involvement in neuronal support and neurite outgrowth subsequent to trauma in the CNS.

While the observation that NGF mRNA is produced in proliferating astrocytes *in vitro* and these levels appear to be regulated by conditions related to the rate of proliferation and cell-cell contact mediated influences, the evidence for astrocyte production of NGF *in vivo* is lacking. Thus the similarity between the expression of NGF during peripheral nerve development and response to injury and the involvement of astrocyte released NGF during similar periods in the CNS is unknown. In light of the apparent widespread role of NGF during these periods in the periphery, the possibility exists that NGF is also elaborated by supporting cells in the CNS. The differential expression of NGF in astrocytes at various stages of growth in culture may indicate that this capacity is related to the embryonic stage of the developing nervous system or the degree of astrocyte proliferation throughout maturation. The close apposition of developing and migrating neurons to a proliferating

population of astrocytes during embryogenesis and the recent indication of NGF and NGF receptor expression during early development indicates a possible involvement of astrocyte derived NGF during development. The presence of phagocytic cells during this period elaborating factors shown to promote astrocyte proliferation and influence NGF expression (see below), may further support this role for astrocytes during ontogeny. The findings showing an exclusively neuronal expression of NGF from early adulthood on may indicate a transition to a target derived network where only those cells innervated by NGF dependent neurons express and secrete NGF from early adulthood throughout life. Glial expression of NGF may only return when astrocytes enter a proliferative state in response to changes in the surrounding microenvironment. These changes may be those associated with CNS trauma or disease. The studies presented here tested the capacity of cultured astrocytes to express and regulate the expression of NGF under various conditions. The objective was to understand if the ability to express NGF mRNA in proliferating astrocytes is found in various brain regions and if this expression can be regulated by a factor present during CNS development and trauma. If astrocyte expression of NGF is a common mechanism to promote neuronal survival during restricted neonatal periods then astrocytes from various brain regions of similar developmental stages would be expected to express NGF mRNA. A second objective was to study the effect of the rate of astrocyte proliferation on NGF expression and the influence of the rate of growth on the capability for regulation of NGF mRNA levels. This may indicate if mature astrocytes share with Schwann cells of the adult animal plasticity with respect to NGF expression and thus also share the capacity to recapitulate aspects of developmental programs. Differences in Schwann cell and astrocyte regulation of NGF in the adult may be one element in the different regenerative capacities of nerves in the central and peripheral nervous systems.

To determine if astrocyte expression of NGF during development may be part of a sequence of events preceding neuronal NGF expression, enriched cultures of embryonic hippocampal neurons were also examined for expression of NGF mRNA. The hypothesis

was that as development proceeds and astrocyte proliferation slows, astrocyte NGF synthesis declines and those neuronal populations remaining dependent upon NGF throughout adulthood derive trophic support from neuronal production in their target fields. Thus neuronal NGF mRNA synthesis may occur following peak astrocyte production. This switch in the sites of synthesis may reflect inherent phenotypic programs of NGF expression and the response to the establishment of neuronal-glial interactions during this period.

Microglia

The various populations of microglia constitute a protective, phagocytic network during maturation and response to disease or injury found within the central nervous system and appear to act as the principal scavenger cells of the CNS. As has been found for most tissues, it has been proposed that two major populations of macrophages are involved in providing phagocytic cells to the mature CNS: one population arises from circulating cells in the blood (monocytes) and the other derived from resident cells within the brain (reviewed in Jordan and Thomas, 1988). Current evidence suggests that monocytic stem cells and/or monocytes enter the brain during embryonic and neonatal development possibly in response to cellular debris generated by growing axons or at sites of an incompletely formed blood brain barrier (Ling *et. al.*, 1982; Innocenti *et. al.*, 1983; Perry *et. al.*, 1985; Perry and Gordon, 1988; Ashwell, 1991). After entering the brain, these phagocytic cells appear to go through a transition from an active phagocytic phenotype displaying a shape and vacuolated cytoplasm similar to peripheral macrophages but with cell surface features (multiple short processes) that set them apart (Giulian and Baker, 1986), to a ramified appearance reflective of a mature, resting microglia. (Ashwell, 1991; Perry *et. al.*, 1985). In culture, ameboid microglia engulf 5 μm polystyrene beads (reflective of active phagocytosis), and show a surface morphology and cytochemistry which distinguish them from other phagocytic cells. In addition, in culture a subgroup of

cultured microglia stop dividing, develop a more ramified morphology, and lose other active macrophage related properties (Giulian and Baker, 1986; Giulian, 1987) Thus these ameboid cells, whose characteristic appearance of filopodia and numerous cytoplasmic lysosomes (Ling, 1976, 1977) likely indicate active phagocytosis, appear during the late stages of embryogenesis at sites of axonal growth and glial proliferation (Ling *et. al.*, 1982, Innocenti *et. al.*, 1983). There is some debate whether the ameboid cells are a transitory form of microglia, appearing only during development and disappearing during the late postnatal period (Jordan and Thomas, 1988), or these cells differentiate into the ramified morphology (Ling *et. al.*, 1982; Murabe and Sano, 1982) only to reappear and proliferate at sites of damage in the mature CNS (Giulian and Baker, 1986; Tseng *et. al.*, 1983). This distinction may be important since the exact nature of the phagocytic cell(s) appearing during injury or degeneration may influence the reaction and survival of glial and neuronal populations.

While the studies outlined above indicate the periodic nature of brain macrophage activity, these morphological descriptions do not indicate the means by which microglia may influence cells and the surrounding microenvironment during various stages of ontogeny. The studies of Giulian during the 1980s have gone far to describe the role of microglia in the regulation of astrocyte proliferation and the coordinated response to trauma (Giulian and Lachman, 1985; Giulian and Baker 1985; Giulian *et. al.*, 1986a, b, c; Giulian,1987; Giulian *et. al.*, 1988a, c ; Giulian *et. al.*, 1989). There is now convincing evidence that major mediators in the microglial response are cytokines, a family of polypeptide factors released by a variety of cells and known to influence many tissues. The cytokine interleukin 1 (IL-1) has been studied in relationship to brain phagocytic cell activity and there is evidence showing that IL-1 is elaborated by microglia in the developing brain (Giulian *et. al.*, 1988a) and is produced at the site of injury in the mature CNS (Nieto-Sampedro and Berman, 1987; Giulian *et. al.*, 1989). Initial studies indicated a protein with IL-1 like activity could stimulate the proliferation of astrocytes isolated from

newborn rat brain and a similar molecule could be isolated from brain of rats following stab wounds to the CNS (Giulian and Lachman, 1985). Later studies examined the involvement of microglia in conjunction with responding circulating mononuclear phagocytes to this lesion paradigm and found that both peripheral and endogenous phagocytic cells respond to this form of injury (Giulian *et. al.*, 1989) Other glial promoting factors (GPF) have been isolated following a similar lesion paradigm (Giulian and Baker, 1985). The source of the IL-1-like (and other GPFs) was further investigated using tissue culture models of isolated populations of glial cells. While earlier studies *in vitro* appeared to indicate astroglia can be induced to produce an IL-1 protein (Fontana *et. al.*, 1982), using stringent purification techniques to remove any microglial component from 'purified' astroglial cultures, Giulian and colleagues have shown neither astrocytes nor oligodendrocytes can respond to lipopolysaccharide (LPS) or fixed *S. aureus*, potent phagocytic signals, and that only microglia respond by producing IL-1 (Giulian and Baker, 1985; Giulian *et. al.*, 1986a). Again the IL-1 isolated from a purified population of microglia induced the proliferation of cultured astrocytes. Prochianz' group has now shown purified amoeboid microglia synthesize IL-1 mRNA and protein and have the protein either associated with their cell membrane or release it into the supernatant in response to LPS (Hetier *et. al.*, 1988). This group also found that elimination of microglia (with leucine-methyl-ester treatment) in 95% pure astrocyte cultures significantly decreased LPS stimulated IL-1 release. In further studies, Giulian has found that injection of IL-1 into the brain can stimulate the proliferation of astroglia and neovascularization at the site of injection (Giulian *et. al.*, 1988b). This group has also found that 2d following a stab lesion of the brain, the peak levels of IL-1 detected in the damaged tissue correlated with the maximal number of low density lipoprotein (LDL)-receptor/non-specific esterase positive cells (markers of phagocytic cells) and these were found to be of peripheral and endogenous origin (Giulian *et. al.*, 1989). This body of work indicates that microglia and other populations of phagocytic

cells are active during periods of CNS development and injury and they secrete IL-1 and other factors which can induce proliferative responses in astroglia.

Interleukin-1

The history of the biology of interleukin-1 involves research focusing on the mediators of host response to infection (for reviews see Dinarello, 1984, 1988). Among the prominent and early reactions to infection, collectively known as the acute-phase response, is the induction of a number of hepatic proteins (the acute phase reactants) and the onset of fever. A plethora of research indicates that these reactions are not a direct consequence of the invading infectious agent but rather related to factors released by responding cells of the host. These studies have shown that products of phagocytic cells of the host, following exposure to the a variety of agents, can alone produce many of the components of the acute phase response. Numerous reports indicate that interleukin-1 plays a leading role in mediating and modulating the host response to infection and inflammation (Dinarello, 1984). Two forms of interleukin-1, IL-1 alpha (IL-1α) and IL-1 beta (IL-1ß) have been isolated. They are encoded by separate genes and, while sharing only ~30% sequence homology, appear to bind to the same receptor and exert similar effects. IL-1α is associated with the outer membrane of monocytes while IL-1ß is secreted (Dinarello, 1988). A very brief review of some the biology of interleukin-1 will be given with a focus on the role of this cytokine during central nervous system response to injury or disease.

The nomenclature of interleukin-1 was coined in the late 1970s to cover a collection of molecules (endogenous pyrogen, leukocytic endogenous mediator, lymphocyte-activating factor, B cell-activating factor, thymocyte proliferation factor) to indicate that these factors, isolated and studied by various laboratories, are the same molecule. This work showed that in response to infection, macrophage (both monocytic and resident) release large quantities of IL-1 which subsequently produce a vast array of effects (fever, hypoferremia, hypozincemia, hypercupremia, granulocytosis, synthesis of fibrinogen, haptoglobin,

ceruloplasmin, C-reactive protein, macroglobulins) (Dinarello, 1984). One of the most significant findings was the involvement of IL-1 in mononuclear phagocyte presentation of processed antigen to T lymphocytes: antigen was found to be presented, in association with the class II major histocompatibility complex, for T-cell recognition and the elaboration of IL-1 by the macrophage was essential to prime or activate T-cells. One result of this stimulation is the synthesis of interleukin-2 (IL-2) and IL-2 receptors on T-cells where IL-2 acts in an autocrine manner to further the proliferative response. IL-1 was found to induce the proliferation or potentiate the proliferative response of thymocytes; the enhancement of the proliferative response (to concanavalin A or phytohemagglutinin) of murine thymocytes remains a standard assay for the presence of IL-1.

Interleukin-1 has been found to mediate a number of parameters associated with inflammation: these include the activation of phospholipase A_2 and formation of prostaglandin E_2 (Chang *et. al.*, 1986; Dayer *et. al.*, 1986), increased synthesis of endothelial cell prostacyclin (Rossi *et. al.*, 1985) and the increased release of histamine from basophils (Subramananian *et. al.*, 1987). In light of the acknowledged antiinflammatory effects of glucocorticoids, a series of reports have recently shown that IL-1 can regulate the hypothalamo-pituitary-adrenal axis (Besedovsky *et. al.*, 1986; Bernton *et. al.*, 1987; Sapolsky *et. al.*, 1987; Berkenbosch *et. al.*, 1987; Rettori *et. al.*, 1987) and may be a significant component of an immunoregulatory circuit to control host response following an immune challenge or tissue damage (del Rey *et. al.*, 1987; Bateman *et. al.*, 1989). These studies were spurred by earlier work showing there is an increased level of circulating glucocorticoids during an immune response (Besedovsky, 1975) and during infectious disease (Beisel, 1975) while glucocorticoids can inhibit the production and action of IL-1 (Wahl *et. al.*, 1975; Snyder and Unanue, 1982). Additional studies indicated that IL-1 exerts this action at the level of the hypothalamus to release corticotropin-releasing-hormone, a peptide most responsible for the release of ACTH from the pituitary (Sapolsky *et. al.*, 1987). The IL-1 induction of CRF release was

corroborated in a parallel study (Berkenbosch *et. al.*, 1987) and supported the Sapolsky finding that IL-1 was selective for CRF and had no significant effect on release of other hypothalamic releasing factors or pituitary hormones. It appears that IL-1ß and not IL-1α can induce this ACTH release and this effect can be blocked with the prostaglandin synthesis inhibitor indomethacin (Katsuura *et. al.*, 1988b). The interaction between IL-1 and glucocorticoids has been supported in a number of reports. Hydrocortisone was shown to block the LPS induction of IL-1 in cultured rat peritoneal macrophages and could block the proliferation of macrophages expressing surface-I-region-associated (Ia) antigens *in vivo* (Snyder and Unanue, 1982). Similar effects have been shown for human blood monocytes. Glucocorticoids may act directly on the gene encoding IL-1 by inhibiting its transcription and decreasing the stability of IL-1 mRNA. Lee (Lee *et. al.*, 1988) found that DEX blocked the induction and reduced the stability and/or degraded the induced IL-1 mRNA in a human promonocytic cell line in response to combined LPS and phorbol 12-myristate (PMA) treatment. There is also evidence that IL-1 and glucocorticoids can interact synergistically to influence responsive tissue. IL-1 possesses activity as a fibroblast growth factor (Schmidt *et. al.*, 1982) and induces interferon-beta$_2$ (IFN-ß$_2$) (Zilberstein *et. al.*, 1986); DEX blocks the IL-1 induced up regulation of IFN-ß$_2$ mRNA and this regulation appears related to the enhanced growth promoting activity of co-treatment of DEX and IL-1 on fibroblasts (Kohase *et. al.*, 1987). The picture thus emerges of balance between IL-1 which mediates events related to immune activation and inflammation and glucocorticoids which act as immunosuppressants and antiinflammatory agents.

The sources and overall physiological role of IL-1 in the central nervous system are poorly understood. The observations that ICV injection of IL-1 can induce slow wave sleep (Krueger *et. al.*, 1984) and analgesia (Nakamura *et. al.*, 1988)., and can lead to the suppression of food intake (Plata-Salaman *et. al.*, 1988) would suggest that IL-1 or IL-1 induced secretion products can have direct effects within the CNS. Fibers containing IL-

1ß-like immunoreactivity (IL-1ß-ir) have been found innervating the hypothalamus of human brain (Breder *et. al.*, 1987). The densest accumulations of fibers was found in periventricular nuclei, the regions that participate in anterior pituitary control. Within this area is the paraventricular nucleus containing the majority of CRF cell bodies in the CNS. IL-1ß-ir fibers were also found projecting to the region of the median eminence containing hypophyseal portal vessels. These findings, together with the observation of fibers in other regions of the hypothalamus involved in various autonomic functions, led to the suggestion that IL-1 may serve as its own intermediate messenger. That is, peripheral IL-1 gains access to the brain at sites where the blood brain barrier is lacking: IL-1 enters the hypothalamus at the organum vasculosum of the lamina terminalis (OVLT) where IL-1 containing neurons in the preoptic area respond by releasing endogenous IL-1 to produce the various acute phase responses (Breder *et. al.*, 1987). While this group has demonstrated the inability of IL-1 to cross the blood brain barrier in the cat (Coceani *et. al.*, 1988), Banks *et. al.* (1989) have shown IL-1α to be actively transported into many brain regions with the hypothalamus having the highest rate (by weight) of uptake (this group does not state explicitly that hypothalamic diffusion through the OVLT does not occur but suggest that other regions, lacking circumventricular organs, displayed uptake that could not be accounted for by diffusion). Dinarello's group (Lechan *et. al.*, 1990) has shown a similar intense immunoreactivity for IL-1 in the rat hypothalamus where IL-1ß-ir was found in fibers and cell bodies (in normal and colchicine pretreated animals); no evidence was given to substantiate neuronal localization. The most intense immunoreactivity was in the hilus of the dentate gyrus and in the molecular layer of fields CA3 and CA4 of the hippocampus proper corresponding to the mossy fiber projection system from dentate granule cells. The presence of IL-1 receptors has been studied on cryostat sections of rat brain where they were found to have similar binding characteristics to those found on fibroblasts and lymphocytes (Farrar *et. al.*, 1987). Widespread binding sites were found throughout the brain, with the highest levels found in the olfactory bulb, the pyramidal cell

layer of the hippocampus, the granule cell layer of the dentate gyrus and the choroid plexus. High levels were also found in molecular layer of the hippocampal formation with few sites found within the hypothalamus. Although the density of staining paralleled neuronal density, the authors could not exclude the possibility of nonneuronal binding sites. Competition analysis of IL-1α and IL-1ß binding sites in membrane preparations of rat brain found the ß form to be 100 times more potent than IL-1α in displacing cold ligand with 4 times as many sites in hypothalamic than cortical membranes (Katsuura *et. al.*, 1988a). A very recent *in situ* hybridization study indicates that IL-1ß mRNA is present in adult rat brain in the pyramidal cell layer of the hippocampus, granule cells of the dentate gyrus and cerebellum and in the olfactory bulb, frontal cortex and ventromedial hypothalamus (Bandlow *et. al.*, 1990).

The panoply of effects displayed by IL-1 and the clear involvement in regulatory processes in the CNS suggests a role for this cytokine in the maintenance of function in central neurons

Response to injury in the central nervous system

A brief summary of the data presented above suggests a sequence of events that may occur following trauma to the CNS. Using the events occurring in the hippocampus following entorhinal cortex damage as a model, subsequent to the lesion phagocytic cells endogenous to the brain and from the periphery invade the lesion site and the degenerating axons in the hippocampal terminal fields (see Cotman, 1985 for review). In addition to removing degenerating myelin and additional cell debris, the phagocytic cells release IL-1 in the region of the damage. An astrocytic reaction in the immediate vicinity occurs soon thereafter and the elevated IL-1 levels appear to be at least partly responsible for this reaction. This lesion also leads to the reactive sprouting of adjacent, uninjured septal fibers which may in turn occupy the terminal fields formally occupied by the injured axons. There has been an extensive body of work conducted recently to characterize the aspects

involved leading to this regenerative response and the factors that may allow intervention in the degeneration of the normal neuronal architecture following injury or disease. In the adult, experiments thus far indicate the basis for the perforant path sprouting is the release of an NGF-like substance from the deafferented zone (Crutcher and Collins, 1986).

The question remains whether there is a CNS population of cells which respond to injury by inducing the synthesis of NGF. One approach to examining this is an extension of the work outlined for cultured astrocytes. That is, do astrocytes in the context of the mature brain respond to IL-1 by the induced production of NGF? Exposure to IL-1 may be considered part of a constellation of factors present during brain injury and the induction of NGF mRNA may be one measure of the plasticity of adult astrocytes. If the adult brain can respond in this manner then it would appear to be able to provide support for at least some populations of neurons. The lack of such a mechanism may be one factor in the inability of CNS neurons to regrow and may provide a point of departure between central and peripheral glia. The response characteristics in the adult brain may also be another gauge of maturation associated changes in astrocytes that may be related to their rate of proliferation. IL-1 was administered intracerebroventricularly to adult male rats and the animals were allowed to survive for a period to preclude the initial phagocytic cell response of the brain. NGF mRNA was measured from hippocampus and compared to saline injected controls. These studies will reflect upon the astrocyte density experiments described above and may indicate the degree mature astrocytes in the intact brain can reiterate response characteristics found in proliferating astroglia.

Methods

Solution hybridization assay

Much of the work presented below was dependent upon detection of the very low levels of NGF mRNA in either cultured cells or fresh frozen tissue from the rat central nervous system. A solution hybridization/nuclease protection assay was used for these studies which allows detection and quantitation of low copy number mRNA and hnRNA in tissue samples (see Blum and Roberts, 1989). The method of analysis is solution hybridization of a high specific activity, antisense ^{32}P-labelled RNA probe (generated from cloned DNA) to total RNA derived from CNS tissues followed by nuclease digestion and gel separation of the hybrids. Sense strand RNA is synthesized from the same clone and is hybridized to probe in each assay to provide a standard curve of known amounts of RNA. Comparison of the signal from tissue to that in the standard curve allows the quantification of tissue levels of the mRNA of interest by regression analysis.

Earlier estimates of NGF mRNA levels in the CNS (Shelton and Reichardt, 1986) indicated approximately 200 fg NGF mRNA/μg poly $(A)^+$ RNA in the rat hippocampus and 95 fg/μg in cortex equivalent to about 10 and 5 fg NGF mRNA/μg total RNA respectively. Preliminary studies on mouse tissue indicated the assay could detect 40 fg NGF mRNA/μg total RNA in mouse hippocampus and 10 fg/μg in cortex. These assays used the cloned mouse NGF cDNA to generate a ^{32}P-labelled RNA probe. This approach could commonly detect a signal of 100 fg NGF mRNA and was linear through at least 5 pg of NGF mRNA. When the analysis of rat tissue was begun, the mouse probe continued to be used since it was known the 3' exon (coding for the entire mature NGF) from the two species share 97.6% sequence homology (Scott *et. al.*, 1983; Whittemore *et. al.*, 1988). Multiple bands were produced in these assays following hybrid digestion. This observation indicates multiple base pair mismatches which are sensitive to ribonuclease

digestion. The rat NGF clone was subsequently obtained and again difficulty was encountered in the isolation of a single band following hybridization/digestion. The resolution of this problem and the entire protocol is given below.

Isolation of RNA

All equipment directly contacting tissue was diethyl pyrocarbonate (DEP) treated for 1 hour (DEP inhibits RNase activity by methylation of the enzyme) and dried overnight at 42°C. Reagents were DEP treated and autoclaved. For isolation of RNA from fresh tissue, animals were sacrificed by decapitation and the brain was rapidly removed and placed on ice. Following 10 minutes of cooling, brain regions were dissected quickly and homogenized in a glass/teflon homogenizer or a sterile 1 ml syringe containing ice cold, RNase-free AT buffer (10 mM Tris-HCl, pH=8.0, 3 mM $CaCl_2$, 2 mM $MgCl_2$, .5 mM DTT, 0.15% Triton X-100, 0.3 M sucrose; 25-50 mg wet weight tissue/ml buffer) gently layered over 400 µl AT buffer containing 0.4 M sucrose. For experiments using primary cultures, the same general procedure was used for isolation of RNA from tissue culture plates. Ice cold 0.3 M sucrose AT buffer was added to the plate (1 ml of buffer to a 60 mm dish or 75 cm^2 flask) and the cells were harvested with a sterile cell scraper (Falcon 3085) and then layered over 0.4 M sucrose AT buffer. The sucrose gradient was spun for 10 minutes at 2500Xg at 4°C to pellet the nuclei The supernatant contains the cytoplasmic RNA and was rapidly processed to destroy endogenous RNases. The pellet contained cell nuclei and was stored at -20°C for isolation of DNA and hnRNA at a later time. The supernatant was incubated in 100 µg/ml Proteinase K in SET buffer (1% SDS, 5 mM EDTA, 10 mM Tris-HCl, pH=8.0, all [final])and incubated at 45°C for 1 hour. The samples were extracted 1:1 (vol/vol) with phenol/chloroform, 0.1 volume 5M NH_4Ac and precipitated with 1 volume isopropanol or 2.5 volume ethanol for a minimum of 2 hours at -20°C. Immediately before the assay, RNA was pelleted by centrifugation (16,000Xg, 15

min. at 4°C), washed in 70% ETOH, lyophilized and resuspended in TE. The RNA concentration was quantitated by optical density at 260 nm on a Beckman DU-61 spectrophotometer.

RNA probes

RNA Synthesis Protocol

Sense and antisense RNA were generated independently from plasmid vectors containing the cDNA template and bacteriophage RNA polymerase promoters. The T3 and T7 promoters are on either side of the template in the Bluescript and Bluescribe vectors allowing generation of both transcripts from the vector. To generate the probe, 187.5 pmoles (~15µl) of alpha-^{32}P-UTP (New England Nuclear) was lyophilized in a 500 µl Eppendorf tube; the probe was then synthesized in the following reagents in a volume of 10 µl: 40 mM Tris-HCl, pH=8.0, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 20 U human placental ribonuclease inhibitor (human recombinant RNasin, Promega), 1 mM each ATP, GTP and CTP, 1 µg linearized plasmid containing cloned DNA and 5U of the appropriate RNA polymerase (T3 and T7 polymerase, Stratagene, La Jolla, CA). All reagents except RNasin, the polymerase and the plasmid are part of the Riboprobe System II buffer kit (Promega). Sense strand was synthesized similarly except that, since this RNA is only labeled to a small degree (for detection during synthesis), the incubation contains 25 nmoles of each rNTP (including UTP) and 6 pmoles of alpha-^{32}P-UTP. The strands were synthesized by incubation for 20 minutes (antisense) or 60 minutes (sense) at 37°C. The reactions were terminated by the addition of DEP H_2O to bring the volume to 100 µl and aliquots were removed to test incorporation on ion exchange (DE81) paper. The DNA template was subsequently digested by incubation of the strands in 50 mM Tris-HCl, pH=8.0, 10 mM $MgCl_2$, 25 U RNasin, and 20 U

(antisense) or 40 U (sense) ultrapure DNase (Worthington). The digestion was incubated at 37°C for 30 minutes and was stopped by the addition of 5 µl of 0.5 M EDTA. The synthesized RNAs were recovered from the unincorporated label on a TE (10 mM Tris-HCl, pH=8.0, 1 mM EDTA) equilibrated Sephadex G-100 (bead size 40-120 µm, Sigma) column. Incorporation of labeled UTP into each strand was used to calculate the amount of synthetic transcript generated. 125-150 ng of antisense strand was synthesized per reaction generating a probe with a specific activity of 1-2x10^9 CPM/µg. The probe was kept on ice and used only on the day of synthesis. 2-4 µg of sense strand was made per reaction, diluted to 100 fg/µl and 1 pg/µl, aliquoted and stored at -80°C.

Synthesis of Specific Probes

A) Mouse NGF: A 900 base pair (bp) Pst I fragment of the cloned mouse cDNA (Ullrich et. al., 1983) was subcloned into the Pst I site of the Bluescript plasmid vector. An 825 bp probe was generated by digestion of the plasmid with Ava II and synthesis with RNA polymerase T3; full length sense strand was produced by digestion with Eco R1 and polymerization with polymerase T7.

B) Rat NGF: A 771 bp BstEII/Pst I fragment of the rat NGF 3' exon was blunt ended and ligated into the Sma I site of the Bluescribe vector (kindly provided by Dr. S. Whittemore, University of Miami; see Whittemore et. al., 1988). A full length probe was produced by linearization with Eco R1 and synthesis with RNA polymerase T3 while sense strand was produced by digestion with Bam H1 and polymerization with polymerase T7. (see Fig 1).

Assay protocol and conditions required to measure rat NGF mRNA

Pelleted sample RNA was resuspended in 5µl TE in 500µl Eppendorf tubes, 20µl of hybridization buffer (60% formamide, 0.9M NaCl, 6 mM EDTA, 60 mM Tris, pH=7.4),

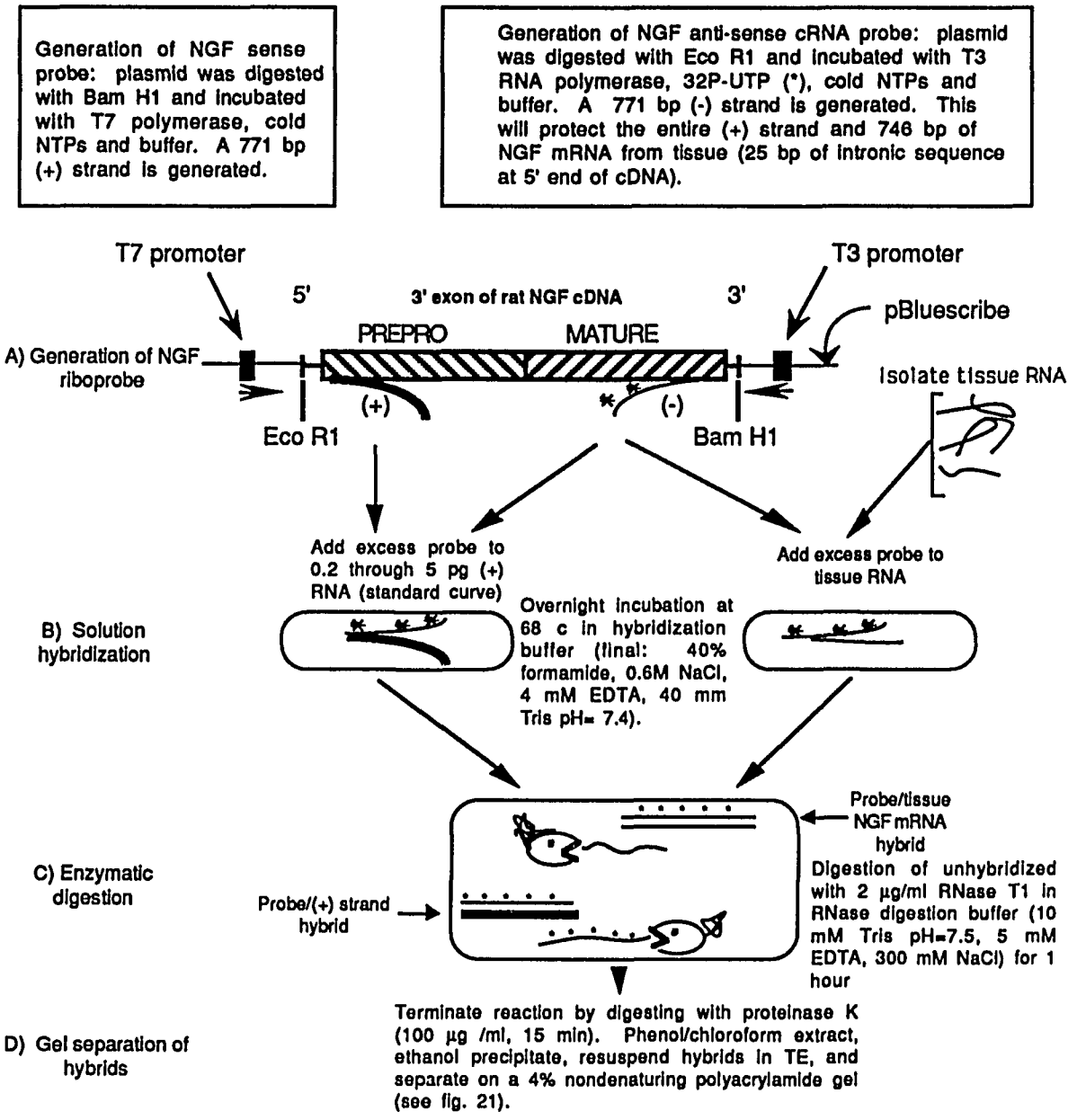


Figure 1. Generation of NGF radiolabeled ribroprobe (3' exon of rat NGF cDNA in pBluescribe, after Whittemore *et. al.*, 1988) and sequence of solution hybridization, nuclease protection assay.

and 2 µl of 150 pg/µl antisense probe was added. Probe was added in at least 10-fold excess of expected sample concentration to ensure hybridization is driven to completion. In addition to the samples, 11 standards containing 0 to 5 pg of sense strand RNA were included. A drop of mineral oil is layered atop the reaction to prevent evaporation, the tubes are placed at 85°C for 5 minutes to heat denature the RNA and were transferred to a 68°C water bath for overnight hybridization. The hybridized samples were transferred to a 1.5 ml Eppendorf tube (leaving the mineral oil behind) and 300 µl digestion buffer was added. A number of different digestions are possible and it was necessary to test various conditions to optimize the assay for rat NGF mRNA. In the initial approach, the hybrids were digested with S_1 nuclease (500 U in 300 mM NaCl, 30 mM NaAc, pH=4.8, 3 mM ZnCl) for 1 hour at 56°C and subsequently extracted with 1:1 (vol/vol) phenol/chloroform, 0.1 vol. 5M NH_4Ac, 1.25 mM EDTA and precipitated using 600 µl isopropanol with 5 µg of carrier yeast total RNA in 1.5 ml Eppendorf tubes for a minimum of 2 hours at -20°C. The hybrids were pelleted by centrifugation (16,000Xg, 15 minutes. at 4°C), washed in 70% ETOH, lyophilized and resuspended in 10 µl TE containing gel loading buffer. The samples were heated for 5 minutes at 68°C to dissolve the pellet and run on a 4% non-denaturing polyacrylamide gel with a ^{35}S- or ^{32}P-labelled BstE II digest of λ DNA or Hae III digest of $\phi\chi174$ DNA (New England Biolabs) serving as double stranded DNA size markers. The gel was dried, exposed for two hours at -70°C to Kodak XAR-5 film with an intensifying screen or overnight at room temperature. The resultant standard curve (0-7 pg (+)RNA) from this assay is shown in the top of figure 2. Three bands are present suggesting the full length hybrid, corresponding to the top band of 771 bp, was partially digested. The two lower bands cumulatively approximate the top band possibly indicating the nuclease was digesting the hybrids at a single base pair or at a limited stretch of poorly hybridized RNA. Similar results were obtained when lower amounts of the S_1 nuclease were used.

