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ALCOHOL-ENHANCED DIFFERENTIATION OF PC12 CELLS

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VITA

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ALCOHOL-ENHANCED DIFFERENTIATION OF PC12 CELLS

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Doctor of Philosophy, December 9, 1994
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Studies were undertaken to elucidate the mechanism(s) by which alcohols potentiate nerve growth factor's (NGF) ability to promote neurite extension in Pheochromocytoma (PC12) cells. The effects of alcohol on NGF receptor parameters were examined using Scatchard analysis. Alcohol treatment did not induce any change in either the high or low affinity form of the NGF receptor.

A morphological study of alcohol's ability to potentiate NGF-induced neurite outgrowth was undertaken to determine if there is a specific window of time during which alcohol exposure, relative to NGF stimulation, has a more pronounced effect on differentiation. Morphological results indicated that alcohol initiated a greater degree of NGF-induced neurite extension in cells pretreated with alcohol for 24 hours compared to cells simultaneously treated with alcohol and NGF, when evaluated after 24 hours of NGF

stimulation. However, this effect was no longer observed as either the length of pretreatment time, or the length of NGF stimulation time, was increased.

Thymidine incorporation studies were undertaken to investigate whether alcohols mediate their effects by altering the proliferative capacity of the cells. Ethanol did not alter the proliferative capacity of PC12 cells. Treatment with either propanol or butanol decreased proliferation after 24 hours. However, this effect was no longer observed by 72 hours of alcohol exposure, suggesting the cells are able to adapt to this effect.

Cell cycle analysis revealed a decrease in the S phase and a concomitant increase in the G_1 phase cells treated for 24 hours with either propanol or butanol (0.4%: 87 mM) indicating the cells are being primed toward a differentiated state. However, a G_1 delay is no longer observed after long term exposure, suggesting adaptation to this effect is occurring.

Lastly, studies were undertaken to analyze G_{M1} content in PC12 cells exposed to alcohol. In cells treated with either, NGF or, NGF and ethanol, increases in G_{M1} were observed. However, treatment with alcohol alone had no effect on G_{M1} content.

This study provides information regarding a specific time period of 24 hours during which alcohol exposure has the ability to modulate NGF-induced neurite extension as well as cell growth and differentiation. An adaptation effect of PC12 cells to alcohol exposure is also alluded to with this study. Furthermore, the enhanced neuritogenesis observed in many neuronal populations of the developing brain due to a single short term exposure to alcohol *in utero* may be caused by alcohol's ability to prime these cells toward a differentiated state.

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INTRODUCTION

Fetal Alcohol Syndrome

The ingestion of alcohol during pregnancy can lead to a number of disturbances in growth and development of the fetus with very consistent manifestations termed Fetal Alcohol Syndrome (FAS) (Jones *et al.*, 1973). It has been estimated that one out of every thousand babies born will be severely affected by FAS, while four to five out of every thousand babies born will be moderately affected with FAS (Sulik *et al.*, 1988). The less severe manifestations of FAS are termed Fetal Alcohol Effects (FAE) (Sulik *et al.*, 1988). A common misconception is that FAS occurs only if the mother drinks consistently throughout pregnancy. However, this observation is not supported by the results of a number of case studies (Smith *et al.*, 1990). It has been documented that ingestion of as few as 2 glasses of beer during the period of rapid brain development can cause significant mental retardation and birth defects (Smith *et al.*, 1990).

There appear to be many factors that govern whether a baby will develop FAS / FAE or not. The variability of the dysmorphologies may be due to genetic susceptibility of both the mother and fetus to the effects of alcohol (Smith *et al.*, 1990). Overall nutritional status of the mother, her pattern of drinking and her age during fetal gestation also seem to correlate with the severity of the defects seen in her offspring (Smith *et al.*,

1990). Dose-dependent as well as fetal chronological age-dependent factors correlate with the severity of the birth defects observed (Smith *et al.*, 1990). The duration of alcohol exposure and the concentrations of alcohol that the fetus is exposed to, will also govern the severity of FAS (Smith *et al.*, 1990). Since there are a number of factors that come into play, it is often difficult to predict the outcome that alcohol exposure will have on a particular fetus.

There are two drinking patterns associated with clinical manifestations of FAS. One type occurs with chronic exposure to ethanol in which the cells become adapted to the effects of ethanol, while the other type occurs with the consumption of large quantities of ethanol for a short period of time (Smith *et al.*, 1990). Both drinking patterns can cause a vast number of birth defects to occur in the developing fetus (Smith *et al.*, 1990). The clinical manifestations of FAS / FAE are varied and have been associated with many structural and organ deformities. Investigation of FAS / FAE has utilized case studies observed in hospitals to describe the syndrome and animal models to reproduce it.

Many structural dysmorphologies have been shown to be related to prenatal exposure to ethanol. There are several craniofacial anomalies which are highly associated with FAS (Jones *et al.*, 1973; Hanson *et al.*, 1976: Jones and Smith, 1973). Small heads with narrow foreheads, short palpebral fissures, small noses, small midfaces, and long upper lips with deficient philtrums are all common abnormalities (Sulik *et al.*, 1988). Most of the FAS babies also exhibit low birth weights and retarded linear growth (Jones *et al.*, 1973; Gottesfeld *et al.*, 1990). Other skeletal abnormalities are also observed. Many FAS children have various joint anomalies and exhibit minimal allowable movement

of the elbow joint, various degrees of hip dislocations and deformities of the interphalangeal joints (Jones *et al.*, 1973).

The growth and development of many organ systems are impaired by prenatal exposure to ethanol. Various forms of congenital heart disease have been observed in FAS / FAE babies. Disturbances to the vasculature itself has also been noted to occur (Ruckman, 1990). The eyes are usually small, closely set and slant downward. Short palpebral fissures are a common finding in FAS babies and in the majority of mice models (Sulik and Johnston, 1983). There have also been reports of either hypoplasia or aplasia of the optic nerve in FAS cases studied (Sulik and Johnston, 1983). The development of the immune system, including both T-cells and B-cells, is impaired in some FAS children (Ammann *et al.*, 1982). In addition, decreased levels of parathyroid hormone which leads to hypocalcemia and hyperphosphatemia, have been observed in four children (Ammann *et al.*, 1982).

Generally, the system that suffers the most damage from ethanol exposure *in utero* is the developing nervous system (reviewed in: Hammer, 1986). FAS children show various degrees of mental retardation, decreased motor skills, increased sensitivity to motor stimuli and many social / behavioral problems (Jones *et al.*, 1973; Jones and Smith, 1973). The severity of brain malformation in FAS babies is often predicted by the degree of underdevelopment of the intermaxillary tissue (Sulik and Johnston, 1983). In general, the more intermaxillary tissue that is missing, the more severe brain damage that is observed (Sulik and Johnston, 1983).

Autopsies performed on FAS children who died shortly after birth showed various degrees of abnormal brain morphology such as; microcephalus, hydrocephalus, cerebral dysgenesis, ventricular enlargement, holoprosencephalus, cerebellar hypoplasia, fusion of the third ventricle, and absence of the olfactory bulbs and corpus callosum (Clarren *et al.*, 1978). Even in mild FAE cases, there are signs of microcephaly which have been associated with impaired development of the brain (Sulik and Johnston, 1983). Whether or not all of these children will exhibit the observable signs of mental retardation is not known.

The vast majority of the research being conducted to elucidate the detrimental effects ethanol upon the developing brain utilizes rat and mouse models. The results of these studies are hard to compare to human development, or to each other, due to use of different methods, inconsistency of blood alcohol concentrations achieved, dose of alcohol given to animals, and species / strain differences (West, 1987). Furthermore, it is very difficult to maintain constant levels of consumption of alcohol by the pregnant animals and this often makes results hard to compare among one another. There are many strains of rats and mice used in research studies which, due to genetic differences, may vary in their ability to metabolize ethanol that is consumed (West, 1987).

Data obtained utilizing rat or mouse models are difficult to extrapolate to humans due to the developmental differences between species. Rats and mice are born at the end of the second trimester when compared to human gestational times (West, 1987). One period of rapid brain growth in humans occurs during the third trimester, while this occurs during the first ten days of postnatal growth in the rat (West and Pierce, 1986). The

degree of microencephaly induced in third trimester equivalent treated rats is dose dependent with the higher doses of alcohol producing greater effects (West and Pierce, 1986).

Microcephaly and microencephaly are common developmental defects observed in FAS babies and in prenatally and early postnatally ethanol exposed rats and mice (West and Pierce, 1986). It has been suggested that the resultant small brain size may, in part, be due to reduced numbers of neurons and supporting cells within the brain (West and Pierce, 1986). In order to truly understand the adverse effect of ethanol on the developing brain, it is necessary to explore what is occurring on a cellular level.

Many cellular changes have been observed in developing neurons exposed to ethanol. However, little is known about the underlying mechanisms which control neuronal cell differentiation. A specific period during fetal development in which the embryo is more sensitive to alcohol-induced effects has not been precisely determined. Studying differential development of various brain areas is difficult due to the vast differences in the time periods of rapid growth spurts within each area of the brain (West and Pierce, 1986). For example, development of the cerebral cortex occurs at a different gestational time than does the development of the hippocampus or the cerebellum (West and Pierce, 1986). There are also many different types of cells in the brain, each developing at different periods. Furthermore, alcohol may not affect each of the developing cell types to the same degree (West and Pierce, 1986). Moreover, alcohol may alter the secretion, expression or response to neurotrophic factors by a given cell type.

Thus, the effects of ethanol on discrete populations of differentiating neuronal cells are currently under study but further investigation is warranted.

The use of a homogeneous population of cells would allow for study into the underlying mechanism(s) by which alcohol may affect neuronal cell growth and differentiation. Pheochromocytoma (PC12) cells are an established cell line which undergo differentiation into sympathetic-like neurons after nerve growth factor (NGF) stimulation (Burstein and Greene, 1978). Alcohol potentiates NGF-induced neurite extension in these cells (Wooten and Ewald, 1991; Messing *et al.*, 1991a). In this study, PC12 cells will be utilized as a model system to evaluate the time frame during which alcohol can alter neurite outgrowth. In specific, experiments were undertaken to determine: 1) whether there is a specific effect of alcohol on NGF receptor binding parameters; 2) whether there are differences in NGF-induced neurite extension based upon the window during which alcohol was administered and relative timing of NGF exposure; 3) whether alcohol exposure alters PC12 cell proliferation; 4) whether culture of PC12 cells in the presence of alcohol changes PC12 cell cycle progression; and 5) whether alcohol enhances NGF effects through a ganglioside dependent mechanism. Taken together, results from this study provide information regarding specific time periods during which alcohol exposure has the ability to alter PC12 cell growth and differentiation. These studies also elude to possible mechanism(s) by which alcohol has the ability to modulate NGF-induced neurite extension as well as a tolerance or adaptation effect of PC12 cells to alcohol exposure.

II. REVIEW OF LITERATURE

Development of the Central Nervous System

The development of the central nervous system has been studied intensely for many years and the information accumulated is vast. For this reason, much of the information presented here was obtained from textbooks on embryology and brain development.

The central nervous system begins as a layer of ectodermal cells in the dorsal aspect of the developing embryo. The primitive mesodermal notochord pushes underneath the layer of ectoderm and causes the cell layer to thicken. The cells are now called neuroectoderm. This induction process continues until the thickened neuroectodermal cells form the neural tube (reviewed in: Carlson, 1981). After closure of the neural tube, the cranial portion becomes markedly enlarged to form the rudimentary brain. Cell division occurs within the central portion of the neural tube to form the neuroepithelium. As the neuroepithelium proliferates, some of the daughter cells become neuroblasts. These precursor cells migrate towards the outer limiting membrane, the rudimentary meninges. These cells will become neurons and various brain nuclei (reviewed in: Carlson, 1981).

As the neural tube begins to close, the lateral edges of the neuroectoderm come in contact with the overlying nonneural ectoderm. The combination of these cells form

bundles which are known as neural crest ectoderm. These cells migrate to form parasympathetic, sympathetic, dorsal root and sensory ganglia. They also develop into the pharyngeal pouches which are responsible for forming many structures in the adult. The cranial division of the neural crest ectoderm in the developing embryo has the capacity for differentiation into the sensory roots of cranial nerves, cranial parasympathetic ganglia, and also certain tissues of the oral cavity (reviewed in: Carlson, 1981). Thus, proper proliferation, migration and differentiation of these cells is important in the overall development of the nervous system and other organs.

Ethanol has adverse effects on the proliferation, survival and migration of neural crest cells in mice prenatally exposed to ethanol (Webster *et al.*, 1983). In early fetal life, ethanol inhibits the production of neural crest cells; thus, the fetus never develops the complete compliment of cells required for proper development. Later in fetal life, ethanol causes neural crest cells to decrease migration, degenerate and die (Webster *et al.*, 1983). Multiple exposures will cause combined defects within the neural crest cell populations which may lead to a variety of organ defects (Webster *et al.*, 1983). Neural crest cells are responsible for the development of many organ systems and craniofacial tissues and thus impairment of their development may be associated with many of the clinical manifestations of FAS.

Although the human brain continues to grow and develop after birth, the architecture is well established by week 11 of human gestation. FAS is associated with varying degrees of mental retardation that are thought, in part, to be brought about by ethanol's interactions with the developing neurons. The growth spurts of various neuronal

populations occur at different gestational periods which makes it difficult to study these effects. Vast research documents that alcohol affects the appearance of the cerebral cortex, the number and appearance of pyramidal neurons in the cerebral cortex and hippocampus, and growth of the cerebellum which will now be further discussed.

The cerebral cortex has numerous functions. This brain region is composed of cell layers which are laid down during the early stages of development. The first neuroblasts to reach this area will migrate to their intended area and extend long processes which will become covered with spines. Other neuroblasts will continue to migrate into this area, push past the ones that arrived earlier and subsequently build the layers of the cerebral cortex (Angevine and Sidman, 1961; Berry and Rogers, 1965). The efferent axons project from the cortex to other areas of the brain for communication (reviewed in: Carlson, 1981). Also during this early period of development, dendritic extensions from the thalamus, corpus callosum and other centers of the brain will begin to grow and communicate with the cerebral cortex (reviewed in: Carlson, 1981). The development of both extracortical and intracortical circuits appears to play a critical role in the overall appearance of the fully developed cerebral cortex (Marin-Padilla, 1978; Pinto-Lord and Caviness, 1979). If the proper connections are not achieved, cell development will be altered.

The cerebral cortex architecture is disorganized and folded following exposure to ethanol on gestational days 14 and 15 in rats (Kolkoskie and Norton, 1988). There is also a delay in cortical lamination and myelination in the sensorimotor part of the cerebral cortex in rat pups exposed to alcohol prenatally (Jacobson *et al.*, 1979). However,

myelination appeared normal at 23 days after birth which suggests that regeneration is possible when the insult is removed (Jacobson *et al.*, 1979). Prenatal exposure to ethanol delayed the generation of cortical neurons by 1 to 2 days and prolonged the time required for neuronal generation (Miller, 1988). Neurons which were generated early in development were found to be smaller in ethanol treated foeti compared to control foeti. Neurons which were generated later, were of normal size but occurred in abnormal positions within the cerebral cortex (Miller, 1988).

The subventricular and ventricular zones of the cerebral cortex are also directly affected by prenatal ethanol exposure (Miller, 1988). On gestational day 21 in rats, ethanol causes an increase in cells appearing in the growth fraction of the subventricular zone of the cerebral cortex, while not affecting the appearance of cells in the same fraction of the ventricular zone (Miller and Nowakowski, 1991). Conversely, the number of proliferating cells within the ventricular zone is decreased (Miller and Nowakowski, 1991). This observation correlates well with previous studies which document that the ventricular zone is significantly thinner with a lower cell packing density in pups prenatally exposed to ethanol (Miller, 1988).

Pyramidal neurons are found in layers II, III, and IV of the somatosensory area and layer V of the motor area of the cerebral cortex (reviewed in: Carlson, 1981). These pyramid shaped neurons contain extensive arrays of dendrites and dendritic spikes utilized for receiving information from other brain areas. During early axonal formation of pyramidal neurons, long entangled spines with synaptic endings protrude from the primary axon. These spines are thought to be responsible for communicating with the neuropil

(Peters and Kaiserman-Abramof, 1970; Purpura, 1975). Once all of the proper connections are made, then the long spines are selectively removed and replaced by mushroom like spikes. At birth, the only areas of these neurons that still contain the long entangled spines are at the most distal portions (Purpura, 1975). Collateral extensions from pyramidal cells will grow to supply intracortical circuits (reviewed in: Carlson, 1981).

The dendritic systems of pyramidal neurons in layer V of the rat cerebral cortex are less extensive in alcohol treated animals than in control animals at birth (Hammer and Scheibel, 1981). Dendrites of the pyramidal neurons of alcohol exposed rat pups also appear twisted, contain less spines and are thinner than control pups (Hammer and Scheibel, 1981). Pyramidal neurons of prenatally alcohol-exposed rat pups still appear long and entangled even at 40 days after birth. In contrast, pyramidal neurons of pair-fed control animals exhibited normal short mushroom like protrusions (Stoltenburg-Didinger and Spohr, 1983). These results indicate a delay exists in maturation of these neurons when the pups are prenatally exposed to alcohol. If improper connections are made, or if these connections are delayed, then it may cause the development of these pyramidal cells to also be delayed. In theory, the interconnectivity of the cerebral cortex may be altered if dendritic systems are not sufficiently developed to sustain synaptic contact (Hammer and Scheibel, 1981). In addition, these abnormalities in dendritic spine appearance and development are very similar to changes observed in mentally retarded children (Marin-Padilla, 1972; Purpura, 1975).

The hippocampus contains three layers, one of which contains pyramidal cells. The hippocampus resembles a ram's horn and is often called the *cornu ammonis*. The separate regions of the hippocampus are designated CA1 through CA4 (Reviewed in: Berne and Levy, 1988). The layer containing pyramidal cell bodies extends to a hilus surrounded by the dentate gyrus containing granule cells (reviewed in: Carlson, 1981; reviewed in: Berne and Levy, 1988). Development of the hippocampus and dentate gyrus are interdependent. Cell migration to the hippocampus occurs in relatively the same manner that it occurs within the neocortical portion of the cerebral cortex (Nowakowski and Rakic, 1979). Much of the dentate gyrus continues to develop after birth and even into adult life in the rat (Bayer, 1982). The dendritic development of granule cells of the dentate gyrus occurs after their migration into the area along the plial surface of the hippocampal fissure (Bayer, 1982). The projections from the granule cells will form input tracts to the pyramidal cells through mossy fibers (reviewed in: Berne and Levy, 1988).

The developing hippocampus has also been studied to examine the effects that alcohol exposure has on these neuronal populations. In mice, the number of pyramidal cells within the hippocampus are decreased following prenatal ethanol exposure (Barnes and Walker, 1981). Hippocampal pyramidal neurons also exhibit decreased numbers of dendritic spines which are shorter in length than those of pair-fed foeti. On the other hand, the number of granule cells of the dentate gyrus, which are intimately involved in development of these pyramidal cells did not decrease (Barnes and Walker, 1981). Behavioral testing of these mice showed them to have a decreased functional capacity in a shock avoidance test as adults (Barnes and Walker, 1981). Therefore, the mental

retardation and decreased motor skills present within FAS children may be explained, in part, by lingering developmental defects within the brain regions affected by prenatal alcohol exposure.

Some populations of pyramidal cells localized within the hippocampus in alcohol-exposed rat foeti appear to be adversely affected while others are not. Exposure to alcohol during gestational days 10-21 ensures that both the CA1 and CA3 pyramidal cells are exposed to ethanol during their period of rapid growth (Bayer, 1980). Prenatal exposure during this time period resulted in a decrease in the number of CA1 cells, but not CA3 cells, in adulthood (West and Pierce, 1986). Artificially reared pups exposed to alcohol from postnatal days 4-10 show a decrease in the number of CA4 cells present, while they exhibit an increase in the number of granule cells of the dentate gyrus (West and Pierce, 1986). Because different pyramidal cell populations are affected only at specific periods of alcohol exposure, these results indicate the importance of timing of ethanol exposure on the developing brain.

Granule cells of the dentate gyrus form the mossy fibers of the hippocampus and form the major excitatory intrahippocampal pathway by innervating the pyramidal cells. These connections are required to ensure proper development of the pyramidal cells (reviewed in: Berne and Levy, 1988). Rats exposed to ethanol throughout gestation exhibited abnormally distributed hippocampal mossy fibers compared to pair-fed individuals. The same abnormal distribution is observed even in adulthood (West *et al.*, 1981). The mossy fibers present in the ethanol-treated rats displayed more extensive and aberrant branching of fibers. In addition, the fibers extended into areas in which they are

not normally found (West *et al.*, 1981). One of the areas where the mossy fibers appeared more dense than normal was at the level of the CA3 pyramidal cells. These aberrant fibers appeared to be a continuation of the normal rostral infrapyramidal band. Small dark granules were also observed within the infrapyramidal area which were not observed in the pair-fed animals (West *et al.*, 1981).

The mossy fibers are the only known functional output of the granule cells of the dentate gyrus and if these are altered, it may indicate that there could be functional abnormalities present within the hippocampus. Since this area of the brain is thought to be partly responsible for memory in humans, it is possible that an alteration of the hippocampus may be linked to the mental retardation observed in children with FAS.

In the rat, cerebellar cortex development is rudimentary at birth and continues growth during the early postnatal period, which is equivalent to the third trimester of human gestation (Hamre and West, 1993). The orientation and branching that occurs in Purkinje cells of the cerebellar cortex are dependent upon afferent neuronal connections from the olivary climbing fibers and granular cell fibers. Immediately after these connections are made, the Purkinje cells rapidly undergo extensive dendritic arborization (Hamre and West, 1993).

Direct changes in the neurons in the cerebellar cortex, including Purkinje cells and granular cells, are affected by prenatal alcohol exposure (Hamre and West, 1993). Purkinje cells, which undergo proliferation just prior to birth, show a decrease in number and increase in cell diameter. Thus, alcohol may also initiate neuronal cell death within this population of cells. Purkinje cell populations formed earlier were more affected than

the more recently formed populations (Hamre and West, 1993). Prenatal exposure of mice to ethanol causes a reduction in the length of the nonphosphorylated neurofilaments in both primary and secondary dendrites of Purkinje cells during early postnatal stages of development (Poltorak *et al.*, 1990). These results indicate that there is a delay in development of these Purkinje cells. Thus, it appears that ethanol exposure during gestation is affecting Purkinje cell growth and development in more than one manner.

Postnatal vapor inhalation of alcohol results in permanent neuronal deficits and decreased cerebellar growth in rats (Bauer-Moffett and Altman, 1975, 1977). There appears to be a window of time occurring between postnatal days 4 through 7, where ethanol exposure has a more profound effect on the loss of Purkinje cells (Hamre and West, 1993). Although the loss of Purkinje cells and granular cells were not equivalent within the 10 lobules of the cerebellar vermis, the patterns of cell loss were similar. Postnatal alcohol exposure in rats causes a decrease in granule cell numbers only when there is a corresponding loss of Purkinje cells (Hamre and West, 1993). Therefore, granular cell loss may be due to the loss of their target cells, the Purkinje cells. Dendritic hypertrophy has also been observed following postnatal alcohol exposure in Fisher rats (Pentney and Quackenbush, 1990).

The presence or absence of neurotropic factors in the developing brain further complicates interpretation of data obtained using rat and mice models to study ethanol's effects on neuronal differentiation. The actual processes that must occur to incite the migration of the neuroblasts, the differentiation of the neuroblasts into neurons, and the mechanisms which control the amount of dendritic branching are still being investigated.

The study of neuronal proliferation, migration and differentiation of the CNS is also difficult because of the interactions between the numerous cell types and their dependence on a variety of neurotrophic factors.

Neurotrophic Factors:

Neurotrophic factors are endogenous freely diffusible molecules that influence the growth, development and general maintenance of neurons (as defined by: Hefti *et al.*, 1989). The molecular mechanisms and direct *in vivo* actions of the known neurotrophic factors are still not completely understood. Neurotrophic factors may be necessary for proper directed axonal projection. The developing growth cone may seek the neurotrophic factor by a chemotactic-like mechanism (Gundersen and Barrett, 1979). Also, in the developing nervous system, a large excess of neurons are produced such that up to 80% of a given population of neurons will undergo degeneration at the time of synaptogenesis. Competition for neurotrophic factor at the target area is thought to be the cause of this degeneration (Oppenheim, 1989). During the past decade, many neurotrophic factors have been identified. Their mechanisms of action, time course for their effects and the precise *in vivo* neuronal populations they affect are still under study. Some of these factors and the *in vitro* neuronal populations they support are briefly described.

Nerve growth factor (NGF) was the first neurotrophic factor discovered and until recently, it was the only factor that met all the requirements to be designated a true neurotrophic factor (Levi-Montalcini, 1987). This was the only factor known that

influenced growth, development and maintenance of nerve cells (Levi-Montalcini, 1987). Because NGF was so specific for the neuronal populations that it affects, it was postulated that there must be other neurotrophic factors that affect other neuronal populations. There are now a whole family of neurotrophic factors which have been isolated that are related to NGF, which have been termed the neurotrophins. This family includes NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and ciliary neurotrophic factor (CNTF) (Yancopoulos *et al.*, 1990).

Brain derived neurotrophic factor (BDNF) was initially purified from pig brain (Barde *et al.*, 1987). This is produced in the brain and is not widely distributed in the peripheral nervous system. This neurotrophic factor appears to have a survival promoting activity on neurons that have a projection in the CNS (Barde *et al.*, 1987). There is some overlap between the neuronal targets for BDNF and NGF. However, the period of development at which these populations are affected by these two neurotrophic factors do not overlap. It is postulated that NGF may be important at early stages of development and then BDNF takes over at a later stage of development (Yancopoulos *et al.*, 1990).

Neurotropin-3 (NT-3) is produced mainly in peripheral nervous tissues and is necessary for the survival of cultured sympathetic neurons and peripheral sensory neurons (Hohn *et al.*, 1990). Nerve growth factor, BDNF and NT-3 have about 50% homology in their amino acid sequence. A reciprocal relationship exists between the expression of BDNF and NT-3 during development of the nervous system (Yancopoulos *et al.*, 1990). It was shown that NT-3 is expressed at remarkably high levels during critical periods in

early neuronal development. After this initial period, NT-3 expression in the CNS decreases whereas BDNF expression increases (Yancopoulos *et al.*, 1990).

Ciliary neurotrophic factor (CNTF) was purified from embryonic chick eye tissue (Barbin *et al.*, 1984). This neurotrophic factor supports the survival of parasympathetic neurons, ciliary ganglia, neural crest derived sensory neurons, and sympathetic ganglia (Barbin *et al.*, 1984). Two forms of CNTF have been described which are present in both chick and rat tissues (Rudge *et al.*, 1987). The highest levels of CNTF expression occur in the eye and in peripheral nerves. Lower levels of CNTF are found in brain and peripheral tissues (Rudge *et al.*, 1987).

There are a number of serine protease inhibitors that have been shown to contain neurotrophic factor-like activity. Cultured rat sympathetic ganglia secrete a urokinase-like protease which blocks further neurite outgrowth. However, if these cells are co-cultured with heart cells, then neurite extension proceeds (Pittman *et al.*, 1987). It has been shown that the heart cells secrete a protease inhibitor which will block the activity of the urokinase protease and thus allow neurite extension (Pittman *et al.*, 1987). Glial derived nexin (GDN), which has a protein sequence similar to known serine protease inhibitors, will promote neurite outgrowth from both neuroblastoma cells and cultured sympathetic neurons (Guenther *et al.*, 1985). GDN is highly expressed within the olfactory system where it is thought to be the main regulatory factor for the rapid turnover of neurons within this system (Reinhard *et al.*, 1988).

The importance of neurotrophic factors and their functions during growth, differentiation and maintenance of neuronal populations within the nervous system is still

being evaluated. Since alcohols potentiate NGF-induced neurite extension in PC12 cells, it may be that the adverse effects of alcohol on developing neuronal structures is due to an interaction between alcohol and neurotrophic factors.

Nerve Growth Factor: History and Biological Properties

Nerve growth factor is the key neurotrophic agent which enhances neuronal differentiation and plays a role in survival of mature neurons. The presence of a neurotrophic agent was first eluded to in 1948 when Bueker grafted fragments of a mouse sarcoma into chick embryos. He discovered sensory nerve fibers had grown into the neoplastic tissue from adjacent dorsal root ganglia. He concluded that the tumor provided a suitable environment for the induction of neurite outgrowth (Bueker, 1948).

Later, Hamburger and Levi-Montalcini repeated these experiments and found that sympathetic and sensory neurons innervated the tumor (Levi-Montalcini and Hamburger, 1951). Furthermore, the nerve fibers present in the tumor were randomly branched and did not contain synaptic endings. With further studies of incorporation of the mouse sarcoma into chick embryos, they found that viscera such as the kidneys, thyroid, parathyroid, and spleen which are not normally highly innervated, became very well innervated (Levi-Montalcini, 1952).

At this point, it was believed that there was some soluble factor present in these tumors that was acting as a neurotrophic agent. Subsequent experiments by Cohen and Levi-Montalcini showed that snake venom and mouse submandibular salivary glands also contained this substance that could cause sensory neurons to grow and differentiate into

bundles of dendrites (Cohen, 1960). This was surprising to them, since these preparations were from completely normal animals.

Nerve growth factor from mouse submandibular glands, snake venom and humans was purified, sequenced and cloned. There is a high degree of homology between all forms. Mouse submaxillary gland NGF has a molecular weight of 130,000 daltons and is composed of three subunits arranged stoichiometrically as $\alpha_2\beta\gamma_2$ (Varon *et al.*, 1967). The α and γ subunits are about 26,000 daltons. The α subunit is not known to possess any biological activity (Varon *et al.*, 1967). The γ subunit is an arginine specific esteropeptidase that appears to function during processing of pro-NGF (Theonen and Barde, 1980). The β subunit is responsible for the biological properties of NGF (Greene *et al.*, 1971). This subunit is composed of two identical peptide chains which are non-covalently linked. Each monomer contains 118 amino acids and has a molecular weight of 13,259 daltons. The β subunit has a structural similarity to the insulin family of peptides (Frazier *et al.*, 1972; Bradshaw, 1978).

Cell types that interact with NGF include many neural crest derivatives, CNS neurons and cells of nonneural origin. Neural crest cells such as sympathetic neurons, paraganglia cells, SIF cells and chromaffin cells are all dependent on NGF in early development. CNS neurons that require NGF include cholinergic, adrenergic, indoleaminergic and peptidergic neurons. Mast cells are nonneural in origin but still interact with NGF (Levi-Montalcini, 1987). In general, cells are maximally responsive to NGF during differentiation and their NGF requirement for survival decreases as the cells mature (Levi-Montalcini, 1987). NGF has been shown to have at least three major roles

in cellular physiology. It has the ability to act as a trophic factor in early development, as a differentiation inducer to begin neurite extension and as a guide for growing or regenerating neurons along its own concentration gradient (Gunderson, 1980; Campenot, 1982).

The Effects of NGF on Cell Growth and Differentiation

NGF is now known to be required for the growth, development, survival and maintenance of many different cell types and areas of the developing embryo (Levi-Montalcini, 1987). NGF and its receptor has been localized to numerous areas of the developing brain and central nervous system by using specific antibodies, *in situ* hybridization and cDNA specific probes (Lu *et al.*, 1989; Lauterborn *et al.*, 1990; Escandon and Chao, 1990; Ojeda *et al.*, 1991). It appears that NGF is necessary in the brain for maintenance and differentiation of cholinergic neurons (Lu *et al.*, 1989; Lauterborn *et al.*, 1990). The majority of cholinergic neurons in the brain are located in the hippocampus and the neocortex (Ojeda *et al.*, 1991). There are now thought to be several populations of neurons in the developing brain that are sensitive to NGF.

Ojeda *et al.*, (1991) hypothesize that NGF may play a broader role in neuronal development in the brain than originally described. This hypothesis is supported by the finding of mRNA's for NGF and NGF-receptors in many areas of the brain that do not contain neurons that are known to be NGF sensitive (Koh *et al.*, 1989). mRNA for NGF has been found in high concentrations in the diencephalon, the postnatal hypothalamus and the olfactory bulb (Lu *et al.*, 1989). These areas have receptors for NGF during prenatal

life but lack these receptors after birth (Lu *et al.*, 1989). Also, during fetal development, almost all areas of the brain have been shown to exhibit mRNA for both NGF and NGF-receptors (Lu *et al.*, 1989). Thus, it has been postulated that in the central nervous system, NGF may act at both local and distant sites during development but later in life it may only act through distant sites for maintenance (Lu *et al.*, 1989).

Extensive information has been generated regarding the mechanism of action of NGF by utilizing an established cell line known as PC12 cells (Mutoh and Guroff, 1989; Vetter *et al.*, 1991; Burstein and Greene, 1978; Garrels and Schubert, 1979). PC12 cells are an established cell line that are derived from a transplantable rat adrenal pheochromocytoma (Greene and Tischler, 1982). When these cells are grown in NGF-free media, they have a morphological appearance of embryonic rat adrenal chromaffin cells and undergo cell division every 48 to 72 hours. These cells respond to NGF by differentiating into cells that resemble sympathetic neurons. PC12 cells are capable of synthesizing, storing, and releasing catecholamine when appropriately stimulated (Fujita *et al.*, 1989; Greene and Tischler, 1976). In addition, fully differentiated PC12 cells also have the capability to generate and transmit active electrical impulses (Greene and Tischler, 1982).

Pheochromocytoma (PC12) cells halt proliferation and begin to undergo neuromorphogenesis within 24 hours upon exposure to NGF. Within seconds after addition of NGF to PC12 cell cultures, a ruffling of the cytoplasmic membrane along with formation of coated pits is observed (Greene and Tischler, 1982). Fluorescent labeling revealed that NGF becomes clustered on the cell membrane at the sites of the coated pits

(Greene and Tischler, 1982). After 24 hours of exposure to NGF, the cells begin to flatten and show cytoplasmic spikes which will become neurites (Greene and Tischler, 1982). After one week, about 90 percent of the cells exposed to NGF will possess neurites (Greene and Tischler, 1982).