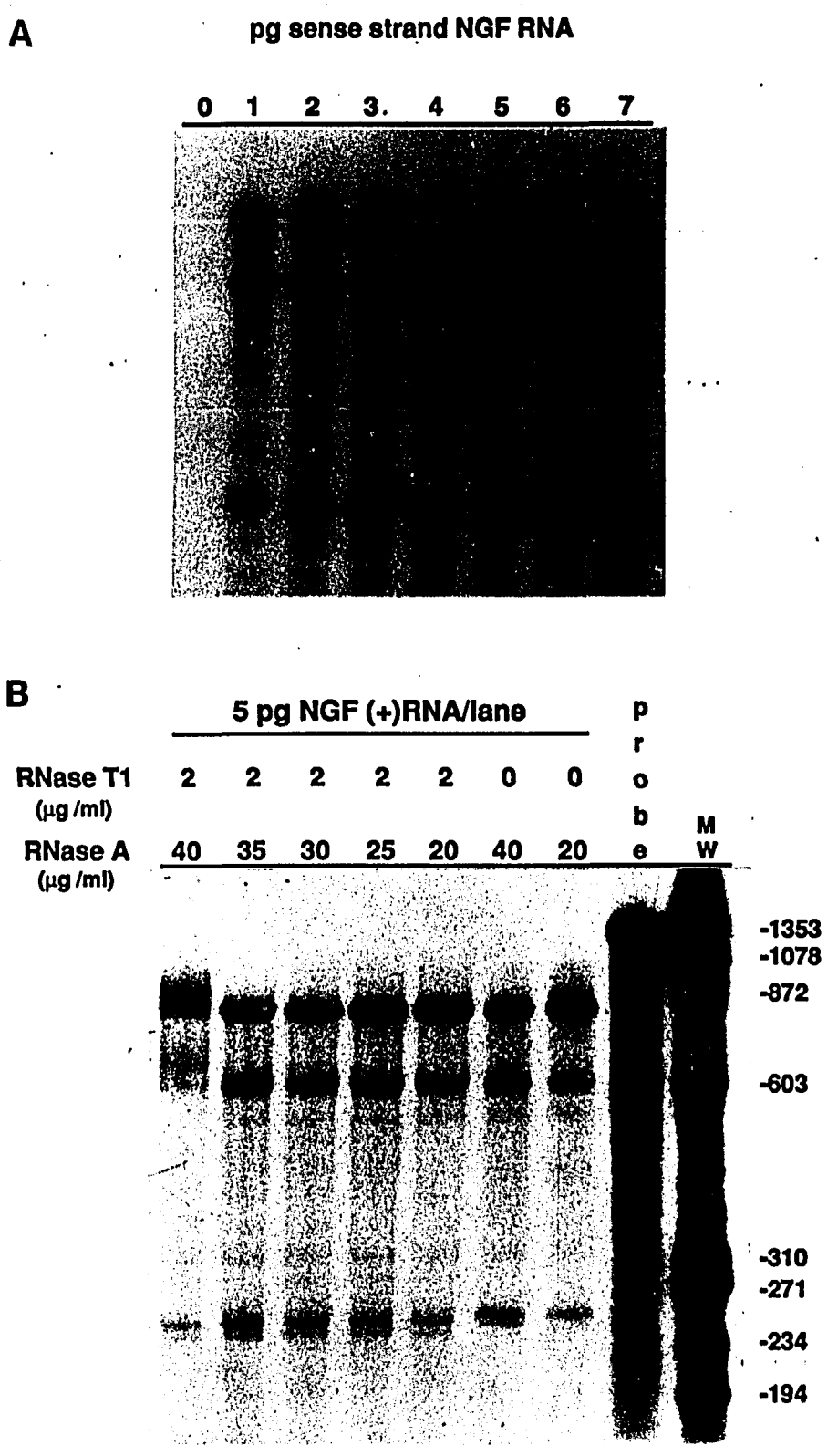
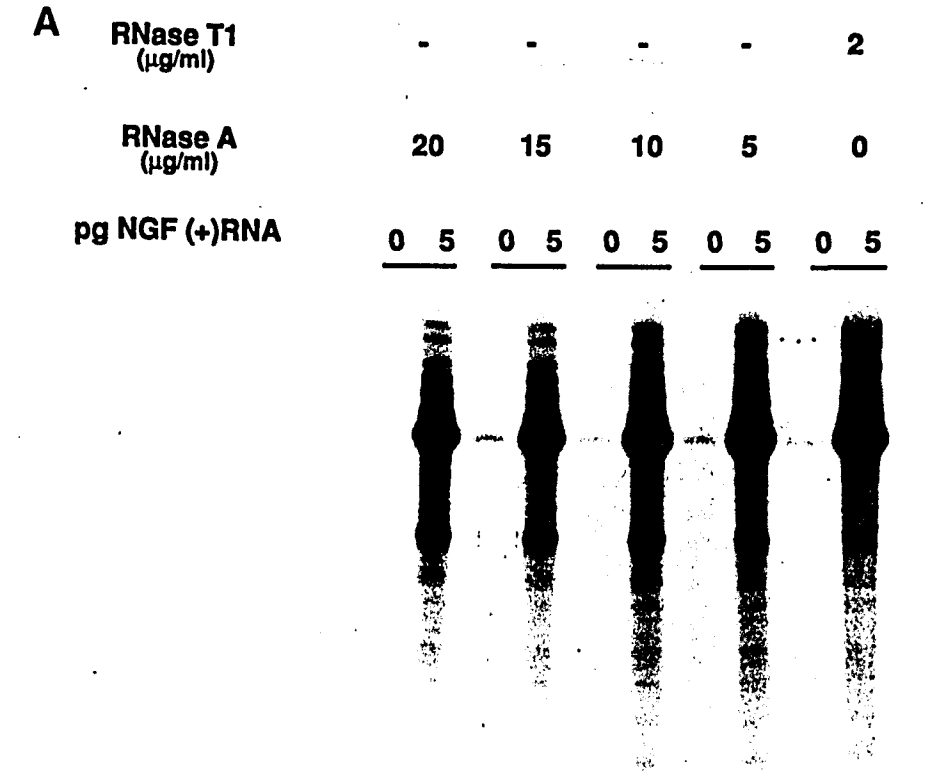


Fig. 2. Tests of digestion conditions for rat NGF probe. A) NGF probe was hybridized to 0 to 7 pg NGF (+) RNA and digested with 500U S1 nuclease. The top band is the full length hybrid while the lower bands represent undesired digestion products. B) Hybrids were digested with various combinations of RNase A and RNase T1 following hybridization with 5 pg (+) RNA. Lower bands are still present.



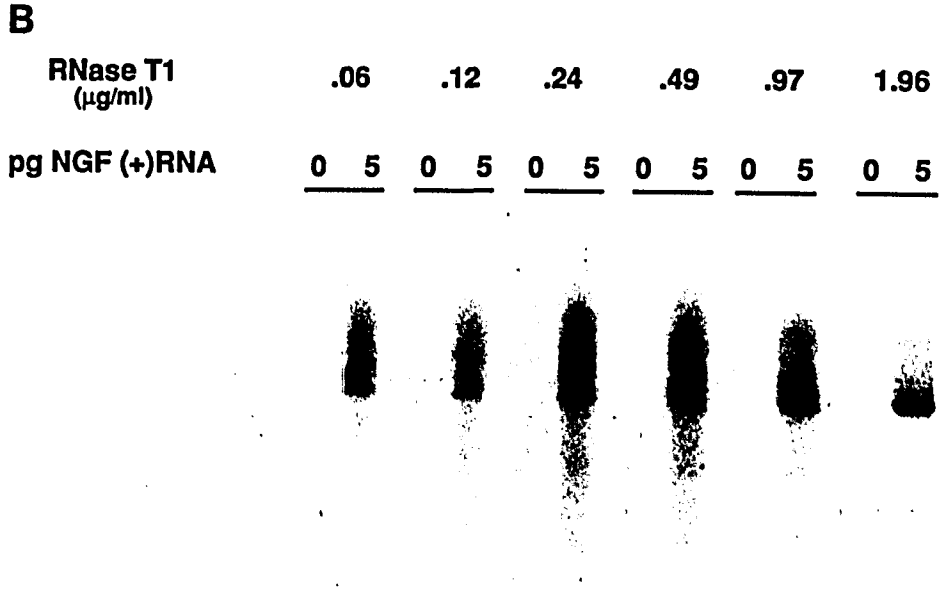


Fig. 3. Additional tests of digestion conditions for NGF probe. A) Titration of RNase A showing diminution of lower band as enzyme concentration is reduced. Digestion with RNase T1 alone produces a single, full length band. B) Titration of RNase T1 to standard assay concentration (~2μg/ml) produces a single band.

Further tests were conducted using a similar hybridization approach but digestion with RNase A and T_1. The advantage of using these enzymes is the ability to manipulate the extent of digestion: whereas S_1 nuclease will digest any single stranded RNA present RNase A digests single stranded RNA only 3' to pyrimidines and RNase T_1 only 3' to G residues (Lee and Costlow, 1987). Thus the two enzymes can be titrated to suit the digestion requirements of a spectrum of RNA hybrids. For digestion with these enzymes, hybridized samples were incubated with the enzymes in 300 µl buffer (10 mM Tris-HCl, pH=8.0, 5 mM EDTA, 300 mM NaCl) at 30°C for 60 minutes. The RNase(s) were subsequently digested by the addition of 15 µl 10% SDS and 5 µl 10 mg/ml proteinase K and incubated at 37°C for 15 min. The remainder of the protocol is identical to that for S_1 nuclease. Various concentrations of RNase A and T_1 were tested against 5 pg of rat NGF RNA-RNA hybrids and the results are shown in figures 2 and 3. The standard concentrations of RNase A and T_1 are 40 µg/ml and 2 µg/ml respectively. As shown in the lower autoradiogram of figure 2, at this concentration the enzymes appeared to over-digest the hybrid (lane 1). At other concentrations of the enzymes, whenever RNase A was present, partial digestion of the hybrid apparently occurred as indicated by the presence of two bands. The upper gel of figure 3 shows the gradual loss of the lower band as the concentration of RNase A is decreased. With no RNase A and 2 µg/ml RNase T_1, a single band was present. Figure 3 (bottom) displays a titration of RNase T_1 (no RNase A) from .06 to 1.94 µg/ml showing the appearance of a single major band as the concentration increases. A standard curve, using RNase T_1 (2 µg/ml) alone, ranging from 200 fg to 5 pg sense strand NGF is shown in figure 4. Based on these results, all assay data presented below used the single enzyme condition for digestion. As a result of this restriction, it was not possible to probe for other RNAs while analysing NGF mRNA levels. This was deduced early in these studies when, in the presence of a second probe, the background in the assay was increased significantly and therefore reduced the ability to resolve the low level of NGF mRNA. Figure 1 presents a condensed diagram of the assay.

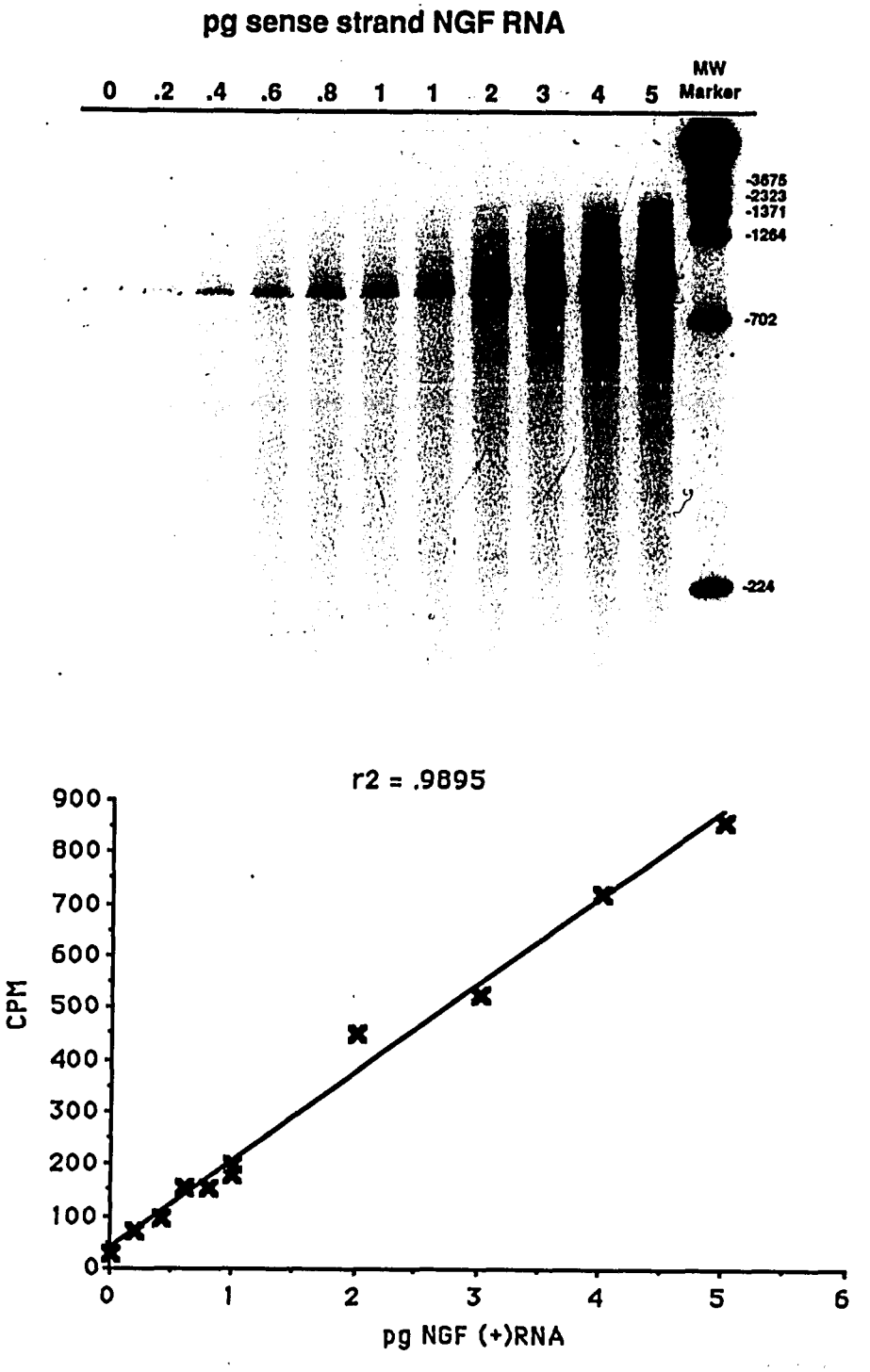


Fig. 4. Standard curve for protection assay using digestion with 2 µg/ml RNase T1. NGF probe was hybridized to .2 to 5 pg NGF (+) RNA and produced a protected band of 771 base pairs. Thus 200 fg of NGF mRNA can be quantified in the assay. Linearity of hybridization is shown in regression plot below.

When samples are included in the assay, the exposed film is aligned to the gel and is used to locate and excise the hybrids from the gel (see figure 21 for sample gel). The radioactivity in the samples is then measured by liquid scintillation. The results are presented as fg NGF mRNA/μg total RNA based upon the regression line generated by the CPMs of the standards.

Astrocyte cultures

Preparation of glial cultures

Timed pregnant Sprague Dawley rats were obtained from Zivic-Miller Labs. Rats were allowed to give birth and rat pups were taken at postnatal day 2 for the preparation of primary cultures of astroglia. Tissue from cerebral cortex, hippocampus, cerebellum, and hypothalamus were dissected, separated from meninges, and broken apart by trituration through a 10 ml pipet. Cells were dissociated in 0.1% trypsin for 30 minutes at 37°C and the digestion was terminated by the addition of Dulbecco's modified Eagle medium (DME, GIBCO), supplemented with 10% heat inactivated fetal calf serum (FCS, J. R. Scientific, Woodland, CA), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO) and 1 mM L-glutamine (Sigma) (culture medium). Dispersed cells were filtered through autoclaved Nitex monofilament polyester mesh and Nitex 130 screens (Tetko, Briarcliff Manor, NY) and pelleted by centrifugation. Cells were resuspended in culture medium, plated at various densities in 60 or 100 mm dishes (Falcon) or 75 cm^2 flasks (Corning) and placed in a 37°C incubator containing a water saturated atmosphere infused with 92% O_2/8% CO_2 (Berkenbosch *et. al.*, 1990). One or two brain regions were dissected per litter. The majority of the work was conducted on primary cultures of cortex where, following a series of early studies, the initial plating density was 3-4 x 10^6 cells per plate (see results). Plating density was less for other areas because of the scarcity of tissue and the need for sufficient plates in the protection assay. Two days before harvest, cultures were fed DME containing 10% charcoal stripped fetal calf serum. Stripped serum was prepared by

incubating FCS in 0.25% (w/v) dextran coated activated charcoal (Sigma) for four hours. All media were filter sterilized (0.45 μM, Nalgene) and stored at 4°C.

Neuron enriched cultures

Rat embryos were removed from pregnant Sprague Dawley rats at the eighteenth day of gestation (E18) and the hippocampus was dissected. Tissue was incubated in 0.1% trypsin at 37°C for 15 minutes, the trypsin was inactivated and cells were dissociated mechanically through a fire-polished Pasteur pipet. The resulting cell suspension was plated on polyornithine coated 35 mm Falcon culture dishes at a density of 5 x 10^5 cells per dish. Cells were allowed to attach to the substrate for three hours in serum-containing medium. Cultures were then switched into serum-free, chemically defined medium and maintained in a 8% CO_2/92% O_2 water saturated incubator. Defined medium contained equal of volumes Minimal Essential Medium (MEM) in Earle's salts (catalog # 320-1090, GIBCO) and Hamm's F-12 nutrient mixture (320-1765, GIBCO) supplemented with 25 mM glucose, 2mM glutamine, 25 mM $NaCO_3$, 15 mM HEPES, 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 20 nM progesterone, and 30 nM sodium selenite (all from Sigma). A variety of plating and culturing parameters were tested to examine the effect of conditions permissive for glial proliferation on NGF mRNA levels. Cells were harvested following various periods up to 5 days *in vitro*. A litter of 12-14 pups produced approximately 20 plates. A minimum of 9 plates was used for detection of NGF mRNA.

Treatment and harvesting of cultures

Medium was never changed during the 48 hours prior to any experimental manipulation as feeding *per se* has been shown to induce NGF mRNA (Spranger *et. al.*, 1990). IL-1ß (Human recombinant interleukin-1ß, Genzyme Corp., Boston) was added directly to the culture medium (total volume added ~0.1% of that in dish). IL-1α (a gift of Hoffman-La

Roche to Dr. Frank Berkenbosch) was used in some of the early studies and in one of the *in vivo* experiments. The dose range tested for IL-1ß was based upon studies conducted in sciatic nerve explants by Thoenen's group (Lindholm *et. al.*, 1987). During the course of the study the supplier of IL-1ß altered the concentration the of product and this corresponded to a period when astrocyte cultures from a series of experiments had shown no response to interleukin-1. The high affinity interleukin-1 receptor has a K_d of 20-100 pM in a variety of mouse cells (Chizzonite *et. al.*, 1989). Prior to the product change, 10 U/ml of IL-1ß was added to the tissue culture flasks and this is equivalent to 2.9 pM. To assure the studies were conducted in the optimal binding range of the receptor, all subsequent experiments used 250 U/ml (71.5 pM) of IL-1ß. Cultures were harvested 3 hours following IL-1ß addition by scraping the cells from the plates. Total cytoplasmic RNA was isolated and NGF mRNA was quantitated using the RNase protection assay as described above.

Immunohistochemistry of cultured cells

To assure the cellular population was similar for flasks harvested for NGF mRNA quantitation and those for antibody staining, flasks from each condition destined for immunocytochemistry were treated identically to those used for RNA isolation. Cells within each flask were fixed 10 minutes in 4% phosphate buffered paraformaldehyde and washed 2x 10' in phosphate buffered saline (10 mM phosphate buffer, pH=7.4, .9% NaCl; PBS). Six or seven discs containing the fixed cells (~35 mm diameter) were subsequently cut out of the flasks and stored at 4°C in PBS until used. Cultures were processed for cell specific markers using a variety of primary antisera (Berkenbosch *et. al.*, 1990). Anti-glial acidic fibrillary protein (GFAP, mouse monoclonal anti-rat IgG (1:50), Fisher) identifies an intermediate filament protein expressed in astrocytes. Anti-galactocerebroside (GC, mouse monoclonal anti-rat IgG (1:4)) recognizes a cell surface marker specific to oligodendrocytes. OX-42 (MRC OX-42, monoclonal mouse-anti-rat

IgG (1:100), Bioproducts for Science, Indianapolis) recognizes the CD-3 complement receptor on macrophage. A_2B_5 (monoclonal mouse-anti-rat IgM (1:4), ascites fluid from hybridoma bearing mice, a gift of Dr. V. Friedrich) identifies a cell surface ganglioside on a subpopulation of mature neurons (Eisenbarth *et. al.*, 1979) as well as O-2A progenitors and type II astrocytes (Raff *et. al.*, 1983). Cells for GFAP immunohistochemistry were permeabilized by treatment in 0.2% Triton X-100 in PBS for 30 minutes and processed as the other antibodies. Discs were washed 3X in PBS and incubated with antibody (in 3% horse serum in PBS) for 2 hours at room temperature or overnight at 4°C. Following washing, A_2B_5 plates were incubated with peroxidase-conjugated anti-IgM (1:100, Amersham) while GFAP, OX-42, and GC were incubated with peroxidase-conjugated sheep anti-mouse Ig in 3% horse serum in PBS for 45 minutes at RT. Color development occurred using the avidin-biotin-peroxidase (ABC) complex method and 3, 3'-diaminobenzidine-tetrahydrochloride as the chromogen, activated by 0.01% hydrogen peroxide (Vectastain, Vector Laboratories Inc.). Discs were sealed using crystal mount liquid coverslipping medium (Biomedia Corp., Foster City, CA).

In Vivo *IL-1 administration*

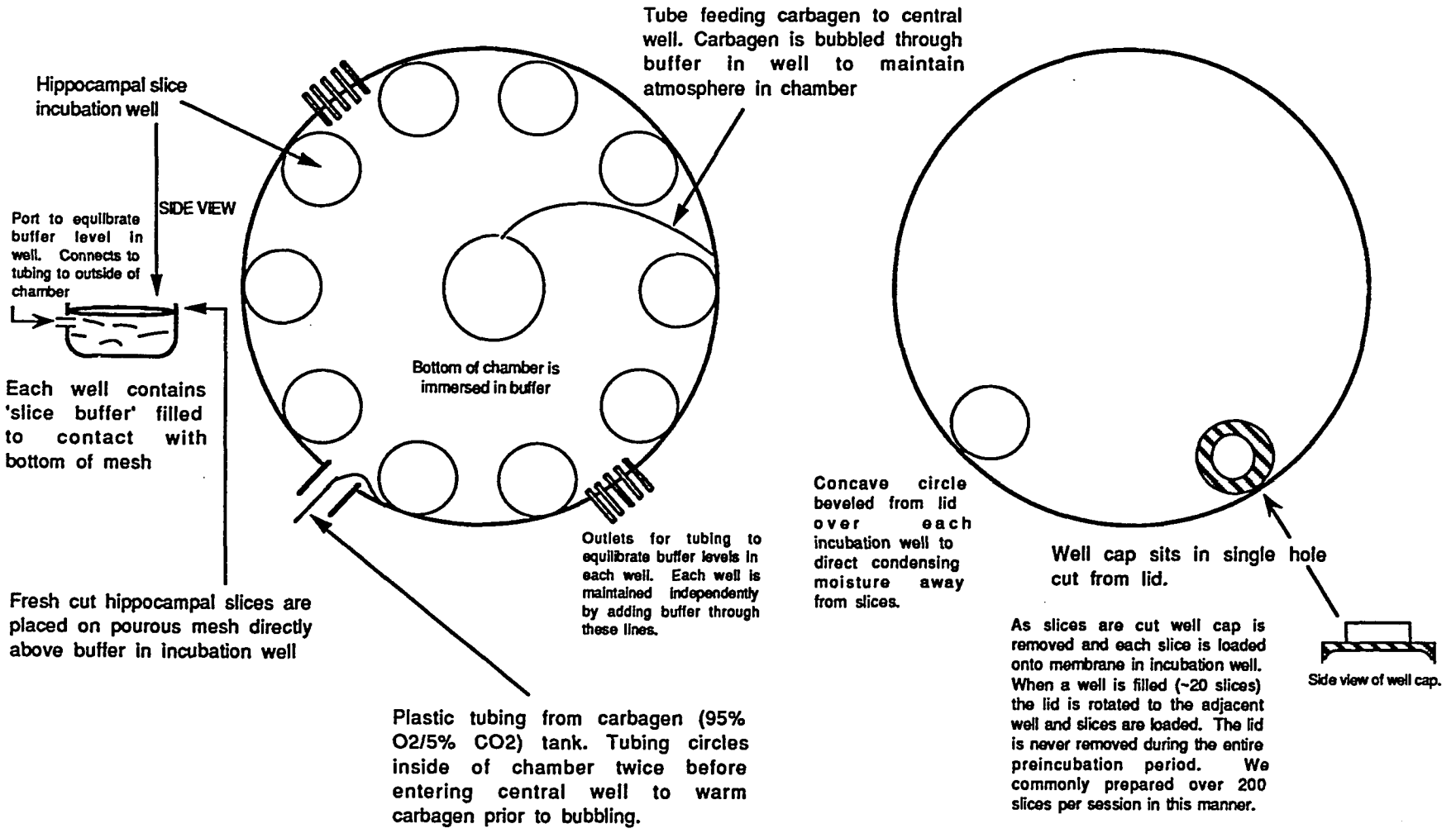
200 g male Sprague Dawley rats were anesthetized with Avertin (60 mg/ml; 1 ml/100 g B.W.) and placed in a Kopf stereotaxic device. A canula was implanted into the lateral ventricle and permanently fixed in place. Interleukin-1 (10U IL-1ß or 100 and 1000U IL-1α in 10 µl PBS) or PBS (saline control) was injected into the lateral ventricle, -1.5 mm from bregma, 2.0 mm lateral to midline and 3.5 mm deep to the ventral aspect of the cranium. Sham animals were placed in the stereotaxic apparatus and prepared for surgery with no penetration of the brain. All animals survived for three hours following treatment. Animals were sacrificed by decapitation, total cytoplasmic RNA was isolated from various brain regions and NGF mRNA was quantitated by the RNase protection assay.

Hippocampal Slices

Preincubation chamber

The general approach using hippocampal slices is to preincubate the tissue in a 'metabolic chamber' where cells recover and accumulate levels of phosphorylated substrates to drive cellular metabolism. Slices remain in this chamber and are subsequently used for a number of experimental paradigms often related to electrophysiological recording studies. During the initial stages of the studies reported here, an incubation chamber was used which had been previously employed to preincubate and record from 400 μm thick slices of rat hippocampus. This circular plexiglass chamber contains a number of wells where the slices incubate. A polycarbonate plastic membrane (12 μm pore; Nucleopore, Anaheim, CA) is pulled taut across a plastic ring and is placed within each well where it acts as an interface between the static buffer (slice buffer: 130 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 15 mM glucose, 2.5 mM CaCl, 1.5 mM $MgSO_4$) filling the well below and the gas phase of a humid, 95% O_2, 5% CO_2 (carbagen) atmosphere above. Polyethylene tubing provides access to each well to maintain buffer levels while a line enters a central well to establish the internal chamber atmosphere. Slices are prepared and placed on the membrane and a lid (containing concave semicircles carved from the inner surface and aligned over each well to direct condensing moisture away from the slices) is secured onto the base of the chamber. For electrophysiological studies, following the preincubation period, electrodes are inserted through a hole in the lid to stimulate and/or record from the slice. These studies indicate slices are viable for a minimum of 8 hours and up to 14 hours.

Our initial studies using this apparatus indicated it was inadequate for our purposes. Whereas studies recording from slices require 10-12 slices per session, preliminary studies quantifying NGF mRNA levels indicated we required over 150 slices for each experiment. In addition, the handling of this number of slices required that the chamber lid be removed



Slice preincubation chamber-base
(built by Joseph Biela, Anaheim, CA)

Slice preincubation chamber-lid

Fig. 5. Chamber for preincubation of hippocampal slices (not to scale).

numerous times thus not allowing the establishment of the enriched atmosphere. We therefore had the preincubation chamber shown in figure 5 built for us by Joseph Biela, Anaheim, CA. This chamber incorporates all of the design elements described above while containing 10 wells and a lid with a hole and removable plug. The hole allows the loading of slices onto the membrane while the lid remains in place and the internal atmosphere is maintained. The lid is simply rotated to the adjacent well when a membrane is full and the plug seals the chamber when loading is complete. This chamber was used for all experiments described below.

Protocol for incubation and harvesting of hippocampal slices

Young adult male Fisher 344 rats (180 g; Charles River) were sacrificed by decapitation, the hippocampi were rapidly dissected on ice and placed in ice cold slice buffer for 10 minutes. Slices were prepared on a McIlwain tissue chopper cutting the hippocampus perpendicular to the septotemporal axis using a standard thickness of 600 μm. We also tested slices at 400 μm and 800 μm and those cut parallel to the longitudinal axis. At 600 μm the two hippocampi from an animal would provide a total of 30-40 slices. Slices were immediately loaded into the preincubation chamber which had been equilibrated with carbagen and maintained at 37°C. Upon completion of loading, the chamber remained undisturbed for various periods during this preincubation phase. The tissue was subsequently transferred to a sterile 12 well tissue culture plate (Falcon) containing 1 ml of slice buffer per well. 20-30 slices were placed in each well; this also represented the average number of slices needed to generate a NGF mRNA signal in the protection assay. The plate was placed on a platform shaker (Mini Orbital Shaker, Bellco Biotechnology, Vineland, NJ) in a water saturated incubator at 37°C in 95% O_2, 5% CO_2 and agitated at low RPM for various times. Transfer to the culture plates was performed to permit direct exposure of slices to a variety of compounds. The tissue was agitated during incubation to encourage dispersion of products of cellular metabolism. Following this

incubation period, slices were collected into either DEP treated, 1 ml glass homogenizers or sterile 1 ml syringes containing 1 ml 0.3 M sucrose AT buffer, and RNA was isolated and processed as described in the RNA isolation procedure. The total RNA was subsequently analyzed for NGF mRNA in the solution hybridization assay.

For the studies using labelled uridine uptake to gauge tissue viability, slices were preincubated in the chamber and transferred to a 12 well plate containing slice buffer and 25 μCi ^{3}H-uridine (5,6 ^{3}H-uridine, 36.5 Ci/mmol, lot 2561-198, New England Nuclear, Boston) and allowed to incubate for various periods. Upon harvesting (1-2 slices per time point), the intact tissue was rinsed once in slice buffer and homogenized in 30 μl 0.3 M sucrose AT buffer. The homogenate was layered over 15μl 0.4 M sucrose AT buffer, centrifuged at 2500g at 4^{o}C for 10 minutes and the upper fraction containing cytoplasmic RNA was tested for uridine incorporation by spotting on DE81 paper and scintillation counting.

Results

Description of the enriched astrocyte cultures

Primary cultures of enriched astrocytes from neonatal rats were the source of tissue for most of the experiments described below. Representative cultures of cortical astrocytes from the protocol employed for the bulk of this work are shown in figures 6, 7, and 8. A composite of phase photomicrographs of astrocytes in culture for 2 days to 25 days is shown figure 6. Cultures were partially characterized using anti-GFAP and anti-GC monoclonal antibodies and antibody A2B5 as shown in figure 7. The broad flattened cells bearing a compressed multipolar or bipolar appearance bind antiserum to GFAP and have been shown to proliferate extensively in culture. These cells correspond to type I astrocytes (for review see Raff, 1989). GFAP+ cells were found in short and longer term cultures (see figures 7A, 7B and 8) and were the majority of cells shown in figure 6 to expand to form the bed layer in cells grown for more than 2 weeks. The smaller, darker GFAP+ cells, often showing a phase bright soma and several branching processes (figures, 6, 7B) correspond to the type II astrocyte. Process bearing cells were clearly visible in the younger cultures growing on the surface of the plate or on top of flattened cells (figure 6) although they did not appear to proliferate. Examination of 6 DIV cultures indicates a proliferation of type 1 astrocytes and a level of type II-like cells similar to that found at 4 DIV. The type II astrocyte arises from the O-2A progenitor cell which shares with the type II the expression of a cell surface sialoganglioside recognized by the A2B5 antibody. Cultures grown for 6 days showed little staining with the A2B5 antibody; older cultures displayed light staining of cells displaying a condensed soma and multiple processes (figure 7E). The complete characterization of a GFAP+ cell as a type II astrocyte must include double staining for the two markers. Oligodendrocytes, which also

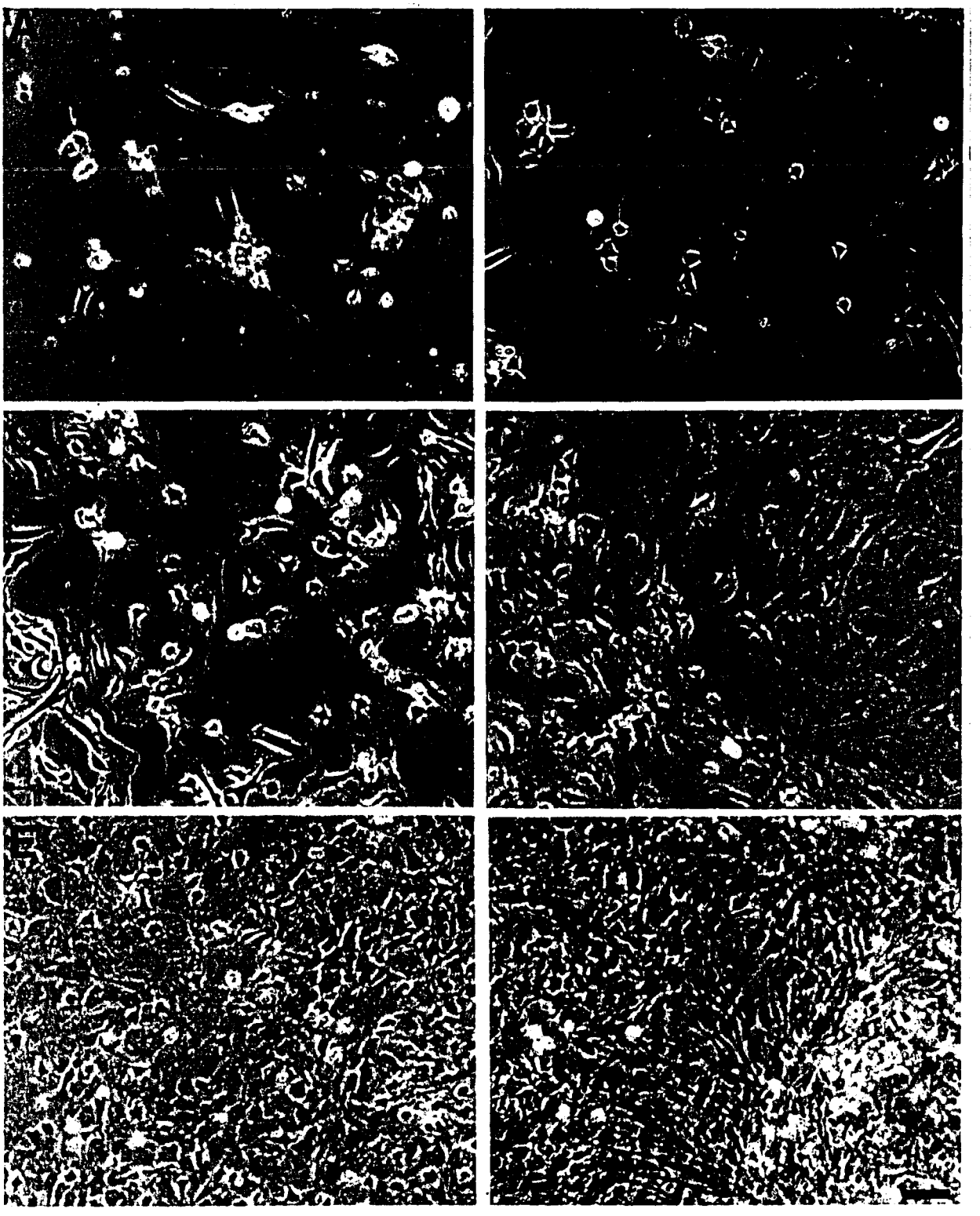


Fig. 6. Phase photomicrographs of development of enriched astrocyte cultures. Astrocytes were isolated from postnatal day 2 rats and plated at 4 x 10E6 cells/plate and photographed following various periods in vitro : A) 2 days; B) 4 days; C) 6 days; D) 8 days; E)10 days; F) 25 days. Bar= 50μM

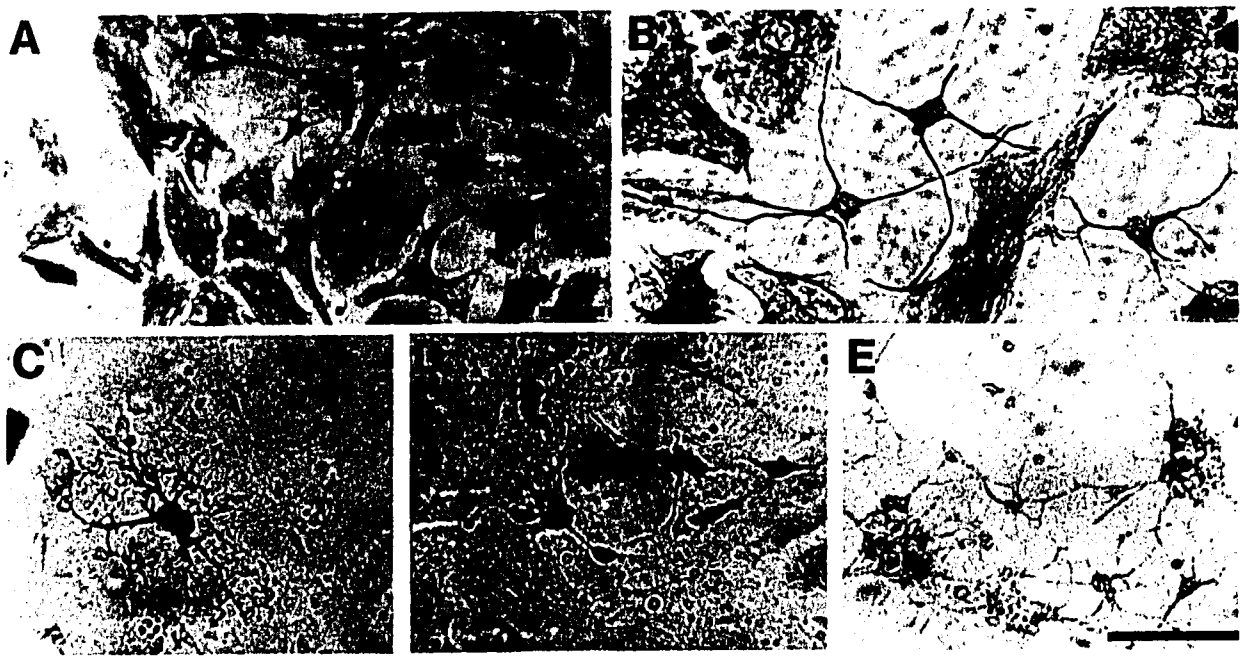


Fig. 7. Phase photomicrographs of enriched astrocyte cultures stained with various antibodies. A & B) Cultures grown for 6 days and exposed to GFAP antisera. The majority of cells were GFAP+ and were composed mostly of flattened cells as seen in (A) and fewer process bearing cells as shown in (B). These correspond to the description of type I and type II astrocytes, respectively. Other cell types present included C) oligodendrocytes displaying GC immunoreactivity (6 day culture), D) OX-42+ macrophage (6 day culture), and E) cells binding the A2B5 antibody indicative of type II astrocytes or a subpopulation of neurons (25 day culture). Bars= 50μM (bar in E applies to C & D).

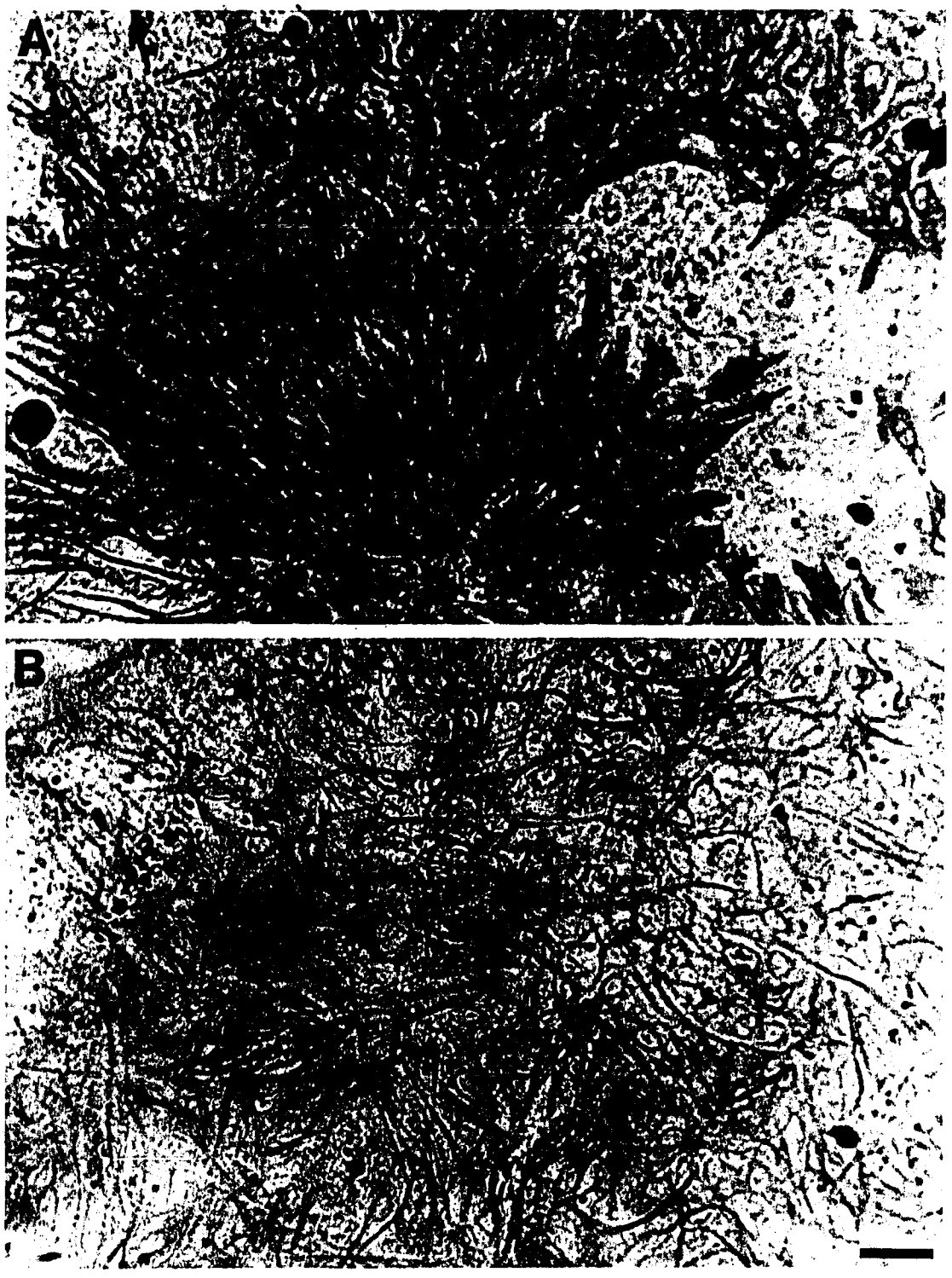


Fig. 8. 25 day cultures exposed to GFAP antisera. GFAP+ cells and processes covered most of the surface of plates but avoided certain circumscribed areas (A). This may be indicate the presence of fibroblasts in these regions. Most areas showed an intricate network of GFAP+ fibers (B). Bar= 50μM.

arise from the O-2A progenitor but are not recognized by A_2B_5 following differentiation, can be identified with antibodies against galactocerebroside (GC), a myelin glycolipid found exclusively in these cells. In cultures of optic nerve, in contrast to the type I astrocyte, oligodendrocytes and type II astrocytes do not proliferate following differentiation. In cultures of various ages a small population of GC+ cells was observed (figure 7C).

In cultures grown for a longer period (figure 8) there was a carpet of flattened but more compact cells where GFAP staining was more diffuse. The majority of these cells are likely to be type I astrocytes. The altered staining pattern may reflect a change in the expression of the protein, a rearrangement of intermediate filaments as astrocytes age or in response to cell to cell contact or the antibody may have greater difficulty penetrating the dense cell layer. This carpet was occasionally interrupted by a circumscribed area unpopulated by GFAP+ cells (figure 8A); this may indicate regions of fibroblast growth which are incompatible with astrocyte expansion.

In addition to glial cells, figure 6 displays a population of small phase bright cells which may correspond to those shown by Giulian (Giulian and Baker, 1986; Giulian, 1987) to be ameboid microglia. In mixed glia cultures isolated and grown in a manner comparable to our approach, this lab found 7 DIV cultures to contain 5% microglia. These cells were always present in our cultures and, in agreement with Giulian, were often on top of type I-like GFAP+ cells. As shown in the bottom panel of figure 6, cultures maintained for more than 2 weeks *in vitro* would commonly show large patches of these phase bright cells on top of a dense bed of cells. The density of these small cells between cultures was variable. Staining 25 DIV cultures with OX-42, an antibody directed against the CD-3 complement receptor found on macrophage, shows a number of positive cells including some with extended thin processes (figure 7D). The number of OX-42 positive cell bodies would indicate a minority of the phase bright cells are macrophage; the remainder may be glial precursors or dying cells. In sum, the cultures used in the experiments described below

contained a variety of cell populations although the vast majority at each stage of growth was represented by GFAP+ astrocytes.

Presence and influence of non-glial cells

A number of assays were performed to gauge the influence of nonglial cells on NGF mRNA levels found in our enriched astrocytes. Cultures used to measure NGF expression in fibroblasts were initially plated as enriched astrocyte cultures and allowed to grow for 1 week. These cultures were then placed on an orbital shaker and agitated overnight in an attempt to remove cells other than type I astrocytes which attach to the culture plate (or to astrocytes). The nonglial cells can be separated from the astrocytes by removal of the culture medium following shaking and replating of the remaining adherent cells. Giulian has shown this procedure to yield highly purified astrocyte cultures. Subsequent to this procedure, we found the cultures to be populated mostly by fibroblasts. We allowed the cells to proliferate for an additional 11 days; at this time the cells had a broad, flattened appearance and phase dark intracellular fibers characteristic of fibroblasts. The cells were then harvested and assayed for NGF mRNA. Cultures prepared in this manner from cortex, hippocampus and hypothalamus expressed NGF mRNA at levels averaging 65% of enriched astrocyte cultures (data not shown). Fibroblasts isolated from meninges of neonatal rat brain expressed similar levels of NGF mRNA to that found in these fibroblast enriched cultures. Yoshida and Gage (1991) have found that fibroblasts isolated from meninges of various brain regions secrete higher levels of NGF than astrocytes plated in a similar manner. While these data indicate fibroblasts from distinct brain regions express NGF mRNA, this approach to fibroblast isolation and the lack of immunocytochemical evidence to judge the percentage of glia remaining in these cultures permits limited interpretation of these data. Examination of cells under phase microscopy indicates very

few non-GFAP+ cells in the 6 DIV (figure 7) cultures and small, circumscribed areas lacking GFAP staining in 25 DIV cells (figure 8).

NGF mRNA measurement in enriched astrocyte cultures

The objective of the work presented in this section was to measure basal NGF mRNA expression in astrocytes isolated from a variety of regions from the neonatal rat brain and in astrocytes grown for various periods *in vitro*. These studies were performed to confirm and expand earlier findings of astrocyte expression of NGF mRNA. Enriched astrocyte cultures were grown for various periods *in vitro* and NGF mRNA was measured in the nuclease protection assay. Table 1 displays representative data for untreated astrocyte cultures across the course of these studies. NGF mRNA levels in whole rat and mouse tissues are included for comparison. A number of methodological considerations should be stated in the compilation and grouping of this data. During the period of these studies there was an evolution in the development of plating techniques (astrocyte isolation procedure, plating density, optimization of feeding medium) and of harvesting approaches to ensure sufficient total RNA for the assay. While the plating procedure was eventually standardized to that of McCarthy and de Vellis (1980), the variability in the yield of this procedure remained an ongoing concern. The issue of yield was critical because of the large number of cells required to detect the NGF mRNA. For example, a typical litter of neonatal rats (10-15 postnatal day 1 pups) produces approximately 4×10^7 and 8×10^6 purified cortical and hippocampal astroglia, respectively. Since one aspect of these studies was to compare levels and regulation of NGF in nonconfluent and confluent glia, we found the optimal, lowest plating density at which cells survived was 4×10^6 cells per 75 mm flask or 100 mm dish. At this density neonatal cortex yields ~10 plates while hippocampus 2-4 plates per litter. To reliably detect NGF mRNA in untreated, nonconfluent glia three flasks were required to provide reasonable quantities of total RNA. While single plates of 50-70% confluent astrocytes could produce a detectable band in the protection assay, the

Region	Days in vitro	fg NGF mRNA/µg total RNA	n
Enriched Astrocyte Cultures			
Cortex	5-25	191.6 (18.4)	16
Hippocampus	7-15	148.5 (13.3)	4
Hypothalamus	6, 7	123.4 (29.7)	2
Cerebellum	6, 7	230.4 (51.4)	2
Fresh Frozen Tissue*			
Rat Hippocampus		43.0 (6.0)	9 (47)
Rat Cortex		8.91 (2.7)	5 (33)
Mouse Hippocampus		55.5 (9.9)	6 (33)
Mouse Cortex		18.4 (3.8)	6 (33)

For the astrocyte cultures, n represents the number of pooled points from separate protection assays, each point comprising one to four 75 mM flasks or 60 mM plates. For the fresh frozen tissue, n represents the number of pooled assays where each assay contained from 2 to 10 samples from the indicated brain regions. For example, data from the rat hippocampus represent assay values of 47 hippocampi collected as the means of 9 separate assays. Data for the fresh, frozen tissue were compiled as control tissues from various experimental paradigms for general comparison purposes only (means are weighted to account for variable sample sizes [± SE]).
* Tissue was dissected from adult animals and either flash frozen in freon chilled to -110°C by liquid nitrogen and stored at -80°C until use or homogenized immediatedly followed by RNA isolation and subsequent storage under ethanol until use.

Table 1. Levels of NGF mRNA per total cytoplasmic RNA in enriched astrocyte cutures from various regions of postnatal day 2 rat brain and in fresh tissue from the adult rat brain.

signal was often too close to background to be reliable. Thus, using three 75 mm flasks per data point and 3 data points per condition, a minimum of 2 litters was required for each control and treatment condition when plating cortex. While the original intent was to perform extensive analysis of hippocampal glia, as these plating parameters became apparent, the focus was switched to neonatal cortical tissue. The choice was also made early in these studies to restrict the analysis to primary cultures: whereas astrocytes will continue to grow in the presence of serum following numerous replatings, the phenotype of the astrocyte population may be further altered by this approach. For example, astrocytes were observed to proliferate at a faster rate following secondary plating in comparison with primary cultures plated at an equivalent density. The majority of the data presented, including all of the immunocytochemistry, are derived from cortical astroglia cultured in an identical manner. However, the initial 1/3 of the work was based upon characterizing NGF mRNA regulation in hippocampal glia and the preliminary analysis of other regions. Table 1 is a compendium of both phases of this study and therefore represents a grouping of data where the plating methodology differed but where the ultimate result was purified, enriched astrocyte cultures.

Expression of NGF mRNA in astrocytes isolated from different areas of the rat CNS

The expression of NGF mRNA was examined in enriched astrocyte cultures derived from different regions of the neonatal rat CNS. The objective was to determine if proliferating astrocytes in culture possess the common trait of NGF expression and would indicate that astroglia from diverse areas of the CNS have the capacity to express the gene during periods of astroglial growth. NGF mRNA was found in nonconfluent cultures of enriched astrocytes isolated from neonatal rat cortex, hippocampus, cerebellum and hypothalamus (figure 9). There was no significant difference between the mean values for untreated astrocytes from these areas. These data indicate that cultured glia from cortex and

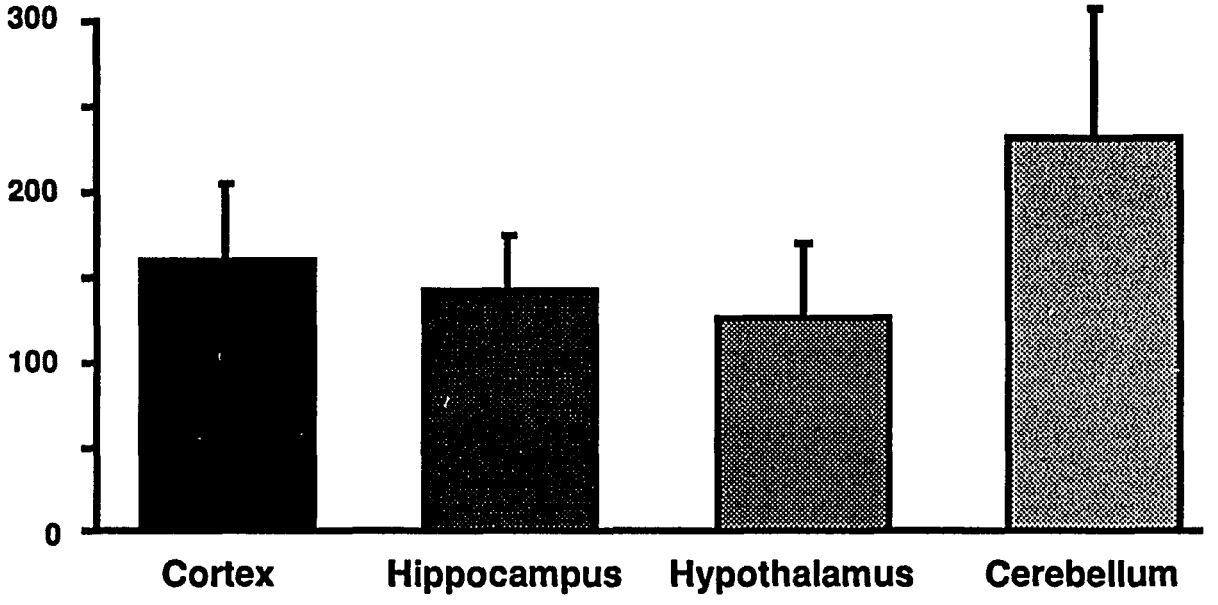
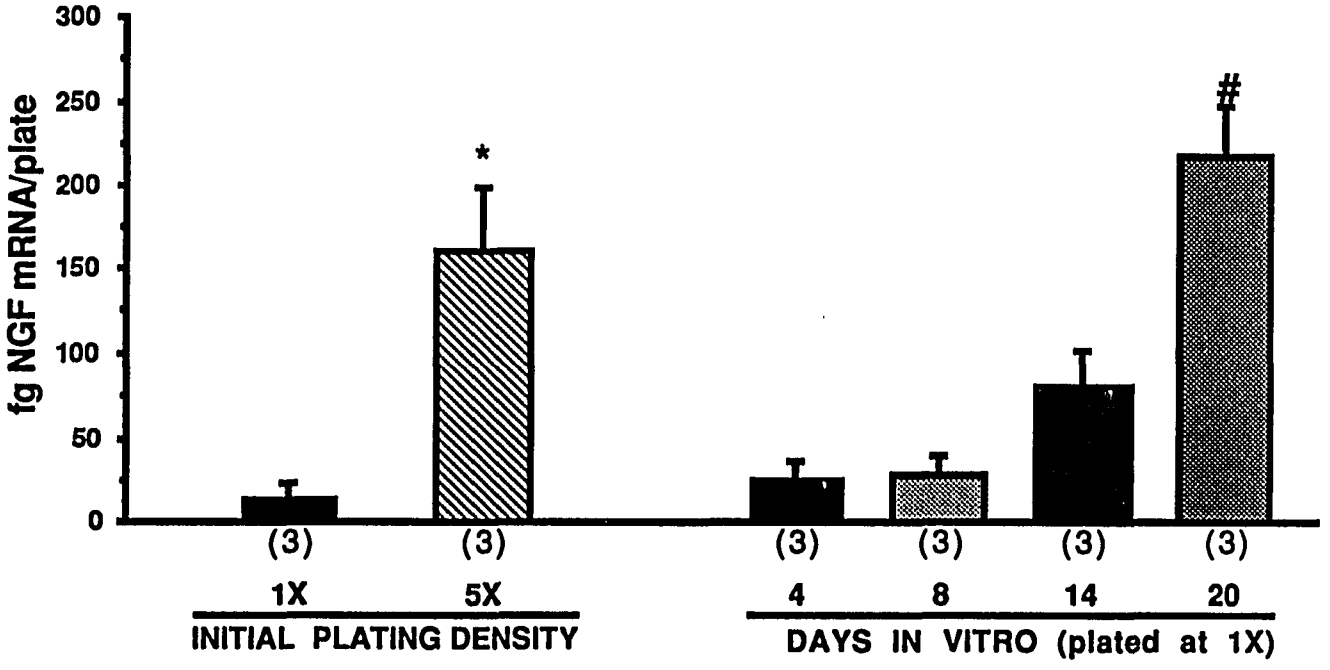


Fig. 9. Representative NGF mRNA levels in enriched astrocyte cultures isolated from regions of postnatal day 1 rat brain. Cells were grown 5-8 days *in vitro* and total cytoplasmic RNA was isolated for screening in the nuclease protection assay. Areas were analyzed in duplicate where 1 to 3 points where examined per area in each assay. All data presented in bar graphs represent mean ± S. E. M. .

hippocampus can express NGF mRNA at levels 3 to 17 times that found in equivalent tissue dissected from adult animals (table 1). This data indicates that astrocytes from a number of brain regions have the capacity to express NGF mRNA and concurs with recent data showing NGF gene expression in astrocytes derived from cerebellum, hippocampus, and basal forebrain (Lu *et. al.*, 1991). This suggests that under certain conditions *in vivo* where astrocyte proliferation is occurring, there may be expression of NGF in astrocytes and this expression may influence the growth and survival of certain populations of neurons in the CNS. The data presented here indicate that astrocytes may provide NGF to a wide variety of neuronal populations during selected periods. Previous studies using *in situ* hybridization histochemistry on cryostat sections from adult animals have shown a very low signal in neurons in the hippocampal formation with no glial expression shown. The lack of glial derived NGF expression in the intact, mature brain suggests that as glia mature and astrocyte proliferation decreases, NGF expression is reduced. The following section examines NGF mRNA levels in astrocytes where parameters associated with the rate of proliferation were examined.

Influence of cell density on expression of NGF mRNA

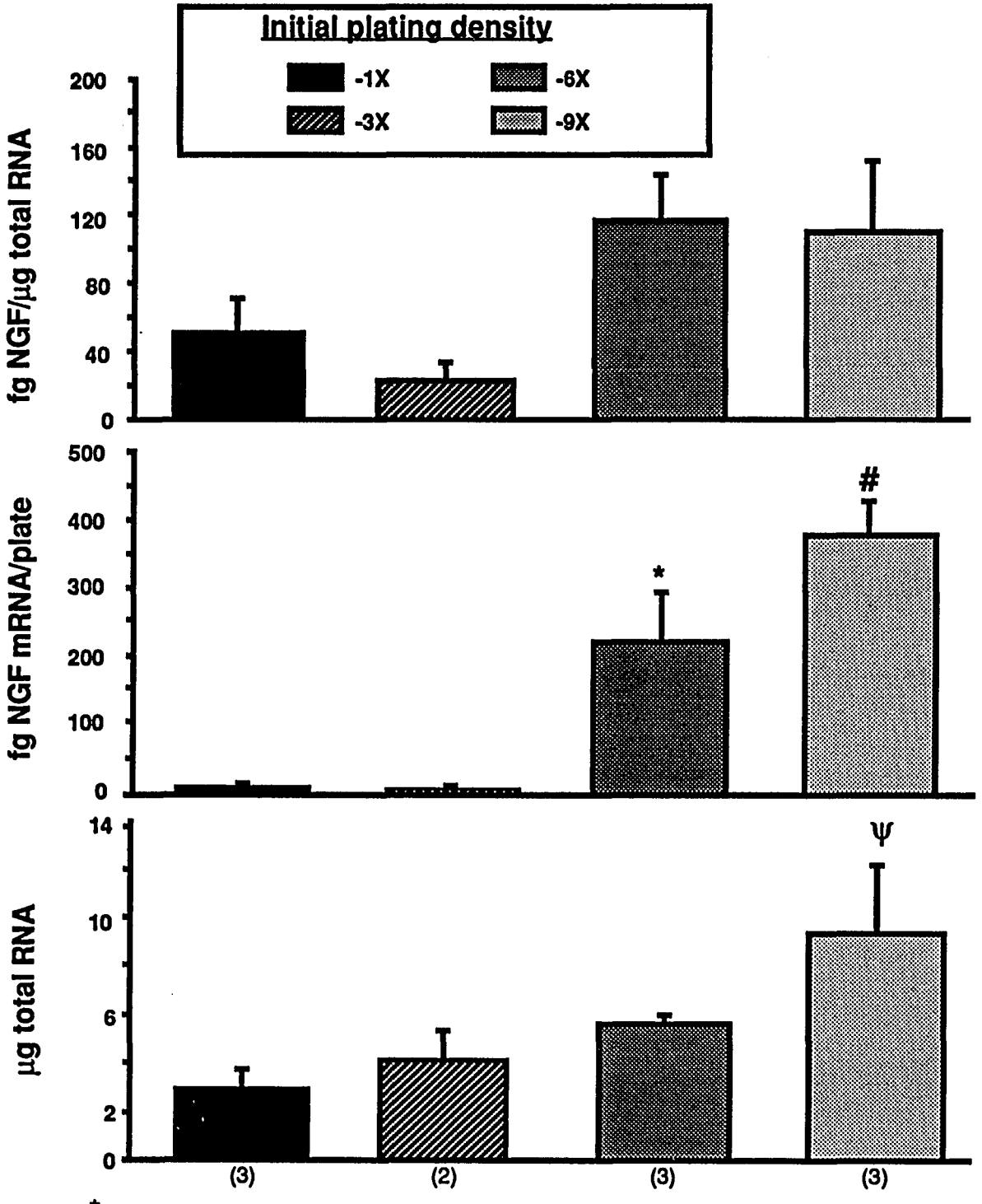
Nonconfluent, rapidly growing astrocytes express high levels of NGF mRNA. Previous studies have shown a significant down regulation of the transcript as the cells reach higher density (Furukawa *et. al.*, 1986, 1987). In light of work showing the addition of serum to enriched astrocyte cultures can induce NGF mRNA (Spranger *et. al.*, 1990; Lu *et. al.*, 1991), there is an apparent correlation between the rate of astrocyte growth and NGF mRNA expression. The influence of cell density on NGF mRNA expression was therefore examined. Cultures were plated at a range of densities and harvested at a single time point or a single plating density was used with a series of survival times. Studies directly comparing plating densities in astrocyte cultures from neonatal rat hippocampus and cortex are shown in figures 10 and 11 respectively. The higher density cultures



* p< .05 (Student's t-test)

\# p< .05 vs all other culture periods, ANOVA (Scheffe post-hoc analysis)

Fig. 10. Effect of plating density and survival period on NGF mRNA levels in enriched astrocyte cultures from neonatal rat hippocampus. For the density experiment, cells were seeded at 1X =1.0 x 10E5 cells/plate and harvested following 7 days *in vitro* Astrocytes were plated at 2.5 x 10E5 cells/plate for the time course study. Note data is expressed on per plate basis.