Burstein and Greene (1978) have studied the difference between generation of neurites and regeneration of neurites in PC12 cells. Generation of neurites occurs within the first 24 hours after exposure to NGF. mRNA synthesis is necessary for novel extension of neurites (Burstein and Greene, 1978). Removal of neurites can be achieved either by mechanical means or by the withdraw of NGF before full differentiation has occurred (Burstein and Greene, 1978). Re-exposure to NGF, stimulates the process of neurite regeneration which occurs without synthesis of mRNA (Burstein and Greene, 1978).

The exposure of neurons or PC12 cells to NGF causes the cells to stop proliferation and begin the process of differentiation. When cells exit the cell cycle to undergo differentiation, they do so after mitosis and usually before entering G_1. This exit is labeled G_0 and the cells may remain in G_0 for the duration of the cells' life (Pardee, 1989). NGF acts on synchronized PC12 cells by allowing them to complete the synthesis (S) phase and mitotic (M) phases of the cell cycle before inducing the process of differentiation (Rudkin *et al.*, 1989). PC12 cells exposed to NGF exit the cell cycle in the G_1 phase to begin the differentiation process (Rudkin *et al.*, 1989). NGF acts on cells in the S and G_2 phase by allowing them to complete their cycle and then exit the cell cycle to undergo differentiation (Rudkin *et al.*, 1989). Thus, the initial proliferative effect that has

been reported by NGF on PC12 cells (Burstein and Greene, 1982), may be due to the completion of the cell cycle of the cells not already in the G_1 phase.

The Effects of Ethanol on Cell Growth and Differentiation

The effect that ethanol has on cell growth and differentiation has been studied for a number of different cell types. Prenatal exposure to alcohol has a profound effect on the numbers of neurons within the neocortex, hippocampus and cerebellum as discussed previously. Proliferation of cells within the ventricular zone (VZ) is decreased causing a thinner and less densely packed VZ (Miller and Nowakowski, 1991; Miller, 1989). There was no alteration in the length of the S or M phase of the cell cycle and thus it is postulated that the increased length of the cell cycle is due to a delay in either G_1 or G_2 (Miller and Nowakowski, 1991).

Ethanol and other short chain alcohols which interact specifically with the cell membrane can promote cells to halt proliferation and arrest cell growth (Cook *et al.*, 1990; Miller and Nowakowski, 1991). For example, ethanol will cause differentiating HL-60 cells to become arrested in the G_1 phase of the cell cycle (Cook *et al.*, 1990). Furthermore, ethanol exposure in the presence of a differentiation inducer causes G_1 arrest representing terminal commitment to differentiation. HL-60 cells exposed to ethanol in the absence of an inducer causes the G_1 arrest to be reversible (Cook *et al.*, 1990).

Ethanol has also been shown to induce growth inhibition in Friend erythroleukemia stem cells (Cook and Keiner, 1991). Cell cycle analysis of these cells also indicates G_1 arrest evident within a few hours after ethanol exposure. Removal of ethanol from these

cells returns cell cycle progression to normal within a few hours (Cook and Keiner, 1991). The C6 cell line, which is derived from a rat astrocytoma, also exhibits an alcohol dose dependent decrease in cell growth (Isenberg *et al.*, 1992).

A decrease in the number of PC12 cells occurs in an alcohol dose dependent fashion (Pantazis *et al.*, 1992). Whether the decrease in cell number is attributed to an alteration in the cell cycle or due to increased cell death is not known. Treatment of PC12 cells with NGF, either prior to or simultaneously with, ethanol exposure will diminish this ethanol-induced reduction in cell number (Pantazis *et al.*, 1992). Therefore, it appears as if NGF is acting through some mechanism to enhance cell survival. Although it has been demonstrated that ethanol will cause a decrease in cell number of PC12 cells in culture, if and when the cell cycle is affected is as of yet unknown.

PC12 cells also provide a well defined model for studying ethanol's effects on neuronal differentiation. PC12 cells exposed to suboptimal NGF and to various short chain alcohols undergo enhanced neuritogenesis. Alcohol stimulates early, rapid cellular differentiation and acquisition of more, longer and highly branched neurites (Wooten and Ewald, 1991; Messing *et al.*, 1991a). A correlation exists between the alcohol's ability to interact with the membrane and the overall effect on neuromorphogenesis. The greater the ability of the alcohol to interact with the membrane, the greater the synergistic effect that alcohol has with NGF on the cell (Wooten and Ewald, 1991). It has not yet been determined whether this enhancement is due to more NGF receptors being exposed within the cell membrane, whether binding parameters of NGF to its receptor are altered or if this is occurring by some alternate mechanism unrelated to the NGF receptors.

NGF Receptors

Two classes of NGF receptors exist on neurons, sympathetic ganglia and PC12 cells (Ojeda *et al.*, 1991; Escandon and Chao, 1990; Kouchalakos and Bradshaw, 1986; Buxser *et al.*, 1990; Sutter *et al.*, 1979). The first is a high-affinity receptor exhibiting slow dissociation kinetics, while the other is a low-affinity receptor which has fast dissociation kinetics. Reported values for these receptors on PC12 cells indicate that for the low-affinity receptor, there are between 10^4 and 10^7 receptors per cell with an affinity in the nanomolar range. The high-affinity receptor form occurs in the range of 10^3 to 10^5 receptors per cell and possesses an average affinity within the 0.1 to 0.01 nanomolar range (Sutter *et al.*, 1979).

There are two current hypotheses surrounding the two types of NGF receptors (Schechter and Bothwell, 1981; Buxser *et al.*, 1990). Data from one group supports the existence of two receptor populations (Schechter and Bothwell, 1981), while other evidence indicates that only the low-affinity receptor occurs which undergoes conversion to a high-affinity form after the binding of NGF (Buxser *et al.*, 1990; Landreth and Shooter, 1980). Landreth and Shooter (1980) imply that naive PC12 cells which have never been exposed to NGF, display only the low-affinity receptor. After a brief lag period during NGF exposure, the cells begin conversion of the low-affinity receptor to a high-affinity form. However, others have not been able to determine a lag phase and provide data to demonstrate that naive PC12 cells actually contain both receptor forms (Schechter and Bothwell, 1981).

Schechter and Bothwell (1981) document two classes of NGF receptors in PC12 cells which can be designated fast (low-affinity) and slow (high-affinity) due to the kinetics exhibited by each type. Both types of receptors are present prior to stimulation with NGF and more fast receptors are present than slow receptors (Schechter and Bothwell, 1981). The fast receptors are distinguished from the slow receptors due to their greater rate of association and dissociation with NGF (Schechter and Bothwell, 1981). Weskamp and Reichardt (1991) have also demonstrated two forms of NGF receptors on PC12 cells and have determined that only the high-affinity receptor is responsible for biological activity. This fact is alluded to with the PC12 clonal line lacking the high-affinity receptor because these cells are unable to undergo morphological differentiation (Green and Greene, 1986).

On the other hand, Buxser *et al.*, (1990) provide evidence to demonstrate that there is really only one class of NGF receptor. They document that fast low-affinity binding is followed by slow high-affinity binding and internalization of the receptors. Also, location of the receptor may influence whether it behaves as a low-affinity or a high-affinity receptor. For example, they suggest that the slow receptor is related to internalization and sequestration; while the fast receptor is free on the cell membrane. They have shown that the fast and slow receptor forms can interconvert by utilizing agents that are capable of changing the positions of receptors within the cells.

The number of NGF cell surface receptor forms present on the cell surface has been further addressed by Kasaian and Neet (1988). Although internalization of the NGF receptor does occur, the amount of receptors internalized is not equal to the amount of high affinity receptors and therefore, there must be two distinct NGF receptor pools on

the cell surface. They actually describe five distinct pools of NGF receptors: 1 and 2) two distinct receptor forms on the cell surface; 3) slow receptors within the cell interior; 4) those receptors within lysozymes and; 5) those that are attached to the cytoskeleton (Kasaian and Neet, 1988). The work of Sutter *et al.*, (1979) also disputes the fact that internalization is the only difference between the receptor forms. The process of internalization is thought to be inhibited at low temperatures. Sutter *et al.*, (1979) showed that both types of receptors are present not only at 37° C but also at 0° C, which implies that differences in receptor forms can not be attributed to internalization alone. However, Hempstead *et al.*, (1988) have cloned the human NGF receptor gene which encodes both the high- and low-affinity species, implying that there is only one receptor. Thus, there still remain unanswered questions about the nature of NGF receptor classes and whether there are two distinct receptors or whether there is only one species which alters its own binding properties after binding to NGF.

The high-affinity form of the NGF receptor has been implicated as the form that is responsible for inducing NGF's biological effects (Green and Greene, 1986; Weskamp and Reichardt, 1991). Many cell surface receptors which begin the process of signal transduction undergo a conformational change after ligand binding (Berridge, 1985). This conformational change then allows internal portions of the receptor to come in contact with transducer and amplifier molecules within the cell membrane. The receptor itself may then become active, such as the insulin receptor which becomes a tyrosine kinase upon ligand binding (Berridge, 1985). The NGF receptor appears to operate in a distinct manner from these other well documented receptor / ligand molecules.

Tyrosine protein kinases were discovered in the 1980's and since this time the family of tyrosine kinases has grown such that there are over 50 members now (reviewed in: Barbacid *et al.*, 1991). Several of the tyrosine kinases are glycoproteins that serve as growth factor receptors (reviewed in: Barbacid *et al.*, 1991). The tyrosine kinase subfamily, known as *trk*, is also thought to be involved in signalling mechanisms as cell surface receptors (reviewed in: Barbacid *et al.*, 1991). The formation of high-affinity form of the NGF receptor appears to require association of the low-affinity form with an accessory protein known as *trk* (Hempstead *et al.*, 1990).

The *trk* proto-oncogene, $p70^{trk}$, was first discovered in a human colon carcinoma (Martin-Zanca *et al.*, 1986). This form of carcinoma occurs due to a somatic rearrangement of the *trk* proto-oncogene. Expression of *trk* in developing mouse embryos revealed that *trk* was expressed in neuronal populations that were derived from neural crest cells (Martin-Zanca *et al.*, 1990; reviewed in: Barbacid *et al.*, 1990). The normal expression of some members of the *trk* proto-oncogene family occurs within certain neurons and sensory structures within the peripheral nervous system (Barbacid *et al.*, 1990). The role of the *trk* subfamily of tyrosine kinases remains to be elucidated, since they show no sequence homology with the known tyrosine kinase receptors that function as cell surface receptors (Barbacid *et al.*, 1990).

It appears that for NGF to bind to the high-affinity receptor, the receptor must first be phosphorylated by a component of the receptor, which is a product of the *trk* proto-oncogene, $gp140^{prototrk}$ (Hempstead *et al.*, 1991). The low-affinity receptor has been named $p75^{NGFR}$. Only cells which co-express both $p75^{NGFR}$ and $gp140^{trk}$ are capable of

expressing the high affinity binding site (Hempstead *et al.*, 1991). These results imply that $p75^{NGFR}$ and $gp140^{trk}$ interact in some fashion to form the high-affinity binding site. Transfection of *trk* cDNA into PC12 mutant cells which lack high-affinity receptor sites enables them to respond to NGF (Loeb *et al.*, 1991). These results indicate that $gp140^{proto trk}$ is necessary for NGF signal transduction and promotion of neuritogenesis.

NGF bound *trk* is not sensitive to trypsin digestion, which is similar to what has been observed for the high-affinity receptor. In contrast, the low-affinity receptor form is sensitive to trypsin digestion (Meakin *et al.*, 1992). The dissociation of NGF bound to *trk* also occurs slowly compared to the low-affinity receptor (Meakin *et al.*, 1992). These results indicate that the *trk* product is similar to the high-affinity NGF receptor. Furthermore, altering NGF at Lys-32 and either Lys-34 or Lys-95 disrupts binding of NGF to $p75^{NGFR}$ without affecting binding to $gp140^{trk}$ or the biological activity of NGF (Ibanez *et al.*, 1992). These results suggest that the two receptor forms may be independent structures and that $gp140^{trk}$ is the high-affinity receptor. Hempstead *et al.*, (1992) have shown that over-expression of $gp140^{trk}$ in an NGF responsive cell line, will accelerate differentiation. In addition, binding studies of these cells revealed both the high-affinity and low-affinity receptor forms increased in number (Hempstead *et al.*, 1992).

Ethanol is known to alter other receptor ligand interactions. For example, ethanol causes desensitization of the nicotinic acetylcholine receptor and increases the affinity of agonist-stimulated induction of these receptors (Held *et al.*, 1991). The acetylcholine receptor is known to be stabilized both metabolically and spatially by muscle activity and

neurotrophic factors (Fumagalli *et al.*, 1990). Therefore, there are two potential mechanisms by which ethanol affects the acetylcholine receptor. Either ethanol depletes the cells of needed neurotrophic factors, thus disrupting acetylcholine receptor synthesis, or ethanol disturbs the acetylcholine receptors' spatial arrangement through its perturbations of the cell membrane. It is possible that ethanol is inducing similar effects on NGF and its receptor. However, no studies have been undertaken to evaluate any changes in either NGF receptor number or affinity due to alcohol exposure.

A potential mechanism for NGF enhanced neurite extension may lie in the ability of ethanol to alter NGF receptor ligand interactions and / or the signal transduction pathways initiated by NGF. Alcohols are both water and lipid soluble and have the potential to disrupt membrane fluidity (Chin and Goldstein, 1977). Decreasing membrane fluidity could allow receptor ligand interactions that may not otherwise occur. The fluidizing effect that ethanol has on cell membranes appears to be related to the amount of gangliosides present within the cell membranes (Harris *et al.*, 1984).

Gangliosides: History and Biochemical Properties

Gangliosides are plasma membrane constituents composed of glycosphingolipids which use ceramide as a building block and contain sialic acid and neutral sugars. The compound ceramide is composed of two fatty acyl chains linked to serine, one through the serine side chain and the second through the serine amino group. When this compound is glycosylated, a glycosphingolipid is formed. Gangliosides are named for the specific sequence of neutral sugars they contain (Svennerholm, 1964). Bovine brain was the first

source from which gangliosides were isolated (Klenk, 1942). Gangliosides are most highly concentrated within neuronal and glial cells (Hamberger and Svennerholm, 1971). Gangliosides have been isolated from every vertebrate tissue and appear to be highly conserved throughout every species.

The synthesis of gangliosides begins within the smooth endoplasmic reticulum, after which they are transferred to the golgi apparatus and then transposed to the cell membrane. These molecules form an integral part of the lipid bilayer of membranes along with other phospholipids, cholesterol and glycolipids. Gangliosides appear to cluster around protein receptor molecules within the membranes to form an intricate part of their microdomain (Klemm, 1989).

Although the majority of the gangliosides present in mammalian cells are found within the cell membrane, other areas of the cell also contain gangliosides. Fractionation of cellular material has revealed the presence of gangliosides within the endoplasmic reticulum, the golgi apparatus and the secondary lysosomes (Critchley *et al.*, 1973; Huterer and Wherrett, 1974; Keenan *et al.*, 1972; Renkonen *et al.*, 1970; Zambrano *et al.*, 1975). The position of gangliosides within the cell membrane allows them to be able to interact with other cells and the extracellular matrix.

The importance of gangliosides as mediators of cell function may be seen in the reaction between G_{M1} and the B subunit of *Vibrio cholera* toxin. The specific binding of this subunit to G_{M1} allows the A_1 subunit of the *Vibrio cholera* toxin to enter the cell and activate adenylate cyclase to produce the adverse effects of this toxin (Fishman, 1982).

This pathway proves gangliosides may be more important mediators of cell function than was once thought.

Gangliosides have recently been shown to play roles in early embryogenesis, neuronal differentiation and cell adhesion. During early development, stage specific embryonic antigens which are spatially regulated oligosaccharides are present on the cell surface. These are thought to be the major regulators for cell adhesion and embryonic compaction. As embryogenesis progresses, there appears to be a shift from this series of glycolipids to the ganglio- and lacto- series of glycolipids (Fenderson *et al.*, 1990).

As embryonal carcinoma cells undergo differentiation due to stimulation with retinoic acid, there is an increase in the amount of G_{D3}, 9-O-acetyl G_{D3} and G_{T3} expression. Concurrently, there is a decrease in the expression of the stage specific embryonic antigens present on these cells. For this reason, it appears that the progression to a differentiated state may be dependent on the expression of these altering carbohydrate structures (Cossu *et al.*, 1985; Cummings and Mattox, 1988).

Developing rat dorsal retinal ganglion cells and their processes appear to contain greater amounts of 9-O-acetyl G_{D3} than do ventral retinal ganglion cells (Constantine-Paton *et al.*, 1986). It is hypothesized that the modified G_{D3} is functioning to somehow direct synapses with their target cells in the midbrain. It may be that the modified ganglioside is functioning as a recognition sequence for proper connections to be formed. This form of modified G_{D3} is also found in many other areas of the developing brain where the pattern of neuronal connections is critical (Mendez-Otero *et al.*, 1988). Therefore, the

function of G_{D3} may be to act as one of the factors governing neurite projection and axonal connectivity.

Gangliosides also may be responsible for cell adhesion (Blackburn *et al.*, 1986). Gangliosides G_{M2}, G_{D3}, and G_{D1a}, and to some extent gangliosides G_{T1b}, G_{M1} and G_{D1b}, support adhesion of embryonic chick retinal cells to a plastic surface when these gangliosides were added to cultures of these cells. In rat brain, a receptor for G_{T1b} was isolated which may be responsible for cell-cell recognition in the brain. The G_{T1b} receptor appears to be associated with oligodendrocytes in the myelinated tracts of the CNS but not within those of the peripheral nervous system. G_{T1b} itself is not expressed in the oligodendrocytes; however, it is expressed in high quantities in the axolemma (DeVries *et al.*, 1980). Therefore, it may be that G_{T1b} is acting to somehow stabilize the interaction between axons, oligodendrocytes and myelin associated with the CNS.

Fibronectin is a plasma protein which is responsible for matrix assembly and cell adhesion. A glycoprotein RGD receptor is responsible for cell adhesion to fibronectin (Speigel *et al.*, 1986). When cells adhere to fibronectin, they undergo cytoskeletal reorganization and fibrillar organization of the fibronectin itself at the cell surface. The process of cytoskeletal reorganization is mediated through the RGD receptor while the fibrillar organization of the fibronectin appears to be mediated by gangliosides (Speigel *et al.*, 1986). The use of antibodies against gangliosides will initiate some inhibition of cell adhesion to fibronectin which implies that gangliosides are also important for the initial binding of cells to fibronectin. However, transformed murine fibroblasts that are deficient in gangliosides are capable of binding to fibronectin, implying that although gangliosides

may have a synergistic effect with the RGD receptor, they are not the primary initiator of cell binding to fibronectin (Speigel *et al.*, 1986). Taken together, all of these results give the impression that gangliosides may play a greater role in cellular functions and cellular differentiation than was once thought.

Many effects on cells in culture have been observed. In many cases, gangliosides appear to decrease the proliferation state of cells. For example, the use of a mouse neuroblastoma cell line (Neuro2a) revealed that synthetic gangliosides and natural gangliosides also induce neuritogenesis in this cell line. This appears to occur through a different mechanism than neurite extension caused by compounds that elevate cAMP (Tsuji *et al.*, 1988). Treatment of neuroblastoma cell lines with neuraminidase results in enhanced neuritogenesis in these cells. This effect is blocked if the cells are exposed to the β subunit of cholera toxin or antibodies to G_{M1} which suggests that G_{M1} plays a role in neurite extension (Wu and Ledeen, 1991). G_{M1} will inhibit the ability of mouse Swiss 3T3 cells to respond to PDGF and EGF stimulated proliferation (Bremer *et al.*, 1984). No effect of ^{125}I-EGF binding was observed which suggests that gangliosides may be inhibiting receptor function further along the pathway (Bremer *et al.*, 1984).

Ganglioside G_{M1} was shown to decrease the number of PC12 cells in culture in the absence of NGF (Katoh-Semba *et al.*, 1984). The addition of G_{M1} to PC12 cells was also shown to enhance the number of PC12 cells in culture which respond to NGF (Katoh-Semba *et al.*, 1984). G_{M1} also enhances NGF-induced neurite extension, increases activation of S6 kinase, stimulates *trk* phosphorylation and decreases [^{3}H]-thymidine incorporation in PC12 cells over that seen with NGF alone (Mutoh *et al.*, 1993). There

was no effect observed on ^{125}I-NGF binding to its receptor in cells treated with the β subunit of cholera toxin (Mutoh *et al.*, 1993). These effects of G_{M1} are very similar to the effects observed with alcohol treatment in PC12 cells.

Ethanol and Ganglioside Interactions

Gangliosides are enriched in CNS plasma membranes and most are located toward the external membrane surface (Svennerholm, 1980). Within these membranes, gangliosides increase membrane order and rigidity of liquid crystalline phospholipids (Curatolo *et al.*, 1977; Tillack *et al.*, 1982). There are two current theories that exist as to how ethanol interacts with cell membranes.

The first theory is based upon the fact that ethanol increases fluidity of the cell membranes by interacting with the lipid matrix. However, this theory has lacked support since the fluidization effects of ethanol are minute at physiologic concentrations (Rubin, 1987). However, increases in ganglioside content will cause membranes to become more rigid and more sensitive to the fluidizing effects of ethanol (Harris *et al.*, 1984). Not each ganglioside has the same membrane ordering effect; $G_{M1} > G_{D1a} > G_{T1b}$ (Harris e*t al.*, 1984). Chronic ethanol exposure decreases lipid metabolism in mice brains and causes an increase in total brain lipids, phospholipids, cerebrosides, gangliosides and sulphatides (Rawat, 1974). Not all gangliosides appear to be affected to the same degree. For example, chronic alcohol exposure initiated an increase in the trisialoganglioside G_{T1b} and a subsequent decrease in the disialoganglioside G_{D1a} in rat brains (Vrbaski *et al.*, 1984). In

contrast, a single acute dose of ethanol causes a decrease in all gangliosides present in adult rat brains (Klemm and Foster, 1986).

Ethanol also induces changes in gangliosides present within the CNS of rat pups that are prenatally exposed to ethanol (Prasad, 1992). Total ganglioside N-acetylneuraminic is increased within the CNS (Prasad, 1992). The alterations in individual gangliosides are brain region specific and some may increase in one area while decrease in other areas of the brain. For example, G_{M1} is increased within the cerebrum and decreased in the brain stem and spinal cord (Prasad, 1992). The alterations in specific gangliosides within specific regions of the developing brain may provide an explanation of the varied effects that ethanol has on different neuronal populations.

In the study of the importance of gangliosides as mediators of ethanol effects, two genetically bred strains of mice have been used. Long sleep mice are more sensitive to ethanol exposure than are short sleep mice (McClearn and Kakihana, 1981; Heston *et al.*, 1974). The major difference in neuronal membrane constituents between these two strains of mice is the monosialoganglioside, G_{M1}. Long sleep mice contain three times the concentration of G_{M1} in cerebellar tissue as do short sleep mice (Ullman *et al.*, 1987).

However, even though long sleep mice possess increased G_{M1} within synaptosomes, surface exposure of G_{M1} in synaptosomes is the same between the two strains of mice (Ullman *et al.*, 1992). Polysialoganglioside hydrolysis by neuraminidase to the G_{M1} endproduct is increased in long sleep mice during acute exposure to alcohol and this causes a decrease in polysialogangliosides present on synaptosomal surfaces (Ullman *et al.*, 1992). In short sleep mice, an increase in surface exposed polysialogangliosides is

observed which is mainly G_{D1a} (Ullman *et al.*, 1992). The variation in G_{M1}, alterations in synaptosomal gangliosides and alterations in surface exposure of gangliosides between these two strains of mice demonstrate the potential contribution of gangliosides in membrane sensitivity to ethanol.

Another theory is that ethanol is dehydrating the membrane by displacing water molecules bound to either outer or inner surfaces of the plasmalemma. Displacing water by more nonpolar molecules, such as alcohols, could potentially alter the conformational relationships of membrane constituents, such as protein receptor molecules. Alcohol does bind in a cooperative manner to the surface synaptic plasma membranes (Kreishman *et al.*, 1985). Butanol binds to dipalmitolphosphotidylcholine (DPPC) and to gangliosides G_{M1} and G_{T1b} forming an alcohol lipid complex which releases bound water molecules (Yurttas *et al.*, 1992). The addition of G_{M1} to membranes composed of DPPC initiates new binding sites for butanol (Yurttas *et al.*, 1992). These results support the alcohol dehydration theory and suggest that gangliosides may be important mediators in the effects that ethanol produces on cellular membranes.

These results indicate that ethanol may affect the presence of gangliosides in brain tissue. But, gangliosides affect ethanol's ability to interact with cell membranes. Thus, the presence and type of gangliosides either already present or induced by ethanol may be important regulators in ethanol fluidization and enhanced neurite extension of PC12 cells.

Alcohol Interactions With Cells

The adverse effects of ethanol have been known to exist for some time but the mechanisms by which ethanol induces these effects still remain to be elucidated. There are two accepted theories to explain how ethanol may elicit its effects. One is via a specific receptor for ethanol and the other is by inducing perturbations in the cell membrane (Chin and Goldstein, 1977). A specific receptor has not yet been found but if there is one, then the process of ethanol binding to this receptor could initiate a number of signal transduction cascades. Even without a specific receptor, ethanol may indirectly affect signal transduction cascades initiated by other ligand receptor interactions.

This effect could potentially utilize a number of second messengers since ethanol has been shown to affect many second messenger systems. Ethanol has been shown to affect protein kinase C (PKC) levels and its translocation (Skwish and Shain, 1990). Ethanol has also been shown to affect cyclic adenosine monophosphate (cAMP) production and calcium fluxes (Hoffmann and Tabakoff, 1990; Brennan *et al.*, 1990).

The other possibility is that ethanol induces perturbations in membrane fluidity due to its unique characteristic of being both water and lipid soluble. Ethanol has been shown to be able to work its way into the membrane and decrease its fluidity (Chin and Goldstein, 1977). New evidence exists for the alcohol-induced membrane dehydration theory, which could also disrupt normal membrane architecture. By decreasing membrane fluidity or altering membrane architecture, it is possible to allow receptor-ligand binding that may not occur otherwise (Chin and Goldstein, 1977). This could potentially allow ethanol to affect the number and / or function of a variety of membrane associated

receptors and induce all of the second messenger changes that have been observed (Held *et al.*, 1991).

There have been many studies that show that alcohol can affect gene expression, protein synthesis, enzyme activity, calcium channels and signal transduction pathways (Rothschild *et al.*, 1987; Hoensch, 1987; Lissemore *et al.*, 1990; Brennan *et al.*, 1989; Mendonca and Zancan, 1989). Many of the effects observed due to ethanol exposure have been shown to be linked to gene regulation in other systems. Therefore, a potential third mechanism by which ethanol might elicit its effects is through a direct effect upon regulation of gene expression. The effect that ethanol has on gene expression is only recently being studied.

This project is designed to explore specific alcohol interactions in PC12 cells related to effects on NGF receptor binding properties, timing of alcohol exposure in relation to NGF exposure, effects of alcohol on cellular proliferation, effects of alcohol on cell cycle regulation and effects of alcohol on ganglioside G_{M1} changes.

III. MATERIALS AND METHODS

Materials

Pheochromocytoma cells (PC12) were purchased from The American Type Culture Collection (ATCC) (Rockville, MD). Rat tail collagen was obtained from Collaborative Biomedical Research (Bedford, MA). Fetal calf serum and horse serum were from JRH Biosciences (Lenexa, KA). FITC-labelled β-subunit cholera toxin was obtained from List Biology Labs Company (Campbell, CA). Nerve growth factor (NGF) (2.5 S), epidermal growth factor (EGF), fibroblastic growth factor (FGF) (recombinant *E. coli*) and insulin like growth factor-1 (IGF-1) (recombinant *E. coli*) were purchased through Bioproducts for Science (Indianapolis, IN). $Na^{125}I$ was obtained from Amersham Corporation (Arlington Heights, IL). [^{3}H]-methyl thymidine was obtained from NEN Dupont (Boston MA). All other chemicals of the highest grade available were obtained from Sigma Chemical (St. Louis, MO).

Cell Culture

Pheochromocytoma cells were grown in RPMI-1640 medium supplemented with 5% (v/v) heat inactivated horse serum, 10% (v/v) heat inactivated fetal calf serum, 50 µg/ml penicillin, and 50 µg/ml streptomycin (Greene and Tischler, 1976). The cells were

grown at 37° C in an 8% CO_2 / 92% humidified air atmosphere on tissue culture ware that was pre-coated with rat tail collagen.

Alcohol Treatment of PC12 Cells

Culture ware was coated with a thin layer of rat tail collagen the day prior to seeding with PC12 cells. Cells were plated into culture dishes and allowed to attach overnight prior to treatment. Alcohols were prepared as two times the final desired concentration in complete medium. Treatments were composed of varying ethanol concentrations (dose), alcohol chain length, and ethanol time course evaluations. Ethanol concentrations ranged from physiologically tolerable levels to pharmacological levels which may induce death in unaccustomed people. Dose treatments within the physiologic range consisted of : 0.1% (w/v) [21.7 mM, 100 mg/dl], 0.2% (w/v) [43.5 mM, 202 mg/dl], 0.4% (w/v) [87.0 mM, 400 mg/dl], and within the pharmacologic range consisted of: 0.6% (w/v) [131.5 mM, 604 mg/dl], and 0.8% (w/v) [173.9 mM, 802 mg/dl] ethanol. Increasing alcohol chain length treatments were evaluated at 0.4% (w/v) [87.0 mM, 400 mg/dl] and consisted of methanol, ethanol, propanol, and butanol. Dose response and alcohol chain length evaluations were performed after either 24 or 72 hours of alcohol exposure. Time course evaluations were conducted with cells exposed to ethanol at 0.4% (w/v) for either 12, 24, 48, or 72 hours. Each plate of cells, including control cells, after treatment for any experiment, was wrapped in parafilm to prevent evaporation of alcohol from the plate and to prevent control cells from being exposed to alcohol.

Iodination of NGF

Iodination of NGF was performed essentially as described by Sutter *et al.*, (1979). To 6.25 µg of 2.5S β-NGF, 20 µl of 0.1 M Phosphate Buffer (Monobasic, pH 7.4), 15 µl of 30 *u*g/ml lactoperoxidase and 1 mCi of $Na^{125}I$ were added. To begin the reaction 15 µl of a $1:10^4$ dilution of 30% H_2O_2 was added. The reaction was gently vortexed and allowed to stand at room temperature for 30 minutes. At this time, another 15 µl aliquot of the $1:10^4$ dilution of H_2O_2 was added and the reaction was allowed to incubate at room temperature for an additional hour. The reaction mixture was then loaded onto a Sephadex G-50 column (30 ml bed volume) to separate iodinated NGF from free ^{125}I. One milliliter fractions were collected. Aliquots of 5 µl were counted on a Beckman gamma counter to identify the iodinated NGF and free iodine peaks which eluted from the column.

Molecular mass was estimated by Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Kouchalakos and Bradshaw (1986). ^{125}I-NGF (1 µg) was mixed with an equal volume of sample buffer (20 mM Tris, pH 6.8, 2% SDS, 2 mM dithiothreitol, 15% sucrose, 0.01% bromphenol blue) and boiled for one minute prior to loading onto a 12% polyacrylamide gel. After electrophoresis, the gel was stained in 0.025% Coomassie Blue, 25% 2-propanol, 10% acetic acid, and destained using 10% acetic acid. The gel was dried and exposed to Kodak X-OMat film overnight at room temperature. Molecular mass markers used were: phosphorylase B (94 kD), bovine serum albumin (63 kD), ovalbumin (42 kD), carbonic anhydrase (31 kD) and soybean trypsin inhibitor (21 kD). A photograph of the autoradiogram is shown in Appendix,

Figure 33. The molecular mass of the ^{125}I-NGF preparation migrated to the area corresponding to 26,000 kD which is the mass of the naive NGF.

Biological Activity of Iodinated-NGF

Pheochromocytoma cells were plated into 60 mm culture dishes and allowed to attach overnight. Iodinated-NGF was added to dishes in quantities equivalent to 25, 50, and 100 ήg/ml. Cells were incubated for 6 days. Photographs of neurite extension were taken at day 3 and day 6. An equivalent degree of neurite extension was observed in PC12 cells treated with ^{125}I-NGF as with native NGF as shown in Appendix, Figure 34. This implies that the biological activity of the iodinated preparation is not altered due to the incorporation of the ^{125}I molecule.

Measurement Of Binding Parameters Hot Titration

Titration of ^{125}I-NGF was performed in order to determine the optimal counts per minute to be used in reaction mixtures for Scatchard analysis. This was performed on every ^{125}I-NGF preparation since the specific activity of each preparation was slightly different. Pheochromocytoma cells were harvested and 3 x 10^5 cells / tube in 200 µl of phosphate buffered gelatin (PBG, 5.75 g Na_2HPO_4, 1.48 g NaH_2PO_4, 0.2 g sodium azide, 1 g gelatin, qs. 1L, pH, 7.6) were added to 24 microfuge tubes. The tubes were divided into four sets of six. To the sets, 2,500, 5,000, 10,000 or 20,000 counts of ^{125}I-NGF were added to respective sets. To half the tubes of each set, 100 ηg of uniodinated NGF was added. The cells were incubated at 37°C for 30 minutes. Post incubation, the reaction

was terminated and processed by layering the reaction mixture onto a two step sucrose gradient prepared in microfuge tubes and consisting of 200 µl of 0.3 M sucrose in PBG (pH, 7.6) as the bottom layer and 150 µl of 0.15 M sucrose in PBG (pH, 7.6) as the top layer. The tubes were centrifuged at 5,000 X g for 5 minutes. The supernatents were drawn off and saved for counting and tips of the microfuge tubes containing the cell pellets were clipped off and placed into another tube for counting. Specific binding was calculated by subtracting the counts obtained from the tubes with the excess unlabelled NGF from those tubes without unlabelled NGF and dividing this number by the total counts added to the reaction tube. The experiment was repeated for each iodinated NGF preparation used. Appendix, Figure 35 depicts a representative graph from one iodinated preparation. For this preparation, the optimum counts per minute were 5,000 cpm and this would be used for Scatchard Analysis performed with this preparation.