* p< .05 vs 1X and 3X, ANOVA (Fisher post-hoc analysis)

\# p< .05 vs all other densities, ANOVA (Fisher post-hoc analysis)

Ψ p< .05 vs. 1X, ANOVA (Fisher post-hoc analysis)

Fig. 11. Effect of density of initial plating on NGF mRNA levels in enriched astrocyte cultures from neonatal rat cortex. Cells were plated at multiples of 2.5 x 10E5/plate and harvested following 6 days *in vitro*. Numbers below bottom graph indicate number of plates per point.

showed higher NGF mRNA levels per plate in cells from both areas after 6-7 days *in vitro*. However, these elevated levels are likely to be a result of increased cell number as reflected by the higher level of total RNA found in the 9X cortical cultures and when expressed on a NGF mRNA/μg total RNA basis (figure 11). The level of NGF mRNA in the 3X cortical cultures was surprisingly low. When hippocampal astrocytes were plated at low density and allowed to grow for 20 days, there was a 6 fold increase in NGF mRNA per plate (figure 10). In studies using cells plated at a higher density (3-4 x 10^6 cells/75 cm^2 flasksflask), a 2 fold increase of NGF mRNA per μg total RNA was observed during the first 17 days in culture. There was a 72% decline in NGF mRNA/total RNA between 17 and 25 days *in vitro* (see control values in figure 17). The trend towards decreased levels of NGF mRNA in the older cultures occurred as the amount of total RNA harvested increased significantly (figure 18). These older cultures may have reached a critical density where cell-cell contact mediated regulation and a reduced rate of proliferation are associated with decreased levels of NGF mRNA. The data from the differential plating study shown in figure 11 may also reflect cell-cell contact regulation of NGF mRNA: the similar levels of NGF mRNA in the 6X and 9X conditions may indicate these cultures had reached a density where further increases in NGF mRNA levels are not observed. These data agree with previous studies showing proliferating astrocyte cultures express NGF mRNA and suggest that events related to elevated cell density are correlated with the down regulation of NGF mRNA.

Interleukin Studies

A series of experiments were conducted using enriched astrocyte cultures to study the regulation of NGF mRNA by interleukin-1. As described above, these experiments were prompted by studies by Thoenen's group suggesting that interleukin-1 is involved in the induction of NGF mRNA in the sciatic nerve following lesion. The general of aim of the

work described here was to evaluate if NGF mRNA is also regulated by interleukin in the central nervous system. The major emphasis was to analyze regulation in cultured astrocytes: the working hypothesis was that cultured proliferating astrocytes, whose mitotic state may serve as a model for the proliferation of astrocytes during development are capable of responding to interleukin-1, a cytokine released by phagocytic cells during ontogeny. One aspect of this response may be the increased expression and release of NGF. A second objective of this work is to determine if slowly proliferating astrocytes, whose role as providers of trophic support in the adult CNS may be diminished (as reflected by the reduced levels of NGF mRNA in confluent cultures) are capable of responding to an agent released during injury by a reiteration of some properties observed during their development. Part of this recapitulation may involve the expression NGF mRNA. This paradigm is analogous to that proposed for the peripheral nervous system: in response to lesion, macrophages invade the site of injury and release interleukin which induces NGF expression and release. NGF may then provide a receptor bound or soluble substrate for the subsequent regrowth of the nerve. The involvement of Schwann cell released NGF in the regrowth of the nerve resembles a similar sequence of events during nerve outgrowth in the development of the PNS. In the CNS, the proliferation of astrocytes during development, the presence of ameboid microglia which are capable of releasing IL-1, the ability of the neonatal brain to respond to IL-1, and the ability of cultured, proliferating astrocytes to express NGF lead to the prospect that NGF may be regulated in astrocytes by IL-1 in the neonatal brain. The observation of increased numbers of peripheral and endogenous macrophage in association with elevated IL-1 levels and changes in the astrocyte population during traumatic brain injury indicates that IL-1 may regulate NGF under these conditions as well. To evaluate this possibility *in vitro*, nonconfluent enriched astrocyte cultures derived from a variety of brain regions were exposed to IL-1 and levels of NGF mRNA were measured in the protection assay. In contrast to the developing CNS, the uninjured adult brain appears to contain astrocytes

whose rate of proliferation is substantially reduced (Korr, 1986). The second hypothesis of this work is that astrocytes in the intact adult brain, lacking the constellation of factors that may permit responsiveness during neonatal periods, are refactory to IL-1 induced regulation of astroglial NGF. This inability to respond may be one reflection of a developmental program in astrocytes which is related to reduced proliferation as these cells mature. This was studied using two approaches: cortical astrocyte cultures were treated with IL-1 following various periods in vitro. At the longest time point the cultures were close to confluence and the basal levels of NGF mRNA expression were reduced. IL-1 was also injected into the intact adult rat brain and total hippocampal RNA was analyzed for changes in NGF mRNA expression. Survival parameters were designed so that microglial response to the trauma was circumvented; thus the regulation observed should be the neural or glial response to the applied IL-1. These latter studies may reflect the ability of slowly proliferating astrocytes *in vivo* to respond to the cytokine. Finally, in view of evidence indicating antagonistic roles of IL-1 and glucocorticoids, an analysis of their interaction in the regulation of NGF mRNA expression in astrocyte cultures was conducted. Preliminary experiments were performed to characterize the influence of glucocorticoids on basal NGF mRNA expression in the cultures.

Influence of glucocorticoids on NGF mRNA levels

Glucocorticoids can reduce the expression of NGF in a fibroblast cell line (Wion *et. al.*, 1986; Siminoski *et. al.*, 1987) and in dissociated cultures from rat hippocampus (Friedman *et. al.*, 1990). In addition, glucocorticoids and interleukin-1 appear to play distinct and often opposing roles in the mediation of a number of physiological systems including regulation of the hypothalamic-pituitary-adrenal axis. Figures 12 and 13 show studies undertaken to analyze the effect of glucocorticoids on NGF mRNA expression in enriched astrocyte cultures. The influence of incubating cultures in charcoal stripped serum (i.e. where glucocorticoids have been removed) was evaluated in anticipation of later studies

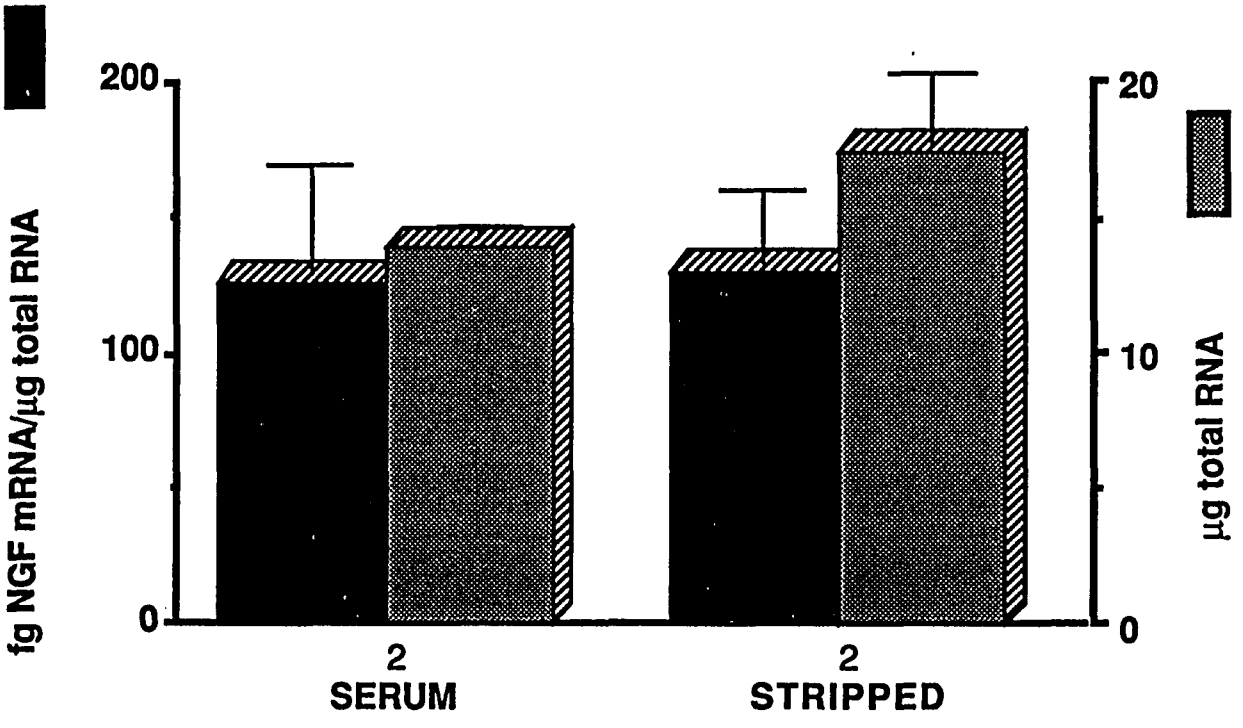


Fig. 12. Effect of incubation in stripped serum on cultures. Astrocytes isolated from PN1 rat cortex were incubated for 3 days (60 mm plates, initial plating density 7.5 x 10E5) in DME containing 10% fetal calf serum (FCS/DME). Cultures were then fed either FCS/DME or DME containing 10% charcoal stripped FCS and harvested following 48 hours additional incubation.

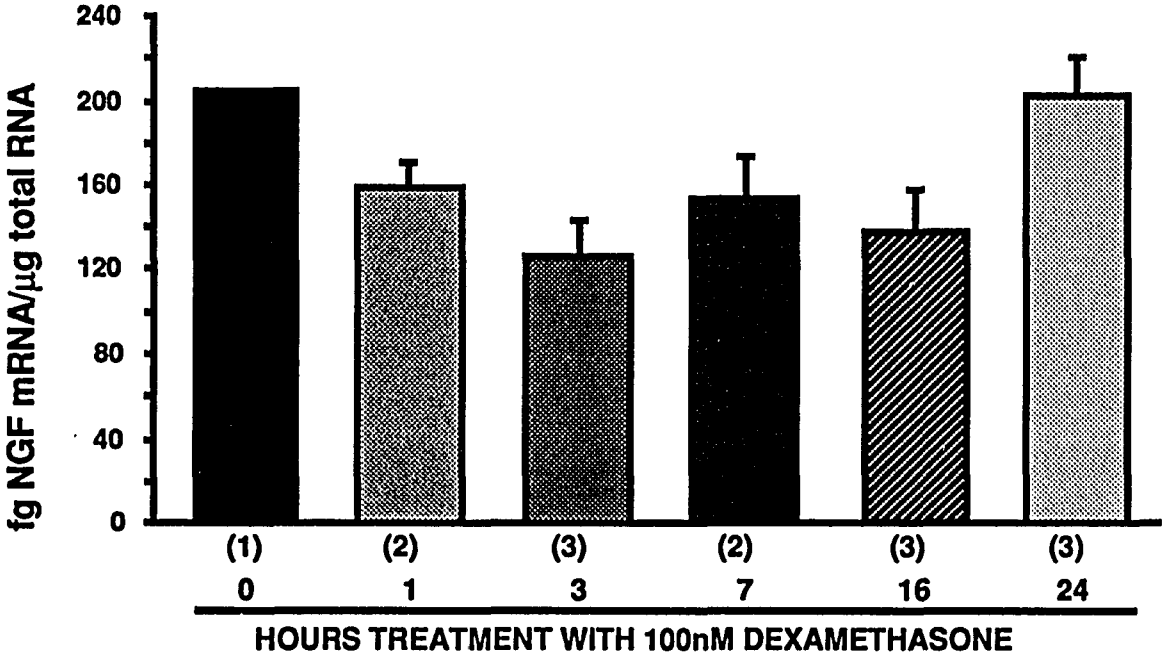


Fig. 13. Effect of dexamethasone addition to glial cultures. Enriched hippocampal astrocyte cultures were incubated 12 days in DME/FCS and then fed DME/stripped FCS an addtional 2 days before harvesting. Dexamethasone was added at the times indicated prior to harvest. Numbers below each bar reprent data points in the protection assay, each point representing pooled RNA from three 60 mm dishes. Means ± S. E. M..

where the presence of glucocorticoids may interfere with IL-1 mediated effects. There was no effect on either NGF mRNA or total cytoplasmic RNA in cortical cultures following 48 hour exposure to charcoal stripped serum (figure 12). Dr. Karen Sheppard has measured glucocorticoid receptor mRNAs in the cultures exposed to 48 hours stripped serum: hippocampal and cortical astrocytes expressed 10.2 and 12.4 pg glucocorticoid receptor (GR) mRNA/ total cytoplasmic RNA, respectively, while only cortical cultures showed a lower level of expression of the mineralocoid receptor (MR) mRNA (2.5 pg/μg total RNA). These levels compare with 8.4 pg mRNA of GR and 15.5 pg mRNA of MR in cytoplasmic RNA isolated from total rat hippocampus. Following adrenalectomy (thus removing circulating glucocorticoids), these levels changed to 13 pg GR mRNA and 12.3 pg MR mRNA per total cytoplasmic RNA. Thus astrocyte cultures express glucocorticoid receptor mRNA levels comparable to those found in whole tissue. Enriched hippocampal astrocytes showed no change in NGF mRNA/μg total RNA following various periods of exposure to 100 nM dexamethasone (DEX) immediately before harvest (figure 13). Cultures were incubated in stripped serum for 48 hours before harvesting and therefore cells were free from glucocorticoid exposure at the time of DEX addition. These data contrast with studies in mixed cultures of hippocampal glia and neurons (Friedman *et. al.*, 1990) where a similar exposure to DEX produced a significant reduction in NGF transcript expression. This may indicate glucocorticoids can regulate NGF expression in cultured neurons or the neuron-glia interaction can induce this sensitivity in glia. Nevertheless, enriched astrocyte cultures express glucocorticoid receptor mRNAs and the removal or the removal and readdition of glucocorticoids had no direct effect on NGF mRNA levels.

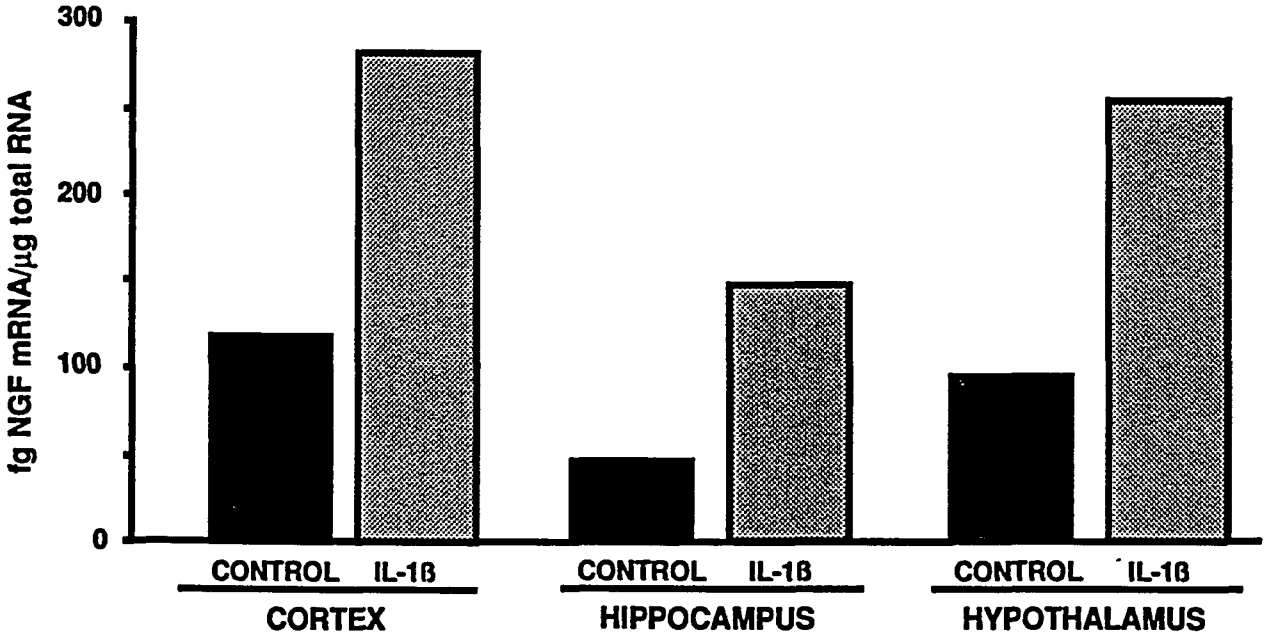
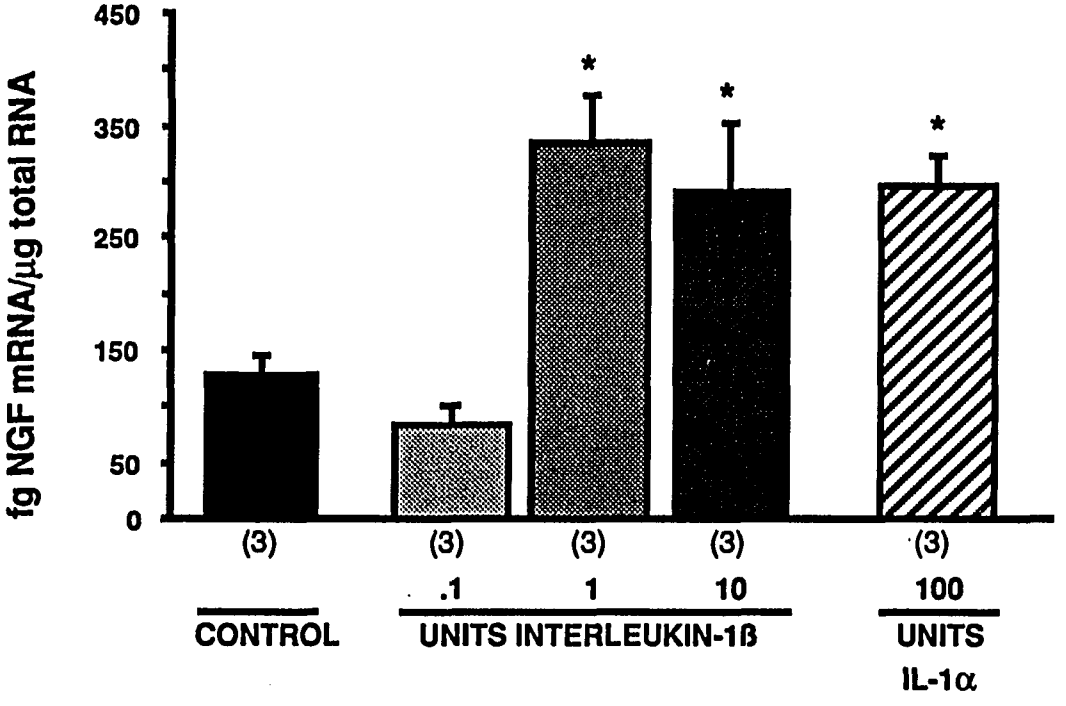


Fig. 14. NGF mRNA levels in glia from various regions and induction by interleukin-1ß. Enriched astrocyte cultures were prepared from PN1 rats and maintained in 10% FCS/DME for 4 days and 3% FCS/DME for 3 days in 60 mm culture dishes. Three hours prior to harvest, 20µl of 1000U/ml human recombinant IL-1ß ([final]=10U/ml) was added to experimental plates. Each bar represents 2 or 3 culture plates and one point within the protection assay.



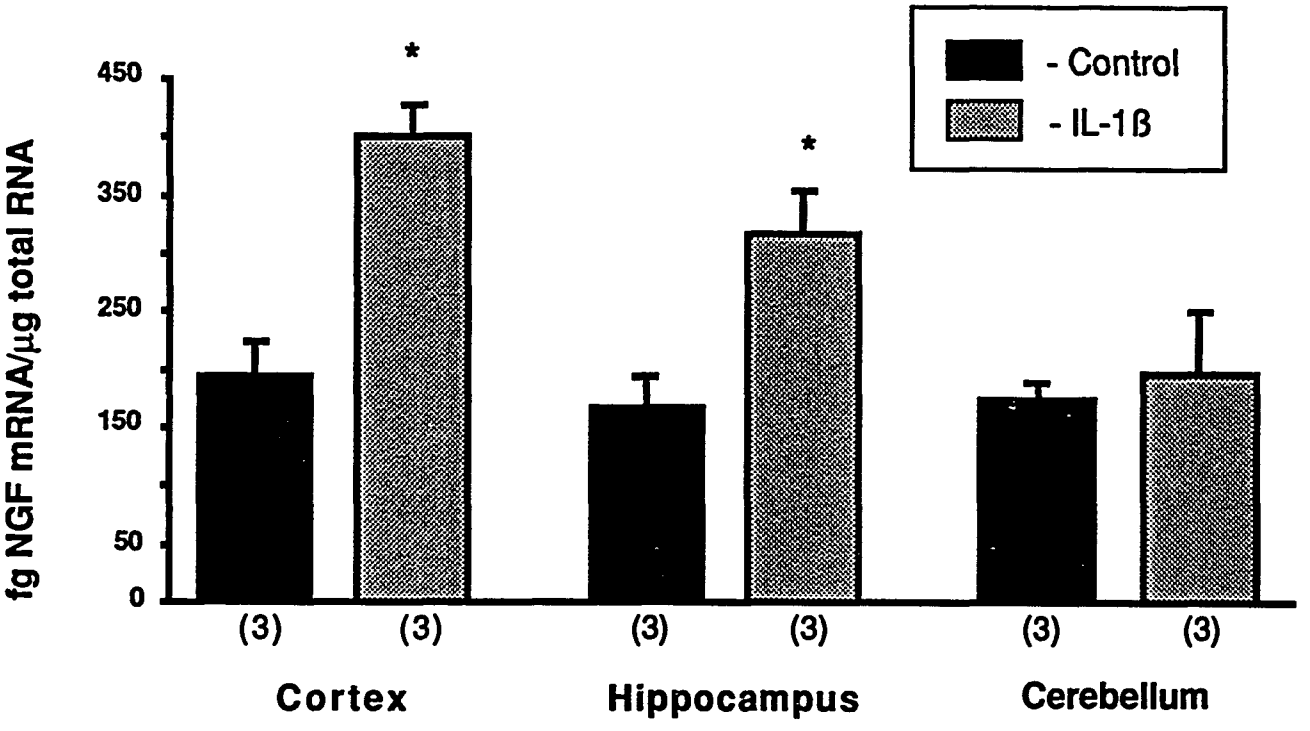
* ANOVA (Fisher, p<.05) versus untreated control

Fig. 15. NGF mRNA levels in enriched hippocampal astrocyte following 3 hours exposure to various amounts of interleukin-1ß or 100 units of interleukin-1α . Cells were seeded at 5 x 10E05 cells/60 mm plate and harvested following 8 days in vitro.

Effect of Interleukin-1 on enriched astrocyte cultures from various brain regions

Figure 14 presents data from early studies where the effect of 10U/ml interleukin-1ß on NGF mRNA levels in enriched astrocyte cultures from various brain regions was examined. These data are single points from the protection assay, each point representing 2-3 60 mm flasks, and thus cannot be statistically compared. The induction shown is 148% in the hippocampal, 253% in the hypothalamic and 282% cortical cultures. In studies using similar culture conditions testing dose-response parameters of IL-1, enriched hippocampal astrocytes displayed a significant induction to 1U and 10U/ml IL-1ß following a 3 hour exposure (figure 15). The effectiveness of IL-1α to produce this induction was also tested and at a dose of 100U/ml a similar induction was found. Further studies at this stage of the work (60 mm plates, plating at a density of 2 x 10^6 cells/plate, treatment following 6 days in vitro) showed a significant, 281% increase in NGF mRNA levels in cortical astroglia following 3 hour exposure to 7.5 U of IL-1ß. These studies were conducted using cultures exposed to 10% fetal calf serum in DME during the entire culture period. To test the possibility that glucocorticoids present in the culture medium can regulate NGF expression and in preparation of testing the interaction of glucocorticoids and interleukin-1 on this expression, cortical astrocyte cultures were cultured for 2 days in normal serum followed by 5 days in charcoal stripped 10% FCS/DME and was compared to cultures exposed the entire period to 10% FCS/DME. NGF mRNA levels were measured following 3 hour exposure to 10U and once again a significant induction was observed in the whole serum maintained cultures (data not shown). A similar degree of induction has been repeatedly observed in cultures exposed to stripped serum for a minimum of 48 hours prior to treatment.

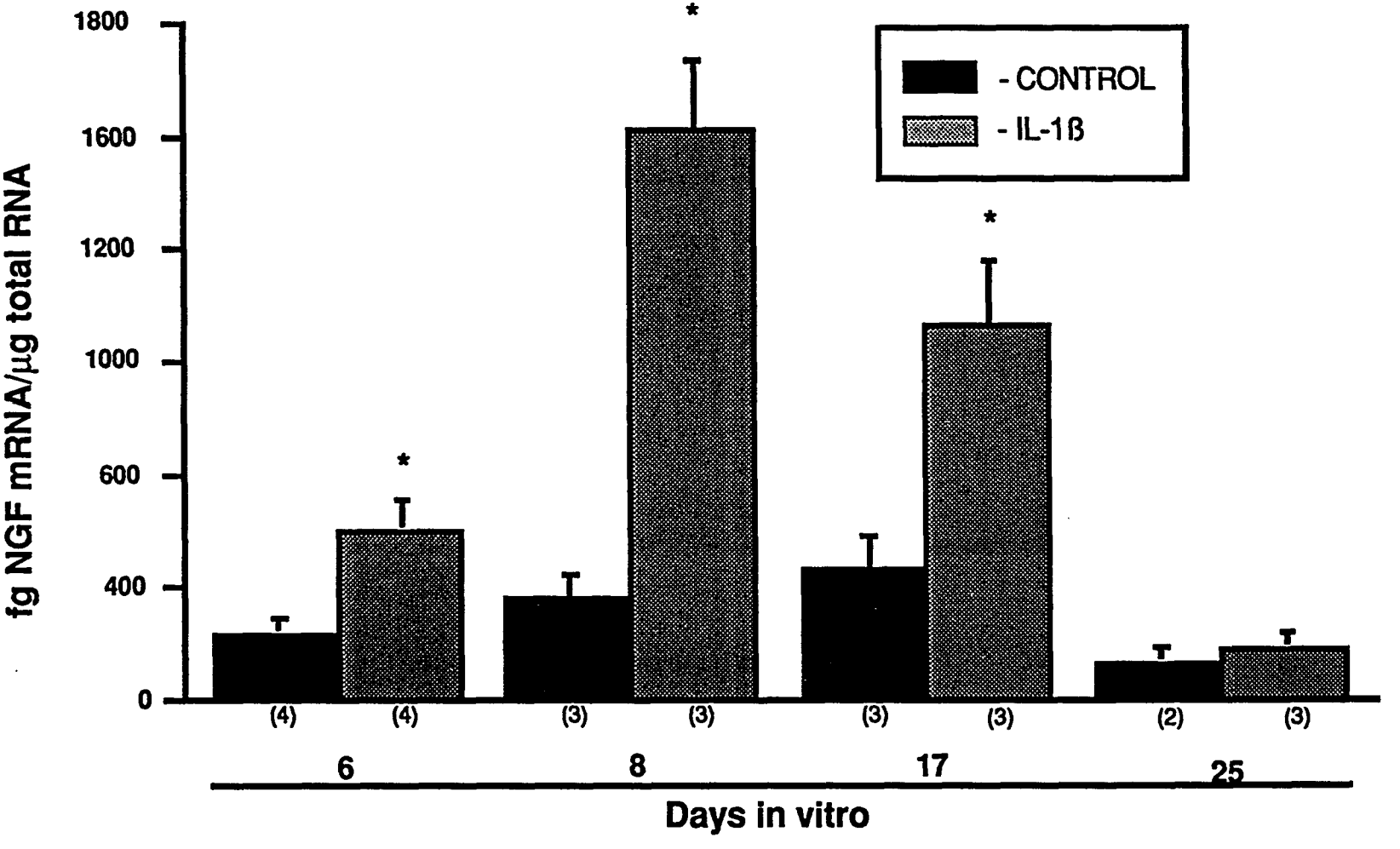
A large series of experiments were conducted where the plating procedure was standardized. It was found that using a plating density of 2-4 x 10^6 cells/75 cm^2 flask



* p<.05 Student's t-test

Fig. 16. Levels and induction by interleulin-1ß of NGF mRNA in enriched astrocyte cultures. Glia were plated at a density of 3.0 x 10E6 cells/75 cm flask and grown for 7 days for cortical cultures and 5 days for hippocampal cultures. Cerebellar cultures were plated at a density of 9.5 x 10E5 per flask and harvested after 8 days in vitro. All cultures were switched to charcoal stripped10% FCS/DME 2 days prior to harvest.

provided the most reliable and efficient culturing approach. The IL-1ß induction experiments were repeated and the results for cortical, cerebellar, and hippocampal glia are shown in figure 16. Significant induction was observed in enriched astrocytes derived from these regions. (A gel displaying the protected NGF mRNA band from RNA isolated from untreated and IL-1 exposed hippocampal astrocyte cultures is shown in figure 21.) No attempt was made to repeat the findings on hypothalamic astrocytes as the yield without replating was insufficient to produce replicate flasks. The cerebellum also provides little tissue in the neonatal rat; when plated at a lower density than hippocampal or cortical astrocytes and allowed to proliferate for 8 days, no induction was observed following 3 hour exposure to IL-1. These data indicate this cytokine can produce a significant elevation of NGF mRNA levels in low density, proliferating cultures of astrocytes from most brain regions and suggests the presence of IL-1 during development plays a role in NGF mediated astrocyte-neuron interactions. The lack of induction in cerebellar glia may be related to the late (in comparison with the hippocampus and cortex), predominantly postnatal development of the cerebellum and may reflect an immature astrocyte population unable to a respond to a mitogen present in the developing brain. NGF and NGF receptor mRNAs have been shown to transiently increase in the postnatal cerebellum (Buck *et. al.*, 1987; Wanaka and Johnson, 1990; Lu *et. al.*, 1991) corresponding to a period of ongoing proliferation in this structure. Thus the capacity for this induction may be related to the stage of development of specific brain regions. While it remains controversial if astrocytes proliferate following injury, a clear astrocytic response is observed near the site of trauma. These 'reactive' astrocytes arise subsequent to the appearance of phagocytic cells and increased brain IL-1 levels. There are currently no reports indicating if IL-1 can induce NGF mRNA expression in the mature brain or if the capacity for induction remains in a slowly proliferating astrocyte population. The following studies were conducted to address these issues.



* p<.05 vs. condition control, Student's t-test

Fig. 17. Control and interleukin-1ß induced NGF mRNA levels in enriched rat cortical astrocytes cultured for various periods *in vitro*. Cells were plated at 3-4 x 10E6/75 cm2 flask in 10% FCS/DME and fed every 3 days. Two days prior to harvest medium was changed to 10% stripped FCS/DME. Interleukin (250U/ml) was given 3 hours prior to harvest.

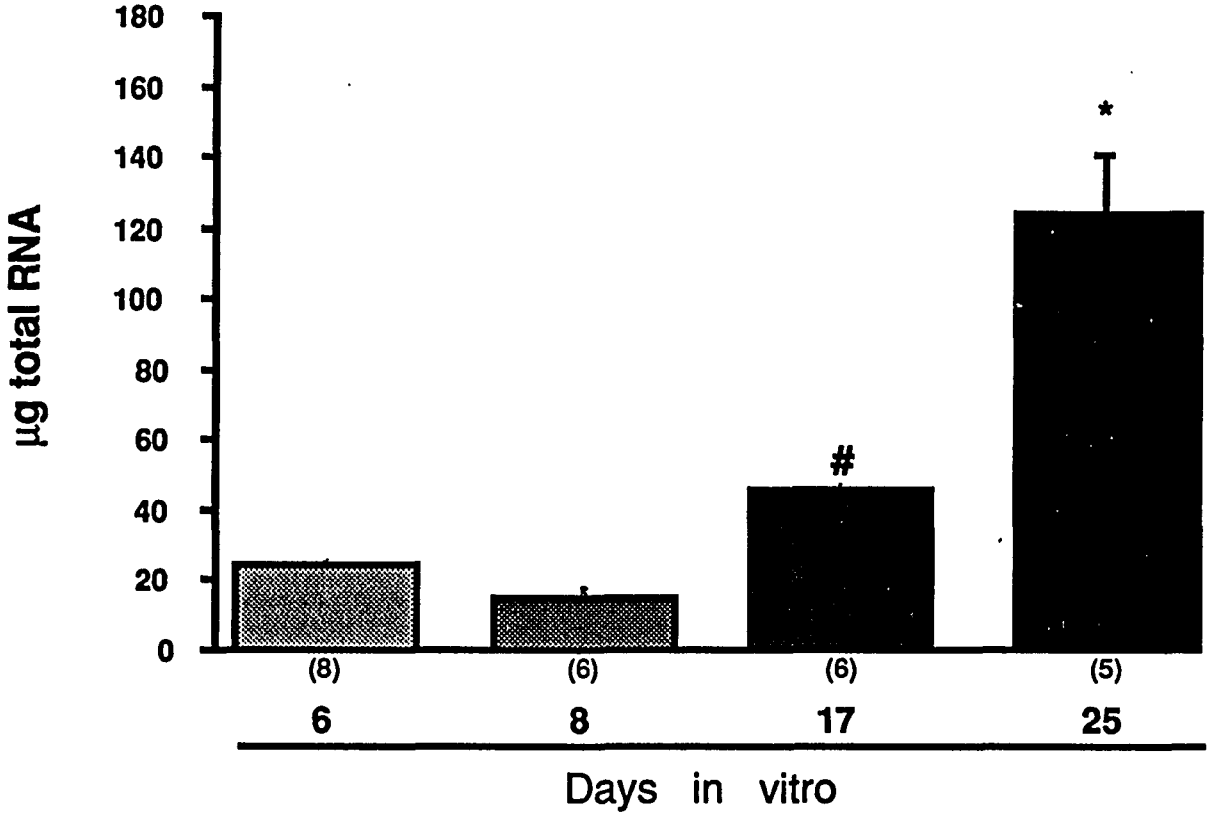


Fig. 18. Total RNA harvested from enriched cortical cultures following various periods in vitro. Each time point represents pooling of control and IL-1 treated flasks.