Cell Number

The number of PC12 cells were titrated in order to determine the optimum cell number to add to Scatchard reactions. Twenty four microfuge tubes were set up for each cell number containing either 1.7, 3.2, 5.4 or 8.1 x 10^5 cells / tube in 200 µl of PBG (pH, 7.6). Each of the sets of 24 tubes of cell numbers were split into groups of six. To each set of six either 2,500, 5,00, 10,000 or 20,000 counts of ^{125}I-NGF was added. To half of the tubes of each set, 100 ηg of uniodinated NGF was added to determine specific binding. The cells were processed as described previously. The experiment was repeated three independent times. Appendix, Figure 36 depicts a representation of cell number

titration. This data showed that binding increases with increasing cell number. Adequate binding was obtained with 3.2 x 10^5 cells per tube and due to tissue culture restraints, this number was chosen to carry out the Scatchard Analysis.

Incubation Time

Incubation times were varied in order to determine the optimum binding time needed for specific binding. Pheochromocytoma cells were set up as described previously using 3.2 x 10^5 cells / tube in 200 µl of PBG (pH, 7.6). Iodinated and uniodinated NGF were added as described previously to determine specific binding and the cells were incubated for either 0.5, 1.0, 2.0 or 3.0 hours prior to processing as previously described. The experiment was repeated three independent times. Appendix, Figure 37 is a representative figure showing the effect of binding time on specific binding. The optimal binding time is 0.5 hours. Binding decreases after this time presumably due to internalization.

Incubation Temperature

Specific binding was evaluated at 37°C and 0°C in order to determine the effect of incubation temperature. Pheochromocytoma cells were set up as previously described using 3.2 x 10^5 cells/tube in 200 µl of PBG (pH, 7.6). Iodinated and uniodinated NGF were added as described previously to determine specific binding and the cells were incubated at either 0° or 37°C for either 0.5 or 2 hours. The cells were processed as

described previously and the experiment was repeated three independent times. Specific binding at 37°C was greater than at 0°C as shown in Appendix, Figure 38.

Competition of ^{125}I-NGF with Uniodinated NGF

Displacement assays were performed to document that ^{125}I-NGF could be displaced from PC12 cells by uniodinated NGF. This would ensure that both forms of NGF were binding to the same cellular receptors and that Scatchard Analysis could be performed. Cells were aliquoted into microfuge tubes at 3 x 10^5 cells / tube in 200 µl of PBG (pH, 7.6). Premixes were made in PBG which contained either 0, 0.1, 0.25, 0.75, 1, 2.5, 5, 7.5, 10, 20, 40 or 100 ηg of uniodinated NGF and 5,000 counts per minute of ^{125}I-NGF in 35 µl. Each uniodinated NGF concentration was done in triplicate. Reaction mixtures were allowed to incubate for 30 minutes at 37°C and were then processed as described previously. The experiment was repeated three independent times. Appendix, Figure 39 is representative of the results obtained with an individual experiment. This proved that NGF could compete with ^{125}I-NGF for NGF receptor binding sites on PC12 cells.

Binding Specificity Of Iodinated-NGF

In order to document the specificity of ^{125}I-NGF binding, experiments were undertaken to displace ^{125}I-NGF away from its receptor(s) by other growth factors. Growth factors used were epidermal growth factor (EGF), fibroblastic growth factor (FGF), insulin like growth factor-1 (IGF-1), insulin and nerve growth factor (NGF).

Premixes of these growth factors were made which contained x ήg of growth factor and 0.01 ήg ^{125}I-NGF per 35 µl (x = 0, 5, 10, 50, 100, 500, 1,000, 2,000, 10,000 ήg). PC12 cells were harvested from plates by gentle pipetting, washed 2X with PBS (pH, 7.1), centrifuged and resuspended at 1 x 10^6 cells / ml in PBG (pH, 7.6). Two-hundred microliters of cell suspension was added to microfuge tubes. The reaction was carried out by adding 35 µl of the appropriate premix and incubating the tubes at 37°C for 30 minutes. Each premix concentration was added to three independent tubes for triplicate samples. The reaction was terminated and processed as described previously. The data are presented as total counts in pellets versus ήg of growth factor. Appendix, Figure 40 depicts the results obtained. The only growth factor that was able to displace ^{125}I-NGF from PC12 cells was uniodinated NGF. This shows that the binding properties of iodinated NGF preparations remain specific for the NGF receptor(s).

Scatchard Analysis

Pheochromocytoma cells were seeded into 100 mm culture dishes previously coated with rat tail collagen and allowed to attach overnight. Alcohol solutions were made as 2X the final desired concentration and added to the appropriate plate. Experiments consisted of dose and alcohol chain length evaluations at 72 hours and a time course evaluation. Scatchard analysis was performed essentially as described by Sutter *et al.*, (1979). Cells were harvested from plates by gentle pipetting and washed two times with PBS (pH, 7.1). After harvest, PC12 cells were resuspended in PBG (pH, 7.6) and the appropriate alcohol at a concentration of 1 x 10^6 cells/ml. Two hundred microliters of

each cell suspension was placed in 42 microfuge tubes. Premixes were made which contained x µg cold NGF and 0.01 ήg iodinated NGF per 35 µl (x = 0, 0.1, 0.25, 0.5, 0.75, 1, 2, 4, 6, 10, 20, 40, 100, or 1000 ήg). At time 0, 35 µl of premix was added to microfuge tubes containing cells. Each determination was conducted in triplicate. The reaction tubes were incubated at 37°C for 30 minutes. The reactions were terminated and processed as described previously.

Each Scatchard analysis was performed a minimum of three times. Initial data analysis was performed by utilizing a computer program known as LIGAND which was specifically designed for the Scatchard procedure (Munson, 1990). The program takes into account the specific activity of the iodinated preparation, the mean total counts added, and the counts of the iodinated preparation that remain bound to the cells with increasing concentrations of unlabeled ligand. Once this information has been entered, the program analyzes the data to determine whether the data fit a one-site model or a two-site model. The data obtained here statistically fit a two-site model better than a one-site model. This is in agreement with reported findings that document two classes of NGF receptors (Sutter *et al.*, 1979). The program then statistically determined the best fit lines through the curvilinear plot obtained from the model and estimated the slopes and x-intercepts of these lines. Each line obtained represents one form of the NGF receptor. The inverse of the slopes of these lines are related to the affinities of NGF receptor forms, while the x-intercepts are related to the receptor numbers. The data obtained were consistent with reported values of affinities and receptor numbers for both classes of NGF receptors (Sutter *et al.*, 1979). The low-affinity receptor had an affinity in the nanomolar

range with between 10^4 and 10^7 receptors per cell while the high-affinity receptor had an affinity between 0.1 to 0.01 nanomolar and between 10^3 and 10^5 receptors per cell (Sutter *et al.*, 1979). Once the affinities and receptor numbers were determined, they were further analyzed using SAS for ANOVA-RCBD (randomized complete block design) and Dunnett's t-test to determine differences between treatment and control means.

Morphology Studies

All morphological studies were carried out in 35 mm culture plates. PC12 cells were seeded at a concentration of 3 x 10^4 cells/plate. The cells were allowed to attach overnight prior to treatment. Alcohol treatments were carried out as previously described for the dose response, side chain evaluation and time course evaluations. NGF stimulation was carried out at 50 ηg/ml. Experimental bias was removed by randomly assigning each plate a number prior to alcohol treatment and then scoring, counting cells and photographing by plate number. Dose and alcohol chain length treatment regimens consisted of PC12 cells which were either pretreated with alcohol for 24 hours prior to NGF stimulation, pretreated with alcohol for 72 hours prior to NGF stimulation or treated simultaneously with alcohol and NGF. Cells were evaluated after either a 24 or 72 hour NGF stimulation endpoint. Two time course evaluation studies were performed. The first was undertaken to determine if there was an alcohol pretreatment time-dependent increase in NGF induced neurite extension. For this study, PC12 cells were pretreated with 0.4% (w/v) ethanol for either 12, 24, 48, or 72 hours and then evaluated at both the 24 and 72 hour NGF stimulation endpoints. The second time course evaluation was to determine if

the rate of NGF-induced neurite extension is altered by pretreatment of cells with alcohol when compared to simultaneous treatment of cells with alcohol and NGF. For this study, PC12 cells were pretreated with 0.4% (w/v) ethanol for either 12, 24, 48, or 72 hours prior to NGF stimulation for either 12, 24, 48 or 72 hours, respectively, or PC12 cells were simultaneously treated with alcohol and NGF for either 12, 24, 48 or 72 hours. Control cells consisted of treatment with only NGF for either 12, 24, 48 or 72 hours.

After treatment incubation, the plates were scored for neurite extension, counted to determine the percentage of cells bearing neurites and photographed to document representative treatment. The morphology of the population of cells was determined by evaluating the entire plate of cells microscopically and assigning a score between 0 to 8 based on the degree of neurite extension. This is a subjective evaluation that was based on the treatment response of the majority of the cells in a given plate. In each plate, there were some cells which did not respond to treatment and some that exhibited an exaggerated response. However, the majority of cells in a given plate generally responded to the same degree. A score of 0 was given to population of cells that appeared round and well attached to plates. A score of 1 indicated that cells were beginning to flatten and extend neurite spikes. A score of 2 indicated that cells possessed neurites whose length was the size of the cell diameter, while a score of 3 was indicative of cells which possessed neurites that were 2X the length of the cell body. A score of 4 was assigned to cells that possessed neurites that were 3X the length of the cell body. Increasing numbers beyond 4 indicated that the cells possess longer and longer neurites. Scoring data are expressed as the mean plate score + / - S. E. M. of the three experiments.

The percentage of cells bearing neurites was determined by counting the total number of cells in three random fields and determining those that possessed neurites. Any cell that possessed a process that was at least 1X the diameter of the cell body was counted as a cell possessing neurites. The percentage was calculated by dividing the number of cells with neurites by the total number of cells and multiplying by 100. The data are expressed as the percentage of cells with neurites. Each experiment consisted of ethanol dose and alcohol chain length responses to the treatment regimens described previously, after 24 and 72 hours of NGF stimulation. Both time course evaluations described previously were also evaluated. Each experiment was repeated 3 independent times. Data from both the score and percentage of cells bearing neurites were analyzed using SAS-RCBD for Dunnett's t-test to compare treatment to control. The dose and time course data were further analyzed using linear regression and linear contrast statements. Percentage of cells bearing neurites data were normalized by using an arc/sin transformation prior to using SAS-RCBD for Dunnett's t-test, linear regression and linear contrast. Photographs depicting representative treatment results were taken to document the degree of neurite extension (Appendix, Figures 41 - 56).

[^{3}H]-Thymidine Incorporation

The inner 24 wells of a 96 well culture plate were coated with rat-tail collagen to be used for seeding cells. A separate 96 well culture plate was used for each alcohol treatment. The cells were seeded at a concentration of 1 x 10^4 cells/well in 100 µl of complete growth medium. Also, 100 µl of medium was placed into all surrounding wells

to prevent evaporation. At the time of treatment, 100 µl of a 2X alcohol solution was added to all wells. At 7 hours prior to the termination of treatment time, 50 µl containing 0.02 µCi/µl [^{3}H]-thymidine stock solution made in complete growth medium was added to each well containing cells. After the 7 hour pulse, the cells were harvested using a Ph.D.^{PM} Microtiter Cell Harvester. Cells were recovered by washing 10X with phosphate buffered saline (PBS, pH, 7.3), subsequently lysed and DNA precipitated with 10 washes of 20% (w/v) trichloroacetic acid (TCA) followed by 10 washes with 95% (w/v) ethanol to dry the filter papers. Filter papers were placed into biovials. After drying overnight, 4 ml of scintifluor was added to each vial. The samples were counted on a Beckman scintillation counter. Data were expressed as a percentage of [^{3}H]-thymidine incorporated into PC12 cells treated with alcohol. This percentage was obtained by dividing the amount of [^{3}H]-thymidine incorporated into PC12 cells treated with alcohol by the amount incorporated into PC12 cells treated with medium alone and multiplying by 100. Each experiment consisted of a dose response at 24 and 72 hours, an alcohol chain length evaluation at 24 and 72 hours, and a time course evaluation. Each experiment was repeated four independent times. Data were analyzed by determining the percent [^{3}H]-thymidine incorporated into ethanol treated versus control cells. After arc/sin transformation, data were evaluated with SAS using a RCBD-ANOVA. Dunnetts' t-test was used to determine significant differences between control and treated cells.

Cell Cycle Analysis

Pheochromocytoma cells were seeded into previously rat tail collagen coated 100 mm culture dishes in 10 ml of complete medium. Alcohol treatments were made at 2X concentrations and 10 ml of alcohol solution was added to dishes at the time of treatment. Cells were harvested from the culture dishes by gentle pipetting. The cells were washed twice in PBS (pH 7.1), resuspended in 5 ml of PBS (pH 7.1) and mixed thoroughly by vortexing. A 50 µl aliquot was removed to determine cell number and cells were aliquoted into microfuge tubes at 1 x 10^6 cells / tube and then centrifuged at 1,000 x g for 5 minutes. The cell pellets were then resuspended in 200 µl of PBS (pH 7.1) and fixed by gently layering the cell suspension onto 800 µl of -80°C methanol alliquoted into microfuge tubes. This was quickly placed into the -20°C freezer for 1 hour. Post fixation, cells were rapidly pelleted at 5,000 x g for 1 minute. The cell pellets were resuspended in 500 µl flow buffer (100 mM Tris-HCl pH 7.4, 40 µg/ml propidium iodide, 1 mg/ml RNAse, 0.1% Triton X-100) to acheive a concentration of 2 x 10^6 cells / ml. The cell suspensions were transferred to 75 mm glass tubes and allowed to incubate on ice for at least 15 minutes. The samples were analyzed on a Coulter Epics Elite flow cytometer using the Multicycle Phoenix Flow System Program.

Data were collected on the flow cytometer until 10,000 individual cells were sampled from each treatment. The Multicycle program output describes the percentage of cells sampled that are in each phase of the cell cycle (ie: G1, S and G2). Each experiment consisted of dose and side chain response at 24 and 72 hours and a time course evaluation (12, 24, 48 and 72 hours). Each experiment was repeated four independent times. The

data obtained were further analyzed by performing an arc/sin transformation and then using SAS for ANOVA-RCBD (randomized complete block design) after performing an arc/sin transformation. Dunnett's t-test was used to determine if there was any statistical difference between the treatment means when compared to the control means.

Ganglioside Studies

Pheochromocytoma cells were seeded onto 60 mm culture dishes previously coated with rat tail collagen and allowed to attach overnight. Alcohol solutions were prepared as 2X the final desired concentration and at the time of treatment, alcohol was added to the dishes. Treatments consisted of a time course evaluation using 12, 24, 36, 48 and 72 hours. Cells were either treated with medium, 0.4% ethanol, 30 ηg/ml NGF or 30 ηg/ml NGF with 0.4% ethanol for the indicated time points. Dose and alcohol chain length evaluations were obtained for 12 hours of 0.4% (w/v) ethanol exposure. The cells were gently harvested by pipetting and placed into 15 ml conical tubes. Cells were pelleted by centrifugation and resuspended in 5 ml of PBS (pH, 7.3). Two tubes for each sample were prepared with 1 x 10^6 cells/tube. Cells were pelleted by centrifugation and resuspended in 50 µl of PBS (pH, 7.1) containing 1% (w/v) sodium azide (Az) and 2% (w/v) fetal calf serum (FCS) (PBS-Az-FCS). To one tube for each sample, 5 µl of FITC labelled cholera toxin β-subunit resuspended in PBS (pH, 7.1) was added. To the other tube for each sample, 5 µl of PBS (pH, 7.1) was added. The tubes were incubated on ice for 30 to 45 minutes and then 2 ml of PBS-Az-FCS was added to each tube. Cells were pelleted by centrifugation and pellets were resuspended in 500 µl of PBS-Az-FCS and

analyzed on a Coulter Epics Elite Flow Cytometer using the Elite Software Program. The program evaluated 10,000 cells in each sample and recorded the log mean of cells containing the fluorescent ganglioside label. The log mean was transformed to the linear mean and this value was used to calculate the Fluorescence Signal to Noise Ratio (FSN). The equation to calculate FSN is as follows:

$$FSN = 10^{[(\text{mean fluorescence exp.} - \text{mean fluorescence control})/\#\ \text{channels/log decade}]}$$

The experiment was repeated four individual times. Data were further analyzed using SAS-RCBD (randomized complete block design) with Dunnetts t-test to compare control means with sample means.

IV. RESULTS AND DISCUSSION

The precise effect which ethanol may have on the growth and differentiation of PC12 cells or the window of time during which cells are most vulnerable to alcohol has not been fully explored. The objectives of this study were to examine: 1) whether the ability of alcohol to potentiate NGF-induced neurite extension is due to a specific effect of alcohol upon NGF receptor affinity, form or number; 2) whether there are differences in NGF-induced neurite extension based upon the window of time during which alcohol was administered in relation to NGF exposure time; 3) whether there is a specific effect of alcohol on PC12 cell proliferation; 4) whether there is a specific effect of alcohol on PC12 cell cycle progression; and 5) whether there is a specific effect of alcohol on the ganglioside G_{M1} which is associated with the process of neurite outgrowth in PC12 cells.

Effects of Alcohol on NGF Receptors: Affinity and Number

Both forms of the NGF receptor are exhibited on PC12 cells (Ojeda *et al.*, 1990; Escandon and Chao, 1990; Kouchalakos and Bradshaw, 1986; Buxser *et al.*, 1990; Sutter *et al.*, 1979). Treatment of PC12 cells with NGF induces the cells to stop proliferation and begin the process of neurite extension (Greene and Tischler, 1982). The high-affinity receptor is thought to be responsible for biological effects of NGF (Green and Greene, 1986; Weskamp and Reichardt, 1991). Thus, alcohols may potentiate NGF's effects by

altering either NGF receptor affinity or directly altering the number of receptors expressed on PC12 cells. Scatchard analysis was employed to investigate receptor binding parameters of PC12 cells exposed to alcohols. Previous studies have shown that alcohols potentiate NGF effects in PC12 cells by 72 hours of simultaneous treatment (Wooten and Ewald, 1991; Messing *et al.*, 1991a), therefore, this time was chosen as the end point for the dose response and alcohol chain length evaluations. The dose range chosen consists of physiologic [0.1% w/v, 21.7 mM (100 mg/dl); 0.2% w/v, 43.5 mM (202 mg/dl) and 0.4% w/v, 87.0 mM (400 mg/dl)] and pharmacologic doses [0.6% w/v, 131.5 mM (604 mg/dl) and 0.8% w/v, 173.9 mM (802 mg/dl)]. Additionally, the effects of methanol, ethanol, propanol and butanol were evaluated at 0.4% (w/v) [87 mM (400 mg/dl)] since NGF induced neurite outgrowth is potentiated in an alcohol chain length dependent manner (Wooten and Ewald, 1991; Messing *et al.*, 1991a).

No specific effect of ethanol dose was observed for the affinity (Figure 1) or number of receptors (Figure 2) for either form of the NGF receptor (Dunnett's t-test; $p < 0.05$). These results indicate that the synergistic effect that ethanol has with NGF is not related to direct alterations within either form of the receptor. In addition, there was no alcohol chain length effect observed in either the low affinity or high affinity form of the NGF receptor, when compared to medium treated cells (Figure 3). Likewise, alcohol chain length did not alter the number of receptors expressed (Figure 4) (Dunnett's t-test; $p < 0.05$). Time course studies were undertaken to examine possible alterations in both receptor parameters (Figures 5,6). No specific alcohol effect was noted in either form of the receptor for either parameter due to any length of exposure time to ethanol (Dunnett's

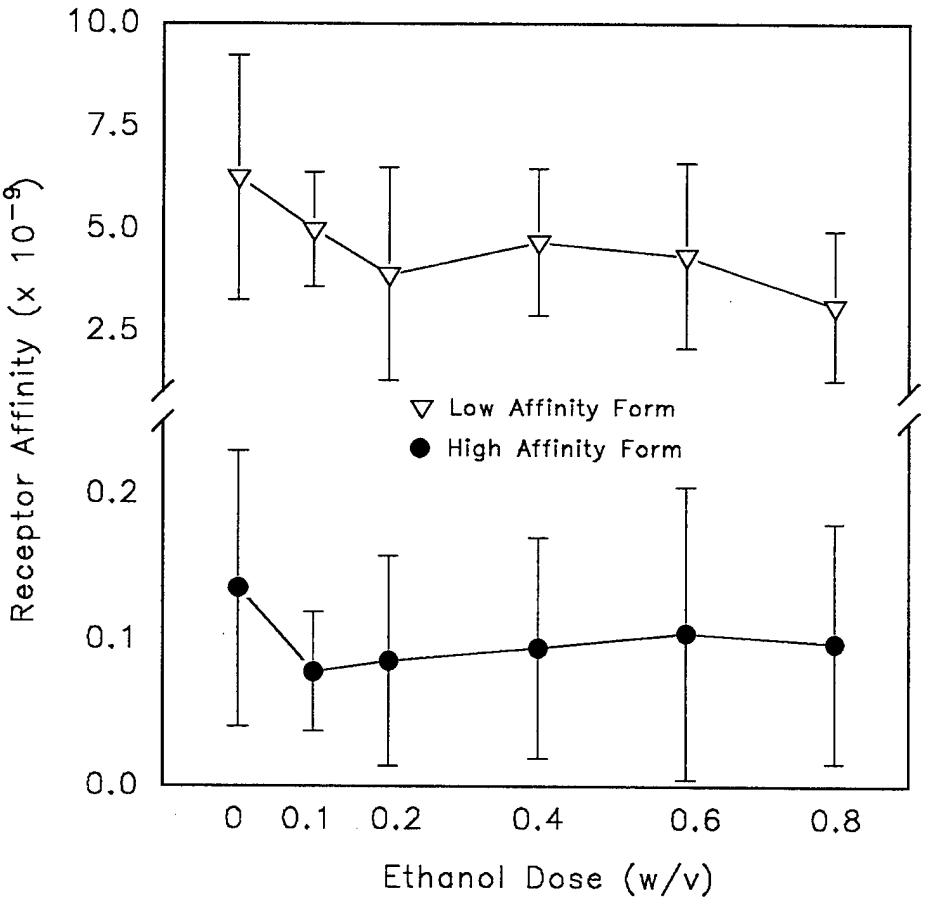

Figure 1. Effect of ethanol on NGF receptor affinity. PC12 cells were treated either with 0, 0.1, 0.2, 0.4, 0.6 or 0.8% (w/v) ethanol for 72 hours prior to harvest. Scatchard analysis was performed on each treatment using triplicate measures at each uniodinated NGF concentration. The curve fitting program, LIGAND, was used to determine the best fit lines. The experiment was repeated three independent times. Receptor affinities, calculated from the slopes of the best fit lines, were further analyzed using a RCBD and Dunnetts t-test ($p < 0.05$) for comparison of treatment to control means. Points represent the mean +/- S. E. (n = 3).

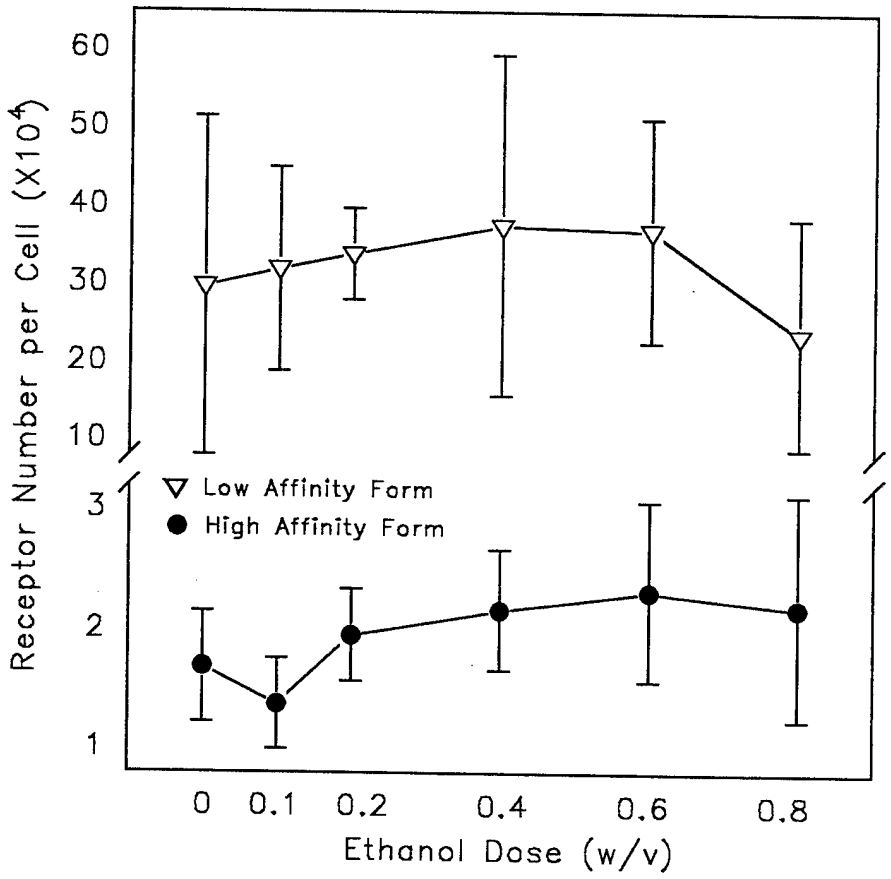

Figure 2. Effect of ethanol on NGF receptor number. PC12 cells were treated either with 0, 0.1, 0.2, 0.4, 0.6 or 0.8% (w/v) ethanol for 72 hours prior to harvest. Scatchard analysis was performed on each treatment using triplicate measures at each uniodinated NGF concentration. The curve fitting program, LIGAND, was used to determine the best fit lines. The experiment was repeated three independent times. Receptor numbers, calculated from the intercepts of the best fit lines, were further analyzed using a RCBD and Dunnett's t-test ($p < 0.05$) for comparison of treatment to control means. Points represent the mean + / - S. E. ($n = 3$).

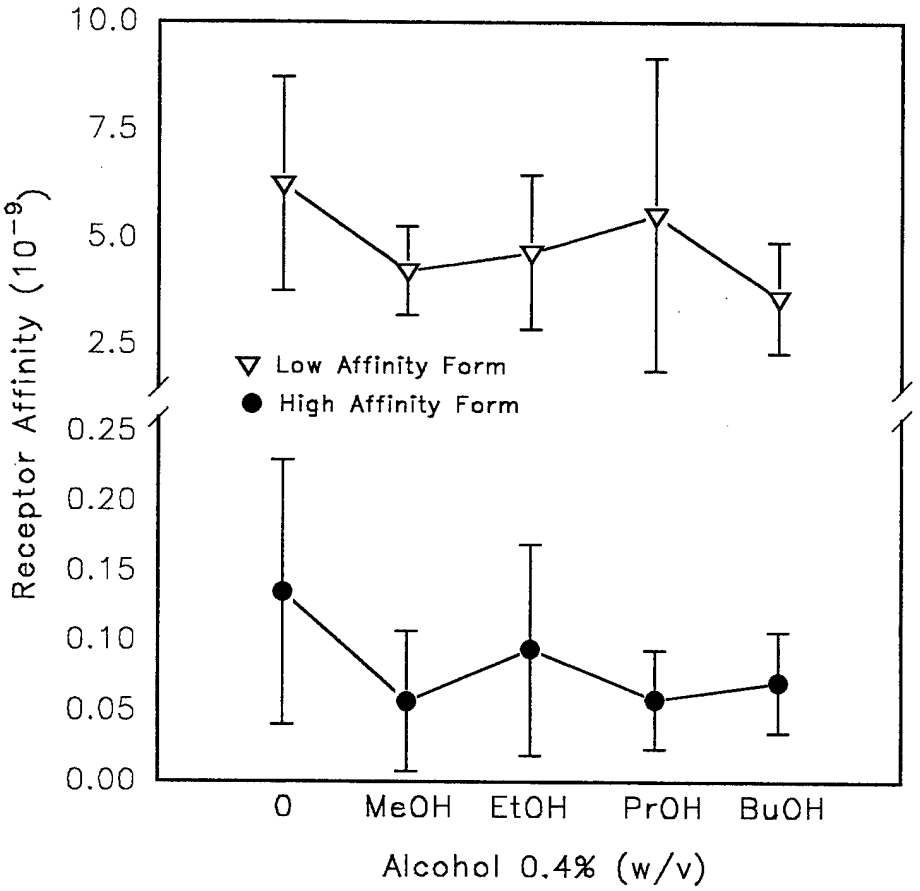

Figure 3. Effect of alcohol chain length on NGF receptor affinity. PC12 cells were treated with either medium or 0.4% (w/v) of MeOH, EtOH, PrOH or BuOH for 72 hours prior to harvest. Scatchard analysis was performed on treatment using triplicate measures at each uniodinated NGF concentration. The curve fitting program, LIGAND, was used to determine the best fit lines. The experiment was repeated three independent times. Receptor affinities, calculated from the slopes of the best fit lines, were further analyzed using a RCBD and Dunnetts t-test ($p < 0.05$) for comparison of treatment to control means. Points represent the mean +/- S. E. ($n = 3$).

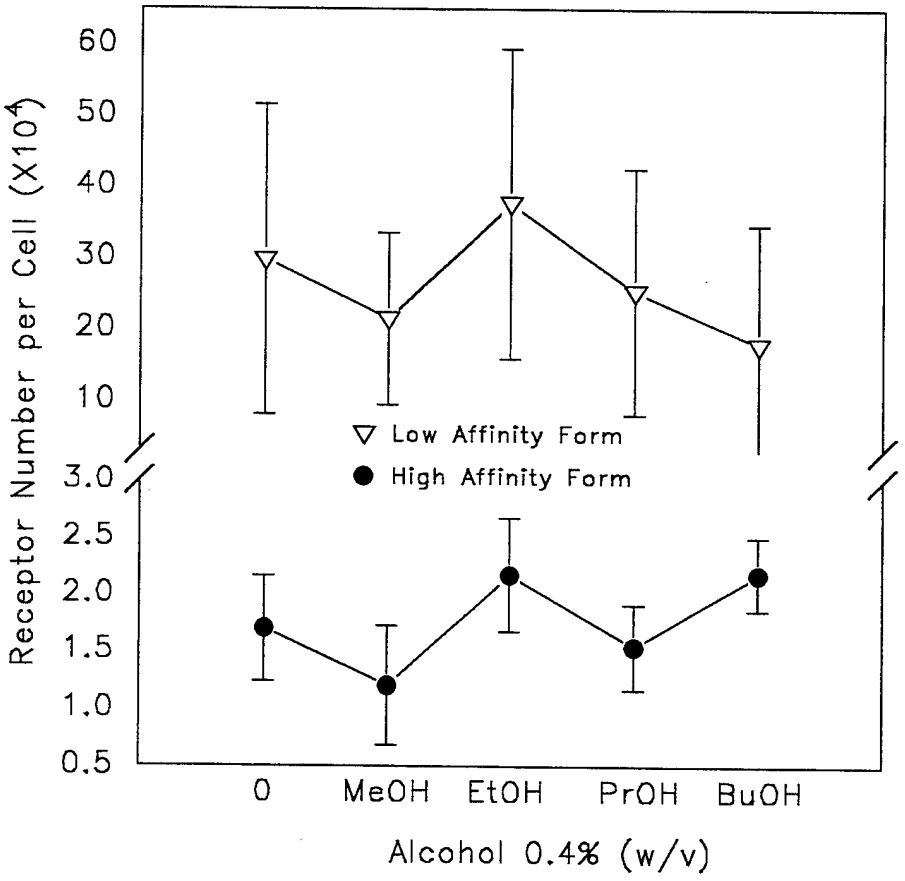

Figure 4. Effect of alcohol chain length on NGF receptor number. PC12 cells were treated with either medium or 0.4% (w/v) of MeOH, EtOH, PrOH or BuOH for 72 hours prior to harvest. Scatchard analysis was performed on each treatment using triplicate measures at each uniodinated NGF concentration. The curve fitting program, LIGAND, was used to determine the best fit lines. The experiment was repeated three independent times. Receptor numbers, calculated from the intercepts of the best fit lines, were further analyzed using a RCBD and Dunnett's t-test ($p < 0.05$) for comparison of treatment to control means. Points represent the mean + / - S. E. ($n = 3$).

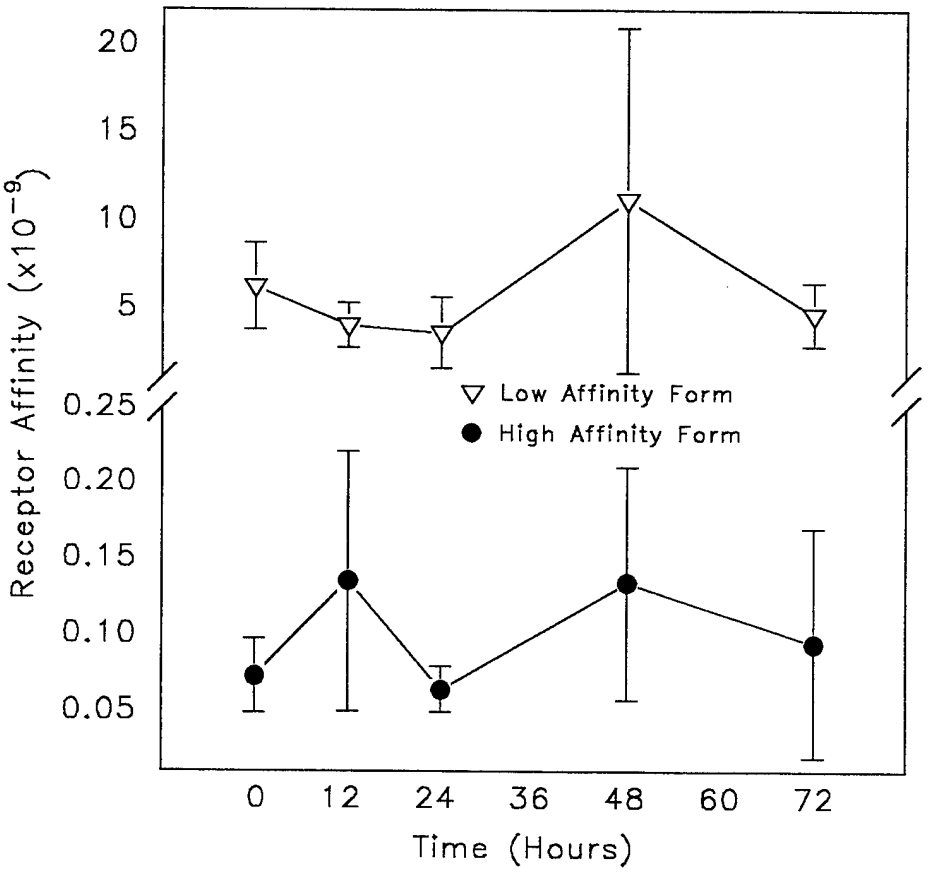

Figure 5. Effect of ethanol on NGF receptor affinity over time. PC12 cells were treated with 0.4% (w/v) of EtOH for either 0, 12, 24, 48 or 72 hours prior to harvest. Scatchard analysis was performed on treatment using triplicate measures at each uniodinated NGF concentration. The curve fitting program, LIGAND, was used to determine the best fit lines. The experiment was repeated three independent times. Receptor affinities, calculated from the slopes of the best fit lines, were further analyzed using a RCBD and Dunnetts t-test ($p < 0.05$) for comparison of treatment to control means. Points represent the mean + / - S. E. ($n = 3$).

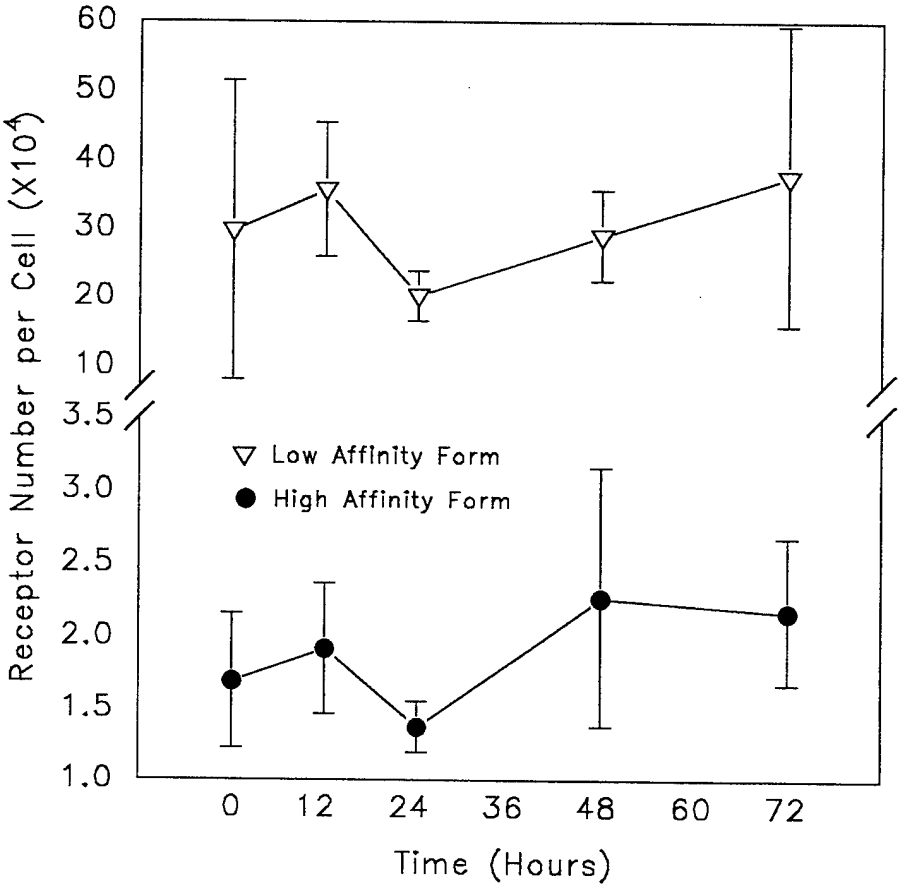

Figure 6. Effects of ethanol on NGF receptor number over time. PC12 cells were treated with 0.4% (w/v) of EtOH for either 0, 12, 24, 48 or 72 hours prior to harvest. Scatchard analysis was performed on each treatment using triplicate measures at each uniodinated NGF concentration. The curve fitting program, LIGAND, was used to determine the best fit lines. The experiment was repeated three independent times. Receptor numbers, calculated from the intercepts of the best fit lines, were further analyzed using a RCBD and Dunnett's t-test ($p < 0.05$) for comparison of treatment to control means. Points represent the mean + / - S. E. ($n = 3$).

t-test; $p < 0.05$). These results imply that the primary effect by which ethanol potentiates NGF-induced neurite outgrowth is not due to a direct alteration in either the affinities, a shift in receptor forms, or the number of NGF receptors.

Effects of Alcohol Exposure on NGF-Induced Neurite Extension

Simultaneously treated PC12 cells with alcohol and NGF for 72 hours possess a greater number of neurites that are also longer and more highly-branched than those cells stimulated with NGF alone. The enhancement effect that alcohol has on NGF-induced neurite extension occurs in a dose dependent and alcohol chain length dependent manner (Wooten and Ewald, 1991; Messing *et al.*, 1991a). The mechanism which underlies this effect is unknown. It is possible that alcohols are acting in a synergistic manner with NGF to enhance the neurotrophic property of NGF, or alternatively, alcohols may prime the cells to differentiate. If alcohols are priming the cells in some manner, then pretreating the cells with alcohols prior to NGF stimulation may enhance the degree of NGF-induced neurite extension over that observed with simultaneous treatment of alcohol and NGF.

Morphology studies were undertaken to determine if there is a specific window of time during which alcohols have a more pronounced effect on NGF-induced neurite extension. In addition, studies were designed to ascertain whether this effect was due to a specific effect of alcohol or a synergistic alcohol / NGF effect. PC12 cells were treated with an alcohol dose range that includes physiologic concentrations (0.1 - 0.4%, w/v) and pharmacologically elevated levels (0.6 - 0.8%, w/v). Time of alcohol exposure was chosen to include various time frames up to 72 hours of exposure. The treatment

regimens chosen were designed in order to determine if pretreatment of PC12 cells prior to NGF stimulation will have a more profound effect on the degree of neurite extension, compared to cells which were simultaneously treated. If there was an enhanced effect on NGF-induced neurite extension when a pretreatment regimen was employed, then this would suggest that alcohol enhances NGF's effects through a priming mechanism. Two time periods of NGF exposure were used to determine if the synergistic effect noticed at 72 hours of NGF stimulation was observed at earlier time points (Wooten and Ewald, 1991; Messing *et al.*, 1991a).

The enhancement of NGF induced neurite extension was evaluated by two independent criteria. The first was a visual observation method where the degree of neurite extension for each plate of cells was scored for an overall population average. The second method was to actually quantitate the number of cells in three individual fields per treatment to determine the percentage of cells bearing neurites. To evaluate the morphological response, several treatment regimens were employed: 1) simultaneous treatment with alcohol and NGF, 2) pretreatment with alcohol for 24 hours prior to addition of NGF and 3) pretreatment with alcohol for 72 hours prior to stimulation with NGF. Additionally, to determine early versus late neurotrophic factor effects, the assay was evaluated either at 24 or 72 hour NGF treatment endpoints.

The degree of neurite extension (plate score) and the percentage of cells bearing neurites were examined as a function of ethanol dose employing the three treatment regimens evaluated at the 24 hour endpoint (Figures 7, 8). Likewise, the cells were evaluated at the 72 hour NGF stimulation endpoint (Figures 9, 10). PC12 cells which

were evaluated at the 72 hour endpoint (Figures 9, 10) possessed a greater degree of neurite extension as well as a greater number of cells possessing neurites when compared to the 24 hour NGF stimulation endpoint (Figures 7, 8), which is attributed to longer exposure to neurotrophic factor. Treatment of cells with alcohol, independent of treatment regimen, using either endpoint evaluation time, resulted in increased neurite extension. Additionally, the percentage of cells bearing neurites increased in a dose dependent fashion when compared to cells stimulated with NGF alone. Linear regression analysis indicates that the degree of neurite extension and the percentage of cells bearing neurites increases in a dose-dependent fashion that is independent of treatment regimen, for both endpoint evaluations ($p < 0.0001$). Linear contrast statements were used to compare the lines generated by each of the treatment regimens to each other. Using this analysis, the dose dependent degree of neurite extension and percentage of cells bearing neurites was unaltered by treatment regimen for either NGF endpoint (Figures, 7, 8, 9, 10). Even though treatment of cells with alcohol alone does not induce these cells to extend neurites, these results suggest that ethanol enhances NGF's effects in a dose dependent fashion which is independent of treatment regimen. Although PC12 cells stimulated with NGF for 72 hours possessed increased numbers of cells which were differentiated, this analysis revealed that no specific alcohol treatment regimen effect could be documented.

The physio-pharmacological effects of the aliphatic alcohols increase as alcohol chain length increases (McCreery and Hunt, 1978). It has been observed that increasing alcohol chain length, increases the degree of NGF-induced neurite extension, and the

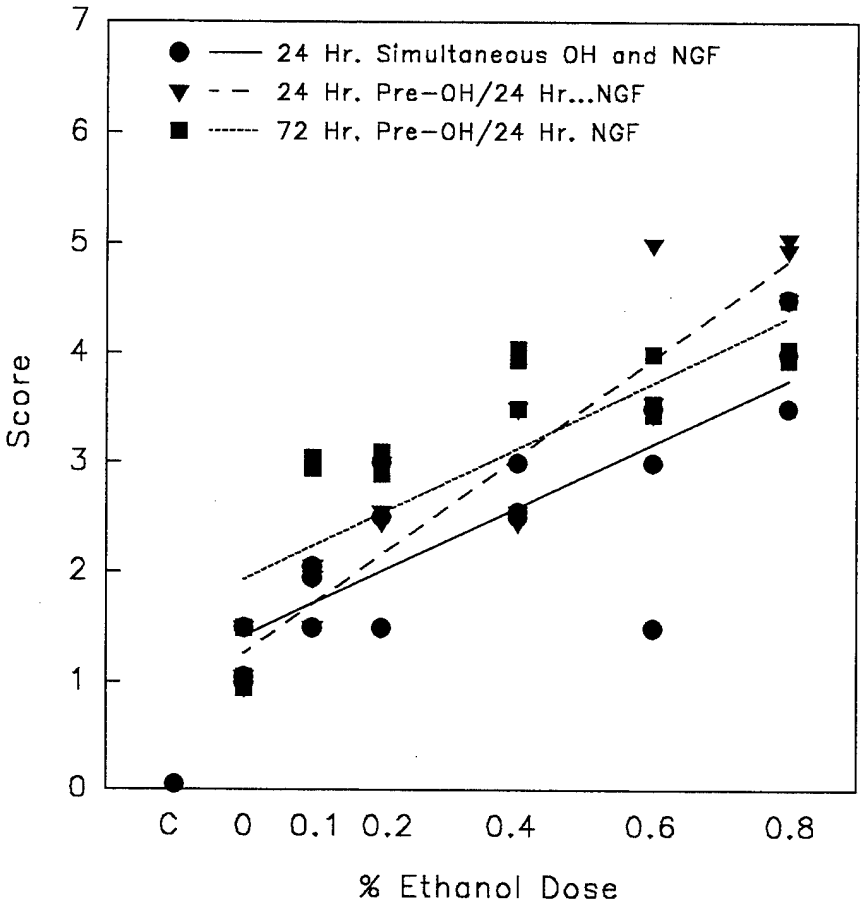

Figure 7. Effect of ethanol dose on neurite extension after 24 hours of NGF stimulation. The degree of neurite extension was evaluated following either; (● ⁻) simultaneous treatment of alcohol and NGF for 24 hours, (▼⁻⁻) pretreatment with alcohol for 24 hours prior to stimulation with NGF for 24 hours, or (■···) pretreatment with alcohol for 72 hours prior to stimulation with NGF for 24 hours. Each point represents the score obtained from an individual plate from one experiment. The lines were obtained from linear regression using the means of the three individual experiments.

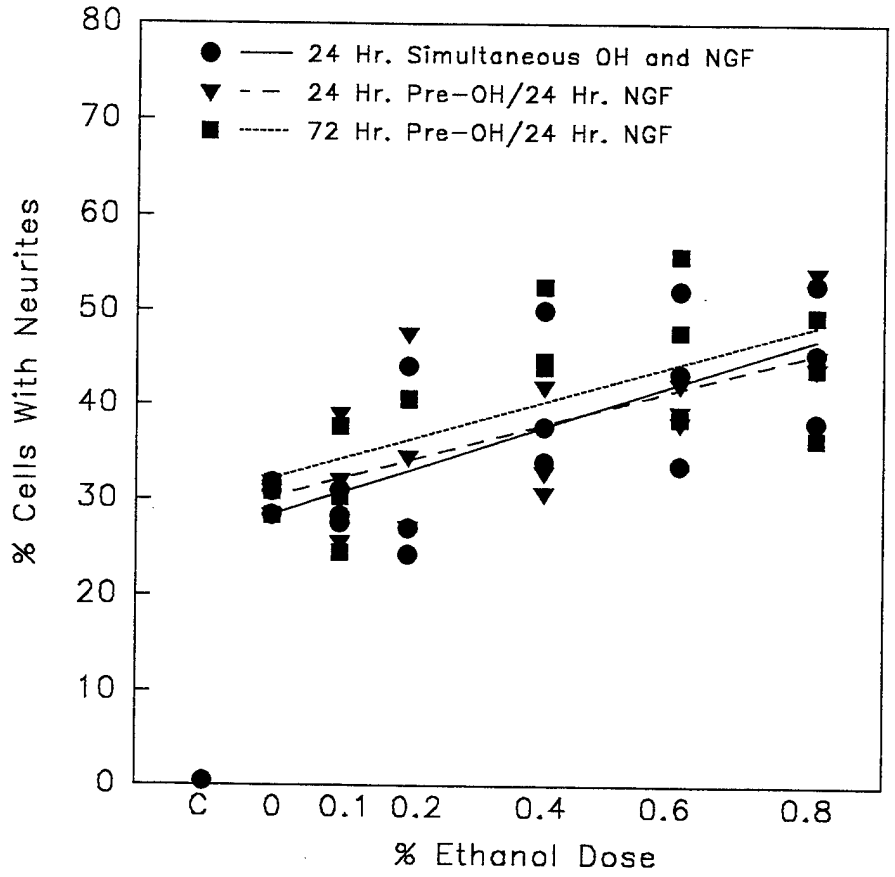

Figure 8. Effect of ethanol dose on the number of cells which possess neurites after 24 hours of NGF stimulation. The number of cells which possess neurites were evaluated following either (● —) simultaneous treatment of alcohol and NGF for 24 hours (▼ –) pretreatment with alcohol for 24 hours prior to stimulation with NGF for 24 hours or (■ooo) pretreatment with alcohol for 72 hours prior to stimulation with NGF for 24 hours. Each point represents the mean of the three counts for each individual plate of PC12 cells; lines indicate linear regression obtained from the means of the mean results of three independent experiments.

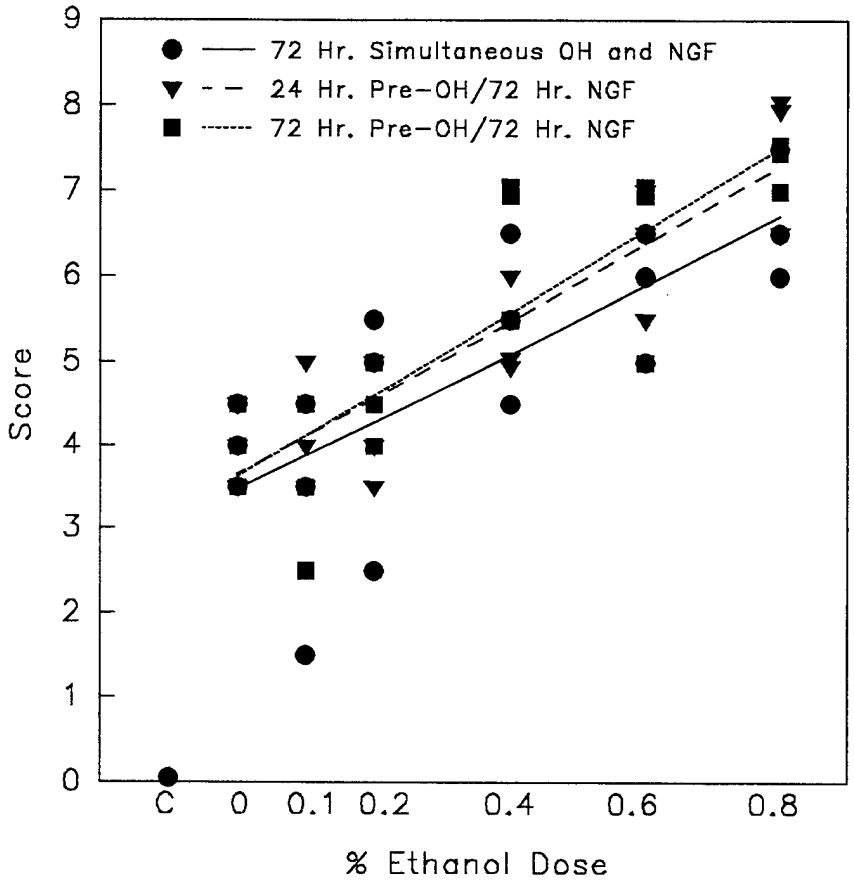

Figure 9. Effect of ethanol dose on neurite extension after 72 hours of NGF stimulation. The degree of neurite extension was evaluated following either; (● ⁻) simultaneous treatment of alcohol and NGF for 72 hours, (▼⁻⁻) pretreatment with alcohol for 24 hours prior to stimulation with NGF for 72 hours, or (■···) pretreatment with alcohol for 72 hours prior to stimulation with NGF for 72 hours. Each point represents the score obtained from an individual plate from one experiment. The lines were obtained from linear regression using the means of the three individual experiments.

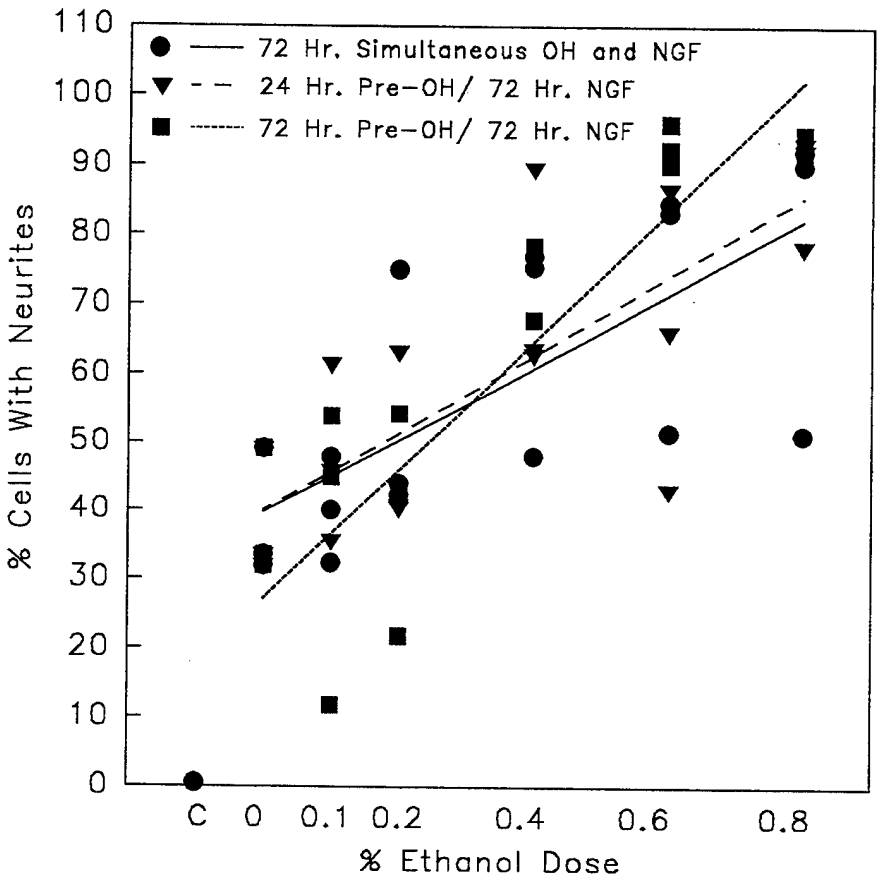

Figure 10. Effect of ethanol dose on the number of cells which possess neurites after 72 hours of NGF stimulation. The number of cells which possess neurites were evaluated following either (● —) simultaneous treatment of alcohol and NGF for 72 hours (▼–) pretreatment with alcohol for 24 hours prior to stimulation with NGF for 72 hours or (■◦◦◦) pretreatment with alcohol for 72 hours prior to stimulation with NGF for 72 hours. Each point represents the mean of the three counts for each individual plate of PC12 cells; lines indicate linear regression obtained from the means of the mean results of three independent experiments.

percentage of cells bearing neurites in PC12 cells treated simultaneously with alcohol and NGF for 72 hours (Wooten and Ewald, 1991; Messing *et al.*, 1991a). Thus, the effects that other alcohol chain lengths have on the degree of neurite extension and the percentage of cells bearing neurites was evaluated using the outlined treatment regimens.

Neurite extension observed within the 24 hour NGF stimulation endpoint, for all of the alcohol chain length treatment regimens tested, was greater than that observed for cells stimulated with NGF alone as indicated by Dunnett's t-test ($p < 0.005$) (Figure 11). There was not a specific trend of alcohol chain length (Tukey's LSD; comparing methanol to ethanol to propanol to butanol). In addition, no effect could be attributed to within treatment regimens for cells treated with either methanol or ethanol (Tukey's LSD). However, within the longer chain length alcohols, propanol and butanol, there was a specific treatment regimen effect on neurite extension. The simultaneous treatment regimen yielded less neurite extension than the 24 hour alcohol pretreatment regimen (Tukey's LSD, $p < 0.05$). Neurite extension induced by the 72 hour alcohol pretreatment regimen fell between the simultaneous regimen and the 24 hour pretreatment regimen.

Likewise, the effect of alcohol chain length was evaluated for the percentage of cells bearing neurites for the 24 hour NGF-stimulation endpoint (Figure 12). The percentage of cells bearing neurites for those cells pretreated with propanol for 72 hours, pretreated with butanol for 24 hours and pretreated with butanol for 72 hours was increased as compared to the percentage of cells bearing neurites for those cells stimulated with NGF alone (Dunnett's t-test; $p < 0.05$). Tukey's LSD revealed no chain length effect; for example; treatment with methanol was not different from treatment with ethanol, from

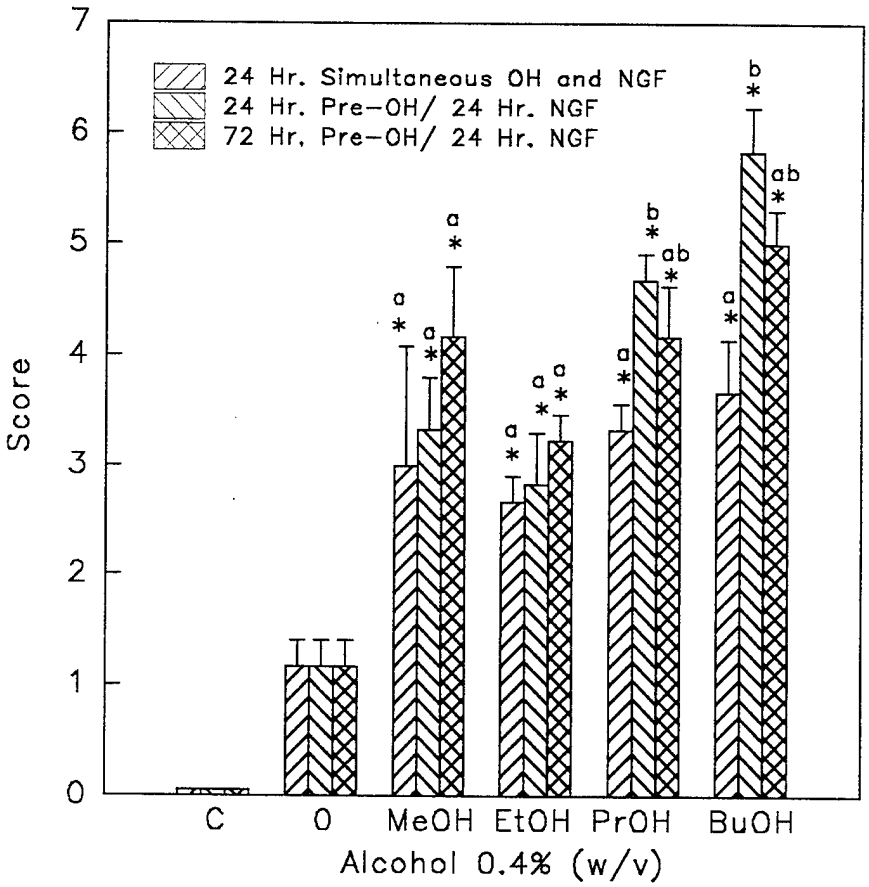

Figure 11. Effect of alcohol chain length on neurite extension after 24 hours of NGF stimulation. The degree of neurite extension was evaluated following either: simultaneous treatment of alcohol and NGF for 24 hours, pretreatment with alcohol for 24 hours prior to stimulation with NGF for 24 hours, or pretreatment with alcohol for 72 hours prior to stimulation with NGF for 24 hours. Each bar represents the mean + / - S. E. M. (n = 3). Bars that are significantly different from cells treated with NGF alone are indicated by *. Letters indicate significant differences within alcohol treatment regimens.

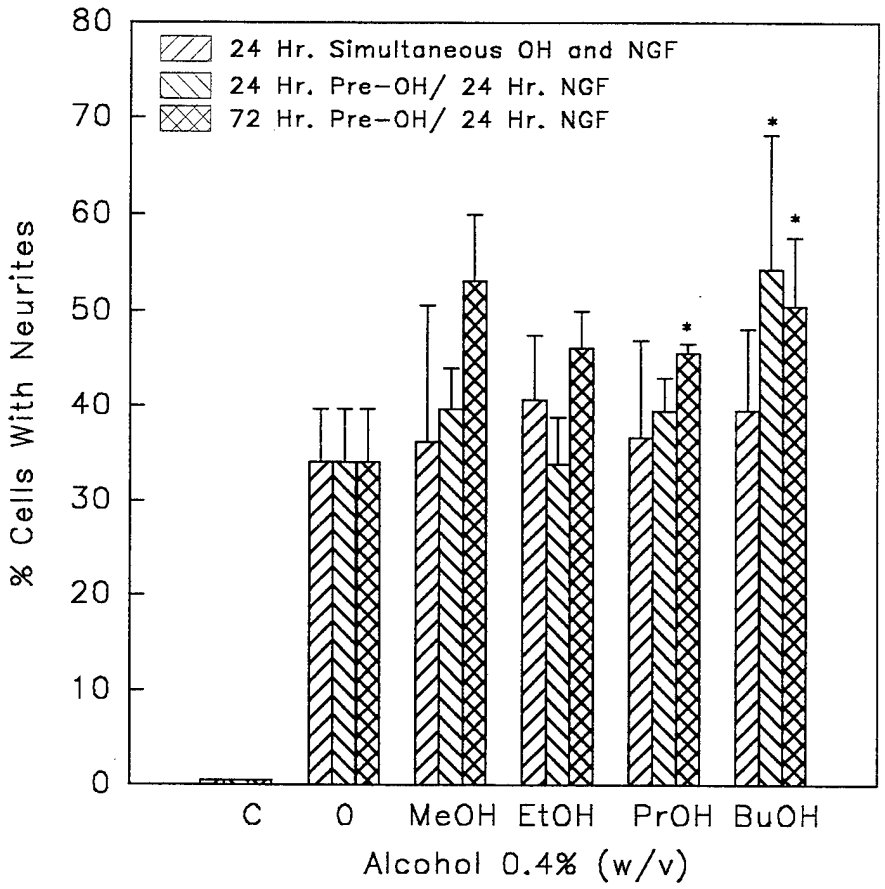

Figure 12. Effect of alcohol chain length on the number of cells which possess neurites after 24 hours of NGF stimulation. The number of cells which possess neurites were evaluated following either: simultaneous treatment of alcohol and NGF for 24 hours, pretreatment with alcohol for 24 hours prior to stimulation with NGF for 24 hours, or pretreatment with alcohol for 72 hours prior to stimulation with NGF for 24 hours. The percentage of cells bearing neurites was obtained from three random fields for each treatment and a mean was obtained. The experiment was repeated three independent times. Each bar represents the mean + / - S. E. M. (n = 3). Those that are significantly different from cells stimulated with NGF alone are indicated by *.

treatment with propanol, from treatment with butanol. There was no specific treatment regimen effect observed within any of the alcohols (Tukey's LSD; $p < 0.05$). The 24 hour pretreatment regimen of the longer chain alcohols did enhance the neurite score, but had no effect on the percentage of cells bearing neurites. This indicates that the cells that responded produced neurites of greater length, but the number of cells that respond is not altered by the treatment regimens.

Accordingly, alcohol chain length treatment was also evaluated after the 72 hour NGF-stimulated endpoint time for both morphological parameters (Figures 13, 14). Cells evaluated at the 72 hour endpoint exhibited greater plate scores and percentages of cells bearing neurites than those evaluated at the 24 hour endpoint (Figures 11, 12, 13, 14). This is attributed to longer exposure to the neurotrophic factor. The degree of neurite extension for the 72 hour ethanol pretreatment regimen, both the 24 hour and 72 hour propanol pretreatment regimens and all of the butanol treatment regimens were greater than the degree of neurite extension induced by NGF stimulation alone (Dunnetts t-test; $p < 0.05$, Figure 13). Treatment regimens for the 72 hour NGF stimulation endpoint (Figure 14) shows that cells pretreated with ethanol for either 24 or 72 hours, cells pretreated with propanol for either 24 or 72 hours and cells treated with butanol, independent of treatment regimen, possessed greater percentages of cells bearing neurites than cells stimulated with NGF alone (Dunnett's t-test; $p < 0.05$).

There was an alcohol chain length trend, such that the average neurite length and the percentage of cells bearing neurites increased with chain length (methanol < ethanol < propanol < butanol) (Tukey's LSD; $p < 0.05$, Figures 13, 14). With all of the alcohols, no

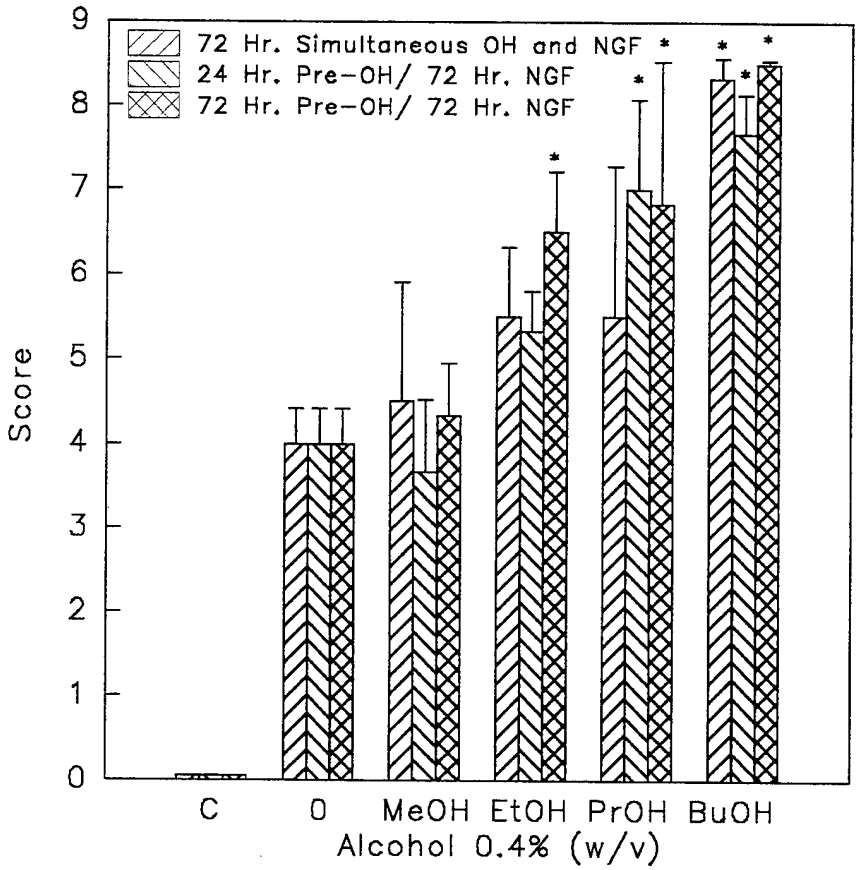

Figure 13. Effect of alcohol chain length on neurite extension after 72 hours of NGF stimulation. The degree of neurite extension was evaluated following either: simultaneous treatment of alcohol and NGF for 72 hours, pretreatment with alcohol for 24 hours prior to stimulation with NGF for 72 hours, or pretreatment with alcohol for 72 hours prior to stimulation with NGF for 72 hours. Each bar represents the mean + / - S. E. M. (n = 3). Bars that are significantly different from cells treated with NGF alone are indicated by *.