Influence of cell density on response of enriched cortical astrocyte cultures to IL-1ß

Figure 17 displays NGF mRNA levels in enriched cortical astrocytes cultures following 3 hour IL-1ß exposure for cells grown for various periods. There was a significant induction following 3 hour exposure to 250U of IL-1ß in the 6, 8 and 17 DIV cultures. In contrast, no induction was found in cortical cultures harvested at 25 DIV. This is the first report associating lack of induction of NGF mRNA by IL-1 in cultured astrocytes with increased cell density. Thus, in addition to the down regulation of basal expression, increased cell density (and other parameters associated with time in culture) appears to be correlated with the blocking of the IL-1ß mediated induction of NGF mRNA. The observation of a ~1d delay between the appearance of phagocytic cells and the astrocyte reaction in the mature brain following trauma may indicate this period is required for mature astrocytes to reenter a proliferative state. However the degree of astrocyte proliferation in the injured CNS is poorly understood. These results may indicate differences in the ability of central and peripheral glia to regulate NGF mRNA levels in response to factors present during injury.

NGF mRNA levels in the adult rat hippocampus following in vivo *exposure to interleukin*

Astrocytes in the intact adult brain proliferate but at a rate significantly lower rate than that found in the young animal. This reduced proliferation may effect the ability of astroglia or neurons to respond to IL-1. Four studies were conducted to determine the effect of *in vivo* administration of interleukin to adult rats on tissue NGF mRNA levels. Following ICV administration of IL-1 or carrier, animals survived for three hours, and tissue RNA was rapidly isolated. Adult animals were chosen so that the effects of IL-1 on the mature glial population could be better understood. The three hour survival period was selected to parallel the period for induction found by Thoenen's group in the explanted

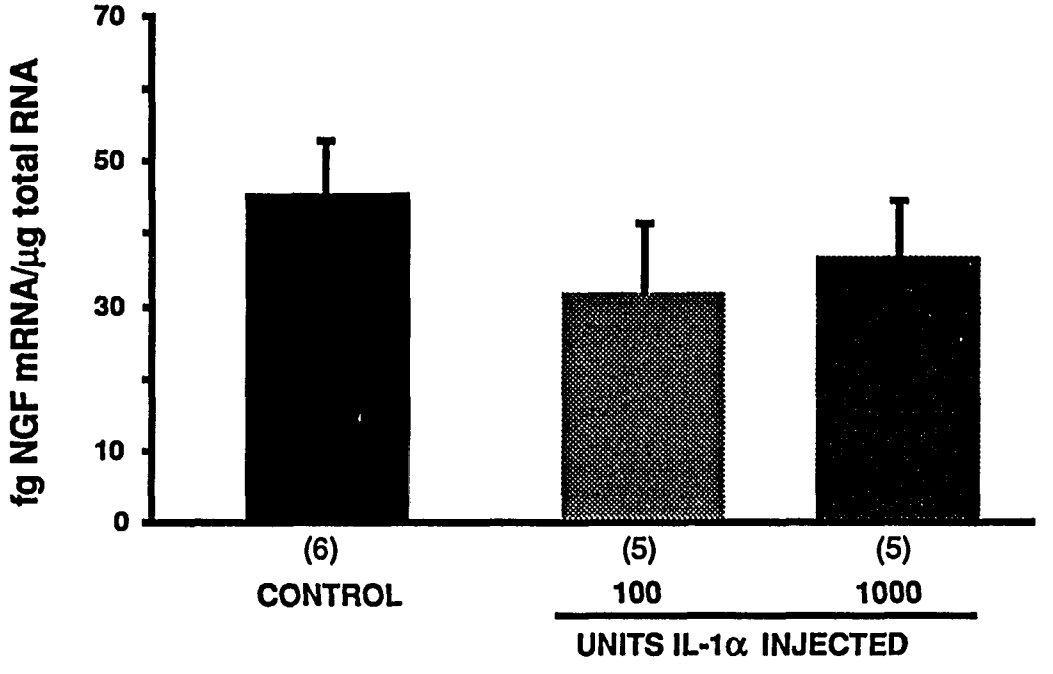


Fig. 19. Hippocampal NGF mRNA levels 3 hours following intracerebroventricular injection of interleukin-1α. IL-1α, in a volume of 10 µl, was injected into the left lateral ventrical of adult male Fisher 344 rats. Control animals received 10 µl .9% saline.

sciatic nerve and to avoid the earliest reported time of macrophage activation in response to brain injury (5-8 hours, Giulian *et. al.*, 1989) and that may occur as a result of the ICV injection. Two of the studies were conducted in concert with glucocorticoid deprivation (physical or pharmacological adrenalectomy) where there was a significant attrition of the experimental groups. The results of two groups of animals receiving 100 or 1000 units of interleukin-1α are shown in figure 19. No significant difference was found between the IL-1α administered and the saline treated group. A similar study using 10U IL-1ß showed no induction in rat hippocampus. These results contrast with those of Spranger *et. al.* (1990) where a 5 fold induction of NGF mRNA in neonatal rat hippocampus was found following ICV injection of IL-1ß. While there are a number of differences between the two experimental paradigms, the difference in ages likely plays a critical role. Younger rats may contain populations of cells, both neurons and glia, susceptible to NGF induction by IL-1. In the context of the current work, astrocytes in the neonate continue to proliferate and thus may be responsive to the cytokine. As the animal matures the rate of proliferation declines and the ability to respond to IL-1 by induction of NGF mRNA falls in parallel. The current studies did not determine if astrocytes can respond following injury in the adult but suggest the abiltiy of astrocytes to respond to IL-1 with elevated NGF mRNA levels may be decreased as the animal matures.

Influence of dexamethasone on response of enriched cortical astrocyte cultures to IL-1ß

As reviewed above, interleukin-1 is involved in numerous aspects of the acute phase response and mediates inflammatory processes. Glucocorticoids are immunosuppressive and antinflammatory and can modify and often block the actions of IL-1. The synthetic glucocorticoid dexamethasone was therefore examined to determine if it could oppose IL-1 effects at the level of NGF mRNA regulation. Figure 20 shows an experiment where enriched cortical astrocytes were incubated for 20 hours in 100 nM dexamethasone prior to

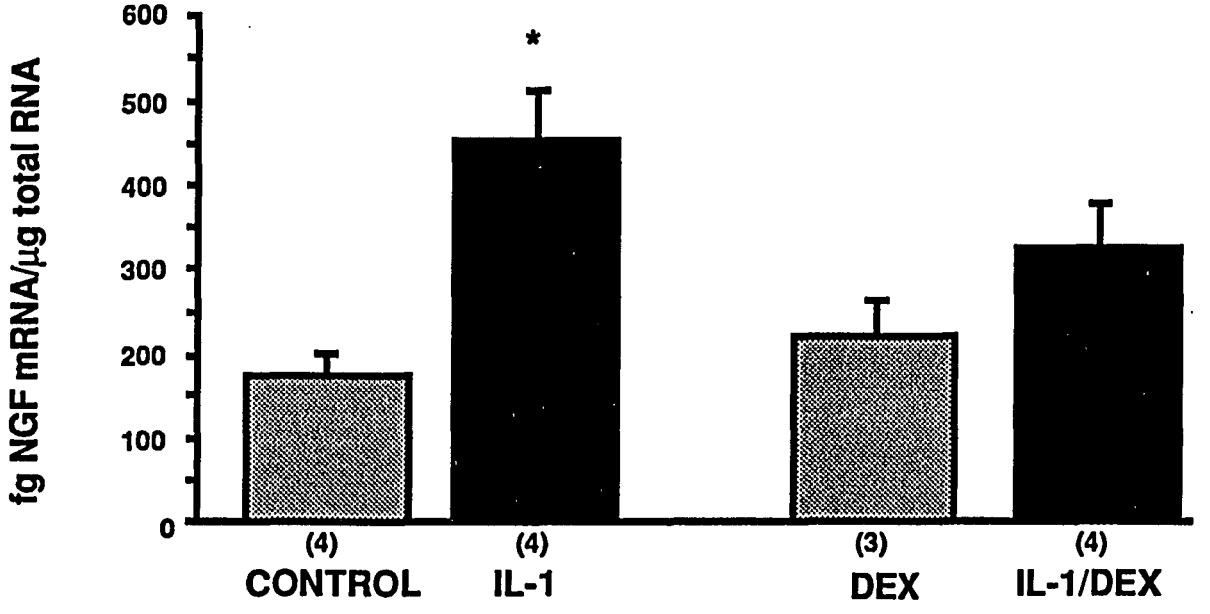


Fig. 20. Effect of dexamethasone on induction of NGF mRNA by interleukin-1ß in enriched cortical astrocyte cultures. Cells were grown for 6 DIV. Two days prior to harvest all cells were fed with 10% stripped serum/DME. Half of the cultures were treated with 100 nM DEX 24 hours later and subsequently exposed to 250U of interleukin-1ß for three hours on the day of harvest as shown.

3 hour exposure to interleukin-1ß. Under this paradigm, there was no elevation in NGF mRNA levels following IL-1ß application in the presence of dexamethasone while DEX had no effect on baseline NGF mRNA levels. One-way analysis of variance with Fisher post-hoc analysis indicates the DEX/IL-1 exposed cultures had significantly lower NGF mRNA levels than cultures exposed to IL-1 alone ($F [3,11] = 8.894$; $p<.05$). This agrees with earlier studies indicating DEX can block the IL-1 mediated induction in cultured rat fibroblasts (Lindholm *et. al.*, 1988) and in mixed cultures from E21 rat hippocampus (Friedman *et. al.*, 1990).

NGF mRNA levels in enriched cultures of E18 hippocampal neurons

Cultures of enriched E18 hippocampal neurons were grown to determine NGF mRNA levels. Five sets of neuronal cultures were harvested and assayed. A minimum of nine 35 mm plates were pooled per point in the assay. As a result of this degree of pooling, there was one data point per condition. In four of the five assays containing neuronal cultures, no NGF mRNA signal was detected. These data comprise cultures grown 1, 2, and 5 days in culture in defined medium. The 5 day cultures include cells exposed to serum for the initial 24 hours *in vitro* (to permit astrocyte proliferation; see Banker, 1980) and then switched to defined medium for the remainder. Phase microscopy of 5 day cultures grown in defined medium (not shown) showed clusters of compact cell soma some displaying a pyramidal shape with long thin processes. Few large, flattened type-1 astrocyte-like cells are observed. Under these culturing conditions, the population of astrocytes, expected to be shown by bipolar or multipolar flattened cells with large flat processes, was sparse. In the assay where a NGF mRNA signal was detected, 60.5 and 31.4 fg NGF mRNA/µg total RNA were present after 1 day and 5 day *in vitro* respectively. Exposure to 10U of IL-1ß for 3 hours produced 100 fg NGF/µg total RNA at 1 day and 40.8 fg NGF/µg total RNA at 5 days (data not shown). These data represent an average of

56.1 CPM above background in the protection assay. Two additional experiments found no NGF mRNA following IL-1 exposure. The most consistent conclusion from these results is that enriched cultures of rat hippocampal neurons at this stage of development do not express and IL-1ß does not induce the expression of NGF mRNA.

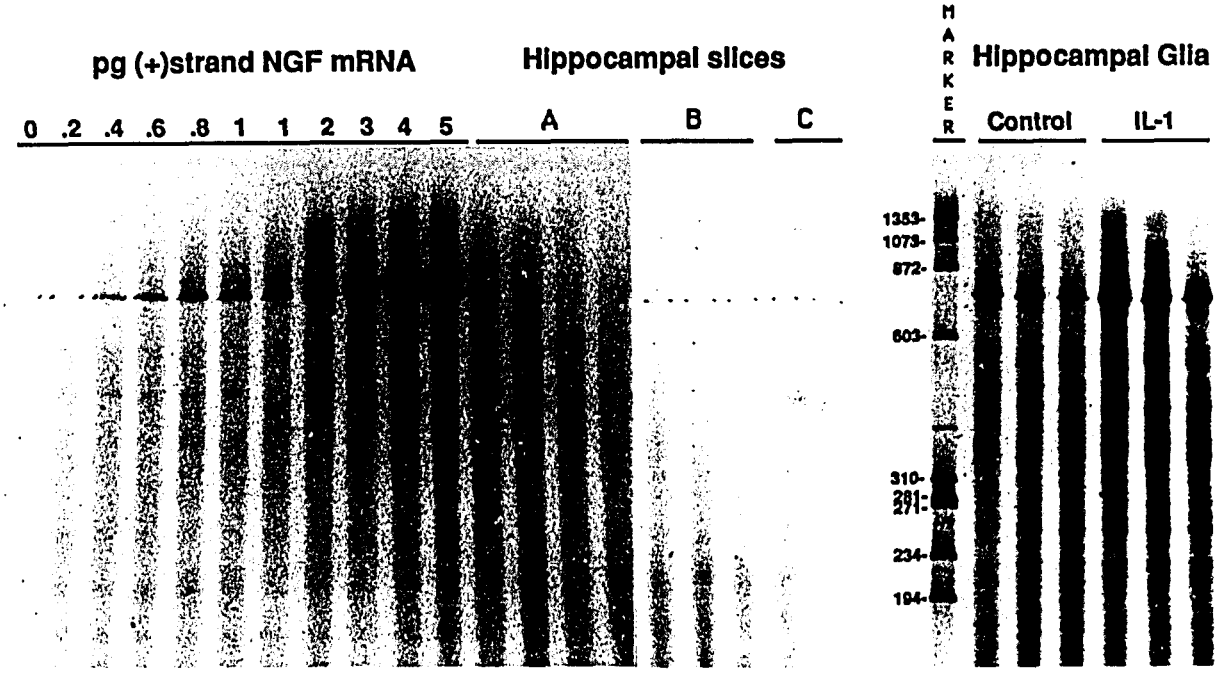


Fig. 21. Protection assay. See methods for solution hybridization/ nuclease protection assay protocol. Hippocampal slice conditions are A) 20 slices/lane, no preincubation or incubation; B) 20 slices/lane, 1.5 hours incubation; C) same as A but 10 slices/lane. Signals in these lanes are at the detection limit of the assay. Enriched hippocampal astrocytes were grown for 6 days in vitro and exposed to 250U IL-1ß for 3 hours. Cultures were immediately harvested and RNA was isolated for the assay. This experiment produced an average 2.5X induction of NGF mRNA in the IL-1 exposed cultures.

Hippocampal slices

The hippocampal slice preparation was used in an attempt to study NGF mRNA levels *in vitro* in tissue where a degree of the anatomy remains intact and where the cellular composition reflects that found in the brain. Slices also have the distinct advantage of evaluating the responses of adult cells *in vitro*. This approach allows the precise control of the environment surrounding the tissue and ensures that compounds under study actually reach the tissue. An effort was made to use slices to study NGF mRNA regulation by IL-1. The objective was to analyze adult tissue responses to IL-1 and to evaluate the site of expression by histochemical techniques if induction was observed. These data could be combined with the culture and *in vivo* studies to determine developmental changes in astrocyte regulation of NGF mRNA expression. This work would also indicate if intact hippocampal neurons could increase NGF transcript levels in response to exogenous IL-1. Initial studies examining the time course of tritiated uridine uptake were conducted to test the viability of the slice preparation. These were followed by an analysis of NGF mRNA levels in slices which had undergone various incubation approaches in a campaign to retain detectable levels of the transcript.

Uptake Studies

Figure 22 displays an experiment using 600 μm sections preincubated for 1.5 hours (in the chamber described in the methods section) and tested for ^{3}H-uridine incorporation following an incubation of 30 minutes to 20 hours. Incubation was conducted in a 12-well tissue culture plate in a water saturated, 37°C, 95% O_2, 5% CO_2 incubator. Slices were harvested twice during the initial hour and approximately every hour thereafter through 6 hours. An additional set of slices was harvested following overnight incubation. This figure indicates that hippocampal slices prepared as described are able to incorporate uridine during extended periods *in vitro* and suggests that the preparation is viable during this period. In agreement with additional incorporation tests conducted, there was an early

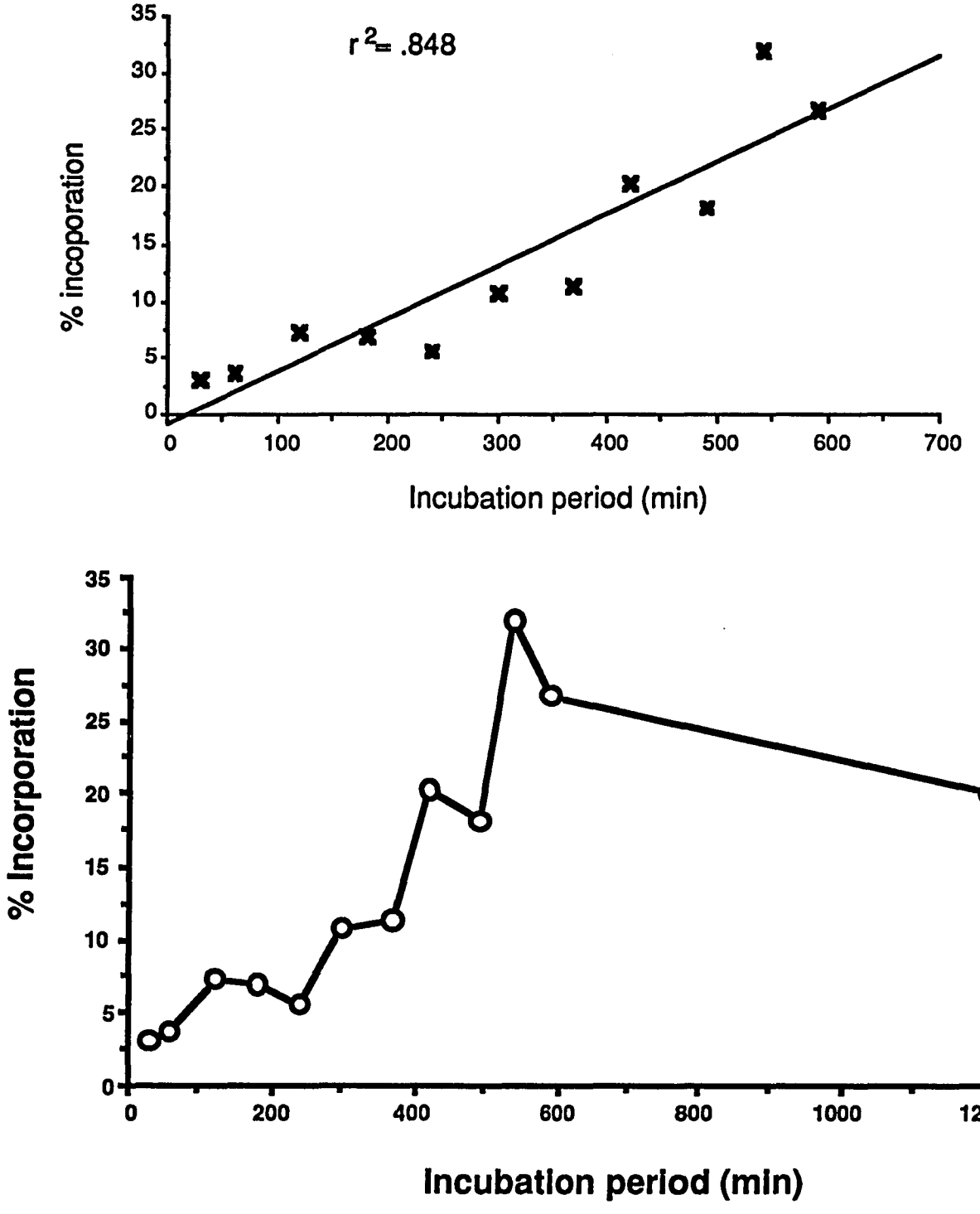


Fig. 22. Incorporation of 3H-uridine into 600 µm thick hippocampal slices over various periods. Slices were preincubated for 1.5 hours and incubated for the times shown in the presence of 25 µCi 3H-uridine. Uptake by total RNA isolated from slices was gauged by measuring incorporated counts and expressing this as a percent of total counts found in the tissue.

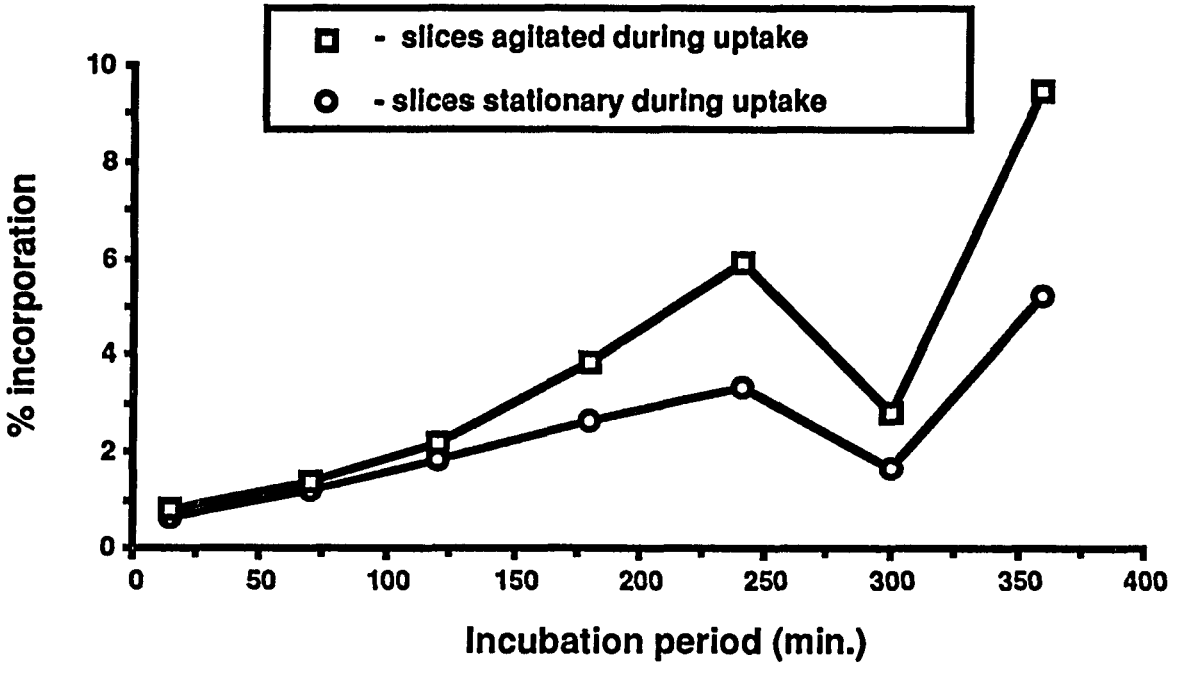
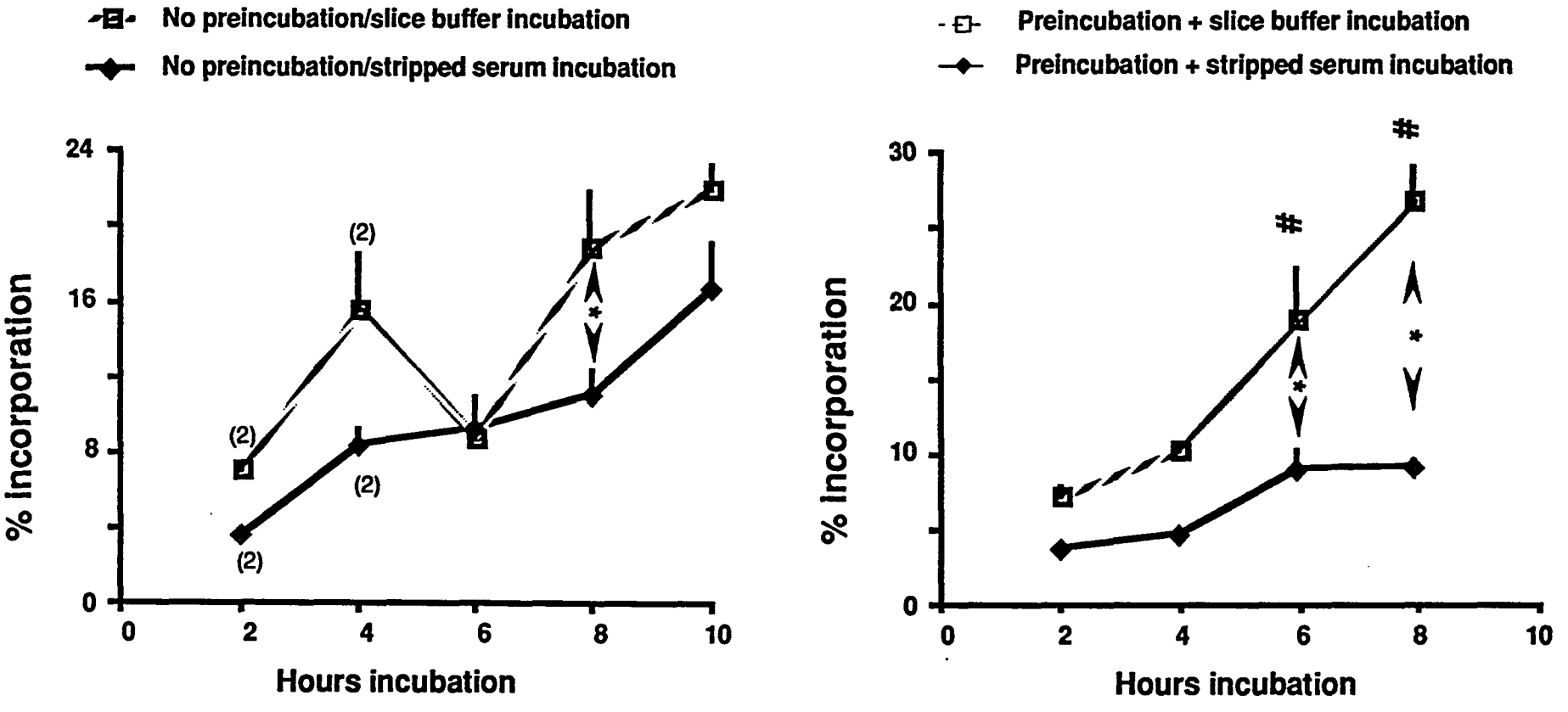


Fig. 23. Uptake of 3H-uridine by hippocampal slices incubated on a shaking platform or stationary. Slices were preincubated for 1.5 hours and incubated for various periods with or without agitation.

phase (~3 hours) where incorporation remained at a relatively low level followed by a period of increased uptake for at least 4 hours. This initial lag period may reflect an ongoing recovery period for cells within the slice or may indicate the time span where intercellular stores of UTP become depleted and the exogenous uridine can compete for incorporation into RNA. This period may also indicate the time necessary for the uridine to penetrate into the deeper layers of the tissue. At these low levels of incorporation, however, detection of continuous uptake may be difficult and the apparent lag phase may constitute the initial stage of linear incorporation. A number of additional uptake studies were conducted to analyze other parameters of the protocol.

Uptake was compared in slices which had been preincubated for 1.5 hours and then incubated either on a shaking platform ('agitated') or stationary on a shelf in the incubator (figure 23). While this study covered 6 hours, a similar trend of increasing uptake was found for both conditions with longer incubations. At the longest time point, uptake in the agitated samples was over 80% greater than that found in the stationary set. This result indicates that gentle shaking during the incubation period may enhance the redistribution of nutrients provided by the buffer and the dispersion metabolic waste products produced by the tissue. The latter issue may be critical as a microenvironment could exist at the interface between the tissue and the buffer/atmosphere where the local pH may be damaging to the survival of nearby cells. In all experiments where slices were incubated for the purpose of measuring NGF mRNA , the tissue was agitated during incubation. One further uptake study was conducted to gauge both the effect of preincubation *per se* and the influence of the incubation medium on uptake parameters (figure 24) This study indicates that slices preincubated and subsequently incubated in slice buffer had the greatest amount of uridine uptake of all conditions after 8 hours of incubation. These are the conditions suggested at the outset of these studies. Regardless of preincubation, slices incubated in slice buffer showed higher levels of incorporation than those maintained in 10% charcoal stripped serum in DME. Preincubation enhances the ability of slices incubated in slice buffer to



* p<.05 (ANOVA, Fisher post-hoc analysis)

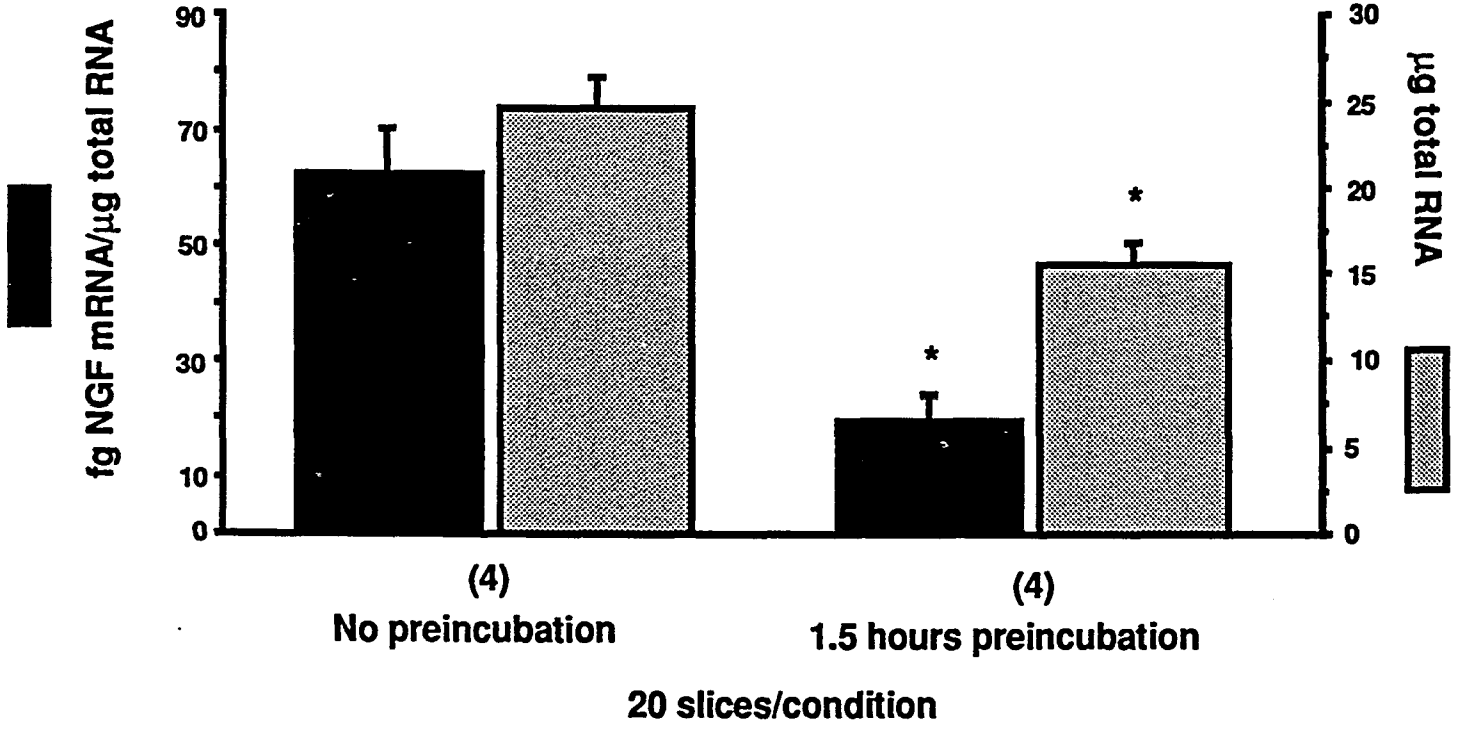
# p<.05 vs. non-preincubated, same incubation parameters (ANOVA, Fisher)

Fig. 24. Comparison of 3H-uridine uptake in hippocampal slices following 1.5 hours princubation or direct incubation and between incubation in slice buffer or 10% charcoal stripped fetal calf serum in DME. All points measured in triplicate unless oterwise indicated.

incorporate uridine into RNA and thus may reflect the value of the metabolic chamber in aiding tissue viability. Incubation in stripped serum appears to reduce the ability of slices to incorporate uridine and may reflect the presence of serum factors which influence the viability of cells in the slice. The majority of our NGF mRNA analysis was conducted in slices incubated in slice buffer.