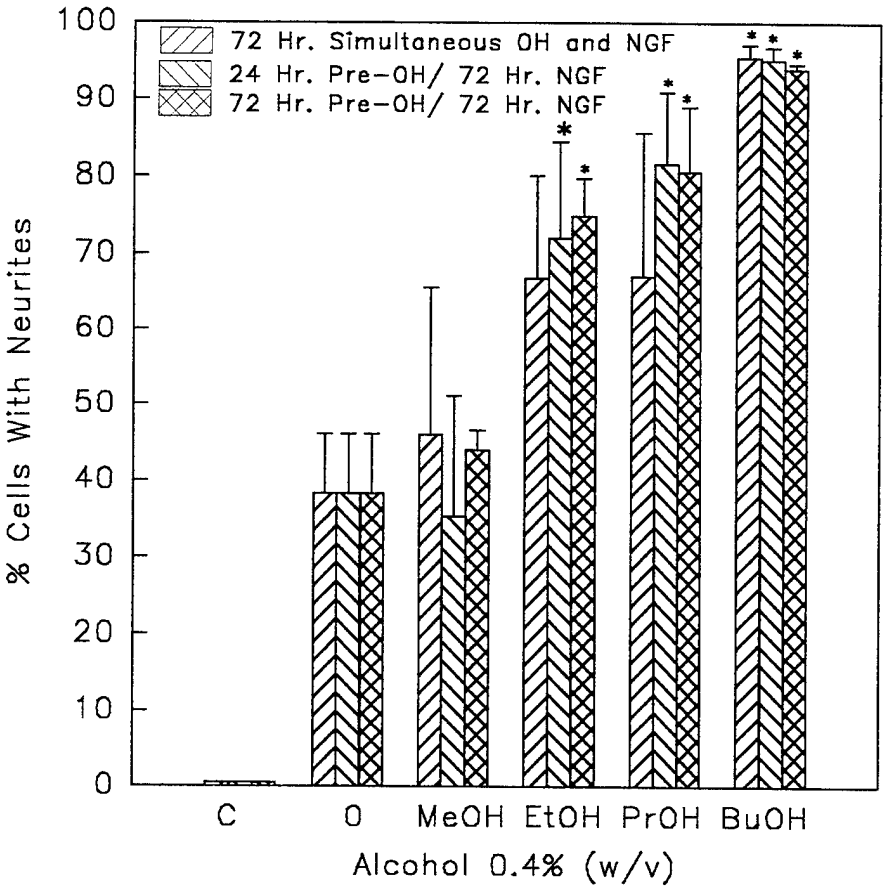

Figure 14. Effect of alcohol chain length on the number of cells which possess neurites after 72 hours of NGF stimulation. The number of cells which possess neurites were evaluated following either: simultaneous treatment of alcohol and NGF for 72 hours, pretreatment with alcohol for 24 hours prior to stimulation with NGF for 72 hours, or pretreatment with alcohol for 72 hours prior to stimulation with NGF for 72 hours. The percentage of cells bearing neurites were obtained from three random fields for each treatment and a mean was obtained. The experiment was repeated three independent times. Each bar represents the mean + / - S. E. M. obtained by using the independent experimental means (n = 3). Those that are significantly different from cells stimulated with NGF alone are indicated by *.

specific treatment regimen effect was observed after 72 hours of NGF stimulation for either morphological parameter as shown by Tukey's LSD ($p < 0.05$). Two responses of PC12 cells to increasing alcohol chain length were altered by the length of time that the cells were stimulated with NGF. First, there was an apparent lack of a trend associated with alcohol chain length effect on the degree of neurite extension and the percentage of cells bearing neurites at the 24 hour NGF endpoint. In contrast, a trend associated with alcohol chain length was observed at the 72 hour endpoint for both morphological parameters. The second observation that was altered by length of culture with NGF is the specific effect which alcohol has on the degree of neurite extension. For example, an apparent 24 hour pretreatment effect was observed with the longer chain alcohols at the early NGF endpoint, but this effect is no longer apparent at the later endpoint. In summary, even though after 24 hours of NGF treatment there was a specific treatment regimen effect on neurite extension with the longer chain alcohols (propanol and butanol), this specific treatment regimen effect was no longer observed after 72 hours of treatment with NGF. Furthermore, while a specific treatment regimen effect on neurite extension was observed at the 24 hour NGF endpoint, no treatment regimen effect was observed with either NGF endpoint for the percentage of cells bearing neurites. This implies that even though the cells are induced to produce longer neurites when exposed to the alcohol prior to NGF stimulation, this pretreatment effect is not altering the number of cells that are capable of the initial response to the neurotrophic factor.

To further examine the effects of ethanol pretreatment on NGF-induced neurite extension and percentage of cells bearing neurites, a time course study was performed in

which the pretreatment time varied and the cells were evaluated at either the 24 or 72 hour NGF stimulation endpoints. In Figures 15 and 16, the results obtained for the degree of neurite extension and the percentage of cells bearing neurites, respectively, for an ethanol pretreatment time course study using 24 and 72 hour NGF stimulation endpoints are shown. This study was undertaken to determine if there was any ethanol pretreatment time-dependent increase in either morphological parameter. PC12 cells stimulated with NGF for 72 hours exhibited a greater degree of neurite extension and percentage of cells bearing neurites than those stimulated with NGF for 24 hours.

Cells pretreated with ethanol and evaluated for neurite extension at the 24 hour NGF stimulation endpoint showed a good fit to a linear model ($r^2 = 0.946$), while cells evaluated at the 72 hour NGF stimulation endpoint did not fit a linear model ($r^2 = 0.605$). Furthermore, cells pretreated with ethanol and evaluated for the percentage of cells bearing neurites at the 24 hour NGF stimulation endpoint had a regression coefficient of $r^2 = 0.853$, while those evaluated after the 72 hour NGF stimulation endpoint had a regression coefficient of only $r^2 = 0.524$. Increasing ethanol pretreatment time prior to NGF stimulation for 24 hours shows an alcohol pretreatment time-dependent increase in NGF-induced neurite extension as well as the percentage of cells bearing neurites. However, after 72 hours of NGF stimulation, there is no longer as great an alcohol pretreatment time-dependent increase in NGF-induced neurite extension or the percentage of cells bearing neurites. Thus, ethanol may have a stimulatory effect on NGF-induced neurite extension as pretreatment time increases during the early periods of NGF stimulation. By the time the cells are stimulated with NGF for 72 hours, much of the

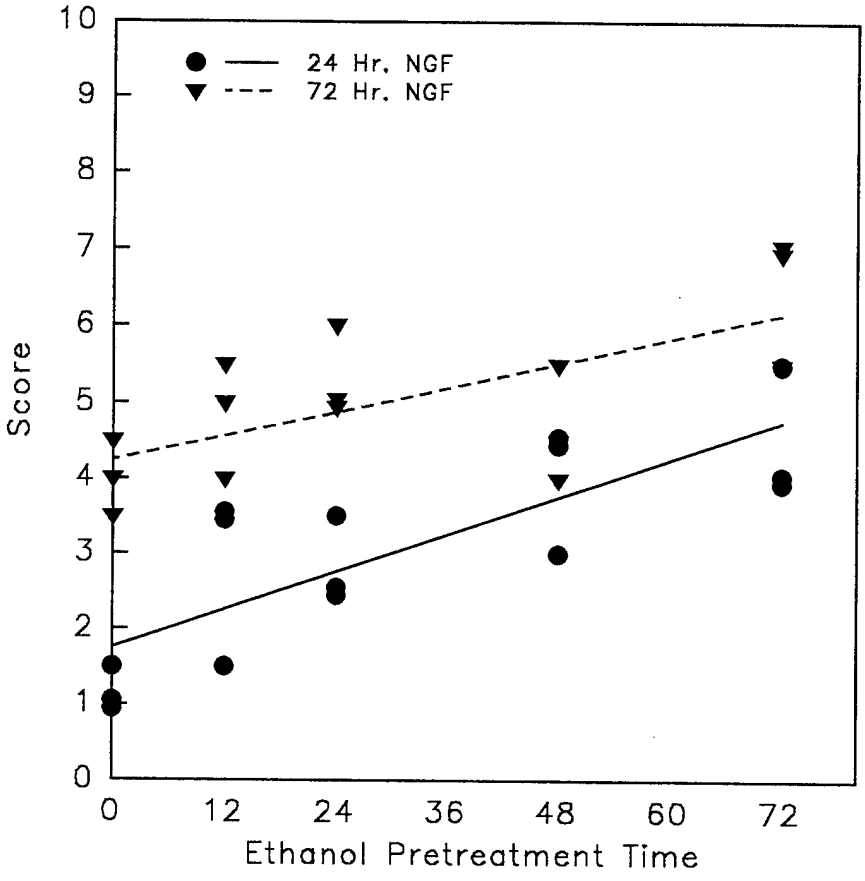

Figure 15. Time course evaluation of ethanol pretreatment time on neurite extension after either 24 or 72 hours of NGF stimulation. PC12 cells were stimulated with 50 ηg/ml NGF alone for either 24 or 72 hours or pretreated with 0.4% (w/v) ethanol for either 12, 24, 48, or 72 hours followed by stimulation with 50 ηg/ml NGF for either 24 or 72 hours. The degree of neurite extension was evaluated at the end of the NGF stimulation. Each point represents one sample. Linear regression analysis of the mean + / - S. E. M. (n = 3) indicated that cells stimulated with NGF for 24 hours fit a linear model well while those stimulated with NGF for 72 hours did not fit a linear model as well.

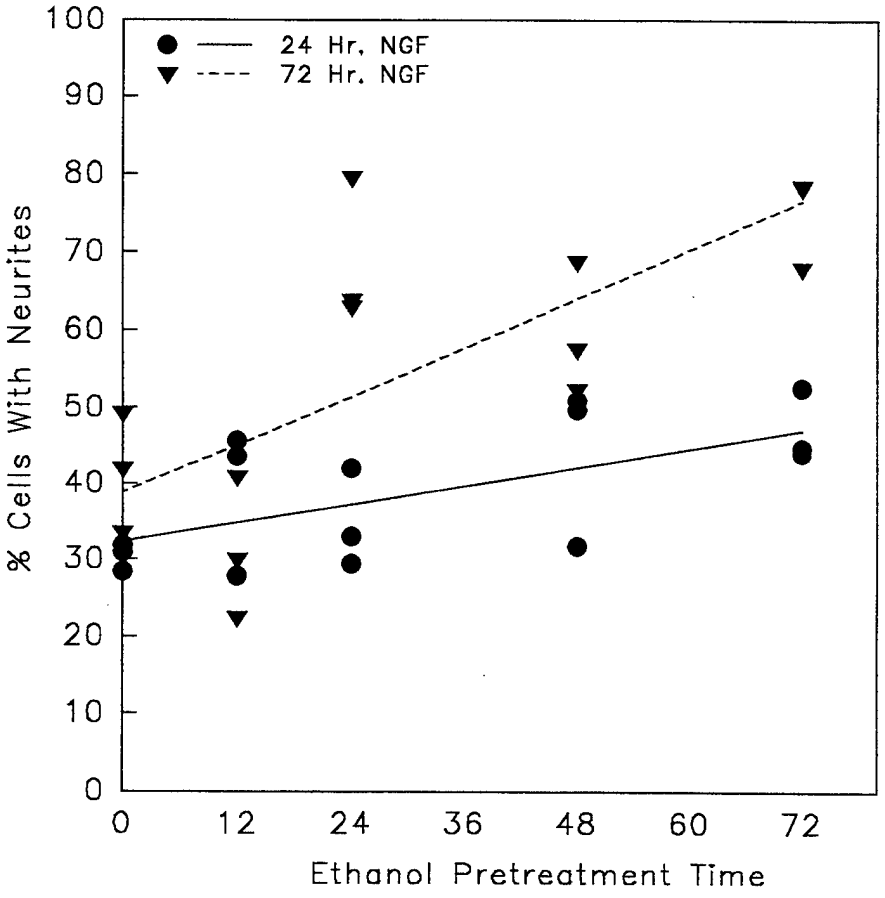

Figure 16. Time course evaluation of ethanol pretreatment time on the number of cells which possess neurites after either 24 or 72 hours of NGF stimulation. PC12 cells were stimulated with 50 ηg/ml NGF alone for either 24 or 72 hours, or pretreated with 0.4% (w/v) ethanol for either 12, 24, 48, or 72 hours followed by 50 ηg/ml NGF stimulation for either 24 or 72 hours. The number of cells possessing neurites were evaluated at the end of the NGF stimulation. The percentage of cells bearing neurites were obtained from three random fields for each treatment and a mean was obtained. The experiment was repeated three independent times. Each point represents the mean of triplicate determinations. Lines were obtained by linear regression analysis using the independent experimental means + / - S. E. M (n = 3). Regression analysis indicated that cells stimulated with NGF for 24 hours fit a linear model well while those stimulated with NGF for 72 hours did not fit a linear model as well.

underlying specific alcohol effect is overpowered by the strong synergistic ethanol / NGF effect.

The second time course evaluation was undertaken to examine whether the rate of NGF-induced neurite extension or the percentage of cells bearing neurites is altered by pretreatment of alcohol, as compared to simultaneous treatment with alcohol and NGF or NGF alone (Figure 17, 18). All three treatment regimens show an NGF stimulation time dependent increase in both morphological parameters. Analysis by linear contrast statements shows that the line which describes the neurite extension for the pretreatment alcohol regimen has a slope of $b = 0.092$ which is greater than either the slope of the line for simultaneously treated cells ($b = 0.070$; $p < 0.01$) or the slope of the line for NGF alone treated cells ($b = 0.057$; $p < 0.0004$). There was no difference between the slopes of the lines for neurite extension with the simultaneously treated cells ($b = 0.070$) and the NGF alone treated cells ($b = 0.057$; $p < 0.14$). The slope of the line for the percentage of cells bearing neurites created by the pretreatment regimen ($b = 9.396$) is greater than the slope of the line for the cells were stimulated with NGF alone ($b = 6.430$; $p < 0.01$). However, there was no difference in the slopes of the percentage of cells bearing neurites for the pretreatment regimen ($b = 9.396$) versus the simultaneous treatment regimen ($b = 8.382$; $p < 0.23$) or for the simultaneous treatment regimen ($b = 8.382$) versus the NGF treatment ($b = 6.430$; $p < 0.11$). The rate of change in neurite extension brought on by pretreating cells with alcohol prior to NGF stimulation is greater than the rate of change of either simultaneously treating cells with ethanol and NGF or stimulating cells with NGF alone. The rate of change in the percentage of cells bearing neurites brought on

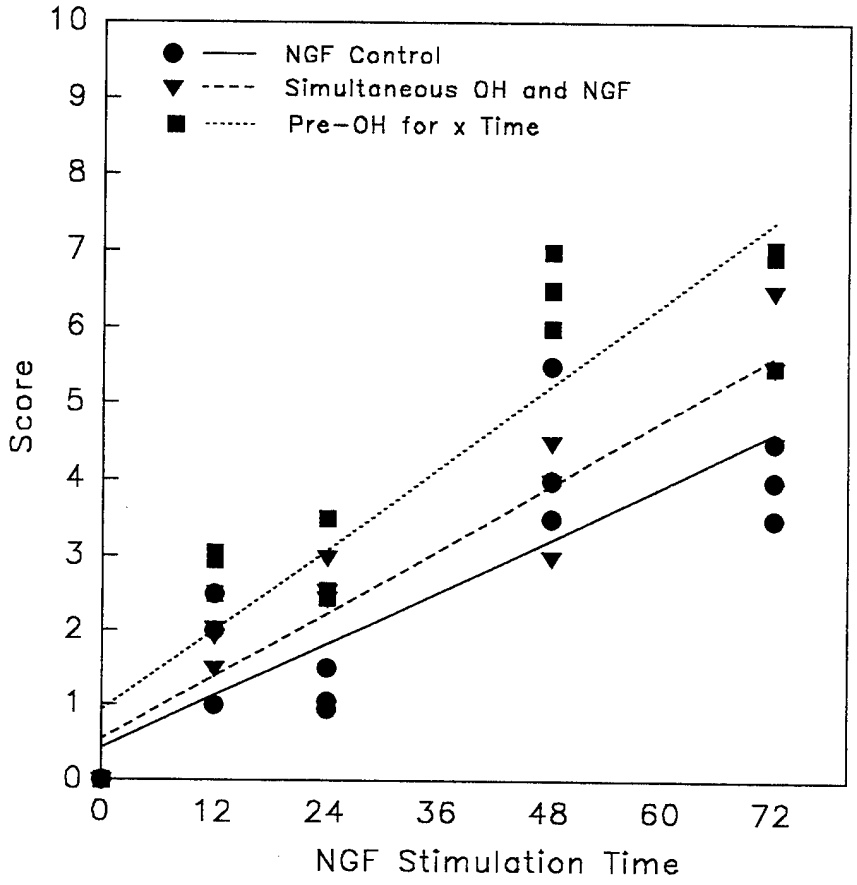

Figure 17. Time course evaluation of ethanol treatment regimen on neurite extension over NGF stimulation time. PC12 cells were either: treated with 50 ηg/ml NGF alone for the indicated time periods, treated with 0.4% (w/v) ethanol and 50 ηg/ml NGF simultaneously for the indicated time periods or, pretreated with 0.4% (w/v) ethanol for the indicated time period followed by 50 ηg/ml NGF treatment for the indicated time periods. Each point represents one sample. Linear regression analysis using the means + / - S. E. M. (n = 3) showed that all treatment regimens fit a linear model. The slope of the line formed by pretreatment with alcohol was greater than the slopes of the other two lines.

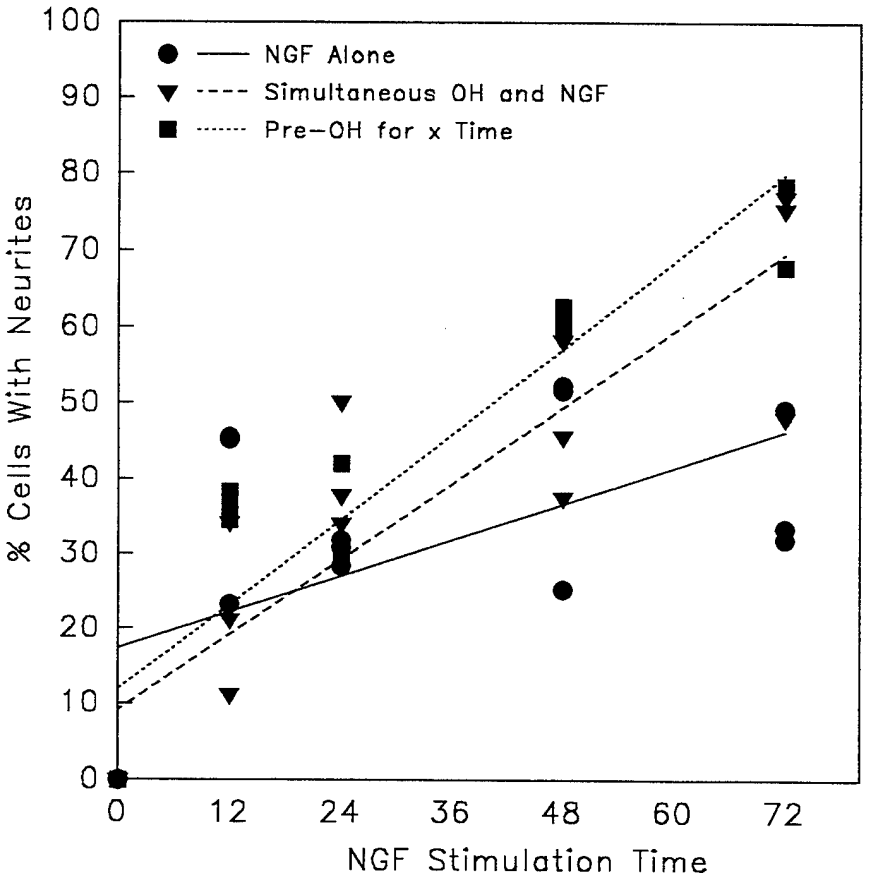

Figure 18. Effect of ethanol on the number of cells which possess neurites with ethanol treatment regimens over NGF stimulation time. PC12 cells were either: stimulated with 50 ηg/ml NGF alone for the indicated time periods, treated with 0.4% (w/v) ethanol and stimulated with 50 ηg/ml NGF simultaneously for the indicated time periods, or pretreated with 0.4% (w/v) ethanol for the indicated time period followed by 50 ηg/ml NGF stimulation for the indicated time periods. The percentage of cells bearing neurites was obtained from three random fields for each treatment and a mean was obtained. The experiment was repeated three independent times. Each point represents one sample. Linear regression analysis using the means + / - S. E. M. (n = 3) showed that all treatment regimens fit a linear model. The slope of the line formed by pretreatment with ethanol was greater than the slopes of the other two lines.

by pretreating cells with ethanol is greater than NGF stimulation alone, but is not greater than simultaneous treatment. Although pretreatment of cells with ethanol has a greater effect than simultaneous treatment on the degree of neurite extension, there is no effect on the percentage of cells bearing neurites with either pretreatment or simultaneous treatment. This is similar to what was observed for the longer chain alcohols where the 24 hour pretreatment regimen induced a greater degree of neurite extension but failed to induce a greater percentage of cells bearing neurites. Pretreatment of PC12 cells with alcohol may prime NGF-induced neurite extension to a greater extent than simultaneous alcohol and NGF treatment in a subtle alcohol-specific manner. It appears as though the alcohol-specific effect alters the rate of neurite extension without affecting the numbers of cells that are capable of responding to the neurotrophic factor. Even though the specific effect of alcohol was not apparent in the individual dose responses tested, there is evidence that a specific alcohol effect exists and that pretreating cells with alcohol increases this response. This further argues for the potential of an underlying specific alcohol effect on NGF-induced neurite extension.

One effect that is not apparent within the presentation of these results is that the alcohol treated cells which do respond to NGF, generally extend more neurites per cell than those cells stimulated with NGF alone. The neurites that are extended in alcohol treated cells also appear more branched with abnormal morphology which is best illustrated with the high concentrations of ethanol and the longer chain alcohols. Appendix figures 41 - 56 show the morphology boards prepared from those treatment regimens just described.

Effects of Alcohol on [^{3}H]-Thymidine Incorporation

Alcohol has been shown to reduce the number of PC12 cells in culture (Pantazis *et al.*, 1992) but it is unclear if this is due to cell death or a decrease in cellular proliferation. Alcohol may also be priming the cells to respond to the neurotrophic factor. To test the possibility of a specific alcohol effect on PC12 cell proliferation, the degree of [^{3}H]-thymidine incorporated into alcohol treated and control cells was evaluated. Ethanol concentrations ranged from low physiologic to high pharmacologic, and the times tested were for short term (24 hours) and long term (72 hours) treatment, similar to the morphology studies. Figures 19 and 20 depict the dose responses obtained after either 24 or 72 hours of ethanol treatment, respectively. The means of each treatment time were compared using Dunnetts t-test ($p < 0.05$) with no differences being observed for any ethanol treatment. These results document that there is no specific effect of ethanol on the proliferation status of PC12 cells at any dose utilized.

The effect of alcohol chain length was also compared using Dunnetts t-test ($p < 0.05$). Cells (PC12) treated with either propanol or butanol for 24 hours showed a significant reduction in the percentage of [^{3}H]-thymidine incorporated (Figure 21). There was an alcohol chain length trend observed for the amount of [^{3}H]-thymidine incorporated into cells treated with these alcohols for 24 hours. However, by 72 hours of alcohol treatment (Figure 22), there were no differences in the amount of [^{3}H]-thymidine incorporation and the alcohol chain length trend was no longer observed. This finding suggests that the longer chain alcohols may induce a specific effect on PC12 cell

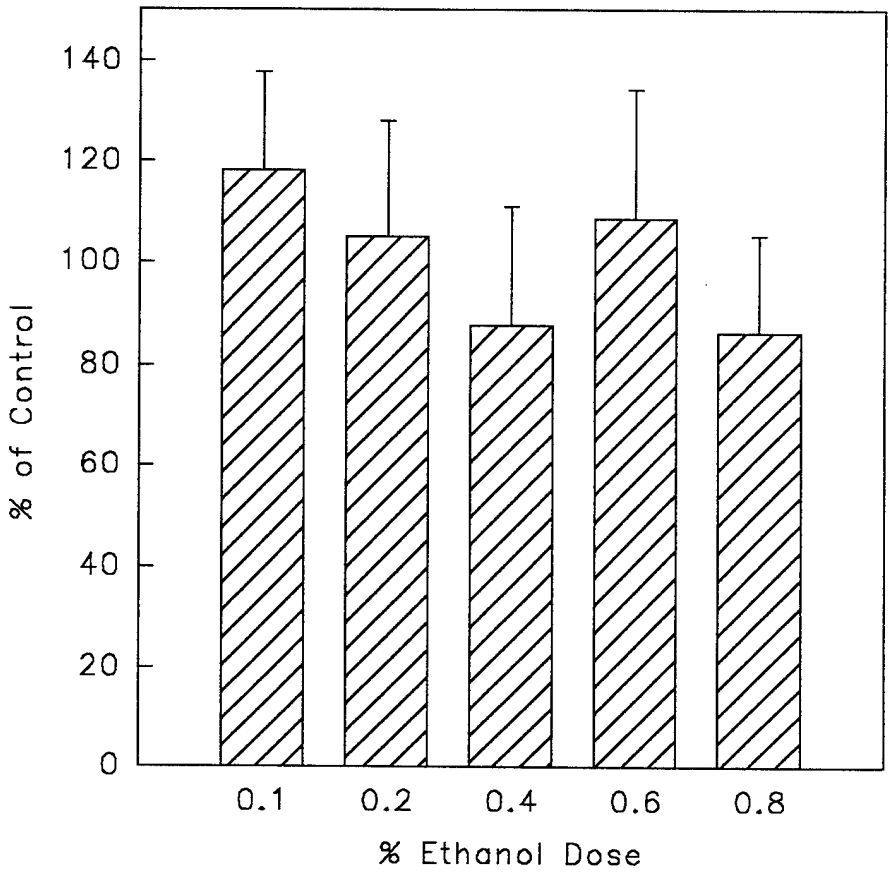

Figure 19. Effect of 24 hour ethanol treatment on proliferation of PC12 cells. The percentage of [^{3}H]-thymidine incorporated into cells which were treated with ethanol at 0.1%, 0.2%, 0.4%, 0.6% or 0.8% (w/v) for 24 hours compared to cells treated with medium for 24 hours. Each treatment of an individual experiment consisted of 24 replicates. The experiment was repeated four independent times. Each bar represents the mean + / - S. E. M. (n = 4). The data was analyzed using a RCBD after performing an arc/sin transformation on the raw percentages.

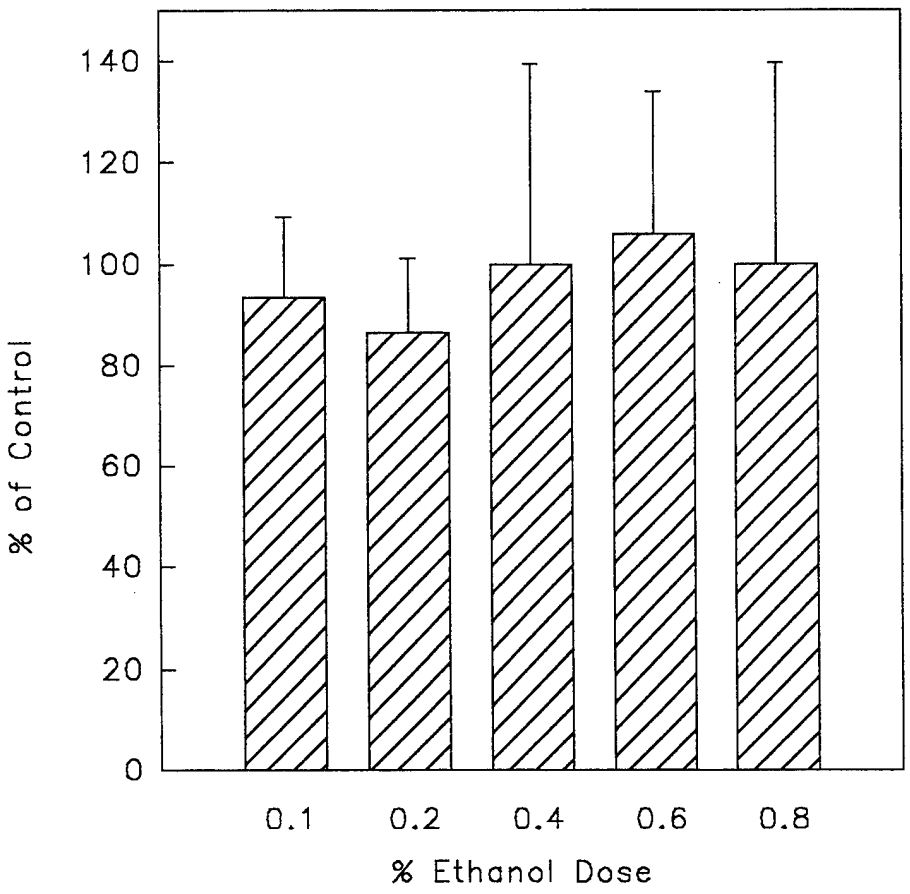

Figure 20. Effect of 72 hour ethanol treatment on proliferation of PC12 cells. The percentage of [^{3}H]-thymidine incorporated into cells which were treated with ethanol at 0.1%, 0.2%, 0.4%, 0.6% or 0.8% (w/v) for 72 hours compared to cells treated with medium for 72 hours. Each treatment of an individual experiment consisted of 24 replicates. The experiment was repeated four independent times. Each bar represents the mean + / - S. E. M. (n = 4). The data was analyzed using a RCBD after performing an arc/sin transformation on the raw percentages.

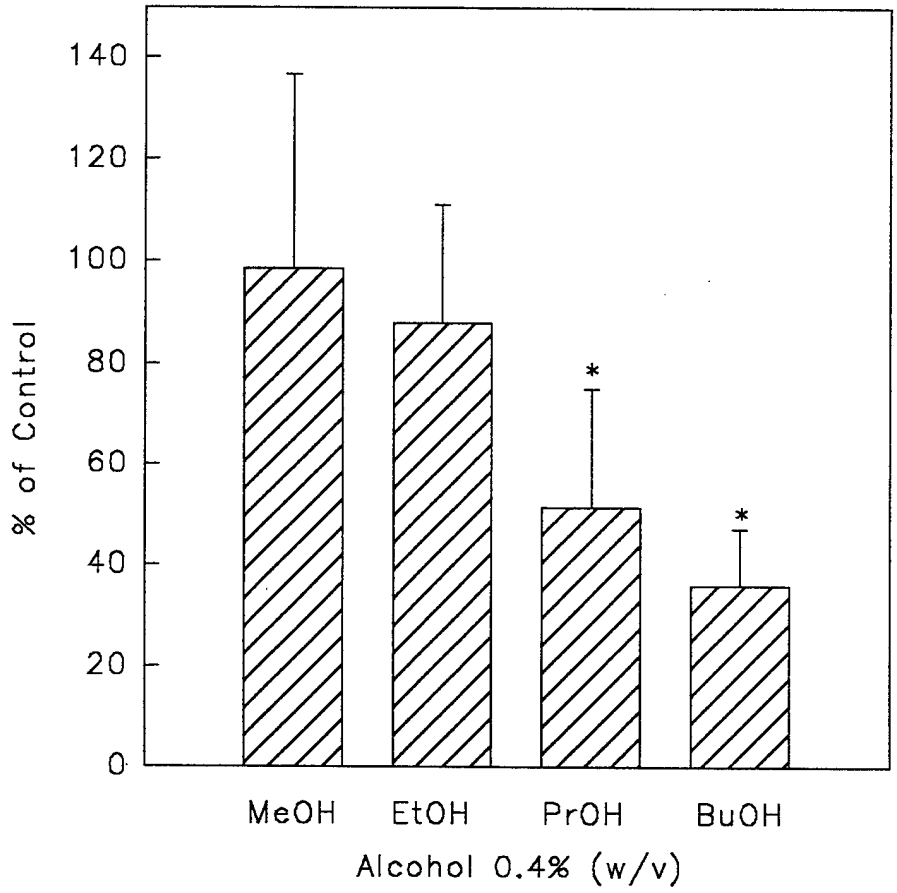

Figure 21. Effect of 24 hour alcohol chain length treatment on proliferation of PC12 cells. The percentage of [^{3}H]-thymidine incorporated into cells which were treated with 0.4% (w/v) MeOH, EtOH, PrOH or BuOH for 24 hours compared to cells treated with medium for 24 hours. Each treatment of an individual experiment consisted of 24 replicates. The experiment was repeated four independent times. Each bar represents the mean + / - S. E. M. (n = 4). The data was analyzed using a RCBD after performing an arc/sin transformation on the raw percentages. Significant differences from control means are denoted by *.