NGF mRNA levels in hippocampal slices

We have conducted many experiments measuring NGF mRNA levels in hippocampal slices under various preincubation and incubation parameters. Figure 25 displays a representative experiment where relative NGF mRNA levels were compared before and after 1.5 hours preincubation (see also figure 21). The level of NGF mRNA found in slices harvested immediately is similar to values found for whole rat or mouse hippocampus (43.0 and 55.5 fg NGF mRNA/μg total RNA respectively). After 1.5 hours of preincubation there was a significant loss in total RNA and a significant, 66% loss of NGF mRNA/μg total RNA. The reduced level of NGF mRNA represents less than double the background CPM level from the protection assay. While it is possible to work in this range, we found some variability in the control levels of the signal often producing an undetectable signal in slices following preincubation/incubation. Further tests indicated there was no additional loss of NGF mRNA following preincubation for incubation periods of up to 6 hours. The 6 hour incubation point corresponds to a time when slices incubated under identical conditions show a significant degree of uridine uptake (Figure 22). The stability of the NGF mRNA was also tested where slices received no preincubation and 3 hours incubation. NGF mRNA/μg total RNA decreased by 78% ($p<.01$ versus unincubated control, Student's t-test). These data display the most frequent finding of a loss of signal during the initial 1-2 hours *in vitro*, regardless of the conditions immediately following slicing, with a stabilization at very low levels thereafter. NGF synthesis may be specifically down regulated since the uptake studies appear to indicate the slices are viable



*p<.05 versus no preincubation (Student's t-test)

Fig. 25. NGF mRNA levels in hippocampal slices harvested immediately or following 1.5 hours preincubation.

and capable of ongoing RNA synthesis. NGF mRNA has a half life of about 90 minutes in cultured fibroblasts following stimulation with IL-1 for 3 hours followed by actinomycin D treatment or washing to remove the cytokine (Lindholm *et. al.*, 1988). The cessation of NGF mRNA synthesis in the hippocampal slice as the result of the slice preparation process would be consistent with this rate of turnover.

We made numerous attempts to enhance the recovery of signal by altering the slicing or harvesting parameters. We analyzed the effect of increasing the amount of total RNA (i.e., increasing the total number of slices) per sample in tissue harvested immediately after slicing. The objective was to increase the NGF mRNA levels assayed per point so that the CPMs above background would produce a more reliable and consistent signal. While three times the amount of total RNA was recovered from 45 slices as from 15 slices (38.6 vs 12.8), the signal remained between 40 and 60 counts above background (45 slices represents the hippocampi from 1-2 animals; when collected directly from an animal without slicing, this amount of hippocampus yields between 75 and 90 μg total RNA and an average signal of 400 CPM above background). This finding is difficult to explain. NGF mRNA was also measured in pooled 400 and 800 μm thick slices and those cut parallel to the longitudinal axis of the hippocampus. The 400 μm slices were difficult to manipulate and often fell apart after handling. In the 800 μm slices, the NGF mRNA/μg total RNA was generally equivalent to that found in whole hippocampus; however the amount of tissue required to generate data using thicker tissue (including the longitudinal slices), hippocampi from more than one animal was needed to produce one data point. In view of these considerations, the use of hippocampal slices for these studies was not considered feasible.

Discussion

Summary

The present studies were undertaken to analyze nerve growth factor expression in cells of the central nervous system and the parameters that may mediate this expression. A sensitive solution hybridization/nuclease protection assay was used to directly quantitate the levels of NGF mRNA in enriched astrocyte cultures from diverse regions of neonatal rat brain. The effect of various culturing conditions, the presence of glucocorticoids and the influence of cell density was examined for their influence on NGF mRNA levels. Work was conducted to study the result of exposure to interleukin-1 in dispersed cultured cells and in the intact rodent brain. IL-1 was chosen because of previous evidence indicating its ability to regulate NGF in the peripheral nervous system and its likely physiological role in the CNS during development and trauma. The data indicate that enriched astrocyte cultures from the neonatal rat cerebral cortex, hippocampus, cerebellum and hypothalamus express NGF mRNA. Cultures from cortex, hippocampus, and hypothalamus exposed to interleukin-1 (IL-1) showed a significant induction of NGF mRNA while no change was observed in cerebellar astrocytes. In contrast, no induction was observed in whole adult rat hippocampus 3 hours following intracerebroventricular injection of IL-1 In addition, reduced NGF mRNA levels and a lack of response to interleukin-1 was observed in cultures allowed to grow 25 days *in vitro*. These results show nerve growth factor is expressed *in vitro* and may be expressed *in vivo* during rapid proliferation of astrocytes in a variety of brain regions. This expression can be enhanced by a cytokine elaborated by cells present in the embryonic and injured central nervous system. The possible role of NGF during brain embryogenesis and subsequent to trauma to the CNS will be discussed in the context of an *in vivo*, astroglial source of release of this trophic agent.

Expression of NGF mRNA in enriched astrocyte cultures from neonatal rat brain

The following sections will provide evidence for a developmental stage specific and region specific astrocyte production of NGF in the CNS. The role for the released NGF would be to provide highly localized trophic support for proliferating neuronal precursors and migrating neurons. This will lay the framework for a discussion of NGF-related astrocyte events occurring following brain trauma which may involve a partial recapitulation of the developmental sequence.

Proliferating, enriched astrocyte cultures express high levels of NGF mRNA (Fig 9). This agrees with earlier reports of cultured glia synthesizing and releasing NGF protein (Lindsey, 1979; Norregen *et. al.*, 1980; Furukawa *et. al.*, 1986; Assouline *et. al.*, 1987; Houlgatte *et. al.*, 1989a; Carman-Krzan *et. al.* 1991). In addition there have now been a number of very recent reports showing NGF mRNA expression in cultures of astrocytes isolated from neonatal rat brain (Yamakuni *et. al.*, 1987; Spranger *et. al.*, 1990; Lu *et. al.*, 1991). Carman-Krzan *et. al.* (1991) found that the ratio of extracellular to intracellular NGF protein in cultured cortical astrocytes was high and characteristic of proteins constitutively secreted with little storage. Thus, in distinction to a number of reports using *in situ* hybridization demonstrating an exclusively neuronal localization of NGF mRNA in mature rodent brain (Ayer-Lelievre *et. al.*, 1988; Whittemore *et. al.*, 1988), the glial component may also provide support for selected populations of cells in the central nervous system through the release of NGF. While direct evidence is not available, the most likely explanation for the different cellular localization of the NGF mRNA in this study versus the histochemistry findings is a developmental switch in the sites of expression (Lu *et. al.*, 1991). Growing and differentiating glia may provide NGF to cells in the embryonic and neonatal CNS. The finding that glial cell lineages and particularly astrocytes appear to coexist within the proliferative zone of the embryonic nervous system of a variety of species (Korr, 1986) supports this possibility. The data presented here showing a decrease

in NGF mRNA levels in 25d enriched astrocyte cultures (see control values, Fig 17) suggests that upon reaching a critical state of confluency or maturation, NGF mRNA is down regulated. This regulation appears to occur at some time after 17 days *in vitro* (under these plating conditions) since cultures at this point expressed high basal levels of NGF mRNA. Work conducted during the early part of these studies evaluated the influence of plating density on NGF mRNA levels in hippocampal and cortical astrocytes (Figs 10 & 11) Under these plating parameters, hippocampal astrocytes expressed increasing levels of the transcript through day 20.

The appearance of the glia after 25 days *in vitro* (figure 6E) was less flattened and more condensed and it was difficult to identify individual glia in the dense carpet of cells. Abney has found that astrocytes in culture, as gauged by their expression of GFAP, develop on a similar time course in vitro as they do in vivo (Abney *et. al.*, 1981). While the data presented here cannot distinguish if the down regulation is a function of time in culture *per se* or final density, the morphological alterations in the glia correlate with a state of confluence where their proliferation has slowed. Hatten has found confluent primary cultures of cerebellar astrocytes to incorporate 2/3 less thymidine than 60-70% confluent cultures (Hatten and Shelanski, 1988). Thus the down regulation of NGF mRNA may correspond to a transition in the developing brain where as ontogeny proceeds astrocyte proliferation is reduced and NGF expression in selected brain regions is predominantly neuronal. The down regulation of basal NGF mRNA levels suggests there is a correlation between the rate of proliferation of these cultures and expression of NGF. This conclusion would agree with a number of studies which examined parameters of proliferation and found the levels of NGF mRNA and NGF protein release to decline as astrocyte cell density increased (Furukawa *et. al.*, 1987; Houlgatte *et. al.*, 1989a; Lu *et. al.*, 1991). Cell to cell contact mediated inhibition appears to play an important role in this regulation. Replating confluent astrocyte cultures where NGF expression has decreased at lower density restores higher level of secretion/synthesis (Furukawa *et. al.*, 1987; Houlgatte *et.*

al., 1989a). The relationship between proliferation and NGF expression is further indicated in transformed astrocyte cell lines where thymidine incorporation continues unaltered as cells reach confluency (Hatten and Shelanski, 1988) and down regulation of NGF secretion occurs at a much higher density than in primary cultures (Houlgatte *et. al.*, 1989a). The replating studies may also indicate the correlation between NGF regulation and the growth potential of astrocytes. Switching rapidly growing non-confluent astrocyte cultures from 10% fetal serum to 0.5% BSA (serum deprivation) reduces cell division and leads to a 90% reduction in NGF secretion (Furukawa *et. al.*, 1987). Addition of serum to either slowly dividing, serum deprived cells (Furukawa *et. al.*, 1987) or simply feeding cells fresh serum (Spranger *et. al.*, 1990) leads to an induction of NGF mRNA and an increase in NGF secretion.

The possible significance of high levels of NGF in proliferating glia may be indicated by the finding that enriched astrocyte cultures from a variety of areas in the rat central nervous system expressed similar high levels of NGF mRNA (Fig 10). Astrocytes from cortex, hippocampus, hypothalamus and cerebellum expressed similar levels of NGF mRNA in non confluent cultures. These findings are in agreement with those Lu *et. al.* (1991) where astrocyte cultures from postnatal day 2 rat hippocampus, cerebellum and basal forebrain expressed NGF mRNA. In addition, astrocytes plated from the hippocampus, striatum, cortex and mesencephalon of the neonatal mouse brain were found to secrete NGF (Houlgatte *et. al.* 1989a). The possibility thus exists that when glia are expanding to similar degree *in vivo* they have the capacity to express and release NGF. If the role of glial NGF is to provide support and guidance for developing neurons then the NGF receptor must be present during embryonic and neonatal stages. Johnson's group (Yan and Johnson, 1988) using receptor immunohistochemistry has found staining in the cerebral cortex from E18-P6 and a signal on day E16 in the developing hippocampal formation. In the cerebellum, receptors appeared on E16 in the cerebellar anlage and increased to a very prominent signal on postnatal day 10 where the surface of Purkinje cell bodies and dendrites and the

premigratory cells of the external granule cell layer showed staining. No signal was found on radial glia. The cerebellar signal disappeared by P15. These results agree with those of Schatteman *et. al.*, (1988) where similar changes in cerebellar NGF-R was observed in developing primate brain with a concomitant significant decrease in total cerebellar NGF-R mRNA during ontogeny. The apparent transient expression in these CNS regions contrasts with the fetal and adult expression of NGF-R immunoreactive cell bodies found in the nuclei of the basal forebrain which coincides with the only CNS population of cells expressing the NGF-R mRNA in the adult (Buck *et. al.*, 1987; Springer *et. al.*, 1990). Additional NGF-R sites in the adult rodent brain in the striatum are visualized by autoradiographic mapping with ^{125}I-NGF (Raivich and Kreutzberg, 1987). The different results may reflect the ability of the autoradiographic approach to detect type I, high affinity NGF-R while immunohistochemistry visualizes the type II, slow dissociating receptor (see Springer, 1988).

The possibility that developing, rapidly dividing glia may provide an early, perinatal source of NGF may be consistent with the observed developmental expression of NGF mRNA. A number of studies have shown low levels of NGF mRNA in the hippocampus and neocortex until about 10 days after birth (Large *et. al.*, 1986; Whittemore *et. al.*, 1986; Auberger *et. al.*, 1987; Lu *et. al.*, 1989). The mRNA increases to 300-400% of that found in the adult at postnatal day 21 and then rapidly decreases to adult levels. This early increase in NGF mRNA is paralleled by a rise in NGF protein in these tissues and is closely followed by an elevation of NGF and choline acteyltransferase (ChAT) in neurons of the basal forebrain (Auberger *et. al.*, 1987; Large *et. al.*, 1986). The cerebellum also shows NGF expression but the pattern is different from regions innervated by central cholinergic neurons and may be reflective of a predominantly local, neonatal role. Local actions for NGF have also been suggested for NGF in the neonatal striatum while an adult role is indicated in lesion studies (Gage *et. al.*, 1989). A common theme in expression patterns from these diverse brain regions is suggested by the work of Lu *et. al.* (1989)

where the low but detectable levels of NGF mRNA in embryonic and neonatal rat hippocampus, olfactory bulb, striatum and cerebellum are transiently associated with elevated expression of NGF-R mRNA in the same structure. This pattern may indicate an early, local mechanism common to a number of developing brain regions followed by a region specific change in NGF expression in association with the maturation of the nervous system. In support of a local, transient role of NGF in many brain regions is the finding of Wanaka and Johnson (1990) where NGF receptor mRNA is expressed at high levels in the proliferating zone of the external granule cell layer of the neonatal rat cerebellum. The receptor transcript was also found in Purkinje cells; both cell types showed a significant loss in signal during the second postnatal week although low level of expression remained in the Purkinje cell layer at postnatal day 30. Cultured embryonic septal (Hefti *et. al.*, 1985; Hartikka and Hefti, 1988a) and Purkinje cells (Cohen-Cory *et. al.*, 1991) can respond to NGF with neurite outgrowth and increased survival though in the latter example excitatory neurotransmitters or depolarizing agents must be present to elicit the effect. Definitive evidence is still lacking that this perinatal induction of NGF is strictly neuronal and, in light of the evidence presented here and elsewhere, astrocytes may contribute to early NGF production. In the mouse hippocampal formation, nearly all of the neurons are post mitotic by embryonic day 18 (Angevine, 1965) and have migrated to their proper destination by early in the first postnatal week, a time when NGF levels are just beginning to rise. Basal forebrain cholinergic cells begin to express NGF-R immunoreactivity 2 weeks before target NGF induction is observed (Koh and Loy, 1989; Yan and Johnson, 1988). The basal forebrain neurons send projections to their targets by E17 (by NGF-R fiber staining, Koh and Loy, 1989) or by the day of birth (by tract tracing methods, Milner *et. al.*, 1983) and thus, if dependent upon NGF during this period, must derive it from a source distinct from the hippocampal neuronal target found later in ontogeny. While the timing of the induction of NGF mRNA, NGF, and ChAT suggests that NGF is involved in the differentiation of the cholinergic phenotype (Large *et. al.*, 1986), studies in the optic

nerve indicate that the predominant astrocyte (type I) found in our cultures appear as early as embryonic day 16 (Raff, 1989) and thus are likely to be present in the hippocampus during neuronal migration and prior to the elevation of NGF expression. In addition, studies by Hatten and colleagues (Hatten, 1989; Gasser and Hatten, 1990) indicate that, in dispersed embryonic hippocampal cultures, the presence of neurons arrested glial proliferation and induced process formation producing a morphology similar to differentiated astrocytes found in older brain. This was found to be a membrane mediated event that could be induced by applying a neuronal membrane fraction to glial cultures. As noted, a change in morphology was also observed in the cell dense 25d cultures reported here where the NGF levels were decreased.

The possibility thus exists that in embryonic brain, rapidly dividing astrocytes provide NGF to a variety of neuronal populations undergoing mitosis and migration. Upon further development and differentiation of the brain, contact between neurons and glia (and between glia and glia) reduces the proliferation of astrocytes and down regulates the astrocytic expression of NGF. This may coincide in time when selected populations of neurons (i.e. hippocampal, neocortical) begin to express NGF thereby serving as trophic support for innervating cholinergic cells. It is noteworthy in this context that Johnson's group (Yan and Johnson, 1988) found that cells in a restricted zone of the subventricular/subependemal layer expressed the NGF receptor for all ages examined (E11 through P10). These cells are mitotically active and give rise to neurons (and glia) and thus might reflect the ability of many early cells of the CNS to respond to NGF. NGF receptor mRNA has been found transiently expressed in meningeal cells in the rat cerebellum during an immediate postnatal period when these cells are critical for the proper morphogenesis of this structure. In the cat and ferret, NGF-R has been found to be briefly associated during mid to late gestation with subplate neurons of the cerebral cortex (Allendoerfer *et. al.*, 1990). These cells are a special class of transient neurons that are generated and become postmitotic early in CNS development where they form a subplate and marginal zone

surrounding the developing cortical plate. Hatten has conducted extensive studies of neuronal migration on astrocytes and found glial guided migration of hippocampal (and cerebellar) neurons plated from E19-E20 rat embryos (Gasser and Hatten, 1990; Hatten, 1990). Thus a number of cell populations in the perinatal brain, including early postmitotic neurons, express the NGF receptor before the availability of significant amounts of target derived NGF. Proliferating glial cells interact closely with at least some of these cells which are likely to be NGF sensitive.

While the preceding evidence may imply a role for NGF during ontogeny, there is evidence it operates in concert with other growth promoting agents. NGF alone does not affect the proliferation, differentiation or survival of cultured E13.5-14.5 striatal neuronal precursors cultured for 2 or 9 days *in vitro*. However, pretreatment or simultaneous exposure with basic fibroblast growth factor (bFGF) led to 17 times more cells expressing an intermediate filament protein found exclusively in proliferating neuroepithelial stem cells (nestin) after 9 days *in vitro* (Cattaneo and Mckay, 1990). There is also evidence of the mitogenic capacity of FGF in neuronal precursor cells in the peripheral nervous system (Birren and Anderson, 1990) where, in agreement with other reports suggesting a role for FGF in proliferating central neuroepithelia (Murphy *et. al.*, 1990), the sequence of expression of the receptors for FGF and NGF suggest alternating roles for these agents in the proliferation and maturation of the CNS (Heuer *et. al.*, 1990).

The above studies and the current findings supplement a model proposed by Hatten (Hatten *et. al.*, 1988) for part of the sequence of events during neuron-glial interactions in embryogenesis. Proliferating astrocytes in the ventricular and subventricular zone would secrete NGF and thereby provide trophic and tropic support to young neurons until the formation of neuron-glia cell-cell contact. This event would inhibit glial cell proliferation, eliminate NGF expression and induce the glia cell to express a morphology conducive to neuronal migration. In turn astrocytes may release growth factors including bFGF (Hatten *et. al.*, 1988) which induces neuronal survival and neurite growth. The ongoing neuronal-

glial contact would suppress glial proliferation while the glia would contribute to the maintenance of the of extracellular environment of the neuron. While this scenario has been studied most extensively for radial glia found predominantly in the developing cerebellum and hippocampus (Hatten, 1985; Gasser and Hatten, 1990), astrocyte involvement in the establishment of axonal pathways has been described for a number of systems in the developing CNS (see Manthorpe *et. al.*, 1986). In sum, the data presented in the current study indicates enriched astrocyte cultures can constitutively synthesize high levels of NGF mRNA during certain periods *in vitro* and suggests that rapidly dividing astrocytes of the neonatal brain may provide trophic and tropic support for neurons in the developing nervous system.

NGF mRNA levels in neuronal cultures from E18 rat hippocampus

High levels of expression of NGF mRNA during periods of proliferation suggest a role for astrocyte released NGF during the genesis of the CNS. The repeated finding of neuronal NGF mRNA expression in the adult may indicate a switch in the cell phenotype expressing NGF during ontogeny. The finding in the current studies of virtually undetectable levels of NGF transcript from cultured neurons of E18 rat hippocampus would support this proposition. Levels of NGF mRNA in these enriched neuronal cultures were at the limit of detection of the assay. One set of cultures, grown for 1 or 5 days *in vitro*, displayed a detectable signal although the amount of CPMs above background were insufficient to produce a reliable estimation of expression. Phase microscopy indicated numerous pyramidal-shaped and small, round cell bodies with an array of long thin processes. Few flattened cells characteristic of type I astrocyte morphology were observed. The neurons harvested after 5 DIV would be equivalent to postnatal day 2 *in vivo*. Low levels of NGF mRNA synthesis by embryonic hippocampal neurons is supported by a recent finding of Houlgatte *et. al.* (1989a). This group has cultured both E17 and E19 hippocampal neurons and measured NGF release. Supernatants from cultures of both ages

began to show detectable NGF levels after the equivalent of about 5d *in vitro* for the cultures reported here. This would agree with the inability to detect NGF mRNA in the 1d cultures and may indicate an inability to detect the transcript induction at the later age. This also suggests that a significant increase in hippocampal neuron NGF mRNA level does not occur until even later in development. These data correspond to *in vivo* studies which failed to detect NGF mRNA before birth with induction occurring during the latter part of the first postnatal week (Large *et. al.*, 1986; *et. al.*, 1986; Auberger *et. al.*, 1987). NGF mRNA expression has also been observed to evolve over the first week *in vitro* in mixed hippocampal cultures from E21 rat (Friedman *et. al.*, 1990) although these cultures contained 10 fold excess of astrocytes to neurons following 1 week in culture. Neurons and astrocytes appeared to express NGF mRNA though combined in situ hybridization and immunohistochemistry was not shown and will be necessary to resolve the site of expression. Postnatal neuronal NGF expression and the detection of low levels of NGF receptor expression prenatally would support a role for astrocyte derived NGF during embryogenesis.

Response of enriched astrocyte cultures to interleukin-1

A series of studies were conducted to evaluate the ability of interleukin-1 to regulate NGF mRNA synthesis in enriched astrocyte cultures from neonatal rat hippocampus and, more extensively, from rat neocortex. An additional set of experiments was conducted in the adult rat to determine if the findings from dispersed neonatal cells could be replicated in the mature, intact central nervous system.

In addition to furthering the understanding of the mechanisms of regulation of NGF in the CNS, these studies were premised on a number of earlier findings. Foremost among the previous work is that conducted by Thoenen's group using the sciatic nerve to study NGF regulation (Heumann, 1987; Heumann *et. al.*, 1987a, b; Lindholm *et. al.*, 1987; Bandlow *et. al.*, 1987). A series of experiments studied the time course and distribution

of NGF and NGF mRNA levels in the adult sciatic nerve following a crush lesion of the nerve trunk. The sciatic nerve carries fibers of NGF responsive (sympathetic and sensory) and unresponsive (motor) neurons; the survival of the responsive cells depends upon trophic support provided by NGF derived from their peripheral target tissues. The crush lesion paradigm permits regrowth from the proximal (ganglion side) nerve stump into the preserved Schwann cell tubes (bands of Büngner) on the distal (peripheral target) side of the lesion (reviewed by Heumann, 1987). The objective of these studies was to determine if the regenerative capacity of the peripheral nervous system was related to the induction of trophic support produced at the site of trauma. Following sciatic nerve lesion, there is a dramatic induction of NGF mRNA and the NGF receptor along various segments of the nerve. The NGF mRNA induction was observed in nonneuronal cells (Schwann cells and fibroblasts including those associated with motor neuron fibers) in the epineural sheath surrounding the nerve (Heumann *et. al.*, 1987a). The induction was found in all parts of the distal nerve fragment but limited to the area immediately adjacent to the injury in the proximal segment. Both the mRNA (Heumann *et. al.*, 1987b) and the receptor protein (Taniuchi *et. al.*, 1986, 1988) are induced in Schwann cells in the entire distal region and unchanged in the proximal part. The timing of these events was found to be revealing. The receptor was induced maximally at 5-7 days following the lesion and returned to near control levels at 3-5 weeks. Blocking the regrowth of the nerve significantly augmented the induction period. This time course corresponds to the regeneration and regrowth period of the nerve and indicate that as the lesioned nerve stump reenters the vacated endoneurium sheath, contact between the regenerating nerve and the sheath down regulated NGF receptor expression on the Schwann cells (Taniuchi *et. al.*, 1988). The induction of NGF mRNA followed a similar time course with a critical difference directly relevant to the current studies: the elevation of the mRNA was biphasic showing a surge at six hours, a subsequent decrease to near control levels and then a second increase at three days where induction was maintained for 2-3 weeks (Heumann *et. al.*, 1987a, b). The second phase

was found to correlate with the period of maximal macrophage infiltration of the wound site. Explanted sciatic nerve produced the primary and not the secondary induction; addition of either peritoneal macrophage conditioned medium or interleukin-1ß, a cytokine released by activated macrophage, gave an induction similar to that found during the second phase. These data suggest that following lesion in peripheral nerve trunks, loss of cell-cell contact between the nerve and Schwann cells, in combination with interleukin-1 (and other factors) released by invading macrophage, induce a NGF laden environment that is permissive for regeneration to occur.

This scenario shares many features related to NGF with events occurring during development. In embryonic Schwann cells in culture and in all perinatal peripheral nerves analyzed (carrying fibers from NGF responsive and nonresponsive cells), NGF-R expression is elevated relative to the adult (Yan and Johnson, 1988). NGF mRNA has also been found elevated in the neonatal sciatic nerve relative to the adult (Bandlow *et. al.*, 1987) while Heumann (1987) cites evidence that during this period of naturally occurring cell death, there are large numbers of macrophage in the nerve. The role of IL-1 in the developing PNS is unknown. Johnson's group (Johnson *et. al.*, 1988) has proposed that the role for Schwann cells in the developing PNS is similar to that following injury: without axonal contact, they express and release NGF which binds to the low affinity receptor on the Schwann cell surface. High affinity NGF receptors on the ingrowing axon are then able to bind the NGF from the Schwann cell sites while contact with the axolema down regulates NGF and NGF-R in the sheath. Thus Schwann cells may provide a NGF substrate for sympathetic and sensory nerve fibers (and likely provides other neurotrophic factors for NGF insensitive peripheral nerve fibers) and directs them to their peripheral target where they receive stable trophic support.

The model for the role of NGF during injury emerging from the peripheral nervous system was the impetus behind the work described here for the study of interleukin-1 in the central nervous system. Interleukin-1 was found to induce NGF mRNA in enriched,

nonconfluent astrocyte cultures from various areas of the neonatal rat brain. Early experiments showed a 2-3 fold induction of NGF mRNA in enriched astrocyte cultures from neonatal rat cortex, hippocampus and hypothalamus following 3 hour exposure to 10U of human recombinant IL-1ß (figure 14). Similar results were observed when 1U IL-1ß or 100U IL-1α was applied to hippocampal astrocyte cultures (figure 15). When using a standardized plating protocol while feeding all the cultures 10% charcoal stripped fetal calf serum 48 hours prior to harvest, induction was once again observed following 3 hour exposure to 250U of IL-1ß in astrocyte cultures from cortex and hippocampus. Under similar culturing conditions, enriched cortical astrocytes tested at 6, 8 and 17 DIV showed a 2 to 5.6 fold increase in NGF mRNA in response to IL-1ß. Carman-Krzan *et. al.* (1991) observed a maximal (2 fold) NGF mRNA induction in cultured astrocytes from rat cortex following 3 hour exposure to 10U IL-1ß with elevated levels maintained for 72 hours. A 2-3 fold increase in NGF protein in the medium was found beginning at 14 hours of exposure and elevated release remained through 72 hours. Protein levels were unchanged following three hours exposure agreeing with findings of the present work where no stimulated NGF protein release was found in 6d or 17d astrocyte cultures (data not shown). This group also observed a 2 fold increase in NGF released from cultured cerebellar astrocytes following a 38 hour exposure; induction was not observed in cerebellar cultures in the current study subsequent to a 3 hour IL-1 exposure (figure 16). Although the plating conditions of the two studies appear similar, slight differences in the temporal parameters related to the developing cerebellum may be of critical importance since this structure contains a large population of cells which continue to proliferate during the postnatal period. This may imply that responsiveness to IL-1 is delayed with respect to other brain regions and the age of our cerebellar cultures predated this sensitivity. Support for this is given by the finding that amoeboid microglia, which can release IL-1 in their developing brain, first appear at postnatal day 2 in the rat cerebellum, 1 week after the initial appearance in the developing cerebral cortex. The maximal stimulated release of IL-1

from mixed glia cultures from these regions also occurs ~10 days later in cerebellum than cortex (Giulian *et. al.*, 1988a).

Cortical cultures grown for 25d *in vitro*, a period when basal NGF mRNA levels are reduced, did not show NGF induction following 3 hour IL-1ß treatment (figure 17). These data would indicate that the ability of IL-1 to induce NGF mRNA in cultured astrocytes shares parameters described above for basal expression: IL-1 responsiveness is observed in astrocytes from a number of brain regions and is associated with conditions where the cells are proliferating. The lack of induction in 25d glia is the first report of a correlation between elevated cell density and reduced responsiveness.

These results indicate that, similarly to the peripheral nervous system, glia of the CNS are able to respond to interleukin-1 through induction of NGF mRNA. Do glia of the brain respond in a similar fashion to the Schwann cell response in the periphery? There are periods during development when IL-1 is present and appears to enhance the proliferation of astrocytes. Ameboid microglia are phagocytic cells found in the brain during late stages of embryogenesis and have been shown to synthesize and secrete IL-1 (Giulian and Baker, 1986; Giulian *et. al.*, 1986b; Giulian *et. al.*, 1988a). These cells are located at sites of axonal growth and glial proliferation during development and may be involved the organization of the nervous system by removal of debris (see Giulian and Baker, 1986). Giulian has found 4 times the level of IL-1 in cerebral cortex homogenates in E18 rat brain than that found in adult. Levels of IL-1 were found to directly correspond to a spike in the proliferation of ameboid microglia. As noted, a similar pattern is observed in the cerebellum but the peak of IL-1 activity is between postnatal days 7 and 10 and corresponds to the delayed maturation of this structure. IL-1 was also found to induce a 7 fold increase in GFAP+ cells in mixed cortical glial cultures of E20 rats. Ameboid microglia appear to differentiate into ramified or process bearing cells and lose macrophage associated properties by the late postnatal period and this is correlated with a reduction in brain levels of IL-1 (Giulian and Baker, 1986; Giulian *et. al.*, 1988a). These amoeboid (or

"reactive") microglia reappear as one component of a phagocytic cell response following injury to the CNS and this is again temporally and spatially correlated with increased IL-1 levels (Giulian *et. al.*, 1989). Thus embryonic astroglial populations appear to be briefly exposed to a mitogen during specific periods of neuronal progenitor proliferation and neuronal migration. In proliferating astrocytes in culture, this mitogen also produces a significant induction of NGF and the NGF receptor is expressed during focal periods of brain development. This suggests that during periods of rapid growth in the perinatal brain, astrocytes have the capacity to respond rapidly to interleukin-1 and part of the response repertoire may be the induction and elaboration of NGF. This induction may occur in a number of brain regions during restricted periods and may be important for local interactions. The lack of induction in the older cultures may reflect cell-cell contact mediated inhibition of response capacity which may be manifested through a down regulation of the IL-1 receptor. Giulian has found a significant astrogliosis 5 days following IL-1 injection into the adult rat brain (Giulian *et. al.*, 1988b). This would indicate IL-1 can either act directly on astrocytes in the mature brain, inducing growth, or indirectly, by stimulating the release of other growth factors. The former possibility would imply mature astrocytes continue to express IL-1 receptors. Nieto-Sampedro and Berman (1987) have shown that extracts from lesioned brain induces 5-10 fold induction of ^{3}H-thymidine incorporation into confluent astrocyte cultures following a 20 hour exposure. This effect was partially blocked with a human monoclonal anti-IL-1 antibody. Astrocytes in a reduced state of proliferation may be more refactory to a brief exposure to IL-1 and thus may respond over a longer time course than proliferating glia. As noted above, cultured cerebellar astrocytes grown to confluency proliferate at 30% the rate of nonconfluent cells. The similarity between a confluent carpet of astrocytes in culture and the low level of astrocyte mitosis in the intact adult brain is unknown.

The role of glucocorticoids in the regulation of NGF mRNA expression

A) Influence of glucocorticoids on basal NGF transcript levels in cultured astrocytes

The presence of glucocorticoids during incubation of the cultures was of concern. Glucocorticoids and NGF can play antagonistic roles on cells in the peripheral nervous system to produce profound effects on the final differentiated phenotype of various neural crest derived cells (Unsicker *et. al.*, 1978; Doupe *et. al.*, 1985; Anderson and Axel, 1986). Glucocorticoids can also specifically affect NGF mRNA levels. Dexamethasone (Wion *et. al.*, 1986) or cortisone (Simonoski *et. al.*, 1986, 1987) can significantly down regulate NGF mRNA and NGF protein release in the mouse L-929 fibroblast cell line. DEX has been shown to reduce NGF transcript levels in primary cultures of rat fibroblasts (Houlgatte *et. al.*, 1989b) and in mixed glial-neuronal cultures from neonatal rat hippocampus (Friedman *et. al.*, 1990). Dexamethasone has also been shown to eliminate the interleukin-1 induction of NGF mRNA in rat sciatic nerve fibroblasts (Lindholm *et. al.*, 1988). The objective of the current work was to determine the ability of glucocorticoids to regulate NGF mRNA expression in cultured astrocytes. The presence of mRNA for the glucocorticoid and mineralocoid receptors in the cortical and hippocampal cultures suggests these cells have the capacity to respond to added steroid. NGF mRNA levels were unchanged in enriched astrocyte cultures from cortex following 48 hour exposure to charcoal stripped 10% fetal calf serum (charcoal stripping removes glucocorticoids and other components from serum). There was also no effect on total RNA levels (figure 12). Exposure of hippocampal cultures to 100 nM dexamethasone for 1 to 24 hours did not significantly reduce expression of NGF mRNA (figure 13). Subsequent studies on cortical astrocytes treated for 20 hours with 100 nM found the amount of NGF transcripts to be identical to control. These results suggest that glucocorticoids do not regulate NGF mRNA expression in nonconfluent cultures of enriched astrocytes. This work is most comparable to that reported by Friedman *et. al.* (1990) where mixed hippocampal cultures

showed a loss of NGF mRNA following overnight exposure to DEX. However, the presence of neurons in the cultures exerted significant influence on the differentiated state of the glia. In addition, the localization of NGF mRNA in neurons following 1 week in culture (and the possible expression of glucocorticoid receptor mRNA in hippocampal neurons similar to that found *in vivo*; see Van Eekeklen *et. al.*, 1988) makes this study quite different from the one reported here. The lack of regulation by DEX suggests that constitutively produced NGF mRNA levels are not under direct control of glucocorticoids in proliferating rat astrocytes. An influence on NGF mRNA levels may be expected however during induction of NGF transcript by IL-1, an agent known to produce biochemical and physiological effects which are often opposed by glucocorticoids.

B) Interaction of glucocorticoids and interleukin-1 on NGF mRNA regulation

The observation that IL-1 rapidly induces NGF mRNA in astrocytes indicates that this capacity may be present during periods of elevated IL-1 levels in brain. The finding that glucocorticoids can block many IL-1 mediated responses may reflect a feedback circuit whereby inflammatory and proliferative responses in disease or injury to the CNS are governed by a balance between the immune and endocrine systems. If the NGF mRNA induction by IL-1 in astrocytes is part of a response cascade during astrocyte proliferation, then it may be subject to a similar balance of regulatory influences. The effects of preincubating astrocyte cultures with glucocorticoids was examined: 20 hour exposure to dexamethasone blocked the IL-1 mediated induction of NGF mRNA in enriched cortical astrocytes (figure 18). These results are in agreement with Lindholm where the observed 35 fold induction of NGF mRNA in cultured rat fibroblasts was blocked by a similar incubation in DEX. These data suggest a common pathway for the DEX attenuation of IL-1 mediated NGF mRNA induction and that glucocorticoids may block signal transduction pathways directly regulated by IL-1. Phospholipase A2 is induced by IL-1 in chondrocytes

(Chang *et. al.*, 1986) while dexamethasone has been shown to stimulate lipocortin, a phospholipase A2 inhibitory protein. Moreover, Lindholm *et. al.*, (1988) has shown that indomethacin and mepacrine, both inhibitors of phospholipase A activity, could block the IL-1 induction of NGF mRNA to a similar degree as dexamethasone. Indomethacin also blocks the IL-1ß induced release of ACTH form the pituitary (Katsuura *et. al.*, 1988b). Thus the data presented here build on a growing body of evidence indicating an interaction between glucocorticoids and interleukin-1.

These results reinforce the view that IL-1 and glucocorticoids have antagonistic effects. Numerous reports show this interaction can occur at multiple levels and is likely reflective of their opposing roles in immune and inflammatory responses. For instance, glucocorticoids can block the proliferation and IL-1 production in LPS stimulated rat peritoneal macrophages (Snyder and Unanue, 1982) and in the human monocytic cell line, U 937 (Baybutt and Holsboer, 1990). In the CNS, glucocorticoid receptors and receptors for IL-1 are expressed in the hippocampus (Farrar *et. al.*, 1987; Van Eekelen *et. al.*, 1988) while IL-1 has been shown to induce release of hypothalamic CRF (Sapolsky *et. al.*, 1987; Berkenbosch *et. al.*, 1987) which is accompanied by elevated levels of circulating ACTH and glucocorticoids. Thus one aspect of response in the CNS to trauma is the direct, reciprocal regulation of immune and endocrine response parameters through the release of soluble factors from the cells of each system. While IL-1 mediates responses related to immune activation, glucocorticoids can suppress the immune system. The data presented showing glucocorticoid blocking NGF induction are consistent with these opposing regulatory processes. An additional segment of this circuit is indicated by the study of Ernfors *et. al.* (1988) where tissues involved in the immune response (spleen, thymus, lymph nodes) expressed high levels of NGF-R mRNA with mononuclear cells apparently responsible for signal in the spleen (see also Thorpe *et. al.*, 1987). There is now a body of evidence indicating that NGF may play a significant role during cellular and humoral immune responses (reviewed in Thorpe *et. al.*, 1988) including a direct and

indirect role in the enhancement of the proliferative response of lymphocytes. An additional level of regulation is introduced by the finding that activated brain macrophage, the component endogenous to brain most likely responsible for the production of IL-1, is also capable of releasing NGF when cultured *in vitro* (Mallet *et. al.*, 1989). The complexity of these various interactions in brain are poorly understood although some initial theories regarding the sequence of events involving immune activation and possible growth factor involvement have begun to emerge (see Fagan *et. al.*, 1990, below).

NGF mRNA levels in rat hippocampus following in vivo *administration of interleukin-1*

Astrocytes continue to proliferate in the aged mouse brain although the rate is about 10% of that found at birth and appears to continue to decline with increasing age (Korr, 1986). Thus the astroglial population is in a distinctly reduced proliferative state in comparison with the neonate. Adult animals were injected with IL-1 to determine if astrocytes (or neurons) in the mature brain can display increased NGF mRNA levels in a time frame similar to that found for sciatic nerve explant (Heumann *et. al.*, 1988a, b; Lindholm *et. al.*, 1987). The objective was to understand if cells of the mature central nervous system can respond in a manner analogous to the periphery with respect to NGF expression thereby recapitulating a cascade that may occur during ontogeny and that may be permissive for neurite regrowth.

These studies were designed to preclude the response related events associated with brain penetration. The work using the lesioned sciatic nerve demonstrated that the elevation in NGF mRNA levels corresponded to time of arrival at the wound site of macrophage i. e. 48-72 hours following the lesion. When the time course of NGF mRNA expression is studied following explantation of the sciatic nerve, this elevation is not observed; if macrophage conditioned medium or 10U IL-1ß is added, a rapid induction of NGF mRNA occurs and this peaks after 3 hours (Heumann *et. al.*, 1987a; Lindholm *et. al.*, 1987).

There are parallels between the reaction following lesion in the PNS and the events subsequent to injury in the CNS. Phagocytic cells appear as early as 5-8 hours following brain injury with the peak production/invasion appearing 2 days following lesion (Giulian *et. al.*, 1989; Fagan and Gage, 1990; see below). Concomitant with the arrival of these cells to a wound site is an elevation in tissue levels of IL-1 in the region of the damage (Giulian *et. al.*, 1989) and studies *in vitro* indicate mononuclear phagocytes are responsible for this IL-1 elaboration (Giulian *et. al.*, 1986c; Nieto-Sampedro and Berman, 1987; Hetier *et. al.*, 1988; Giulian *et. al.*, 1989). Subsequent to the appearance of the phagocytic cells, an astrocytic response occurs as shown by increased GFAP immunoreactivity at the site of injury. These studies demonstrate a sequence of events begins to occur as early as 5 hours following trauma to the CNS and this cascade includes an astroglial reaction. The studies presented here used a 3 hour exposure period both to circumvent these reactive processes and to determine if the temporal response characteristics in the CNS are similar to that found in the sciatic nerve. The choice of dose of IL-1 was similarly based on the sciatic nerve studies.

No effect was observed in hippocampal NGF mRNA levels 3 fours following intracerebroventricular injection of IL-1α or IL-1ß into the adult rat (IL-1α data shown in figure 19). This would indicate the intact, adult brain does not respond to increased IL-1 levels by induction of NGF mRNA. This result may be consistent with our tissue culture data: older, slowly proliferating astrocytes do not respond to IL-1 with NGF mRNA induction. These data may also be supported by a recent study by Spranger *et. al.* (1990) where, following ICV injection of IL-1 into 10 day old animals, a five fold induction of hippocampal NGF mRNA was observed. Again, the IL-1 induction of NGF mRNA observed in proliferating astrocytes in the current study may be consistent with the *in vivo* induction in neonates where astrocyte proliferation is greater than in the mature animal. However, some caution must be applied in the comparison of these studies as the neonate experiment employed an 8-9 hour survival period and a dose of IL-1 over 300X that given

in the current study. As described above, the longer survival period may encompass the initial response events in brain that may allow astroglia to become responsive to IL-1. It is unknown what role the higher dose or differences in the diffusion of IL-1 in brain parenchyma of neonates versus adults may have played.

The differences found in the responses to IL-1 by neonatal and adult animals, and the lack of induction in slowly proliferating astrocytes may also be reflected in lesion studies in the CNS where changes in NGF mRNA were measured. Whittemore has shown that 10 days following a unilateral fimbria fornix transection in the neonate NGF mRNA levels are elevated in the terminal fields of the hippocampus (Whittemore *et. al.*, 1987). Control levels were found 30 days after the lesion while ablation of the entorhinal cortex, another source of hippocampal afferents, did not produce induction. In contrast, a number of studies have shown no change in NGF mRNA levels following fimbria fornix lesion in the hippocampus in the adult rat (Goedert *et. al.*, 1986; Whittemore *et. al.*, 1986; Korsching *et. al.*, 1986). The results imply that a population of cells remains in the neonatal brain which is able to respond to injury by expressing NGF. This population may lose this capacity during ontogeny and this may be related to the observation in this work that reduced proliferation of astrocytes is correlated with a lost ability to respond to IL-1 with induced expression of NGF mRNA.

Possible role of IL-1 and NGF in the adult, damaged CNS

The view that glia may provide support during neuronal development and regeneration after injury may be consistent with the current findings of growth factor expression in proliferating astrocytes and induction subsequent to IL-1 treatment. The presence of mediating factors such as IL-1 during critical periods following injury may lead to the elaboration of a number of trophic agents from glia which may intervene in the degenerative process. The experiments in the sciatic nerve demonstrate that the immune response following nerve damage may play a key role in the establishment of a suitable environment

permissive for the regeneration of the nerve. An analogous response cascade may occur in the central nervous system. Amoeboid microglia, which secrete IL-1 (Giulian *et. al.*, 1986), invade and/or are activated in the brain following lesion and produce elevated levels of IL-1 and other cytokines (Nieto-Sampedro and Berman, 1987; Giulian, 1987) which lead to astrogliosis and neovascularization (Giulian and Lachman, 1985; Giulian *et. al.*, 1988). The release of IL-1 (and other factors) may have a concerted effect on astrocytes with respect to NGF expression: it may induce a proliferative state thereby making astrocytes amenable to NGF mRNA induction by IL-1. The current work suggests rapid astrocyte proliferation, cell-cell contact mediated events or other characteristics of the cultures prior to approximately the third week *in vitro*, are permissive for IL-1 mediated NGF induction. The presence of trauma related NGF may stabilize responsive neuron populations or may induce the receptor in previously nonsensitive cells to provide trophic support.

The involvement of NGF, astrocytes, and IL-1 has recently come under study in well studied lesion paradigms which produces classic sprouting responses. Denervation of the hippocampus through lesion of the fimbria-fornix (FF) or perforant path (PP) removes input from medial septum and entorhinal cortex, respectively. One result of the lesion is degeneration of a mostly cholinergic, NGF-R expressing cell population in the medial septum; numerous studies have now shown that intervention with exogenously applied NGF can increase cell survival in the medial septum (Hefti *et. al.*, 1986; Williams *et. al.*, 1986; Kromer, 1987; Gage *et. al.*, 1988b) This observation, the high levels of NGF mRNA in a number of target regions of other basal forebrain neurons expressing a similar phenotype, and a large amount of experimental work have suggested that these cells are dependent upon NGF as a target derived trophic agent through adulthood (see Batchelor *et. al.*, 1989). A secondary response to FF lesion is the ingrowth of fibers to the denervated zone of the hippocampus. The majority of this sprouting arises from the sympathetic innervation of the hippocampal vasculature and is derived from neurons in the superior

cervical ganglion (SCG) which express NGF-R throughout life (Loy and Moore, 1977; Stenevi and Björklund, 1978). NGF appears to be important since injection of anti-NGF antibodies into the adult rodent following lesion eliminates the response (Springer and Loy, 1985). One interpretation has been that the denervated hippocampal target cells continue to elaborate NGF producing elevated levels of the protein in the hippocampus (Collins and Crutcher, 1985; Gasser *et. al.*, 1986; Larkfors *et. al.*, 1987) and sympathetic fibers are able to respond to this new source of trophic support. Support for this view comes from the observation that the sprouted fibers preferentially occupy sites similar to those vacated by the lost input (Crutcher *et. al.*, 1987). There is no induction of NGF mRNA in the hippocampus following lesion in the adult (Korsching *et. al.*, 1986). Following PP lesion, septal cholinergic fibers sprout into the deafferented outer molecular layer of the dentate gyrus and this response may also involve NGF (Crutcher and Collins, 1986). An alternative (or additional) view for the source of NGF takes into account the constellation of events occurring following lesion. As shown by work in Gage's lab (Gage *et. al.*, 1988a; Fagan and Gage,1990), there appears to be sequence of events involving microglia, IL-1 and astrocytes during the immediate post-lesion period. Perforant path lesions produced an overall increase in the number cresyl violet stained cells in the denervation zone. An increase of OX-42+ cells and IL-1 immunopositive cell bodies in a similar region on an adjacent section was observed within 24 hours of lesion. The appearance of this staining coincided with degenerative events of the lesioned fibers while increased IL-1 immunoreactivity may correspond to increased IL-1 levels found in brain following injury (Giulian and Lachman, 1985; Nieto-Sampedro and Berman, 1987). Cortical stab wound studies by Giulian (Giulian *et. al.*, 1989) indicate that both endogenous brain microglia and invading, blood borne macrophage are present at a CNS lesion site within hours of injury and peak 2 days following trauma. Peak IL-1 levels in the injury site directly corresponded to the time of the greatest number of macrophage present. Morphological evidence indicates there is also a conversion of brain microglia to a reactive form during this period.

Gage's group found that three days after the perforant path lesion GFAP+ cells appear to be hypertrophic and there is an apparent small increase in cell number in the dentate gyrus molecular layer. This corresponds to Giulian's work where a five fold induction of prominent GFAP+ cells also appeared three days following lesion. Interestingly, while this astroglia induction occurred within 300 μm of the wound site and near phagocytic cells, daily IP injection of chloroquin, in inhibitor of IL-1 secretion, significantly reduced the number of GFAP+ cells. There appears to be little consensus if adult brain astrocytes respond to injury by mitosis, differentiation into a 'reactive' phenotype or both (Katz *et. al.*, 1990b). Using cultured cells from injured brain, this group has provided evidence that most of the proliferative activity following lesion can be attributed to cells of macrophage/monocyte origin with astrocyte activation involving hypertrophy, terminal differentiation and little proliferation. While the increased GFAP+ cells found by Giulian following IL-1 injection correlated with elevated glutamine synthetase activity (an astroglial enzyme), the methodology could not distinguish between migration, proliferation or altered expression to account for changes in GFAP+ cell number. These studies show that while the origin and actual increase in cell *number* (in contrast to increased immunoreactivity for a preexisting population) of microglia and astrocytes remains unresolved, the change and sequence of appearance of these phenotypic markers suggests a microglial activation followed by reactive events in astrocytes which may be mediated by IL-1 in response to brain trauma.

The present data suggest that the model for the sequence of events leading to the reexpression of NGF (and NGF receptor) in the peripheral nervous system may not apply to the events subsequent to injury in the CNS. That is, whereas IL-1 induces NGF expression in rapidly proliferating astrocytes, this degree of proliferation does not appear to occur in the damaged, mature brain and the temporal sequence of IL-1 mediated NGF induction also does not occur. The observation that damage to the adult striatum induces the NGF receptor in cholinergic interneurons (Gage *et. al.*, 1989) indicates that injury

related events may lead to increased NGF responsiveness in many brain regions. This effect is potentiated by the simultaneous application of NGF. Infusion of NGF also produces NGF receptor induction and cholinergic neuron hypertrophy in the basal forebrain of unlesioned neonatal or adult animals (Cavicchoili *et. al.*, 1989; Higgins *et. al.*, 1989). Thus while mechanisms to respond to elevated NGF appear to exist in the mature brain, the lack of NGF induction in slowly proliferating astrocytes in culture and the lack of response in the adult suggests that mature astrocytes differ from their peripheral counterparts in the ability to reiterate a developmental program and respond to IL-1 by synthesizing and releasing NGF. If the capacity to regulate NGF synthesis is related to the proliferative state of astrocytes, how capable are astrocytes in the adult brain to respond by proliferation? Korr (1986) cites studies in the adult rat where, following salt injestion, a significant increase in proliferating pituicytes and astrocytes solely in the supraoptic nucleus occurs. An enriched environment can also influence astrocyte cell number: rats raised in a stimulating environment showed 27% greater astrocytes in their visual cortex than unstimulated rats. Thus regionally and functionally specific proliferative plasticity appears to be an astrocytic property. The adaptive characteristics of astrocytes would appear to make them a tool for further research into their ability to supply trophic agents to the mature CNS. Many parameters of adult *in vivo* IL-1 application remain to be elucidated. Certainly longer survival periods and dose response characteristics need to studied.

While it is clear astrocyte plasticity is displayed following experimental lesion paradigms, increased markers for astrocyte (and microglial) reactivity are also present during neurodegeneration such as in Alzheimer's disease (Vijayan *et. al.*, 1991; Itagaki *et. al.*, 1989). Although elevated levels of IL-1 are also found in the Alzheimer brain (Griffin *et. al.*, 1989) there appears to be no induction of NGF mRNA in the hippocampus of Alzheimer patients (Goedert *et. al.*, 1986). Thus, while collateral sprouting does occur in the adult brain, there is still a large degree of ignorance as to whether the inability of axons in the mature central nervous system to display significant regrowth is a function intrinsic

to postmitotic CNS neurons or if the environment in the mature brain is not compatible to regrowth. A number of factors in addition to the proposed inability of astrocytes to recapitulate an ontogenetic cascade may play a role. As reviewed by Lindsey (1986), these could include the inability of the adult CNS to express other factors such as extracellular components and cell surface guidance cues to provide a permissive environment. In addition, one product of the astrocytic, monocytic and microglial response to CNS trauma is the formation of a 'glial scar.' This collection of cells surrounds the damaged area and may act as a physical barrier to regrowth. The mature CNS may also produce inhibitory factors which are elaborated initially in development to prevent further axonal growth in the adult CNS. Part of this developmental process may include the down regulation of astroglial derived growth factors. Examining the mechanisms behind this regulation will increase the understanding of the differences between the neonatal and mature CNS and may lead to therapeutic approaches to produce controlled regeneration in neurodegenerative diseases.

References

Abney, E. R., P. P. Bartlett, and M. C. Raff (1981). Astrocytes, ependymal cells, and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic rat brain. *Dev. Biol.* 83: 301-310

Allendoerfer, K. L., D. L. Shelton, E. M. Shooter and C. J. Shatz (1990). Nerve growth factor receptor immunoreactivity is transiently associated with the subplate neurons of the mammalian cerebral cortex. *Proc. Natl. Acad. Sci.* 87: 187-190

Aloe, L. and R. Levi-Montalcini (1979). Nerve growth factor-induced transformation of immature chromaffin cells *in vivo* into sympathetic neurons: Effect of antiserum to nerve growth factor. *Proc. Natl. Acad. Sci.* USA 76: 1246-1250

Altin, J. G. and R. A. Bradshaw (1990). Production of 1,2-diacylglycerol in PC12 cells by nerve growth factor and basic fibroblast growth factor. *J. Neurochem.* 54: 1666-1675

Anderson, D. J. and R. Axel (1986). A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. *Cell* 47: 1079-1090

Angeletti, R. H., and R. A. Bradshaw (1971). Nerve growth factor from mouse submaxillary gland: amino acid sequence. *Proc. Natl. Acad. Sci.* USA 68: 2417-2421

Angevine, J. B. Jr. (1965). Time of neuron origin in the hippocampal region. An autoradiographic study in the mouse. *Exp. Neurol.* Suupl. 2 1: 1-70

Armstrong, D. M., R. D. Terry, R. M. Deteresa, G. Bruce, L. B. Hersh, and F. H. Gage (1987) Response of septal cholinergic neurons to axotomy. *J Comp.Neurol* 264: 421-436

Ashwell, K. (1991). The distribution of microglia and cell death in the fetal rat forebrain. *Dev. Br. Res.* 58: 1-12

Assouline, J., P. Bosch, R. Lim, I. S. Lim, R. Jensen, and N. J. Pantazis (1987). Rat astrocytes and Schwann cells in culture synthesize nerve growth factor-like neurite-promoting factors. *Dev. Br. Res.* 31: 103-118

Auburger, G., R. Heumann, R. Hellweg, S. Korsching, and H. Thoenen (1987). Developmental changes in nerve growth factor and its mRNA in the rat hippocampus: Comparison with choline acetyltransferase. *Dev. Biol.* 120: 322-8

Ayer-LeLievre, C., L. Olson, T. Ebendal, A. Seiger, H. Persson (1988). Expression of the ß-nerve growth factor gene in hippocampal neurons. *Science* 240: 1339-1341

Baetge, E. B., B. B. Kaplan, D. J. Reis, and T. H Joh (1981). Translation of tyrosine hydroxylase from poly(A)-mRNA in pheochromocytoma cells is enhanced by dexamethasone. *Proc Natl. Acad. Sci. USA* 78: 1269-1273

Bandlow, C. E., M. Meyer, D. Lindholm, M. Spranger, R. Heumann, and H. Thoenen (1990). Regional and cellular codistribution of interleukin 1ß and nerve growth factor mRNA in the adult rat brain: possible relationship to the regulation of nerve growth factor synthesis. *J. Cell Biol.* 111 1701-1711

Bandlow, C. E., R. Heumann, M. E. Schwab, and H. Thoenen (1987). Cellular localization of nerve growth factor synthesis by *in situ* hybridization. *EMBO J.* 6: 891-899

Banker, G. A. (1980). Trophic interactions between astroglial cells and hippocampal neurons in culture. *Science* 209: 809-810

Banks, W. A., A. J. Kastin, and D. A. Durham (1989). Bidirectional transport of interleukin-1 alpha across the blood-brain barrier. *Br. Res. Bull.* 23: 433-437

Batchelor, P. E., D. M. Armstrong, S. N. Blaker, and F. H. Gage (1989). Nerve growth factor receptor and choline acetyltransferase co-localization in neurons within the basal forebrain: response to fimria-fornix transection. *J .Comp. Neurol.* 284: 187-204

Bateman, A., A. Singh, T. Kral, and S. Solomon (1989). The immune-hypothalamic-pituitary-adrenal axis. *Endocr. Rev.* 10: 92-102

Baybutt, H. N. and F. Holsboer (1990). Inhibition of macrophage differenttiation and function by cortisol. *Endocrinology* 127: 476-480

Beisel, W. R. (1975). Metabolic response to infection. *Ann. Rev. Med.* 26: 9-20

Berkenbosch, F., J. Vans Oers, A. del Rey, F. Tilders, and H. Besedovsky (1987). Corticotrophin-releasing factor-producing factor neurons in the rat activated by Interleukin-1. *Science* 238: 524-526

Berkenbosch, F., L. M. Rofolo, V. L. Friedrich Jr., D. Casper, M. Blum, and N. K. Robakis (1990). The Alzheimer's amyloid precursor protein is protein is produced by type 1 astrocytes in primary cultures of rat neuroglia. *J. Neurosci. Res.* 25: 431-440

Bernton, E. W., J. E. Beach, J. W. Holliday, R. C. Smallridge, and H.G. Fein (1987). Release of multiple hormones as a direct result of interleukin-1 on pituitary cells. *Science* 240: 321-324

Besedovsky, H. O., E. Sorkin, M. Keller, and J. Muller (1975). Changes in blood hormone levels during the immune response. *Proc. Soc. Exp. Biol. Med.* 150: 466-470

Besedovsky, H., A. del Rey, E. Sorkin, C. A. Dinarello (1986). Immunoregulatory feedback between interleukin-1 and glucocoticoid hormones. *Science* 233: 652-654

Birren, S. J. and D. J. Anderson (1990). A v-myc-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not by NGF. *Neuron* 4: 189-201

Breder, C. D., C. A. Dinarello, and C. B. Saper (1988). Interleukin-1 immunoreactive innervation of the human hippocampus. *Science* 240: 321-324

Buck, C. R., H. J. Martinez, I. B. Black, and M. V. Chao (1987) Developmentally regulated expression of the nerve growth factor receptor gene in the periphery and brain. *Proc. Natl. Acad. Sci.* USA 64: 787-794

Carman-Krzan, M., X. Vige, and B. Wise (1991). Regulation by interleukin-1 of nerve growth factor secretion and nerve growth factor mRNA expression in rat primary astroglial cultures. *J. Neurochem.* 56: 636-643

Cattaneo, E. and R. McKay (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature* 347: 762-765

Cavicchioli, L., T. P. Flanigan, G. Vantini, M. Fusco, P. Polato, G. Toffano, F. S. Walsh, and A. Leon (1989). NGF amplifies expression of NGF receptor messenger RNA in forebrain cholinergic neurons of rats. *Eur. J. Neurosci.* 1: 258-262

Chang, J., S. C. Gilman, and A. J. Lewis (1986) Interleukin 1 activates phospholipase A_2 in rabbit chondrocytes: a possible signal for IL-1 action. *J. Immunol.* 136: 1283-1287

Chizzonite, R., T. Truitt, P. L. Kilian, A. S. Stern, P. Nunes, K. P. Parker, K. L. Kaffka, A. O. Chua, D. K. Lugg, and U. Gubler (1989). Two high-affinity interleukin 1 receptors represent separate gene products. *Proc. Natl. Acad. Sci.* 86: 8029-8033

Coceani, F., J. Lees, and C. A. Dinarello (1988). Occurence of interleukin-1 in cerebral spinal fluid of the conscious cat.. *Br. Res.* 446: 245-250

Cohen-Cory,S., C. F. Dreyfus, and I. B. Black (1991). NGF and excitatory neurotransmitters regulate survival and morphogenesis of cultured cerebellar purkinje cells. *J. Neurosci.* 11: 462-471

Collins, F. and C. A. Crutcher (1985). Neurotrophic activity in the adult rat hippocampal formation: Regional distribution and increase after septal lesion. *J. Neurosci.* 5: 2809-2814

Cotman, C. and M. Nieto-Sampedro (1985). Progress in facilitating recovery of function after central nervous system trauma. *Ann. NY Acad. Sci.* 457: 83-104

Crutcher, K. A. (1987). Sympathetic sprouting in the central nervous system: a model for studies of axonal growth in the mature mammalian brain. *Br. Res.*12: 203-233

Crutcher, K. A. and F. Collins (1986). Entorhinal lesions result in increased nerve growth factor-like growth promoting activity in medium conditioned by hippocampal slices. *Br. Res.* 399: 383-389

Davies, A. M., C. Bandlow, R. Heumann, S. Korsching, H. Roher, and H. Thoenen (1987). Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. *Nature* 326: 353-357

Dayer, J.-M., B. De Rochemonteix, B. Burrus, S. Demczuk, and C. M. Dinarello (1986). Human recombinant interleukin-1stimulates collagenase and prostaglandin E_2 production by human synovial cells. J. Clin. Invest. 77: 645-654

del Rey, A., H. Besedovsky, E. Sorkin, C. A. Dinerarello (1987). Interleukin-1 and glucocorticoid hormones integrate an immunoregulatory feedback circuit. *Ann. NY Acad. Sci.* 496: 85-90

Dinarello, 1984. Interleukin-1. *Rev. Infect. Dis.* 6: 51-95

Dinarello, C. A. (1988). Biology of Interleukin. *FASEB J.* 2: 108-115

Doupe, A. J., P. H. Patterson, and S. C. Landis (1985). Small intensely fluorescent cells in culture: Role of glucocorticoids and growth factors in their development and interconversions with other neural crest derivatives. *J. Neurosci.* 5: 2143-2160

Edgar, D. H. and H. Thoenen (1978). Selective enzyme induction in a nerve growth factor-responsive pheochromocytoma cell line (PC12). *Brain Res.* 154: 186-190

Edwards, R. H., M. J. Selby, and W. J. Rutter (1986). Differential RNA splicing predicts two distinct nerve growth factor precursors. *Nature* 319: 784-787

Eisenbarth, G. S., F. S. Walsh, and M. Nirenberg (1979). Monoclonal antibody to a plasma membrane antigen of neurons. *Proc Natl. Acad. Sci.* 76: 4913-4917

Ernfors,P., F. Hallbook, T. Ebendal, E. M. Shooter, M. J. Radeke, T. P. Misko, and H. Persson (1988). Developmental and regional expression of ß-nerve growth factor receptor mRNA in the chick and rat. *Neuron* 1: 983-996

Fagan, A. M. and F. H. Gage (1990). Cholinergic sprouting in the hippocampus: a proposed role for IL-1. *Exp. Neurol* 110: 105-120

Farrar, W. L., P. L. Killian, M. R. Ruff, J. M. Hill, C. B. Pert (1987). Visualization and characterization of interleukin-1 receptors in brain. *J. Immunol.* 139: 459-463

Fontana, A., F. Kristensen, R. Dubs, D. Gemsa, and E. Weber (1982). Production of prostaglandin E and an interleukin-1 like factor by cultured astrocytes and C6 glioma cells. *J. Immunol* 129: 2413-2419

Friedman, W. J., L. Larkfors, C. Ayer-LeLievre, T. Ebendal, L. Olson, And H. Persson (1990). Regulation ß-nerve growth factor expression by inflammatory mediators in hippocampal cultures. *J. Neursci. Res.* 27: 374-382

Furukawa, S., Y. Furukawa, E. Satoyoshi, and K. Hayashi (1986). Synthesis and secretion of nerve growth factor by mouse astroglial cells in culture. *Biochem. Biophys. Res. Comm.* 136: 57-63

Furukawa, S., Y. Furukawa, E. Satoyoshi, and K. Hayashi (1987). Synthesis/secretion of nerve growth factor is associated with cell growth in cultured mouse astroglial cells. *Biochem. Biophys. Res. Comm.* 142: 395-402

Fusco, M., B. Oderfeld-Nowak, G. Vantini, N. Schiavo, M. Gradkowska, M. Zaremba, and A. Leon (1989). Nerve growth factor affects uninjured, adult rat septohippocampal cholinergic neurons. *Neuroscience* 33: 47-52

Gadient, R. A., K. C. Cron, and U. Otten (1990). Interleukin-1ß and tumor necrosis factor-a synergistically stimulate nerve growth factor (NGF) release from cultured astrocytes. *Neurosci. Lett.* 117: 335-340

Gage, F. H., K. Wictorin, W. Fisher, L. R. Williams, S. Varon, and A. Björklund (1986). Retrograde cell changes in medial septum and diagonal band following fimbria-fornix transection: Quantitative temporal analysis. *Neurosci.* 19: 241-55

Gage, F. H., P. Olejniczak, D. M. Armstrong (1988a). Astrocytes are important for sprouting in the septohippocampal circuit. *Exp. Neurol.* 102: 2-13

Gage, F., D. M. Armstrong, L. R. Williams and S. Varon (1988b). Morphological response of axotomized septal neurons to nerve growth factor. *J. Comp Neurol.* 269: 147-155