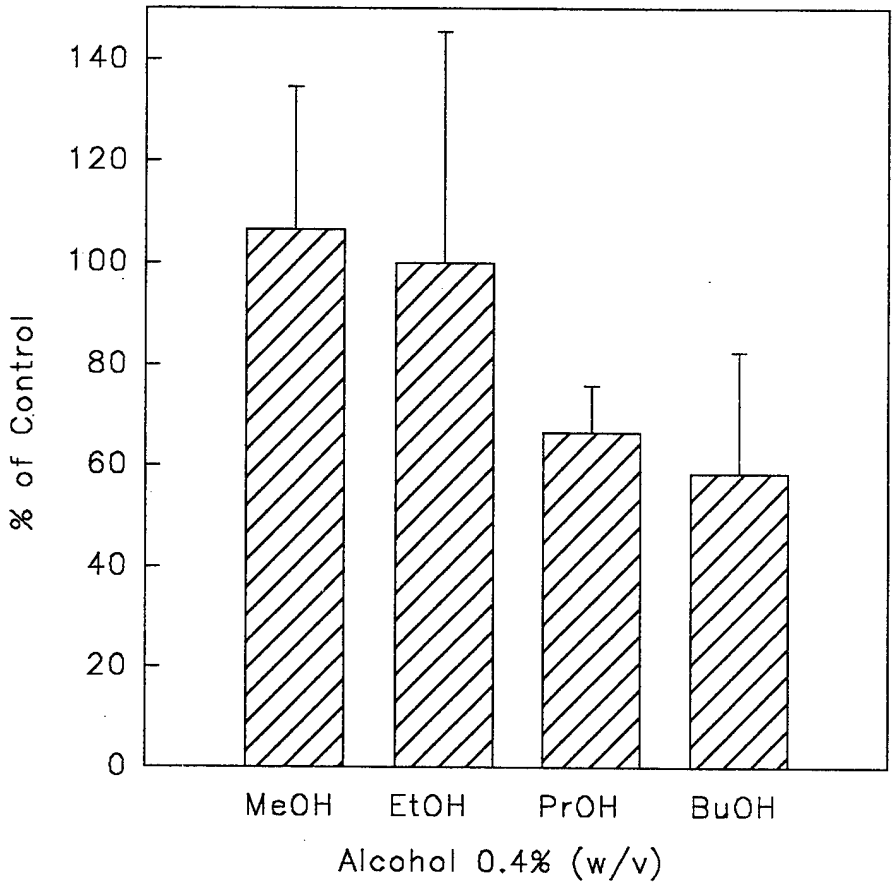

Figure 22. Effect of 72 hour alcohol chain length treatment on proliferation of PC12 cells. The percentage of [^{3}H]-thymidine incorporated into cells which were treated with 0.4% (w/v) MeOH, EtOH, PrOH or BuOH for 72 hours compared to cells treated with medium for 72 hours. Each treatment of an individual experiment consisted of 24 replicates. The experiment was repeated four independent times. Each bar represents the mean + / - S. E. M. (n = 4). The data was analyzed using a RCBD after performing an arc/sin transformation on the raw percentages.

proliferation at early time points which is overcome at later time points, as the cells are able to adapt and recover their proliferation status by 72 hours.

Figure 23 depicts the results obtained from a time course evaluation. There were no differences in the percentages of [^{3}H]-thymidine incorporated at each time point when compared to control cells. This further suggests that ethanol is not working through a mechanism that is halting proliferation of PC12 cells in order to induce enhanced PC12 cell differentiation. Moreover, ethanol may be priming the cells to be able to respond to the neurotrophic factor without noticeably interfering with proliferation. The longer chain alcohols may exert a greater priming effect, which allows an alteration in the proliferative status of cells treated with these alcohols to be observed.

Effects of Alcohol Exposure on Cell Cycle Regulation

Ethanol has been shown to cause a delay in the progression of G_1 in C6 and HL-60 cells (Isenberg *et al.*, 1992; Cook *et al.*, 1990). To examine the effect that alcohols may have on cell cycle progression in PC12 cells, the same ethanol dose range, chain length alcohols and alcohol exposure time was evaluated as used for morphology and proliferation studies. A decrease in the S phase of the cell cycle was observed in cells treated with 0.8% ethanol for 24 hours compared to untreated cells (Dunnett's t-test; $p < 0.05$; Figure 24). An increase in the G_2 phase of the cell cycle was observed also in those cells treated with either 0.2% or 0.8% ethanol compared to control cells (Dunnett's t-test; $p < 0.05$). There was no alteration in the G_1 phase of the cell cycle for cells treated with ethanol for 24 hours (Figure 24). At high pharmacological concentrations of ethanol,

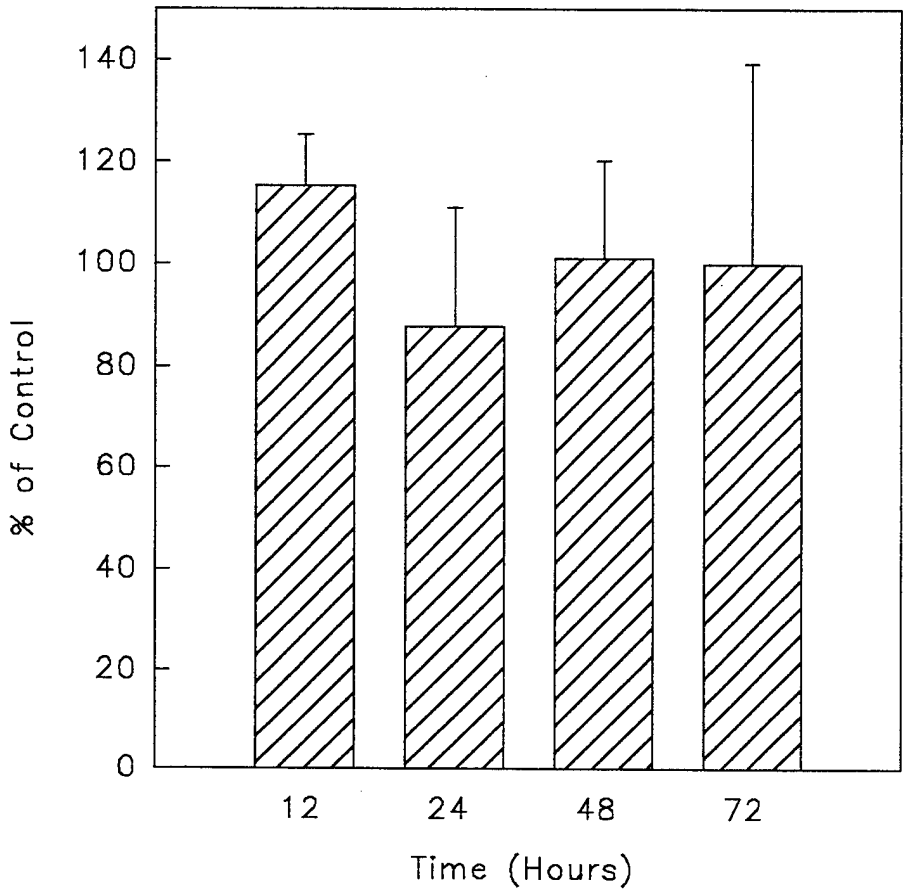

Figure 23. Effect of ethanol treatment time on PC12 cell proliferation. Cells were treated with 0.4% (w/v) ethanol for the indicated time periods while control plates were treated with medium at the same time. Data are shown as the percentage of [^{3}H]-thymidine incorporated into the treated samples at each time point compared to the [^{3}H]-thymidine incorporated into the control samples at each time point. Each treatment of an individual experiment consisted of 24 replicates. The experiment was repeated four independent times. Each bar represents the mean + / - S. E. M. (n = 4). The data was analyzed using a RCBD after performing an arc/sin transformation on the raw percentages.

there is a decrease in the synthesis phase of the cell cycle which should relate to the proliferation status of the cells. However, there was no decrease observed in proliferation of these cells. The evaluation of the cell cycle may be a more sensitive indicator of cellular proliferation.

In contrast, cells treated with the indicated concentrations of ethanol for 72 hours showed no significant differences in either G_1, G_2 or S phases of the cell cycle from control cells (Figure 25). These findings agree with the proliferation studies. The effects that ethanol has on PC12 cells appears to occur within an early window of time. Moreover, longer periods of exposure appear to lead to adaptation or recovery from any effects observed earlier.

Changes in cell cycle were also examined as a function of alcohol chain length at either 24 or 72 hours (Figures 26, 27). At 24 hours of alcohol treatment, there appears to be an alcohol chain length effect such that there is an increase in the percentage of cells in G_1 and a decrease in the percentage of cells in S as chain length increases. Cells treated with propanol or butanol for 24 hours displayed a significant increase in the percentage of cells within the G_1 phase of the cell cycle, indicating a delay in the progression of G_1 (Dunnett's t-test; $p < 0.05$; Figure 26). The same cell populations showed a significant decrease in the percentage of cells within the S phase of the cell cycle (Dunnett's t-test; $p < 0.05$). However, only cells treated with butanol for 24 hours possessed a significant increase in the percentage of cells within the G_2 phase of the cell cycle when compared to control cells (Dunnett's t-test; $p < 0.05$). These data suggest that the longer chain alcohols may induce a delay in the progression through the cell cycle. Butanol delays G_2 as well as

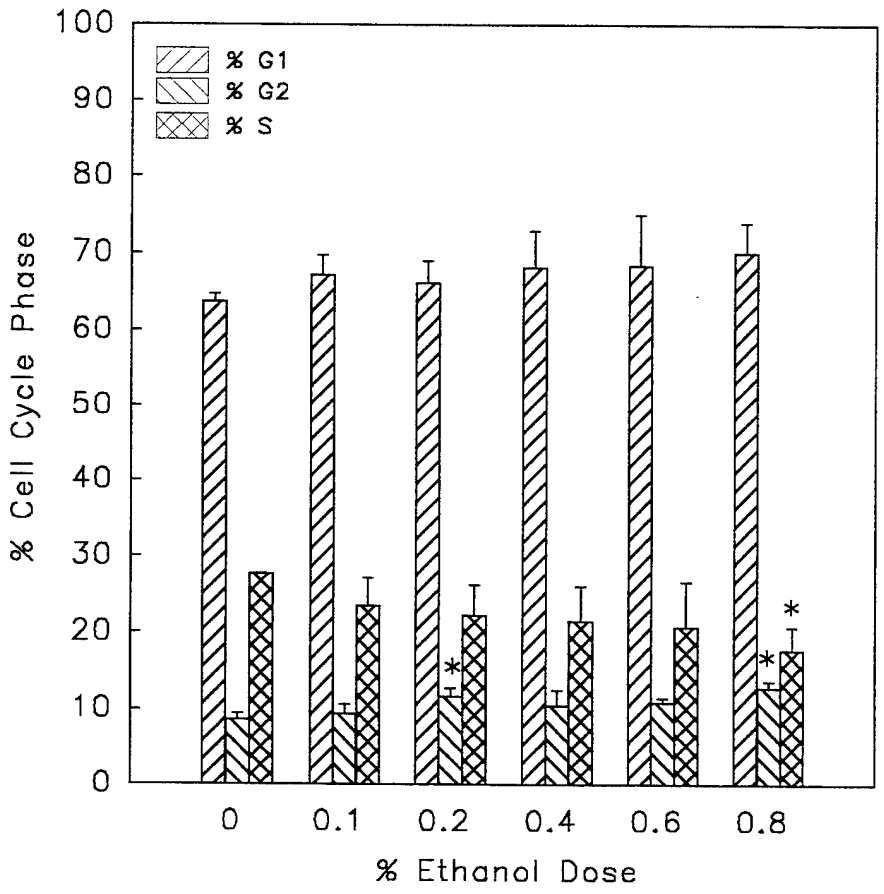

Figure 24. Effect of 24 hour ethanol treatment on PC12 cell cycle progression. PC12 cells were treated with either 0.1, 0.2, 0.4, 0.6 or 0.8% (w/v) ethanol for 24 hours. Percentages of cells in G_1, S and G_2 are given as output from the Multicycle program after sampling of 10,000 individual cells in a sample. Each experiment was repeated four independent times. Data was analyzed using a RCBD after performing an arc/sin transformation. Each bar represents the mean + / - S. E. M. (n = 4). Significant differences from control cells are denoted by *.

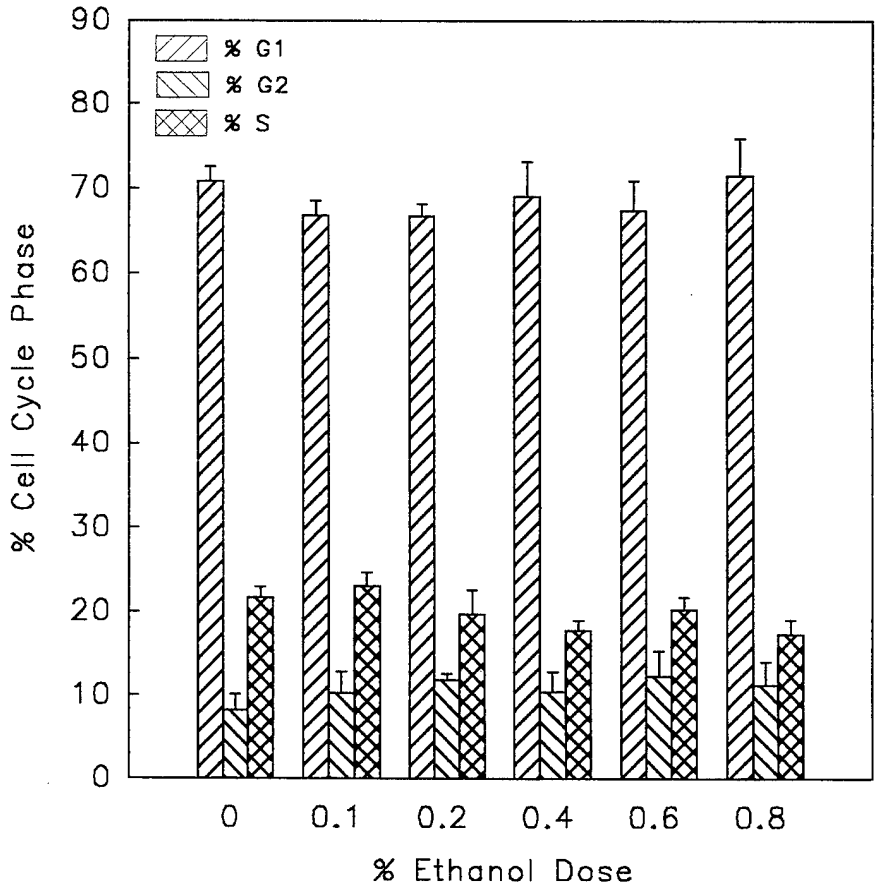

Figure 25. Effect of 72 hour ethanol treatment on PC12 cell cycle progression. PC12 cells were treated with either 0.1, 0.2, 0.4, 0.6 or 0.8% (w/v) ethanol for 72 hours prior to harvesting. Percentages of cells in G_1, S and G_2 are given as output from the Multicycle program after sampling of 10,000 individual cells in a sample. Each experiment was repeated four independent times. Data were analyzed using a RCBD after performing an arc/sin transformation. Each bar represents the mean + / - S. E. M. (n = 4). Significant differences from control cells are denoted by *.

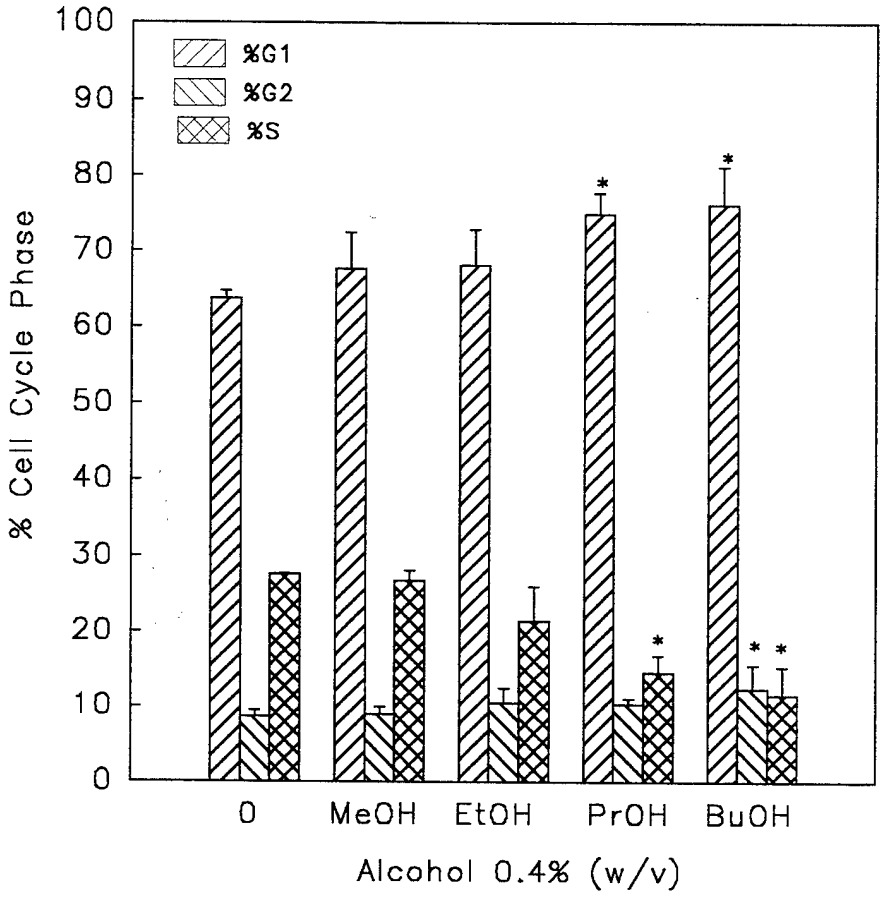

Figure 26. Effect of 24 hour alcohol chain length treatment on PC12 cell cycle progression. PC12 cells were treated with either 0.4% (w/v) MeOH, EtOH, PrOH or BuOH for 24 hours. Percentages of cells in G_1, S and G_2 are given as output from the Multicycle program after sampling of 10,000 individual cells in a sample. The experiment was repeated four independent times. Data were analyzed using a RCBD after performing an arc/sin transformation. Each bar represents the mean + / - S. E. M. (n = 4). Significant differences from control cells are denoted by *.

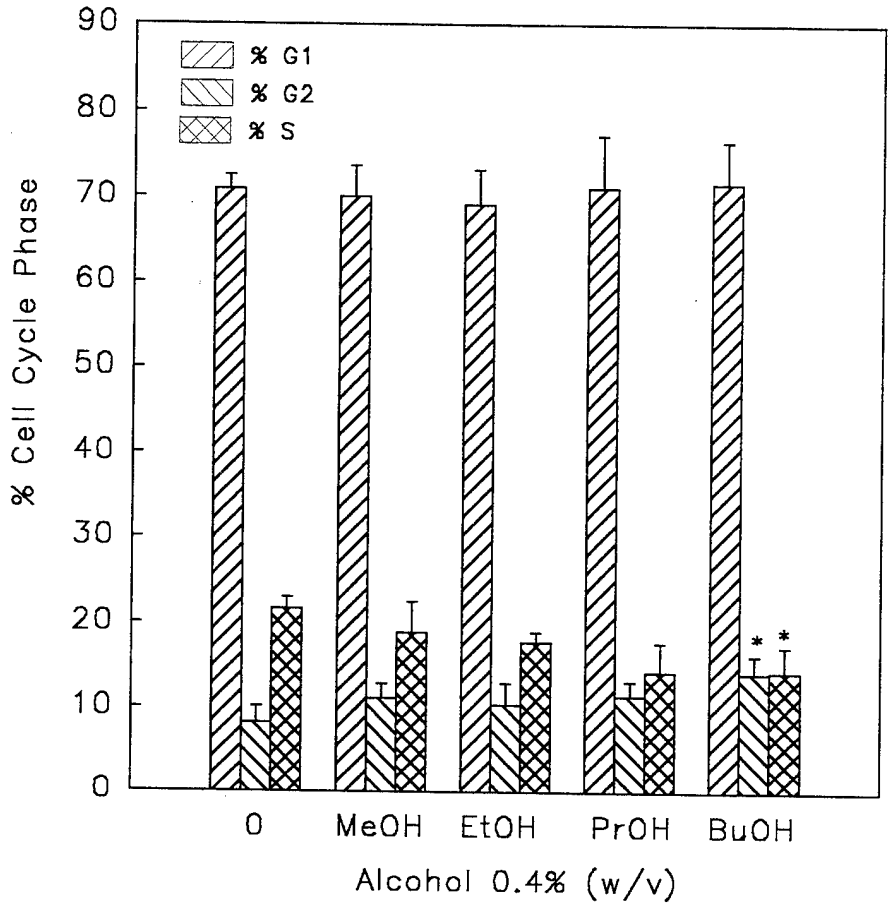

Figure 27. Effect of 72 hour alcohol chain length treatment on PC12 cell cycle progression. PC12 cells were treated with either 0.4% (w/v) MeOH, EtOH, PrOH or BuOH for 72 hours prior to harvesting. Percentages of cells in G_1, S and G_2 are given as output from the Multicycle program after sampling of 10,000 individual cells in a sample. The experiment was repeated four independent times. Data were analyzed using a RCBD after performing an arc/sin transformation. Each bar represents the mean + / - S. E. M. (n = 4). Significant differences from control cells are denoted by *.

G_1, which could imply that there are two specific periods within the cell cycle that this alcohol is affecting. The decrease in the synthesis phase of the cell cycle seen in both propanol and butanol treated cells agrees well with the proliferation studies performed. Treatment of cells with butanol for 72 hours showed a significant decrease in cells within the S phase of the cell cycle and a significant increase in cells within the G_2 phase of the cell cycle when compared to control cells (Dunnett's t-test; $p < 0.05$; Figure 27). None of the other alcohols caused any significant change in cell cycle progression when treated for 72 hours compared to untreated cells (Figure 27). By 72 hours of alcohol exposure, there did not appear to be an alcohol chain length trend in either G_1 or S, which was observed in cells treated with alcohol for 24 hours. While the 24 hour treatment effects on cell cycle progression seen with propanol treated cells is absent by 72 hours, some of the butanol effects remain. This could imply that there are two separate effects. The effect that induces a G_1 delay in butanol treated cells is no longer observed by 72 hours, however, the effect that induces a delay in G_2 remains. Only butanol and very high concentrations of ethanol showed the ability to delay G_2. Thus, this may be an effect that is specific to butanol and high concentrations of ethanol in PC12 cells. Both butanol and high concentrations of ethanol are known to initiate greater amounts of cell death in PC12 cell cultures (Bridges, 1994; Thesis). Therefore, it may be that the delay in G_2 observed here is related in some manner to cell death.

A time course evaluation was performed for cells treated with 0.4% ethanol (Figure 28) and no significant difference in any phase of the cell cycle was observed for any of the tested time periods when compared to medium treated cells at those time

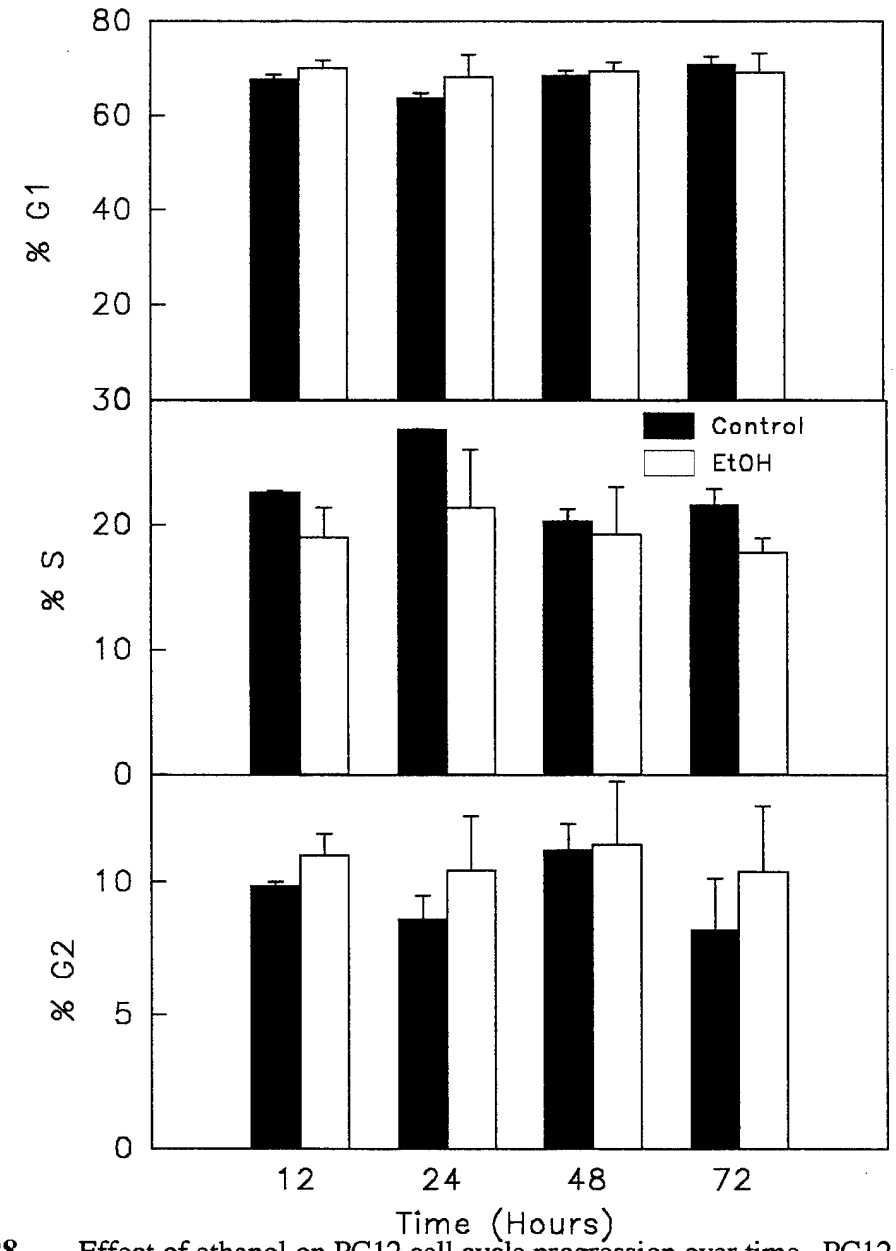

Figure 28. Effect of ethanol on PC12 cell cycle progression over time. PC12 cells were treated with 0.4% (w/v) ethanol for the indicated time periods. The percentages of treated and control cells in the G_1, S and G_2 phases of the cell cycle, given as output from the Multicycle program after sampling of 10,000 individual cells in a sample, are shown for the time points indicated. The experiment was repeated four independent times. Data was analyzed using a RCBD after performing an arc/sin transformation. Each bar represents the mean + / - S. E. M. (n = 4).

points. This also agrees well with the proliferation study and indicates that no other cell cycle parameter is affected in this clonal line PC12 cells due to 0.4% (w/v) ethanol exposure.

Effect of Ethanol on Ganglioside GM_1

Gangliosides, when added to culture media of PC12 cells, have been shown to inhibit cell proliferation and enhance neuritic outgrowth. This enhancement of neurite outgrowth occurs without alteration of the binding parameters of the NGF receptors (Mutoh *et al.*, 1993). These effects are similar to what has been observed with ethanol on PC12 cells. Simultaneous addition of Cholera Toxin B (CTB), which specifically binds to G_{M1} with a high affinity, and NGF increases inhibition of [^{3}H]-thymidine incorporation into PC12 cells over NGF alone (Mutoh *et al.*, 1993). Gangliosides also enhance the membrane disordering actions of ethanol (Harris *et al.*, 1984). Due to these observations, one of the possible mechanisms by which ethanol enhanced neurite outgrowth may occur is due to a change in the amount of G_{M1} present in the ethanol treated cells compared to untreated cells. To test this possibility, cells treated with 0.4% ethanol (w/v) over time were tested for the amount of G_{M1} present in the cells as compared to control cells.

There was no significant change in G_{M1} for any ethanol treated cells at any time point. Treatment with NGF alone greatly enhanced the amount of G_{M1} present in the cells over those cells treated with ethanol and control cells (Figures 29, 30). The use of ethanol along with NGF showed no effect over those observed with NGF alone (Figure 30). Figures 31 and 32 depict ethanol dose and alcohol chain length evaluations performed at

12 hours, respectively. There was no effect observed at any ethanol concentration or with any alcohol tested. Furthermore, the dose response does not fit a linear model. These results imply that alcohols do not alter the amount of G_{M1} present within PC12 cells.

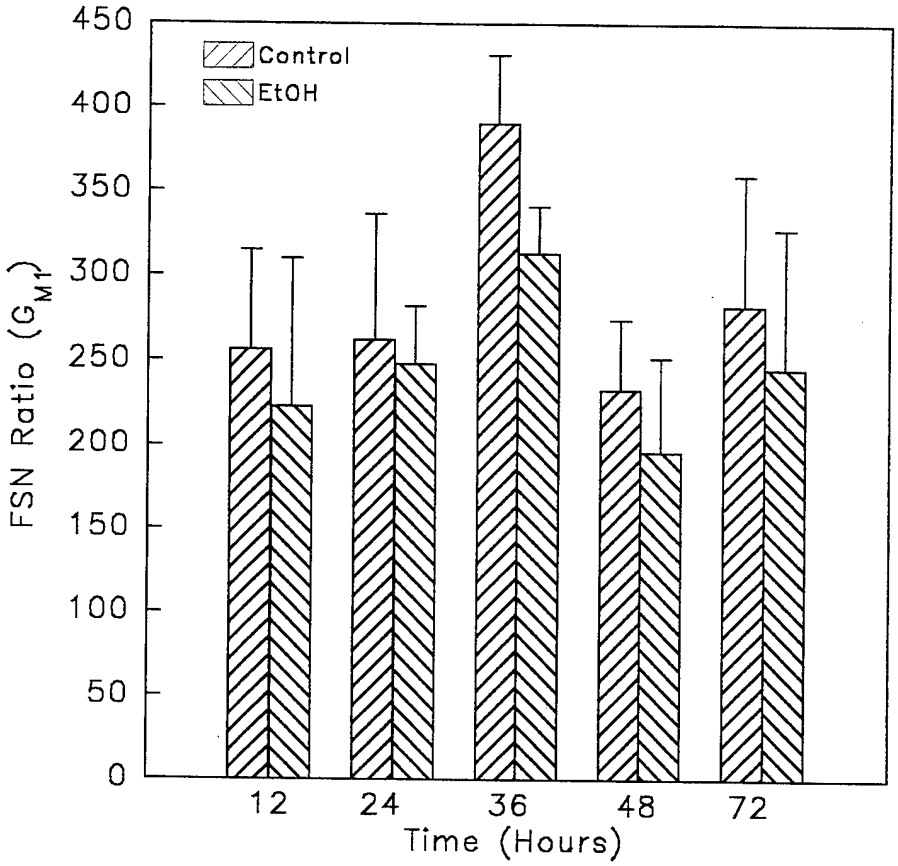

Figure 29. Effect of ethanol on G_{M1} over time. PC12 cells were grown in the presence or absence of 0.4% (w/v) ethanol for the indicated time points. For FITC-labelling procedure refer to Methods and Materials. For each sample, the fluorescence of 10,000 individual cells was used to determine the log mean fluorescence. This value was converted to the linear mean. The fluorescence signal to noise ratio (FSN) was calculated by using the formula; $FSN = 10^{[(\text{intensity exp-intensity ctl})/\#ChD]}$. Each experiment was repeated 4 independent times. The data are plotted as the mean + / - S. E. M. FSN versus time (n = 4). Data was analyzed using a RCBD and a students t-test ($p < 0.05$) to determine differences between medium and ethanol treated cells at a given time.

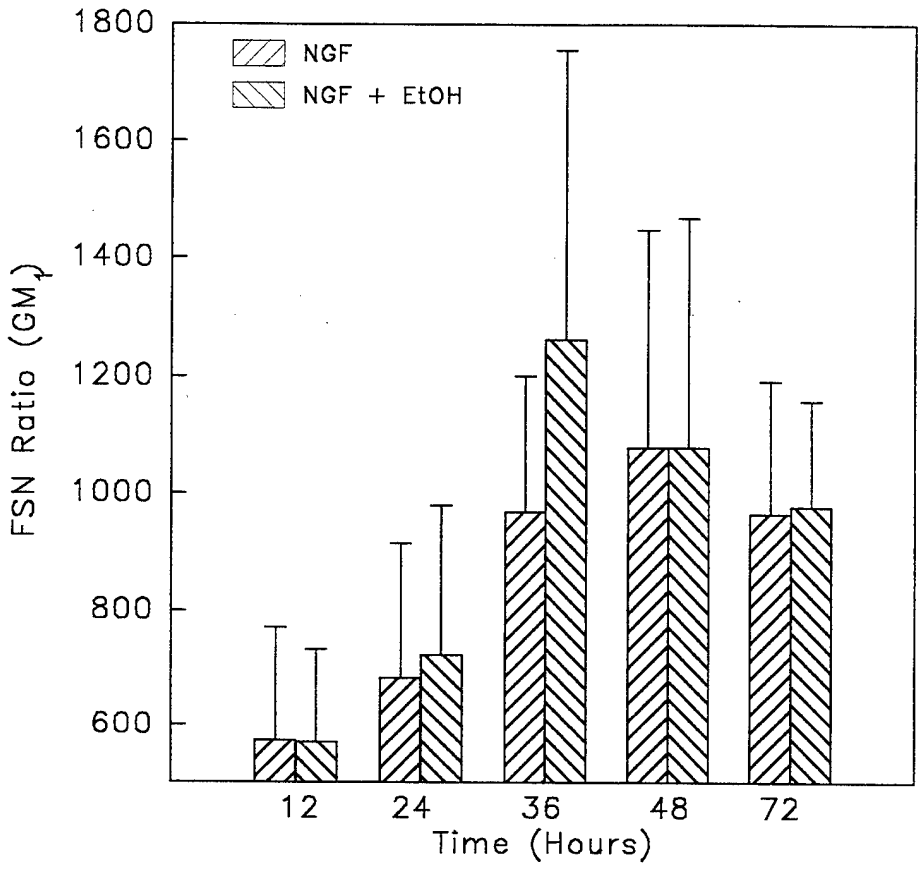

Figure 30. Effect of ethanol and NGF on G_{M1} over time. PC12 cells were grown in the presence of either NGF or NGF with 0.4% (w/v) ethanol for the indicated time points. For FITC-labelling procedure refer to Methods and Materials. For each sample, the fluorescence of 10,000 individual cells was used to determine the log mean fluorescence. This value was converted to the linear mean. The fluorescence signal to noise ratio (FSN) was calculated by using the formula; $FSN = 10^{[(intensityexp-intensityctl)/\#ChD]}$. Each experiment was repeated four independent times. The data are plotted as the mean + / - S. E. M. FSN versus time (n = 4). Data was analyzed using a RCBD and a students t-test ($p < 0.05$) to determine differences between NGF and NGF with ethanol treated cells at a given time.