Gage, F., Batchelor, P. K. Chen, D. Chin, G. A. Higgins, S. Koh, S. Deputy, M. B. Rosenberg, W. Fischer, and A. Björklund (1989). NGF receptor reexpression and NGF-mediated cholionergic neuronal hypertrophy in the damaged adult neostriatum. *Neuron* 2: 1177-1184

Gall, (1991). Kainic acid-induced seizures stimulate increased expression of nerve growth factor mRNA in rat hippocampus. *Mol. Br. Res.* 9 113-

Gasser, U. E., G. Weskamp, U. Otten, and A. R. Dravid (1986). Time course of the elevation of nerve growth factor (NGF) content in the hippocampus and septum following lesions of the septohippocampal pathway in rats. *Br. Res.* 376: 351-356

Gasser, U. E. and M. E. Hatten (1990). Neuronal-glia interactions of rat hippocampal cells in culture *in vitro*: glial-guided neuronal migration and neuronal regulation of glial differentiation. *J. Neurosci.* 10: 1276-1285

Giulian D.and L.B. Lachman, (1985). Interleukin-1 stimulation of astroglial proliferation after brain injury. *Science* 228: 497-499

Giulian, D. and T. J. Baker, (1985). Peptides released by ameboid microglia regulate astroglial proliferation. *J. Cell Biol.* 101: 2411-2415

Giulian, D. and T. J. Baker (1986). Characterization of ameboid microglia isolated from developmental mammalian brain. *J. Neurosci.* 6: 2163-2178

Giulian, D., R. L. Allen, T. J. Baker, and Y. Tomozawa (1986a). Brain peptides and glial growth. I. Glia-promoting factors as regulators of gliogenesis in the developing and injured central nervous system. *J. Cell Biol.* 102: 803-811

Giulian, D. and D. G. Young (1986b). Brain peptides and glial growth. II. Identification of cells that secrete glia-promoting factors. *J. Cell Biol.* 102: 812-820

Giulian, D., T. J. Baker, L.-C. N. Shih, and L. B. Lachman (1986c). Interleukin-1 of the central nervous system is produced by ameboid microglia. *J. Exp. Med.* 164: 594-604

Giulian, D. (1987). Ameboid microglia as effectors of inflammation in the central nervous system. *J. Neurosci. Res.* 18: 155-171

Giulian, D., D. G. Young, J. Woodward, D. C. Brown, and L. B. Lachman (1988a). Interleukin-1 is an astroglial growth factor in the developing brain. *J. Neurosci.* 8: 709-714

Giulian, D., J. Woodward, D. G. Young, J. F. Krebs, and L. B. Lachman (1988b). Interleukin-1 injected into the mammalian brain stimulates astrogliosis and neovascularization. *J. Neurosci.* 8: 2485-2490

Giulian, D., J. Chen, J. E. Ingeman, J. K. George, and M. Noponen (1989). The role of mononuclear phagocytes in wound healing after traumatic injury to adult mammalian brain. *J. Neurosci.* 9: 4416-4429

Gnahn, H., F. Hefti, R. Heumann, M. E. Schwab and H. Thoenen (1983). NGF-mediated increase of choline acetyltransferase (ChAT) activity in the neonatal rat forebrain: Evidence for a physiological role of NGF in the brain ? *Dev. Br. Res.* 9: 45-52

Goedert, M., A. Fine, S. P. Hunt, and A Ullrich (1986). Nerve growth factor mRNA in peripheral and central rat tissues and in the human central nervous system: Lesion effects in the rat brain and levels in Alzheimer's disease. *Mol. Br. Res.* 1: 85-92

Gorin, P.D. and E.M. Johnson, Jr. (1980). Effects of exposure to nerve growth factor antibodies on the developing nervous system of the rat: an experimental autoimmune approach. *Dev. Biol.* 80: 313-323

Greene, L. A. and E. M. Shooter (1980). The nerve growth factor: Biochemistry, synthesis, and mechanism of action. *Ann Rev. Neurosci.* 3: 353-402

Greene, L. A. and A. S. Tischler (1982). PC12 pheochromocytoma cultures in neurobiological research. *Adv. Cell. Neurobiol.* 3: 373-414

Griffin, S., L. C. Stanley, C. Ling, L. White, V. Macleod, L. J. Perrot, C. L. White III, and C. Araoz (1989). Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 86: 7611-7615

Hartikka, J. and F. Hefti (1988a). Development of septal cholinergic neurons in culture: Plating density and glial cells modulate effects of NGF on survival, fiber growth, and expression of transmitter-specific enzymes. *J. Neurosci.* 8: 2967-2985

Hartikka,J. and F. Hefti (1988b). Comparison of nerve growth factor's effects on development of septum, striatum, and nucleus basalis cholinergic neurons in vitro. *J. Neurosci. Res.* 21: 352-364

Hatanaka, H. and H. Tsukui (1986). Differential effects of nerve growth factor and glioma-conditioned medium on neurons cultured from various regions of fetal rat central nervous system. *Dev. Br. Res.* 30: 47-56

Hatten, M. E. and R. H. K. Liem (1981). Astroglial cells provide a template for the positioning of developing cerebellar neurons in vitro. *J. Cell Biol.* 90: 622-630

Hatten, M. E. (1985). Neuronal regulation of astroglial morphology and proliferation in vitro. *J. Cell Biol.* 100: 384-396

Hatten, M. E. (1987). Neuronal inhibition of astroglial cell proliferation is membrane mediated. *J. Cell Biol.* 104: 1353-1360

Hatten, M. E. and M. L. Shelanski (1988). Mouse cerebellar granule neurons arrest the proliferation of human and rodent astrocytoma cells in vitro. *J. Neurosci.* 8 1447-1453

Hatten, M. E., M. Lynch, R. E. Rydel, J. Sanchez, J. Joseph-Silverstein, D. Moscatelli, and D. B. Rifkin (1988). In vitro neurite extension by granule neurons is dependent upon astroglial-derived fibroblast growth factor. *Dev. Biol.* 125: 280-289

Hatten, M. E. (1989). Neuronal regulation of astroglial proliferation and differentiation. In: *The assembly of the nervous system* pp 151-166; Alan J. Liss New York

Hatten, M. E. (1990). Riding the glial monorail: a common mechanism for guided neuronal migration in different regions of the developing mammalian brain. *Trends Neurosci.* 13: 179-184

Hefti, F., A. Dravid, and J. Hartikka (1984). Chronic intraventricular injectiopns of nerve growth factor elevate choline actetlytranferase activity in adult rats with partial septo-hippocampal lesions. *Br. Res.* 293: 305-311

Hefti, F., J. Hartikka, F. Eckenstein, H. Gnahn, R. Heumann, and M. E. Schwab (1985). Nerve growth factor increases choline acetyltransferase but not survival or fiber outgrowth of cultured fetal septal cholinergic neurons. *Neuroscience* 14: 55-68

Hefti, F. (1986a). Nerve growth factor (NGF) promotes the survival of septal cholinergic neurons after fimbrial transection. *J. Neurosci.* 6: 2155-2162

Hefti, F., J. Hartikka, A. Salvatierra, W. J. Weiner, and D. C. Mash (1986b). Localization of nerve growth factor receptors in cholinergic neurons of the human basal forebrain. Neurosci Lett. 69: 37-41

Hendry, I. A. and J. Campbell (1976). Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. *J. Neurocytol.* 5: 351-360

Hetier, E., J. Ayala, P. Denefle, A. Bousseau, P. Rouget, M. Mallet, and A. Prochiantz (1988). Brain macrophages synthesize interleukin-1 and interleukin-1 mRNA in vitro. *J. Neurosci. Res.* 21: 391-397

Heuer, J. G., C. S. von Bartheld, Y. Kinoshita, P. C. Evers, and M. Bothwell (1990). Alternating phases of FGF receptor and NGF receptor expression in the developing chicken nervous system. *Neuron* 5: 283-296

Heumann, R. (1987). Regulation of the synthesis of nerve growth factor. *J. Exp. Biol.* 132: 133-150

Heumann, R., Lindholm, D., C. Bandtlow, M. Meyer, M. J. Radeke, T. P. Misko, E. Shooter, and H. Thoenen (1987a). Differential regulation of mRNA nerve growth

factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: Role of macrophages. *Proc Natl. Acad. Sci.* 84: 8735-8739

Heumann, R., S. Korsching, C. Bandtlow, and H. Thoenen (1987b). Changes in nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection. *J. Cell Biol.* 104: 1623-1631

Heumann, R., S. Korsching, J. Scott, H. Thoenen (1984). Relationship of levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. *EMBO J.* 3: 3183-3189

Higgins, G. A., S. Koh, K. S. Chen, F. H. Gage (1989a). NGF induction of NGF receptor gene expression and cholinergic neuronal hypertrophy within the basal forebrain in the adult rat.. *Neuron* 3: 247-256

Houlgatte, R., M. Mallet, P. Brachet, and A. Prochiantz (1989a). Secretion of nerve growth factor in cultures of glial cells and neurons derived from different regions of the rat brain. *J. Neurosci. Res.* 24: 143-152

Houlgatte, R., D. Wion, P Brachet (1989b). Levels of nerve growth factor and its mRNA secreted by rat primary fibroblasts and iris transplants are influenced by serum and glucocorticoids. *Dev. Br. Res.* 47: 171-179

Innocenti, G. M., S. Clarke, H. Koppel (1983). Transitory macrophages in the white matter of developing visual cortex. II. Development and relations with axonal pathways. *Dev. Br. Res.* 11: 55-66

Itagaki, S., P. L. McGeer, H. Akiyyama, S. Zhu, and D. Selkoe (1989). Relationship of microglia and astrocytes to amyloid deposits of Alzheimer's disease. *J. Neuroimmuno.* 24: 173-182

Johnson, E. M. Jr., M. Taniuchi, and P. S. Distefano (1988). Expresion and possible function of nerve growth factor receptors on Schwann cells. *Trends Neurosci.* 11: 299-304

Johnston, M. V., J. Lynn Rutkowski, B. W. Wainer, J. B. Long, and W. C. Mobley (1987). NGF effects on developing forebrain cholinergic neurons are regionally specific. *Neurochem. Res.* 12: 985-994

Jordan, F. L. and W. E. Thomas (1988). Brain macrophages: questions of origin and interrelationship. *Br. Res. Rev.* 13: 165-178

Katsuura, G., P. E. Gottshall, and A. Arimura (1988a). Identification of a high-affinity receptor for interleukin-1 beta in rat brain. *Biochem. Biophys. Res. Comm.* 156: 61-67

Katsuura, G., P. Gottshall, R. Dahl, and A. Arimura (1988b). Adrenocorticotropin release induced by intracrerbroventricular injection of recombinant human interleukin-1 in rats: possible involvement of prostaglandin. *ENDO* 122: 1773-1779

Katz, D. M., M. Erb, R. Lillis, K. Neet (1990a). Trophic regulation of nodose ganglion cell development: evidence for an expanded role of nerve growth factor during embryogenesis in the rat. *Exp. Neurol.* 110: 1-10

Katz, I. R., L. M. Iacovitti, and D. R. Reis (1990b). In vitro studies on the characterization of cellular proliferation following neuronal injury in the adult rat brain. *J. Neuroimmunol.* 29: 33-48

Koh, S. and R. Loy (1989). Localization and development of nerve growth factor-sensitive rat basal forebrain neurons and their afferent projections to hippocampus and neocortex. *J. Neurosci.* 9: 2999-3018

Kohase, M., D. Henriksen-Destefano, P. B. Sehgal, and J. Vilcek (1987). Dexamethasone inhibits feedback regulation of the mitogenic activity of tumor necrosis factor, interleukin-1, and epidermal growth factor in human fibroblasts. *J. Cell. Physiol.* 132: 271-278

Korr, H. (1986) Proliferation and cell cycle parameters of astrocytes. In : *Astrocytes. Cell Biology and Pathology of Astrocytes.* Vol. 3. S Federoff and A. Vernadakis, eds. Academic Press, Orlando, pp. 77-127

Korsching, S., G. Auburger, R. Heumann, J. Scott, and H. Thoenen (1985). Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *EMBO J.* 4: 1389-1393

Korsching, S., R. Heumann, H. Thoenen, and F. Hefti (1986). Cholinergic denervation of the rat hippocampus by fimbrial transection leads to transient accumulation of nerve growth factor without a change in NGF mRNA content. *Neurosci.Lett.* 66: 175-180

Kromer, L. F. (1987). Nerve growth factor treatment after brain injury prevents neuronal death. *Science* 235: 214-217

Krueger, J. M., J. Walter, C. A. Dinarello, S. M. Wolff, and L. Chedid (1984). Sleep-promoting effects of endogenous pyrogen (interleukin-1). *Am. J. Physiol* 246 R994-R999

Landis, S. C. and P. H. Patterson (1981). Neural Crest cell lineages. *Trends Neurosci.* 4: 172-175

Large, T. H., S. C. Bodary, D. O. Clegg, G. Weskamp, U. Otten, and L. F. Reichardt (1986). Nerve growth factor gene expression in the developing rat brain. *Science* 234: 352-355

Larkfors, L., I. Stromberg, T. Ebendal, and L.Olson (1987). Nerve growth factor protein level increases in the adult rat hippocampus after a specific cholinergic lesion. *J. Neurosci. Res.* 18: 525-531

Lechan, R. M., R. Toni, B. D. Clark, J. G. Cannon, A. R. Shaw, C. A. Dinarello, and S. Reichlin (1990). Immunoreactive interleukin-1 beta localization in the rat forebrain. *Br. Res.* 514: 135-140

Lee, J. J. and N. A. Costlow (1987). A molecular titration assay to measure transcript prevalence levels. *Methods in Enzymology* 152: 633-648

Lee, S. W., A.-P. Tsou, H. Chan, J. Thomas, K. Petrie, E. M. Eugui, and A. C. Allison (1988). Glucocorticoids selectively inhibit the transcription of the interleukin 1ß gene

and decrease the stability of interleukin 1ß mRNA. *Proc. Natl. Acad. Sci. USA* 85: 1204-1208

Levi-Montalcini, R. and B. Booker (1960). Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve growth protein. *Proc. Natl. Acad. Sci. USA* 46: 384-391

Levi-Montalcini, R. and P. U. Angeletti (1966). Immunosympathectomy. *Pharmacol. Rev.* 18: 619-628

Levitt, S. P. and P.Rakic (1980). Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the deveoping monkey brain. *J. Comp. Neurol.* 193: 815-840

Liesi, P., D. Dahl, and A. Vaheri (1983). Laminin is produced by early rat astrocytes in primary culture. *J. Cell Biol.* 96: 920-924

Lindholm, D., R. Heumann, B Hengerer, and H. Thoenen (1988). Interleukin 1 increases stability and transcription of mRNA encoding nerve growth factor in cultured rat fibroblasts. *J. Biol. Chem.* 262: 16348-16351

Lindholm, D., R. Heumann, M. Meyer, and H. Thoenen (1987). Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. *Nature* 330: 658-659

Lindsay, R. M. (1979) . Adult rat brain astrocytes support the survival of both NGF dependent and NGF-insensitive neurons. *Nature* 282: 80-82

Lindsay, R. M. (1986). Reactive Gliosis. In : *Astrocytes. Cell Biology and Pathology of Astrocytes*. Vol. 3. S Federoff and A. Vernadakis, eds. Academic Press, Orlando, pp. 231-262

Ling, E. A. (1976). Some aspects of amoeboid microglia in the corpus collosum and neighboring regions of neonatal rats. *J. Anat.* 121: 29-45

Ling, E. A. (1977). Light nad electron microscopic demonstration of some lysosomal enzymes in the amoeboid microglia in neonatal rat brain. *J. Anat.* 123: 637-648

Ling, E. A., C. Kaur, and W. C. Wong (1982). Light and electron microscopic demonstration of non-specific esterase in amoeboid microglial cells in the corpus callosum in post natal rats: a cytochemical link to monocytes. *J. Anat.* 135: 385-394

Loy, R., and R. Y. Moore (1977). Anomalous innervation of the hippocampal formation by peripheral sympathetic axons followingmechanical injury. *Exp. Neurol.* 57: 645-650

Lu, B., C. R. Buck, C. F. Dreyfus, and I. B. Black (1989). Expression of NGF and NGF receptor mRNAs in the developing brain: evidence for local delivery and action of NGF. *Exp. Neurol.* 104: 191-199

Lu, B., M. Yokoyama, C. F. Dreyfus, and I. B. Black (1991). NGF gene expression in actively growing glia. *J. Neurosci.* 11: 318-326

Mallet, M., R. Houlgatte, P. Brachet, and A. Prochiantz (1989). Liposaccharide-stimulated brain macrophage release NGF in vitro. *Dev. Biol.* 133: 309-311

Manthorpe, M., E. Engvall, E. Ruoslahti, F. M. Longo, G. E. Davis, and S. Varon (1983). Laminin promotes neurite regeneration from cultured peripheral and central neurons. *J. Cell Biol.* 97: 1882-1890

Manthorpe, M., J. S. Rudge, and S. Varon (1986). Astroglial cell contributions to neuronal survival and neuritic growth. In: *Astrocytes. Biochemistry, Physiology, and Pharmacology of Astrocytes.* Vol,. 2. S Federoff and A. Vernadakis, eds. Academic Press, Orlando, pp. 315-376

Martin, D. P., R. E. Schmidt, P. S. Distefano, O. H. Lowry, J. G. Carter, and E. M. Johnson (1988). Inhibitors of protein synthesis and RNA synthesis prevent neuronal cell death caused by nerve growth factor deprivation. *J. Cell Biol.* 106: 829-844

Martinez, H. J., C. F. Dreyfus, G. M. Jonakait, and I. B. Black (1987). Nerve growth factor selectively increases cholinergic markers but not neuropeptides in rat basal forebrain in culture. *Br. Res.* 412: 295-301

Milner, T. A., R. Loy, D. G. Amaral (1983). An anatomical study of the development of the septo-hippocampal projection in the rat. *Dev. Br. Res.* 8: 343-371

Mobley, W. C., J. L. Rutkowski, G. I. Tennekoon, J. Gemski, K. Buchanan, and M. V. Johnston (1986). Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. *Mol. Br. Res.* 1: 53-62

Mobley, W. C., J. L. Rutkowski, G. I. Tennekoon, K. Buchanan, and M. V. Johnston (1985). Choline acetyltransferase in striatum of neonatal rats increased by nerve growth factor. *Science* 229: 284-287

Murabe, Y. and Y. Sano, (1982). Morphological studies on neuroglia. VI. Postnatal development of microglial cells. *Cell Tissue Res.* 225: 469-485

Murphy, M., J. Drago, and P. F. Bartlett (1990). Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells in vitro. J. Neurosci. Res. 25: 463-475

Nakamura, H., K. Nakanishi, A. Kita, and T. Kadokawa (1988). Interleukin-1 induces analgesia in mice by a central action.. *Eur. J. Pharm.* 149: 49-56

Nieto-Sampedro, M. and M. Berman (1987). Interleukin-1-like activity in rat brain: Sources targets, and effect of injury. *J. Neurosci. Res.* 17: 214-219

Norrgren, G., T. Ebendal, M. Belew, C.-O. Jacobson, and J. Porath (1980). Release of nerve growth factor by human glial cells in culture. *Exp. Cell Res.* 130: 31-39

Noble, M., J. Fok-Seang, and J. Cohen (1984). Glia are a unique substrate for the in vitro growth of central nervous system neurons. *J. Neurosci.* 4: 1892-1903

Otten, U., G. Weskamp, M. Hardung, and D. K. Meyer (1987). Synthesis and release of nerve growth factor by rat macrophages. *Soc. Neurosci. Abstr.* 13: 184

Otten, U., M. Goedert, M. Schwab, and J. Thibault (1979). Immunization of adult rats against 2.5 nerve growth factor: effects on the peripheral nervous system. *Brain Res.* 176: 79-90

Perry, V. H., D. A. Hume, and S. Gordon (1985). Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. *Neuroscience* 15: 313-326

Perry, V. H., M. C. Brown, and S Gordon (1987). The macrophage response to central and peripheral nerve injury. *J. Exp. Med.* 165: 1218-1223

Perry, V. H. and S. Gordon (1988). Macrophages and microglia in the nervous system. *Trends Neurosci.* 11: 273-277

Plata-Salaman, C. R., Y. Oomura, and Y. Kai (1988). Tumor necrosis factor and interleukin-1ß: suppression of food intake by direct action in the central nervous system. *BR. Res.* 448 106-114

Prochiantz, A. and M. Mallet (1988). Astrocyte diversity. *Ann. NY Acad. Sci.* 540: 52-63

Purves, D. and J. Lichtman (1985). *Principles of neural development.* Sunderland, MA: Sinauer Assoc.

Raff, M. C. (1989). Glial cell diversification in the rat optic nerve. *Science* 243: 1450-1455

Raff, M., R. H. Miller, M. Noble (1983). A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on the culture medium. *Nature* 303: 390-396

Raivich, G. and G. W. Kreutzberg (1987). The localization and distribution of high affinity ß-nerve growth factor binding sites in the central nervous system of the adult rat. A light microscopic autoradiographic study using [125I]ß-nerve growth factor. *Neuroscience* 20: 23-36

Rettori, V., J. Jurcovicova, S. M. McCann (1987). Central action of interleukin-1 in altering the release of TSH, growth hormone, and prolactin in the male rat. *J. Neurosci. Res.* 18: 179-183

Rose, G., G. Lynch, and C. Cotman (1976). Hypertrophy and redistribution of astrocytes in the deafferented dentate gyrus. *Br. Res. Bull* 1: 87-92

Rossi, V., F. Brevario, P. Ghezzi, E. Dejana, and A. Mantopani (1985). Interleukin-1 induces prostacyclin in vascular cells. *Science* 229: 1174-1176

Sapolsky, R., C. Rivier, G. Yamamoto, P. Plotsky and W. Vale (1987). IL-1 stimulates the secretion of hypothalamic corticotrophin releasing factor. *Science* 238: 522-524

Schatteman, G. C., L. Gibbs, A. A. Lanahan, P. Claude, and M. Bothwell (1988). Expression of the NGF receptor in the developing and adult primate central nervous system. *J. Neurosci.* 8: 860-873

Schmidt, J. A., S. B. Mizel, D. Cohen, and I. Greene (1982). Interleukin 1, a potent regulator of fibroblast proliferation. *J. Immunol.* 128: 2177-2182

Schwab, M. E., U. Otten, Y. Agid, and H. Thoenen (1979). Nerve growth factor (NGF) in the rat CNS: Absence of specific retrograde axonal transport and tyrosine hydroxylase induction in locus coeruleus and substantia nigra. *Brain Res.* 168: 473-483

Schwab, M. E. and H. Thoenen (1985). Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. *J. Neurosci.* 5: 2415-2423

Schwartz, J. P. and K. Mishler (1990). ß-adrenergic receptor regulation, through cyclic AMP, of nerve growth factor expression in rat cortical and cerebellar astrocytes. *Cell. Mol. Neurobio.* 10: 447-457

Scott, J., M. Selby, M. Urdea, M. Quiroga, G. I. Bell, and W. J. Rutter (1983). Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. *Nature* 302: 538-540

Seiler, M. and M. E. Schwab (1984). Specific retrograde transport of nerve growth factor (NGF) from neocortex to nucleus basalis in the rat. *Brain Res.* 300: 33-39

Selby, M. J., R. Edwards, F. Sharp, and W. J. Rutter (1987). Mouse nerve growth factor gene: structure and expression. *Mol. and Cell. Biol.* 7: 3057-64

Shelton, D. L. and L. F. Reichardt (1984). Expression of ß-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc Natl. Acad. Sci.* 81: 7951-7955

Shelton, D. L. and L. F. Reichardt (1986). Studies on the expression of the ß-nerve growth factor gene (NGF) in the central nervous system: Level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons. *Proc. Natl. Acad. Sci.* 83: 2714-2718

Siminoski, K., J. Bernanke, C. Kay, and R. A. Murphy (1986). Steroids and tiiodothyrodine reduce nerve growth factor concentrations in medium conditioned by L-929 fibroblasts. Endocrinology 118: 1417-1425

Siminoski, K., R. A. Murphy, P. Rennert, and G. Heinrich (1987). Cortisone, testosterone and aldosterone reduce levels of nerve growth factor messenger ribonucleic acid in L-929 fibroblasts. *Endocrinology* 121: 1432-1437

Smith, J. and M. Fauquet (1984). Glucocorticoids stimulate adrenergic differentiation in cultures of migrating and premigrating neural crest. *J. Neurosci.* 4: 2160-2172

Snyder, D. S. and E. R. Unanue (1982). Corticosteroids inhibit murine macrophage Ia expression and interleukin-1 induction. *J. Immunol.* 129: 1803-1805

Spranger, M., D. Lindholm, C. Bandlow, R. Heumann, H. Gnahn, M. Naher-Noe, and H. Thoenen (1990). Regulation of nerve growth factor (NGF) synthesis in the rat central nervous system: comparison between the effects of interleukin-1 and various growth factors in astrocyte cultures and in vivo. *Eur. J. Neurosci.* 2: 69-76

Springer, J. E. and R. Loy (1985). Intrahippocampal injections of antiserum to nerve growth factor inhibit septohippocampal sprouting. *Br. Res. Bull.* 15: 629-34

Springer, J. E., S. Koh, M. W. Tayrien, and R. Loy (1987). Basal forebrain magnocellular neurons stain for nerve growth receptor: correlation with cholinergic cell bodies and effects of axotomy. *J. Neurosci. Res.* 17: 111-118

Springer, J. E. (1988). Nerve growth factor receptors in the central nervous system. *Exp. Neurol.* 102: 354-365

Springer, J. E., E. Robbins, S. Meyer, F. Baldino, Jr., and M. E. Lewis (1990). Localization of nerve growth factor receptor mRNA in the rat basal forebrain with in situ hybridization histochemistry. *Cell Mol. Neurobiol.* 10: 33-39

Stenevi, U. and A. Björklund (1978). A pitfall in brain lesion srudies: growth of vascular sympathetic axons into the hippocampus after fimbrial lesions. *Neurosci. Lett.* 7: 219-274

Subramananian, N. and M. A. Bray (1987). Interleukin1 releases histimine from human basophils and mast cells in vitro. *J. Immunol.* 138: 271-275

Taniuchi, M., H. B. Clark, J. B. Schweitzer, and E. G. Johnson Jr. (1988). Expression of nerve growth factor receptors by schwann cells of axotomized peripheral nerves: Ultrastructural localization, suppression by axonal contact, and binding properties. *J. Neurosci.* 8: 654-681

Taniuchi, M., J. B. Schweitzer, and E. M. Johnson (1986). Nerve growth receptor molecules in rat brain. *Proc. Natl. Acad. Sci.* 83: 1950-1954

Tarris, R. H., M. E. Weichsel , and D. A. Fisher (1986). Synthesis and secretion of a nerve growth-stimulation factor by neonatal mouse astrocytes in vitro. *Pediatr. Res.* 20: 367-372

Thoenen, H. and Y.-A. Barde (1980). Physiology of Nerve growth factor. *Physiol. Rev.* 60: 1284-1335

Thoenen, H., S. Korsching, R. Heumann, and A. Acheson (1985). Nerve growth factor. *Ciba Found. Symp.* 116: 113-128

Thorpe, L. W., R. W. Stach, B. Morgan, and J. R. Perez-Polo (1988). The biology of nerve growth factor. Its interactions with the immune system. *In* Neural Control of Reproductive Function. J. Lakowski, D. Rassin, J. R. Perez-Polo, Eds.: 351-369. Alan R. Liss Inc. New York

Thorpe, L. W., R. W. Stach, G. A. Hashim, D. Marchetti, and J. R. Perez-Polo (1987). Receptors for nerve growth factor on rat splenic mononuclear cells. *J. Neurosci. Res.* 19: 349-356

Tiercy, J. M. and E. M. Shooter (1986). Early changes in the synthesis of nuclear and cytoplasmic proteins are induced by nerve growth factor in differentiating rat PC12 cells. *J. Cell Biol.* 103: 2367-2378

Tseng, C. Y., E. A. Ling, and W. A. Wong (1983). Scanning electron microscopy of amoeboid microglial cells in the transient cavum septum pellucidum in pre- and postnatal rats. J. Anat. 136: 251-263

Ullrich, A., A Gray, C. Berman, and T. Dull (1983). Human ß-nerve growth factor gene sequence highly homologous to that of mouse. *Nature* 303: 821-825

Unsicker, K. B. Krisch, U. Otten, and H. Thoenen (1978). Nerve growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: impairment by glucocorticoids. *Proc. Natl. Acad. Sci. USA* 75: 3498-3502

Van Eekelen, J. A. M., W. Jiang, E. R. De Kloet, and M. C. Bohn (1988) Distribution of the mineralocoid and glucocorticoid receptor mRNAs in the rat hippocampus. *J. Neurosci.* Res. 21: 88-94

Vernadakis, A. (1988). Neuron-glia interactions. In: *International Review of Neurobiolog.* Vol. 30, pp 149-219

Vijayan, V. K., J. W. Geddes, K. J. Anderson, H. Chang-Chui, W. G. Ellis and C. W. Cotman (1991). Astrocyte hypertrophy in the Alzheimer's disease hippoocampal formation. *Exp. Neurol.* 112: 72-78

Wahl, S. M., L. C. Altman, and D. L. Rosenstreich (1975). Inhibition of *in vitro* lymphokine sythesis by glucocorticoids. *J. Immunol.* 115: 476-481

Walker, P., M. E. Weichsel, Jr., S. B. Hoath, R. E. Poland, and D. A. Fisher (1981). Effect of thyroxine, testosterone and corticosterone on nerve growth factor and epidermal growth factor concentrations in adult female submaxillary gland: Dissociation of NGF and EGF responses. *Endocrinology* 109: 582-587

Wanaka, A. and E. M. Johnson, Jr. (1990). Developmental study of nerve growth factor mRNA expression in the postnatal rat cerebellum. *Dev. Br. Res.* 55: 288-292

Weskamp, G., U. Gasser, A. Dravid, and U. Otten (1986). Fimbria-fornix lesion increases nerve growth factor content in adult rat septum and hippocampus. *Neurosci. Lett.*, 70 121-126

Whittemore, S. R. and A. Seiger (1987). The expression, localization and functional significance of beta-nerve growth factor in the central nervous system. *Br. Res. Rev.* 12: 439-64

Whittemore, S. R., L. Larkfors, T. Ebendal, V. R. Holets, A. Ericsson, and H. Persson (1987). Increased ß-nerve growth factor messenger RNA and protein levels in neonatal rat hippocampus following specific cholinergic lesions. *J. Neurosci.* 7: 244-251

Whittemore, S. R., P. L. Friedman, D. Larhammar, H. Persson, M. Gonzalez-Carvajal, and V. R. Holets (1988). Rat ß-nerve growth factor sequence and site of synthesis in the adult hippocampus. *J. Neurosci. Res.* 20: 403-410

Whittemore, S. R., T. Ebendal, L. Larkfors, L. Olson, A. Seiger, I. Stromberg, and H. Persson (1986). Developmental and regional expression of ß nerve growth factor messenger RNA and protein in the rat central nervous system. *Proc. Natl. Acad. Sci.* USA 83: 817-821

Williams, L. R., S Varon, G. M. Peterson, K. Wictorin, W, Fischer, A. Bjørklund, and F. H. Gage (1986). Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. *Proc. Natl. Acad. Sci.* USA 83: 9231-9235

Wion, D., R. Houlgatte, and P. Bratchet (1986). Dexamethasone rapidly reduces the expression of the ß-NGF gene in mouse L-929 cells. *Exp. Cell Res.* 162: 562-565

Wood, S. J., J. Pritchard, and M. V. Sofroniew (1990). Re-expression of nerve growth factor receptor after axonal injury recapitulates a development event in motor neurons: differential regulation when regeneration is allowed or prevented. *Eur. J Neuro.* 2: 650-657

Yamakuni, 1987. Expression of ß-nerve growth factor mRNA in rat glioma cells and astrocytes from rat brain. FEBS Lett. 223: 117

Yan, Y. and E. M. Johnson, Jr. (1988). An immunohistochemical study of the nerve growth factor receptor in developing rats. *J. Neurosci.* 8: 3481-3498

Yanker, B. A. and E. M. Shooter (1982). The biology and mechanism of action of nerve growth factor. *Ann. Rev. Biochem.* 51: 845-868

Yoshida, K and F. H. Gage (1991). Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. *Br. Res.* 538: 118-126

Zilberstein, A., R. Ruggieri, J. N. Korn, and M. Revel (1986). Structure and expression of cDNA and genes for human interferon-beta2, a distinct species inducible by growth stimulatory cytokines. *EMBO J.* 5: 2529-2537