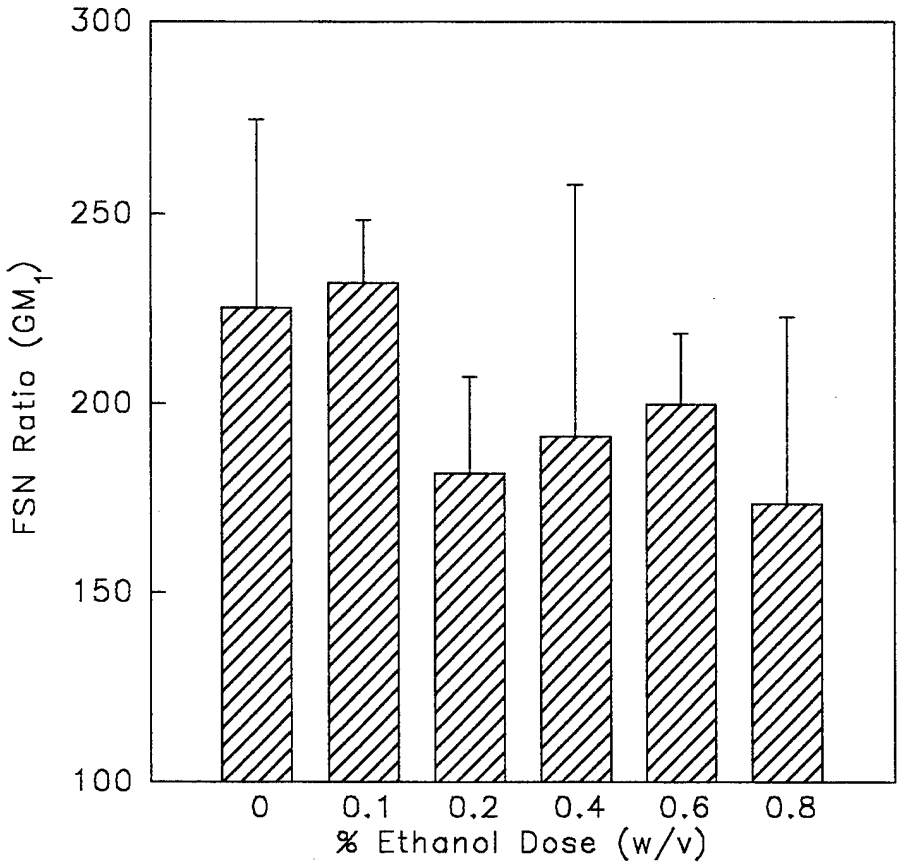

Figure 31. Effect of ethanol dose on G_{M1} at 12 hours. PC12 cells were grown in the presence of 0, 0.1, 0.2, 0.4, 0.6, or 0.8% (w/v) ethanol for 12 hours. For FITC-labelling procedure refer to Methods and Materials. For each sample, the fluorescence of 10,000 individual cells was used to determine the log mean fluorescence. This value was converted to the linear mean. The fluorescence signal to noise ratio (FSN) was calculated by using the formula; $FSN = 10^{[(intensityexp-intensityctl)/\#ChD]}$. Each experiment was repeated four independent times. The data are plotted as the mean + / - S. E. M. FSN versus time (n = 4). Data was analyzed using a RCBD and a students t-test ($p < 0.05$) to determine differences between NGF and NGF with ethanol treated cells at a given time.

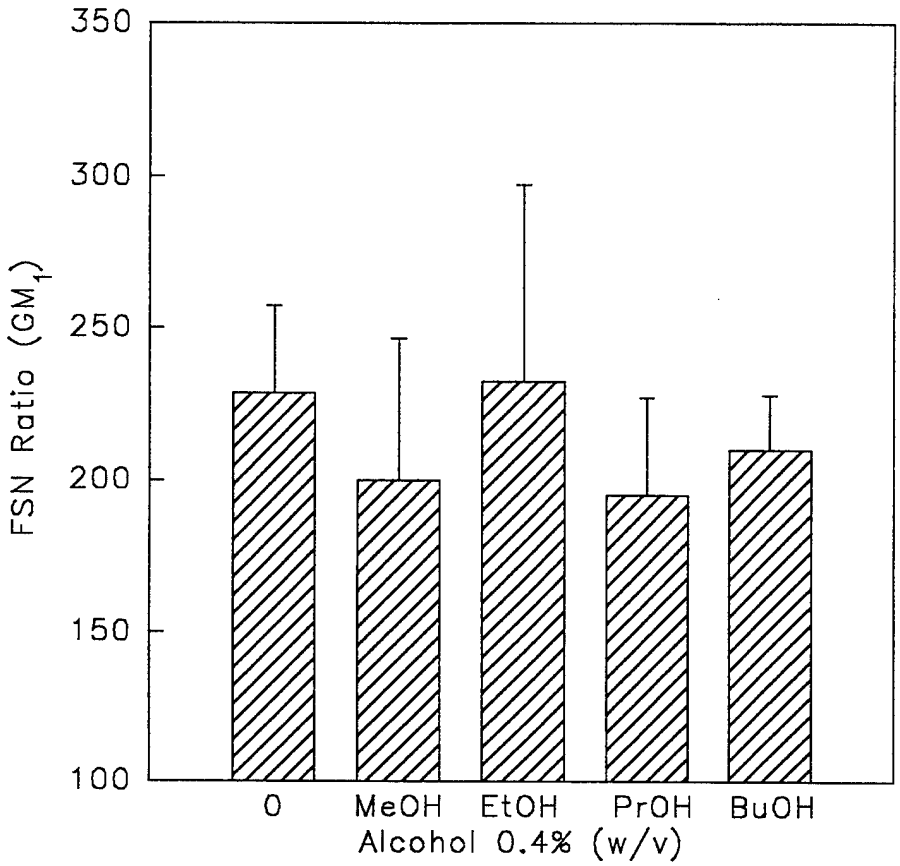

Figure 32. Effect of alcohol chain length on G_{M1} at 12 hours. PC12 cells were grown in the presence of medium, MeOH, EtOH, PrOH or BuOH at 0.4% (w/v) for 12 hours. For FITC-labelling procedure refer to Methods and Materials. For each sample, the fluorescence of 10,000 individual cells was used to determine the log mean fluorescence. This value was converted to the linear mean. The fluorescence signal to noise ratio (FSN) was calculated by using the formula; $FSN = 10^{[(intensityexp-intensityctl)/\#ChD]}$. Each experiment was repeated four independent times. The data are plotted as the mean + / - S. E. M. FSN versus time (n = 4). Data was analyzed using a RCBD and a students t-test ($p < 0.05$) to determine differences between NGF and NGF with ethanol treated cells at a given time.

V. CONCLUSIONS

Ethanol has been shown to have a differential effect on neuronal populations within the developing brain. Some neuronal populations appear to have reduced neuritic extensions in addition to a reduction in the number of cells due to prenatal alcohol exposure (Hammer and Scheibel, 1981). In comparison, rat hippocampal mossy fibers and Purkinje dendritic extensions exhibit an increase in length as well as branching pattern with alcohol exposure during gestation (West et al., 1981; Pentney and Quakenbush, 1990). Therefore, it appears that neuronal populations may exhibit differential sensitivities to ethanol. Specific time frames and windows of vulnerability, during which ethanol may result in brain damage to the developing fetus are being investigated. However, due to numerous developing neuronal populations and differences between animal species being evaluated, the collection of these data is very difficult. Although it is known that neurotrophic factors are necessary for proper development of the nervous system, the expression times of the various neurotrophic factors required for CNS development are still being investigated. Also, the interaction between ethanol and neurotrophic factors should be evaluated more closely. Neurotrophic factors are involved in cell survival, growth and differentiation, and ethanol appears to affect many of these parameters.

Previous studies have indicated that alcohols have the ability to act synergistically with NGF to potentiate NGF-induced differentiation of PC12 cells (Wooten and Ewald,

1991; Messing *et al.*, 1991a). Although treatment of PC12 cells with alcohol alone will not induce these cells to begin the process of neurite outgrowth, it is unclear whether the enhancement effect on NGF-induced neurite outgrowth is due to an underlying alcohol specific effect or an effect that is dependent on the presence of both alcohol and NGF. The mechanism(s) which underlie the ability of alcohol to enhance NGF-induced neuromorphogenesis has not been fully explored. PC12 cells were used as a model to evaluate the effect of timing of treatment regimens in relation to the neurotrophic factor, NGF, to gain insight into the mechanism(s) inducing these effects.

Data presented here suggests that there are at least two distinct mechanisms by which alcohols have the ability to enhance NGF-induced neurite outgrowth in PC12 cells. Exposure of cells to both alcohols and NGF, independent of treatment regimen, causes the cells to undergo enhanced differentiation compared to cells treated with NGF alone. These observations provide support for a synergistic alcohol / NGF mechanism. However, there is also evidence which suggests an alcohol specific mechanism. The alcohol related effect occurs during a 24-hour window of pretreatment time, which allows PC12 cells to undergo enhanced differentiation over cells treated simultaneously with alcohol and NGF. In contrast, by 72 hours of pretreatment time, alcohol no longer exhibits the ability to enhance differentiation compared to cells treated simultaneously with both substances. When the 72 hour pretreated cells are then treated with NGF, they respond in a manner analogous to those simultaneously treated with alcohol and NGF. This finding implies that longer periods of alcohol pretreatment may allow the cells to undergo adaptation leading to tolerance to the alcohol-specific effect while not altering the

synergistic mechanism. Furthermore, the length of exposure to NGF appears to be critical to the alcohol-specific mechanism, without affecting the synergistic effects. For example, only cells evaluated during the early window of NGF treatment showed evidence of the alcohol-specific effect. Taken together, these results suggest the presence of a synergistic alcohol / NGF mechanism which is not affected by alcohol pretreatment or length of culture with NGF, as well as, a specific alcohol mechanism that is affected by alcohol pretreatment time and length of exposure to NGF.

The above observations also appear to be dependent upon the chain length of the alcohol and may be related to their physio-pharmacological properties (McCreery and Hunt, 1977). Pretreatment of PC12 cells for 24 hours with either propanol or butanol induced a greater degree of neurite extension than either alcohol pretreatment of cells for 72 hours or simultaneous treatment with alcohol and NGF at the 24 hour evaluation time. The alcohol-specific mechanism alluded to with propanol and butanol may also occur with the shorter chain alcohols, but because these alcohols do not interfere with membrane properties to as great an extent, the effect may be hidden by the overpowering alcohol / NGF synergistic effect. There was no alteration in the percentage of cells bearing neurites with these alcohols, thus implying that alcohol is not affecting the number of cells responding to NGF but is enhancing the NGF responsiveness of the cells to NGF. Even though these cells are an established cell line and, therefore, they generally represent a homogeneous population, it may be that only certain cells within this population are able to undergo the priming effect of alcohols.

PC12 cell differentiation is initiated by NGF binding to its receptors. Therefore, the mechanism underlying either of the two alcohol-induced mechanisms or the tolerance effect alluded to might be due to a possible alteration in the binding properties on the NGF / NGF receptor complexes. An increase in NGF receptor binding parameters which showed up in all time frames tested may indicate that NGF receptor interactions were a potential mechanism behind the synergistic effect. Conversely, if the NGF receptor interactions were greater during short term alcohol exposure (24 hours) and then decreased with long term alcohol treatment (72 hours), it would explain both the alcohol-specific effect and tolerance effect that were observed. However, no changes in the affinity of NGF for its receptor, the types of receptors expressed or receptor number were observed for either form of the NGF receptor were observed upon exposure to alcohols. The phosphorylation state of the *trk* proto-oncogene is thought to be related to the biological activity induced by the high affinity receptor form (Green and Greene, 1986; Weskamp and Reichardt, 1991). The phosphorylation state of *trk* was found to be directly proportional to the affinity of the high affinity NGF receptor (Hempstead *et al.*, 1992). Thus, since there was no change in the affinity of this receptor, there would likely be no change in the phosphorylation state of *trk*. The findings from these studies document that direct changes in the NGF receptor are not responsible for either the priming effect of alcohols or the tolerance effect.

Gangliosides are important mediators of cellular function. G_{M1} has been shown to potentiate NGF-induced neurite extension (Mutoh *et al.*, 1993). For this reason, it was postulated that alcohols may increase the amount of G_{M1} within PC12 cells which might, in

turn, contribute to enhanced neuromorphogenesis. PC12 cells stimulated with NGF for time periods as short as 12 hours showed a significant increase in G_{M1}. However, no affect in the amount of G_{M1} was observed with any alcohol treatment tested in PC12 cells. It could be that while G_{M1} is increased with NGF stimulation, there is another ganglioside, which was not evaluated in this series of experiments, that plays a role in alcohol enhanced neurite outgrowth.

There is a window of alcohol pretreatment time, around 24 hours, where the cells are more vulnerable to alcohol effects. Alterations in cell cycle regulation and proliferation may be responsible for the priming effect of alcohols in PC12 cells. Ethanol exposure has been shown to affect the cell cycle regulation of a number of different cell types (Cook *et al.*, 1990; Miller and Nowakowski, 1991; Cook and Keiner, 1991; Isenberg *et al.*, 1992). In cell lines that retain the ability to undergo differentiation, ethanol appears to prime the cells toward terminal commitment to differentiation. For example, this effect has been observed in HL-60 cells (Cook *et al.*, 1990). When these cells are exposed to ethanol alone, they become reversibly arrested in the G_1 phase of the cell cycle. However, when exposed to ethanol in the presence of a differentiation inducer, the differentiation process is enhanced (Cook *et al.*, 1991). Short term exposure to ethanol may be priming PC12 cells to respond to the differentiation inducer, NGF, in a similar fashion.

The window of 24-hour alcohol exposure time for a specific alcohol effect on PC12 cells was also observed in cell cycle studies and [^{3}H]-thymidine incorporation assays. In both of these studies, differences from control cells were observed in alcohol

treated cells which were exposed to high concentrations of ethanol and the longer chain alcohols for 24 hours. These results further support the existence of a window of time where cells are more vulnerable to alcohol's effects. The disappearance of the cell cycle and proliferation effects with long term alcohol exposure gives credence to the idea of a tolerance or adaptation mechanism in PC12 cells.

The longer chain alcohols caused a noticeable increase in the percentages of cells in the G_1 phase of the cell cycle as well as a decrease in proliferation. The effect was only observed at 24 hours of alcohol treatment and was no longer present in the cells exposed to alcohol for 72 hours. This increase in G_1 and decrease in proliferation may be a contributing factor in the enhancement of NGF-induced neurite outgrowth observed in cells pretreated with alcohol for 24 hours prior to NGF stimulation. Ethanol causes a delay in G_1 progression in HL-60 cells which is related to the initiation of differentiation in this cell line (Cook *et al.*, 1990). The disappearance of this effect in PC12 cells may be responsible for the cellular adaptation leading to tolerance to the alcohol specific mechanism. Both butanol and 0.8% (w/v) ethanol induce a build up in the G_2 phase of the cell cycle which could be due to the higher degree of cell death that these two alcohols cause in PC12 cells (Bridges, 1994; Thesis).

Nerve growth factor also induces a delay in the G_1 phase of the cell cycle of PC12 cells (Rudkin *et al.*, 1989). Thus, alcohol treatment for 24 hours is causing a delay in G_1 and then stimulation with NGF at this time may further enhance this elevation in G_1. If NGF-induced differentiation is occurring due to a G_1 arrest, then the exaggeration of G_1 induced by alcohol exposure could potentially lead to the enhancement of NGF-induced

neurite extension observed in these cells. However, by 72 hours of alcohol treatment, there is no longer an alcohol induced delay up in G_1, so that now when the cells are stimulated with NGF, there is only the NGF-induced increase in G_1. Thus, the cells respond as if they are being exposed to alcohol and NGF simultaneously.

Both the proliferative ability of cells and regulation of cell cycle are affected by gene expression and signal transduction pathways. Priming effects of alcohols may result from alterations in gene expression, RNA synthesis, protein synthesis or signal transduction pathways.

The priming effect of ethanol in PC12 cells may be due to an alteration in gene expression induced by alcohols. Ethanol has been shown to alter gene expression in a number of different cell types. For example, in the neuronal cell line, NG108-15 neuroblastoma x glioma cells, four hours of ethanol exposure increases transcription of the Hsc70 gene (Miles *et al.*, 1990). This protein is a member of the heat shock family and is responsible for molecular trafficking of proteins to be secreted (Miles *et al.*, 1990). Treatment of HL-60 cells with ethanol also induces the cells into a more differentiated phenotype (Cook *et al.*, 1990). A transient down regulation in gene expression of c-*myc* and c-*myb* occurs in HL-60 cells due to ethanol exposure within 6 hours and returns to normal cellular levels by 24 hours of ethanol exposure (Datta *et al.*, 1990). A decrease in c-*myc* mRNA has been shown to be associated with differentiation of HL-60 cells (Watanabe *et al.*, 1985). Tumor necrosis factor (TNF) gene expression is also altered in HL-60 cells due to alcohol exposure (Datta *et al.*, 1990). Gene expression of TNF is increased within 24 hours of ethanol treatment and returns to normal levels by 72 hours of

ethanol exposure (Datta *et al.*, 1990). Tumor necrosis factor plays a major role in destruction of tumor cells, but also has a minor physiological role in regulation of normal cell growth (Datta *et al.*, 1990). Pentylenetetrazol (PZT) is a convulsant that is known to affect the GABA-benzodiazepine receptor complex (Rehavi *et al.*, 1982). This compound induces c-*fos* gene expression in rat and mice brains (Rehavi *et al.*, 1982). Pretreatment of rats with ethanol for 10 to 60 minutes prior to PZT administration showed a significant reduction in the amount of c-*fos* mRNA expression normally induced by PZT (Le *et al.*, 1990). Although ethanol itself did not directly affect c-*fos* regulation in this experiment, it may still have an effect on other receptor complexes that are coupled to c-*fos* expression.

In PC12 cells, ethanol has been shown to induce an early transient increase in c-*fos* and c-*jun* expression occurring within 60 minutes (Wooten and White, 1993). Analysis of cell cycle responses in PC12 cells showed that c-*fos* was induced in the G_1 phase (Rudkin *et al.*, 1989). Thus, if short term alcohol exposure is initiating a delay in the G_1 phase of the cell cycle, it may lead to the increase in expression of c-*fos*. However, with longer periods of ethanol treatment, there is no longer a delay in G_1 and subsequently no increase in c-*fos*. The proto-oncogenes c-*fos*, c-*jun*, c-*myc* and c-*myb* all regulate cellular growth and differentiation and ethanol's effects on gene expression of these cellular moieties could lead to the priming effect observed by ethanol in PC12 cells.

Another mechanism for a delay in cell cycle progression could involve inhibition of RNA synthesis. Ethanol has been shown to inhibit RNA synthesis in other cells, such as rat hepatocytes (Poso and Poso, 1981). Administration of alcohol for 24 hours in rats induces a decrease in protein synthesis occurring in the liver (Morland and Bessesen,

1977). Regulation of protein synthesis could potentially be responsible for the effects of alcohol observed in this study. Cellular growth and differentiation are dependent upon the synthesis and secretion of many proteins, including kinases and other enzymes. Thus, alcohol may not only effect cellular proteins at the level of gene expression, but may also effect cellular proteins at the level of transcription.

A delay in cell cycle progression could also be due to changes in signal transduction pathways. A number of membrane-induced signal transduction pathways utilize cAMP as a second messenger (Berridge, 1985). Adenylate cyclase, when activated, is the enzyme that produces cAMP (Berridge, 1985). The two G-proteins, which are coupled to adenylate cyclase, have identical β and γ subunits and distinctly different α subunits. Gαs is stimulatory to the pathway while Gαi is inhibitory (Berridge, 1985). Many cell types have been shown to respond to alcohol treatment by changes in cAMP production. For example, lymphocytes and platelets removed from alcoholics have a decreased response to receptor stimulated cAMP production (Diamond *et al.*, 1987; Tabakoff *et al.*, 1988). The decrease in cAMP production could be due to ethanol affecting either the receptors or the G-proteins coupled to adenylate cyclase. The cell line NG108-15, shows an increase in cAMP production after ethanol treatment for 10 minutes and normal levels of cAMP after 48 hours of culture in ethanol (Gordon *et al.*, 1986; Mochly-Rosen *et al.*, 1988). Ethanol treatment for 4 days also causes a reduction in adenylate cyclase activity and reduces cAMP content in PC12 cells (Rabin, 1990).

The addition of NGF to PC12 cells has been shown to increase cAMP production (Cremins *et al.*, 1986). Addition of N^6, O^2-dibuturyl adenosine 3':5' cyclic monophosphate

(βt_2cAMP), the cell permeable analog of cAMP, to PC12 cells will initiate neurite extension independent of mRNA synthesis, which differs mechanistically from neurites induced by NGF (Gunning *et al.*, 1981). Neurite outgrowth produced by βt_2cAMP is not maintained after this compound is removed. Furthermore, the simultaneous addition of βt_2cAMP and NGF showed a synergistic effect on neurite extension (Gunning *et al.*, 1981; Richter-Landsberg and Jastorff, 1986). Since short term ethanol exposure may lead to increased levels of cAMP within PC12 cells, it is possible that the enhanced neurite extension observed with the 24-hour pretreatment regimen could be due to ethanol-induced increases in cAMP. Long term ethanol stimulation results in decreased levels of cAMP and therefore by the 72 hour pretreatment regimen, the synergistic role of cAMP may no longer be present.

The other manner by which ethanol has been shown to affect cAMP is through heterologous desensitization of receptors coupled to adenylate cyclase (Mochly-Rosen *et al.*, 1988). There is evidence that the amount of αs mRNA and protein are decreased after 48 hours of ethanol exposure in NG108-15 cells (Mochly-Rosen *et al.*, 1988). Receptor mediated events involving adenosine and prostaglandin E both induce cAMP production in these cells. After 48 hours of alcohol treatment, the amount of cAMP that these ligands could induce was shown to be far less than in control cells (Mochly-Rosen *et al.*, 1988). During short term alcohol exposure, adenylate cyclase may be activated to a greater extent through receptor mediated events, and therefore cause an increase in cAMP. This could act synergistically with the NGF stimulation to cause a greater degree of neurite extension. However, after long term ethanol exposure, there may be

heterologous desensitization of the NGF receptor complex such that the amount of cAMP that is produced is far less.

At the 24 hour NGF stimulation endpoint, pretreatment of cells with either propanol or butanol for 24 hours induced a greater degree of neurite extension than in cells simultaneously treated with alcohol and NGF. However, pretreatment of cells for 72 hours with these alcohols did not show a greater degree of neurite extension than the simultaneously treated cells. The fact that the longer pretreatment time of 72 hours did not enhance neurite extension above simultaneous treatment, while the 24 hour pretreatment time does, may suggest that longer periods of alcohol treatment allows the cells to adapt to the alcohol specific effect. There is morphological evidence that long term exposure of PC12 cells to alcohols causes these cells to respond to NGF stimulation in the same manner that simultaneously treated cells do. This is in contrast to the short term exposure to alcohols, which appears to potentiate neurite extension above that of simultaneously treated cells. These results indicate that the cells are somehow able to adapt or become tolerant to the specific alcohol effects observed with short term treatment. Changes in cell cycle regulation and the proliferation state of PC12 cells that were observed at 24 hours were no longer apparent by 72 hours. These results further lend support to an underlying tolerance effect that is achieved by longer periods of alcohol exposure.

Cell cycle analysis, proliferation assays and the morphological data all suggest that treatment with ethanol for longer periods of time causes the cells to revert to a physiological state that is equivalent to untreated cells. For example, at 24 hours of

alcohol exposure, there are effects observed within the cell cycle regulation and [^{3}H]-thymidine incorporation. By 72 hours of culture with alcohol, these effects are not observed. Furthermore, cells pretreated with alcohol for 72 hours respond in the same morphological manner as those cells which are exposed to alcohol and NGF simultaneously. This is in contrast to cells which were pretreated for only 24 hours which exhibited a greater degree of neurite extension. These data suggest that the cells are adapting to the specific affects of alcohols by the 72 hour culture time point.

It is well known that chronic alcoholics build up a tolerance to the effects of alcohol such that consumption levels must increase in order to achieve the same mind altering effects. The mechanism behind neuronal adaption and alcohol dependency are poorly understood. Studies are being undertaken to evaluate this effect, and include alcohol effects on gene expression, RNA and protein synthesis and signal transduction pathways.

Chronic alcohol consumption leads to tolerance, dependence and serious withdrawal symptoms. Many of the behavioral responses of both acute and chronic exposures to ethanol appear to be induced by catecholamine changes (Gayer *et al.*, 1991). Tyrosine hydroxylase (TH) plays a role in the formation of monoamines by hydroxylating tyrosine and forming dihydrophenylalanine (reviewed in; Berne and Levy, 1988). Ethanol has been shown to increase TH mRNA levels in N1E-115 neuroblastoma cells in a time dependent fashion beginning at 12 hours of ethanol exposure (Gayer *et al.*, 1991). Within the TH promotor, there is a specific *cis*-acting sequence that is responsible for regulation of TH gene expression in response to ethanol (Gayer *et al.*, 1991). Increased TH activity

could induce an increase in catecholamines and monoamines present in the CNS which may lead to the development of dependency and tolerance. It has been shown that interruption of adrenergic neurotransmissions by either destroying the neurons, blocking catecholamine synthesis or blocking receptor activity will inhibit the formation of alcohol tolerance but increases the severity of withdrawal symptoms and seizures (Gayer *et al.*, 1991). PC12 cells are capable of synthesizing, storing and secreting catecholamines, mainly norepinephrine. Therefore, PC12 cells exposed to ethanol may increase gene expression of TH which may lead to higher levels of TH within the cells. TH activity is known to be enhanced by NGF (Theonen *et al.*, 1971). With more of the TH enzyme present due to ethanol stimulation, NGF exposure may then lead to more catecholamines being synthesized within the ethanol treated cells. This may play a role in the loss of the specific alcohol effect after longer periods of NGF stimulation. The mechanism behind increases in catecholamines leading to tolerance and dependency is unknown.

Signal transduction pathways may also play a role in the tolerance or adaptation observed for the specific alcohol mechanism. Ethanol exposure has been shown to affect a number of signal transduction pathways in many cell types. The cAMP pathway has been previously discussed for its possible role in both the alcohol specific mechanism and its relation to the tolerance effect. Other signal transduction pathways may also play a part in these effects. For example, ethanol treatment of astroglial cells for as little as 30 seconds, causes translocation of protein kinase C (PKC) from the cytoplasm to the membrane (Skwish and Shain, 1990). PC12 cells possess 6 isoforms of PKC. Ethanol exposure increases PKC activity in a time-dependent fashion in PC12 cells (Messing *et al.*, 1991b).

It has been shown that ethanol exposure for 6 days in PC12 cells up-regulates β, δ and ϵ isoforms of PKC (Messing *et al.*, 1991b). The phorbol ester, phorbol 12-myristate, 13 acetate (PMA) down regulates both the δ and ϵ isoforms and inhibits the enhancement of ethanol induced neurite outgrowth, suggesting that these two isoforms play a role in regulation of neurite outgrowth (Roivainen *et al.*, 1993).

NGF stimulation of PC12 cells is known to increase PKC activation within minutes after NGF binds to its receptors (Hama *et al.*, 1986). Alcohol exposure for 6 days induces increases in the PKC isoforms δ and ϵ which may mediate ethanol's enhancement of NGF-induced neurite outgrowth (Roivainen *et al.*, 1993). No studies have been performed to determine the regulation of these isoforms in response to treatment with both NGF and alcohol at the same time. Thus, it may be that PKC activation in the presence of both substances may be enhanced compared to either NGF or alcohol alone. If this occurs, then the enhanced activation of PKC by the combination of these substances may be important for the synergistic mechanism between alcohol and NGF. The loss of the alcohol-specific mechanism with increased NGF stimulation time could potentially be overcome by synergistic regulation of PKC activation.

Alcohols appear to have a variety of effects on gene expression and signal transduction pathways that can be related to cellular growth and differentiation. Mechanisms underlying the differential effects that alcohol has on the various neuronal populations may be due to combinations of these effects. Hippocampal mossy fibers and Purkinje cells appear to undergo similar responses to alcohol exposure as do the *in vitro* model, PC12 cells. Thus, it is possible that for these cells to undergo the enhanced

differentiation observed with ethanol exposure, the presence of a neurotrophic factor may be required within a certain time frame in relation to ethanol exposure.

Nerve growth factor and NGF receptor mRNA are co-expressed during development in the cerebellum, perinatal olfactory bulb and hippocampus (Lu *et al.*, 1989). Cerebellar levels of mRNA for both NGF and the NGF receptor increase rapidly in early neonatal life, peak around post-natal day 10 and then decrease dramatically (Lu *et al.*, 1989). Adverse effects of ethanol on cerebellar Purkinje cell development are most pronounced during embryonic days 4 - 7 (Hamre and West, 1993). Thus, NGF would be present within this population of cells at the time that these rat pups were exposed to ethanol. Therefore, it may be that the presence of NGF at the time of ethanol exposure is causing these cells to undergo the neuronal hypertrophy observed. However, ethanol exposure at this period of embryonic development also causes a reduction in the number of Purkinje cells (Hamre and West, 1981), implying that ethanol may be affecting these cells in more than one way.

During embryonic days 16 - 22, high levels of NGF and NGFR (receptor) mRNA are found within the hippocampus. However, the postnatal hippocampus shows expression of NGF mRNA, but no longer expresses mRNA for the NGF receptor (Lu *et al.*, 1989). The hippocampus also expresses brain derived neurotrophic factor (Ernfors *et al.*, 1990; Hofer *et al.*, 1990). Prenatal and postnatal ethanol exposure causes aberrant branching of dendrites in hippocampal mossy fibers (West *et al.*, 1981). Thus, there is potential for more than one neurotrophic factor to influence the response of these neurons to ethanol during development. No studies have been conducted to evaluate changes in

either NGF or NGF receptor mRNA within the developing nervous system in response to alcohol exposure.

Taken together, the results of this study indicate that the response of PC12 cell neurite extension due to ethanol exposure is complex and occurs through at least two independent mechanisms. Since alcohol has been shown to affect gene expression and a number of signal transduction cascades, each mechanism may be controlled by a different pathway. Clinical studies have determined that brain damage can occur in children whose mothers ingested alcohol only once during gestation. For example, a single short term ethanol insult in rat and mice pups during the third trimester equivelent of human gestation has been shown to cause enhanced neurite extension and branching in hippocampal mossy fibers (Hamre and West, 1993). The appearance of a certain window of time during which ethanol may cause the cells to undergo an enhanced response to a differentiation inducer could potentially explain how a short term exposure to alcohol may initiate such vast and significant changes in the dendritic pattern of these cells. This study revealed a specific alcohol mechanism which is observed only during a certain time frame which may therefore account for the some of the adverse effects on brain development in those children exposed to alcohol for only a short period of time.

Although PC12 cells become tolerant to the specific alcohol mechanism with long term exposure, they do not appear to be able to overcome the synergistic effect of alcohol and the neurotrophic factor. Thus, the varying degrees of neuronal dysgenesis may be accounted for by the stage of growth of the neurons, as well as their responsiveness and requirement for neurotrophic factors, at the time of the ethanol insult. The timing of

neurotrophic factor presence appears to be important in the enhanced neuritogenesis of PC12 cells. The study of how alcohol exposure affects the expression and secretion of neurotrophic factors within the developing brain has yet to be discovered. The timing of alcohol exposure in relation to neurotrophic factor expression, and the cells responsiveness to the neurotrophic factor, may then relate to the varying degrees of neuronal dysgenesis observed. This in turn, may be responsible for the varying degrees of mental retardation observed in Fetal Alcohol Syndrome (FAS) children. Thus, *in vivo* studies to determine alcohol effects on developing neurons in relation to timing of the expression, abundance and possible alterations of neurotrophic factors are warranted.

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APPENDIX

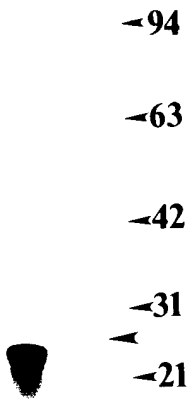

Figure 33. Molecular mass of iodinated NGF preparation. NGF was iodinated with $Na^{125}I$ and 5 µl of the preparation was analyzed by electrophoresis on a 12% SDS-PAGE gel. The molecular mass markers used were phosphorylase B (94 kD), bovine serum albumin (63 kD), ovalbumin (42 kD), carbonic anhydrase (31 kD) and soybean trypsin inhibitor (21 kD). The molecular mass of the iodinated NGF occurs at approximately 26 kD which reflects the molecular mass of the dimer form.

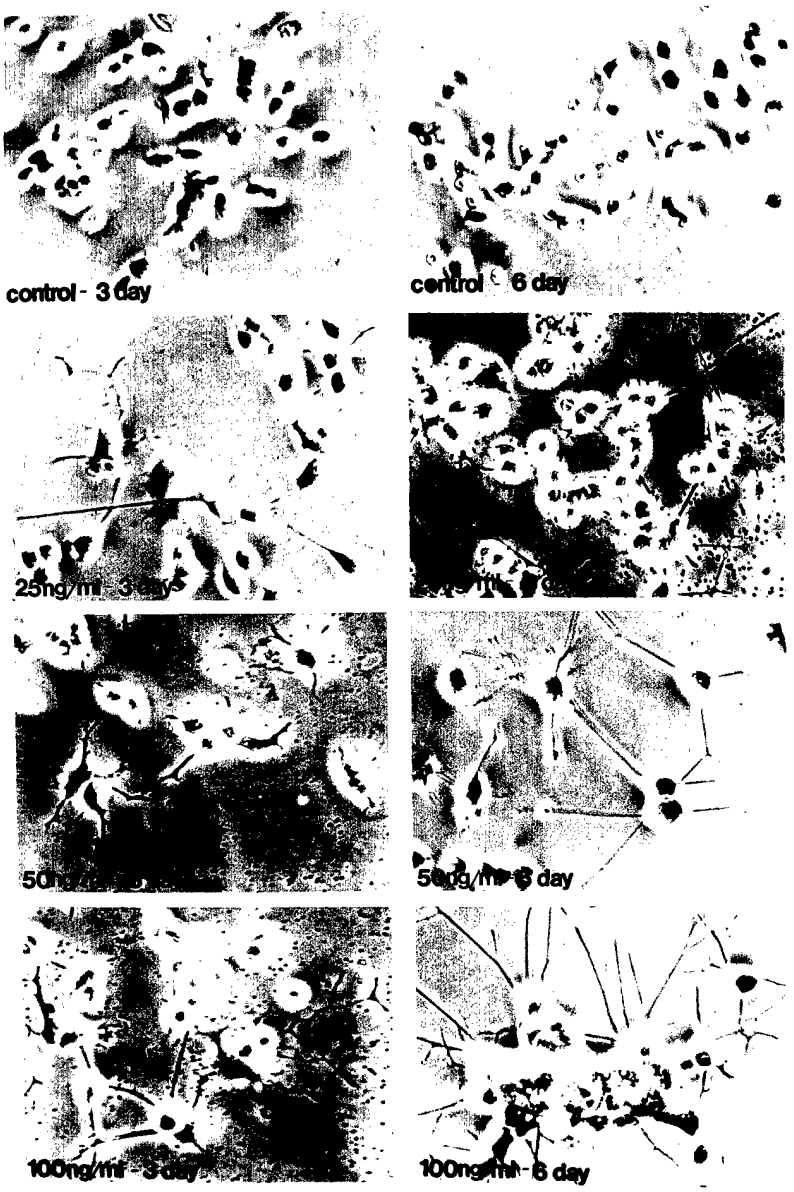

Figure 34. Neurite extension induced by ^{125}I-NGF. PC12 cells were stimulated with ^{125}I-NGF for either 3 or 6 days. Treatments: a) three day control cells, b) six day control cells, c) 25 ηg/ml ^{125}I-NGF at three days, d) 25 ηg/ml ^{125}I-NGF at six days, e) 50 ηg/ml ^{125}I-NGF at three days, f) 50 ηg/ml ^{125}I-NGF at six days, g) 100 ηg/ml ^{125}I-NGF at three days, h) 100 ηg/ml ^{125}I-NGF at six days.

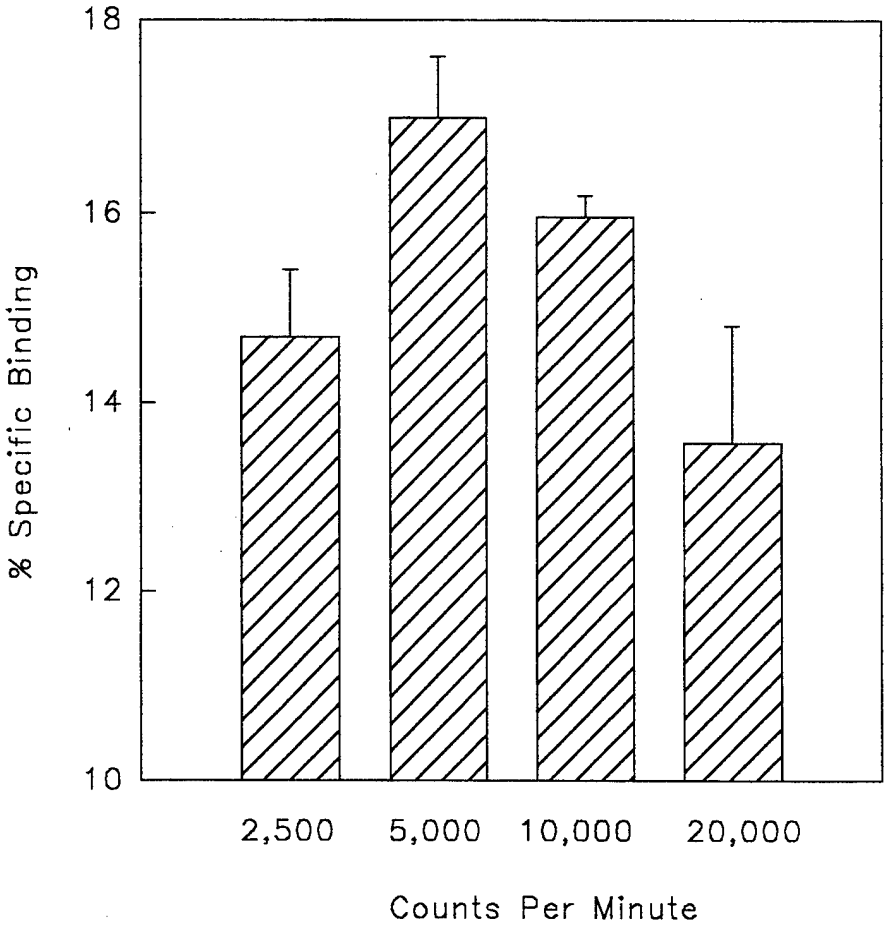

Figure 35. Titration of ^{125}I-NGF to determine counts per minute to add to assays. PC12 cells were aliquoted into microfuge tubes at 3 x 10^5 cells/tube in 200 µl of phosphate buffered gelatin (PBG). 35 µl of premix containing either 2,500, 5,000, 10,000 or 20,000 counts per minute of ^{125}I-NGF with or without 1 µg of uniodinated NGF was added to the tubes. Reaction mixtures were incubated at 37°C for 30 minutes and then processed by centrifugation through a two-step sucrose gradient. The counts per minute bound to the cell pellets was determined. Percent specific binding was calculated by subtracting the counts per minute of the samples containing uniodinated NGF from those without uniodinated NGF and dividing by the total number of counts added to the reaction mixture. Each bar depicts the mean + / - S. E. M. of three samples. These data are representative of four independent experiments.

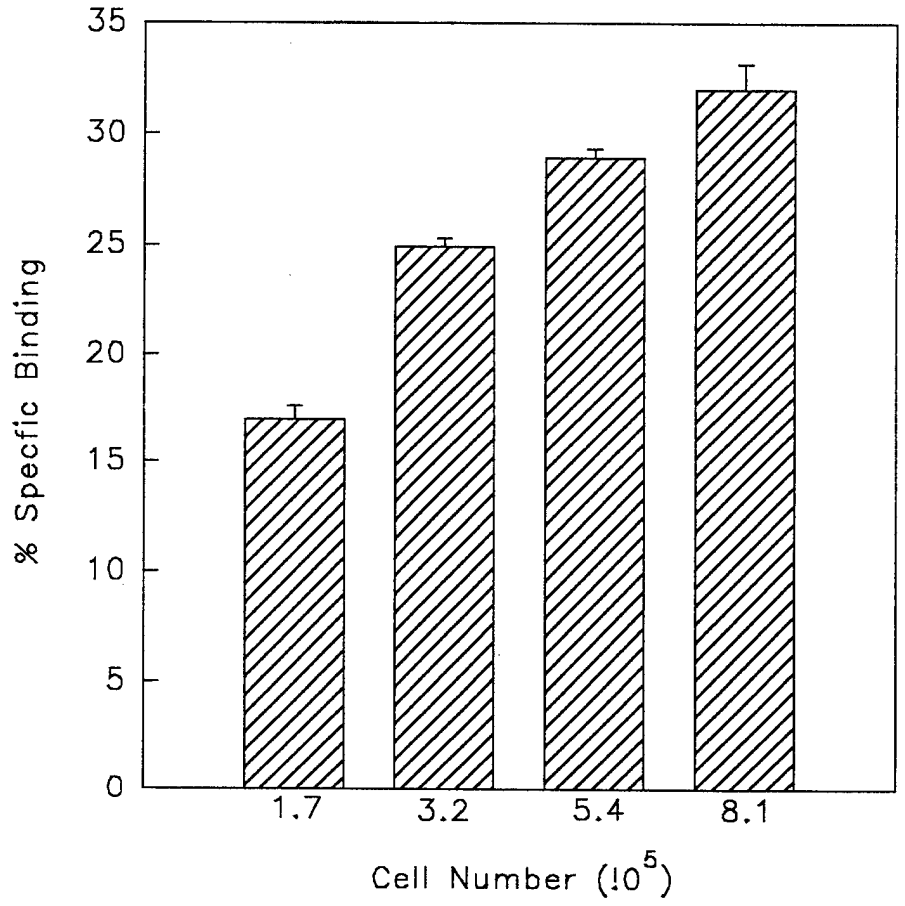

Figure 36. The effect of cell number on specific binding. PC12 cells were aliquoted into microfuge tubes at either 1.7, 3.2, 5.4 or 8.1 x 10^5 cells/tube in 200 µl of phosphate buffered gelatin (PBG). 35 µl of premixes containing 5,000 counts per minute of ^{125}I-NGF with or without 1 µg of uniodinated NGF was added to the tubes. Reaction mixtures were incubated at 37°C for 30 minutes and then processed by centrifugation through a two-step sucrose gradient. The counts per minute bound to the cell pellets was determined. Percent specific binding was calculated by subtracting the counts per minute of the samples containing uniodinated NGF from those without uniodinated NGF and dividing by the total number of counts added to the reaction mixture. Each bar depicts the mean + / - S. E. M. of three samples. These data are representative of four independent experiments.

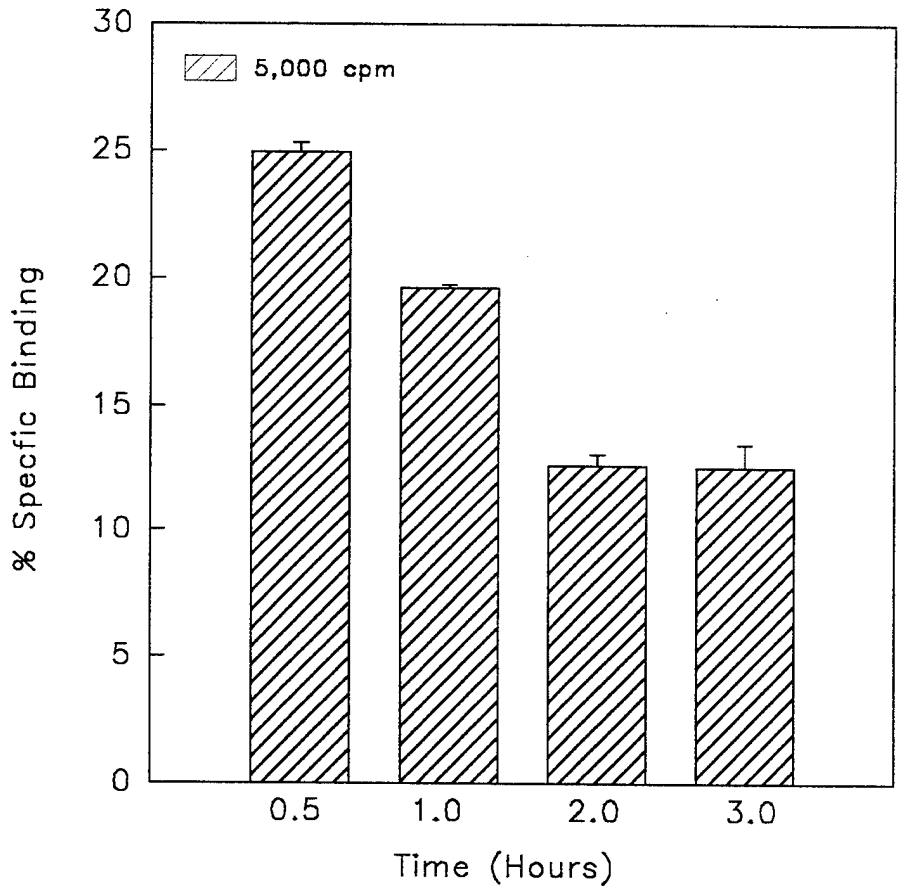

Figure 37. The effect of incubation time on specific binding. PC12 cells were aliquoted into microfuge tubes at 3.2 x 10^5 cells/tube in 200 µl of phosphate buffered gelatin (PBG). 35 µl of premixes containing 5,000 counts per minute of ^{125}I-NGF with or without 1 µg of uniodinated NGF was added to the tubes. Reaction mixtures were incubated at 37°C for either 0.5, 1, 2 or 3 hours and then processed by centrifugation through a two-step sucrose gradient. The counts per minute bound to the cell pellets was determined. Percent specific binding was calculated by subtracting the counts per minute of the samples containing uniodinated NGF from those without uniodinated NGF and dividing by the total number of counts added to the reaction mixture. Each bar depicts the mean + / - S. E. M. of three samples. These data are representative of four independent experiments.

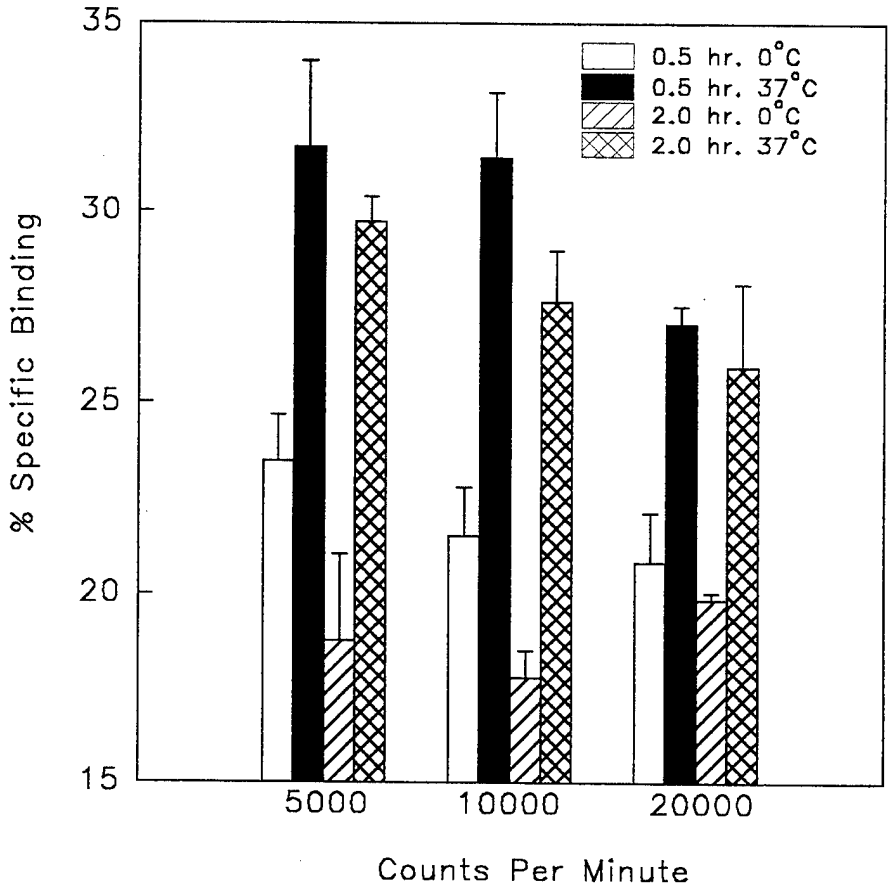

Figure 38. The effect of incubation temperature on specific binding. PC12 cells were aliquoted into microfuge tubes at 3.2 x 10^5 cells/tube in 200 µl of phosphate buffered gelatin (PBG). 35 µl of premixes containing either 5,000, 10,000 or 20,000 counts per minute of ^{125}I-NGF with or without 1 µg of uniodinated NGF was added to the tubes. Reaction mixtures were incubated at either 0°C or 37°C for either 0.5 or 2 hours and then processed by centrifugation through a two-step sucrose gradient. The counts per minute bound to the cell pellets was determined. Percent specific binding was calculated by subtracting the counts per minute of the samples containing uniodinated NGF from those without uniodinated NGF and dividing by the total number of counts added to the reaction mixture. Each bar depicts the mean + / - S. E. M. of three samples. These data are representative of four independent experiments.

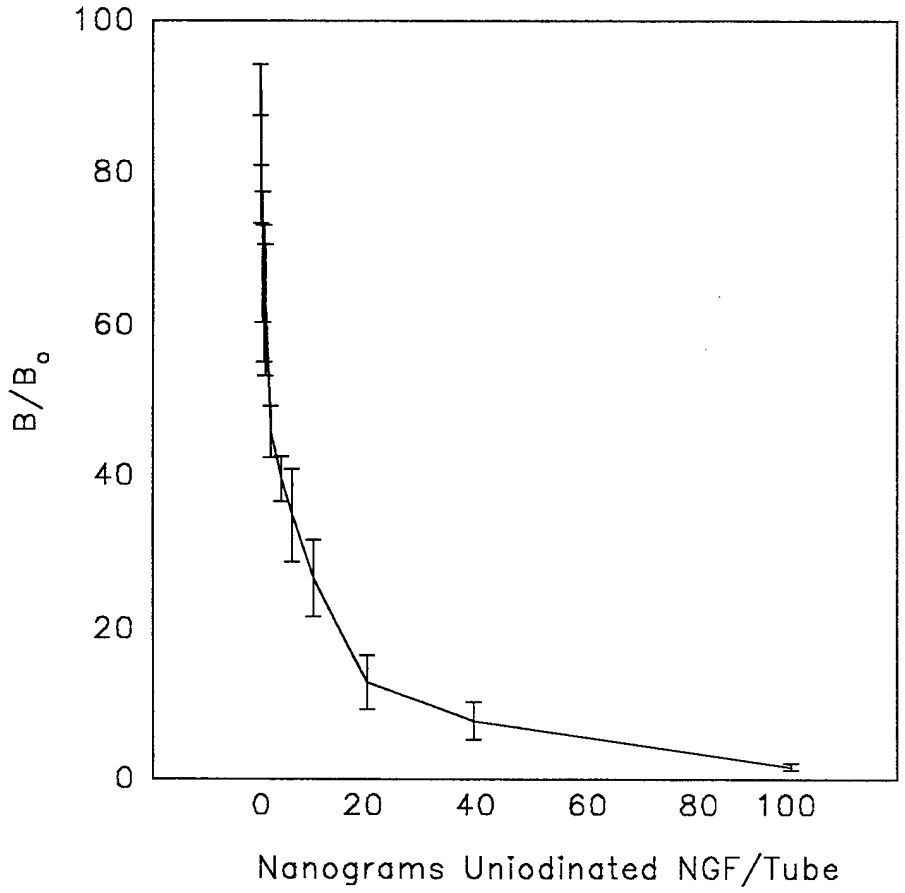

Figure 39. Displacement of ^{125}I-NGF by uniodinated NGF. PC12 cells were aliquoted into microfuge tubes at 3.2 x 10^5 cells/tube in 200 µl of phosphate buffered gelatin (PBG). Premixes containing either 0, 0.1, 0.25, 0.75, 1, 2.5, 5, 7.5, 10, 25, 50 or 100 ηg uniodinated NGF and 5,000 counts per minute of ^{125}I-NGF in 35 µl was added to the tubes. Reaction mixtures were incubated at 37°C for 30 minutes and then processed by centrifugation through a two-step sucrose gradient. The counts per minute bound to the cell pellets was determined. The amount of ^{125}I-NGF bound to cell pellets and the amount of free NGF was calculated. Data are plotted as bound ^{125}I-NGF / total ^{125}I-NGF versus uniodinated NGF. Points depict the mean + / - S. E. M. of three samples. These data are representative of four independent experiments.

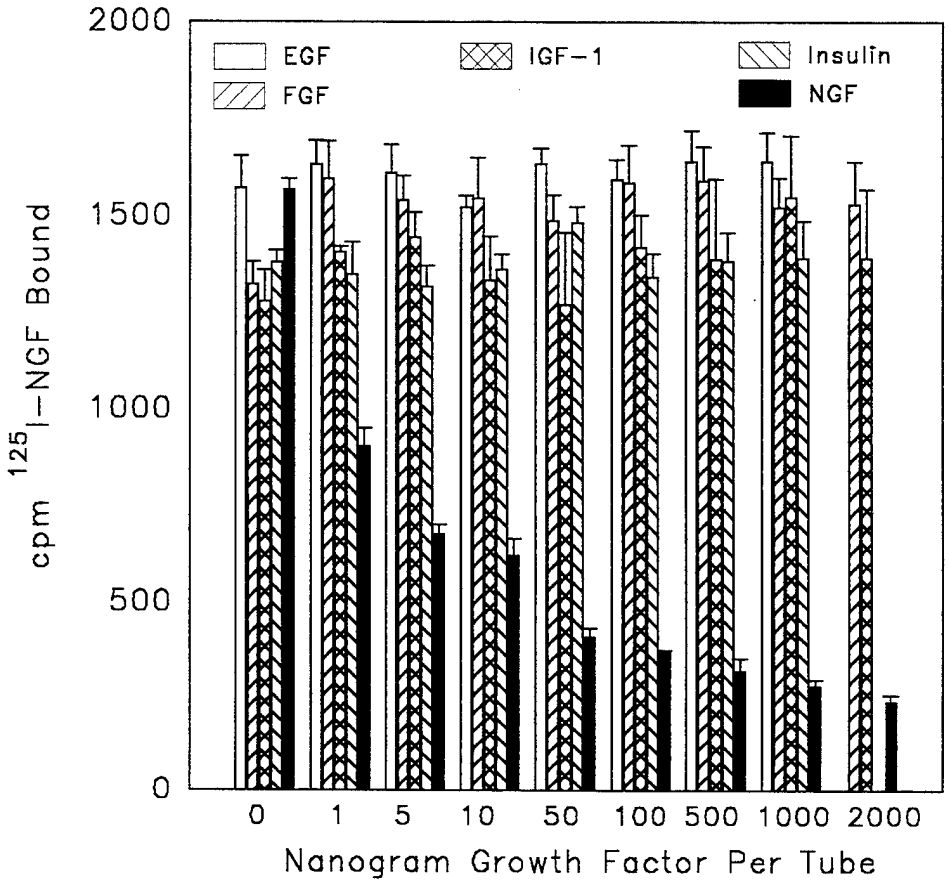

Figure 40. Specificity of ^{125}I-NGF binding. PC12 cells were aliquoted into microfuge tubes at 3.2 x 10^5 cells/tube in 200 µl of PBG. 35 µl of premixes containing either 0, 1, 5, 10, 50, 100, 500, 1000, 2000 or 10,000 ηg uniodinated epidermal growth factor (EGF), fibroblastic growth factor (FGF), insulin like growth factor - 1 (IGF-1), Insulin or nerve growth factor (NGF) and 5,000 counts per minute of ^{125}I-NGF was added to the tubes. Reaction mixtures were incubated at 37°C for 30 minutes and then processed by centrifugation through a two-step sucrose gradient. The counts per minute of ^{125}I-NGF bound to the cell pellets was determined. Bars depict the mean + / - S. E. M. of three samples.

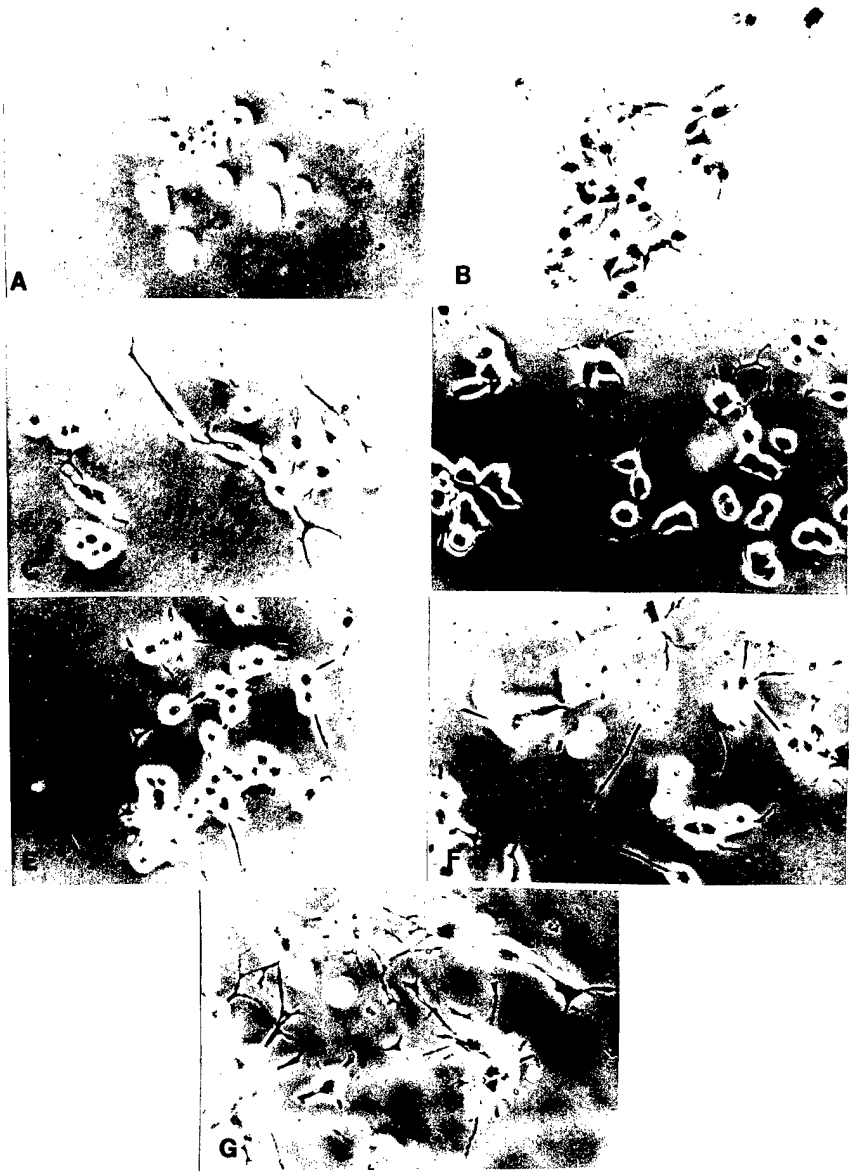

Figure 41. Effect of simultaneous treatment with ethanol and NGF on NGF induced neurite extension. PC12 cells were simultaneously treated with varying concentrations of ethanol (0.1 - 0.8 % w/v) and stimulated simultaneously with 50 ηg/ml NGF for 24 hours. Treatments: a) none, b) none, c) 0.1%, d) 0.2%, e) 0.4%, f) 0.6%, g) 0.8%, b - g also contained 50 ηg/ml NGF.

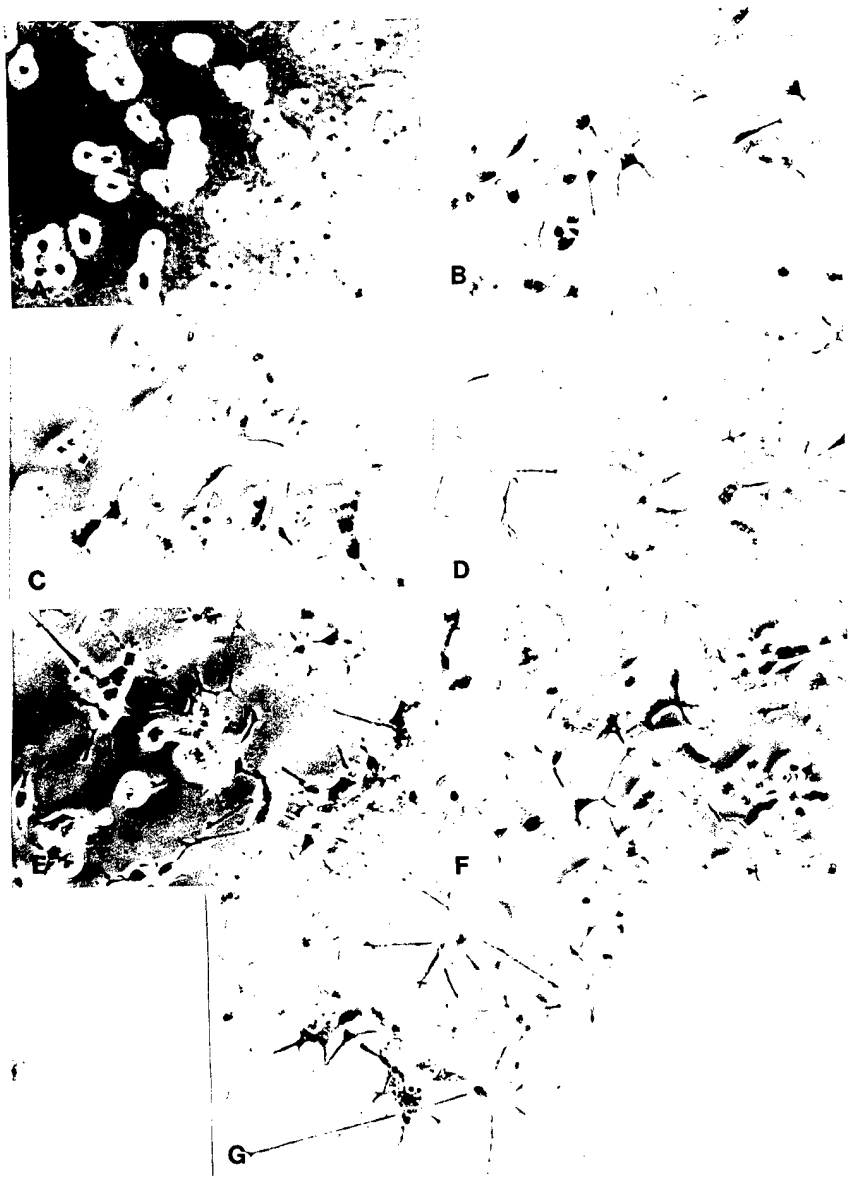

Figure 42. Effect of ethanol pretreatment prior to NGF stimulation on NGF induced neurite extension. PC12 cells were treated with varying concentrations of ethanol (0.1 - 0.8% w/v) and allowed to incubate for 24 hours prior to stimulation with 50 ηg/ml NGF for 24 hours in the continued presence of ethanol. Treatments: a) none, b) none, c) 0.1%, d) 0.2%, e) 0.4%, f) 0.6%, g) 0.8%, b - g were followed by 50 ηg/ml NGF for 24 hours.

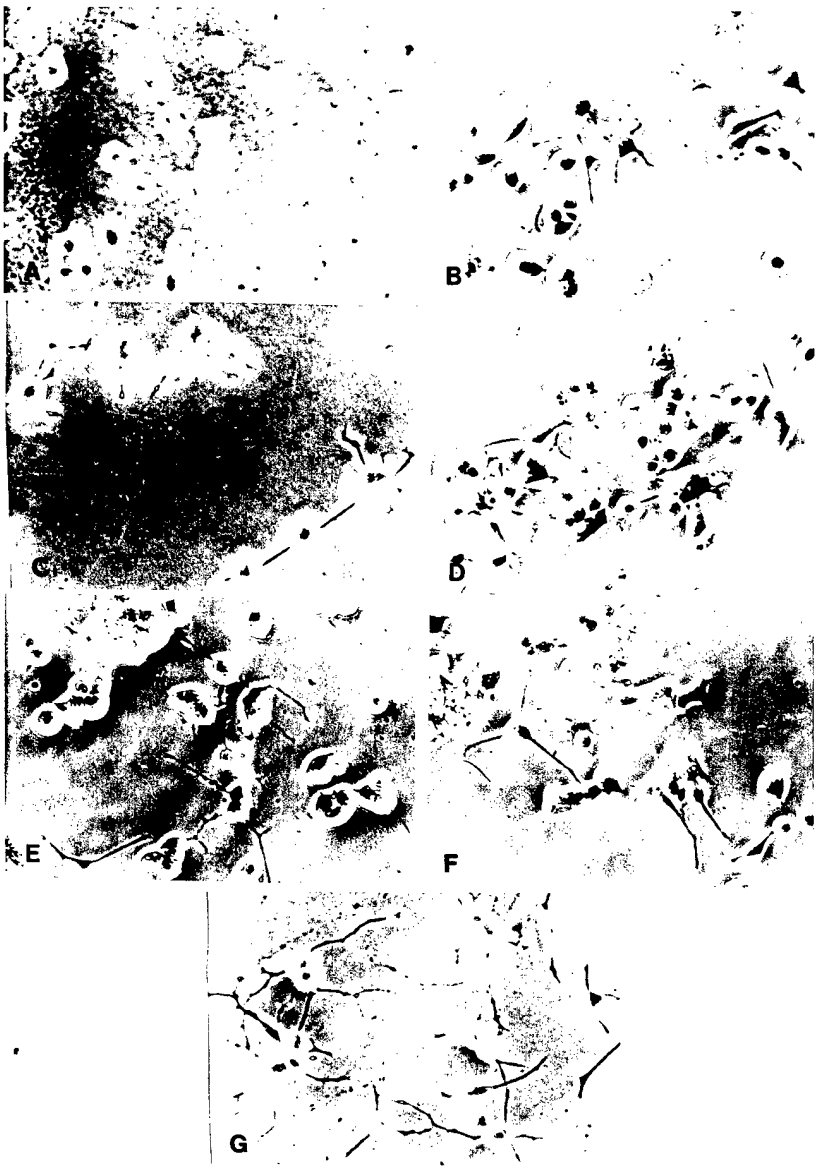

Figure 43. Effect of ethanol pretreatment prior to NGF stimulation on NGF induced neurite extension. PC12 cells were treated with varying concentrations of ethanol (0.1 - 0.8% w/v) for 72 hours prior to stimulation with 50 ηg/ml NGF for 24 hours in the continued presence of ethanol. Treatments: a) none, b) none, c) 0.1%, d) 0.2%, e) 0.4%, f) 0.6%, g) 0.8%, b - g were followed by 50 ηg/ml NGF for 24 hours.

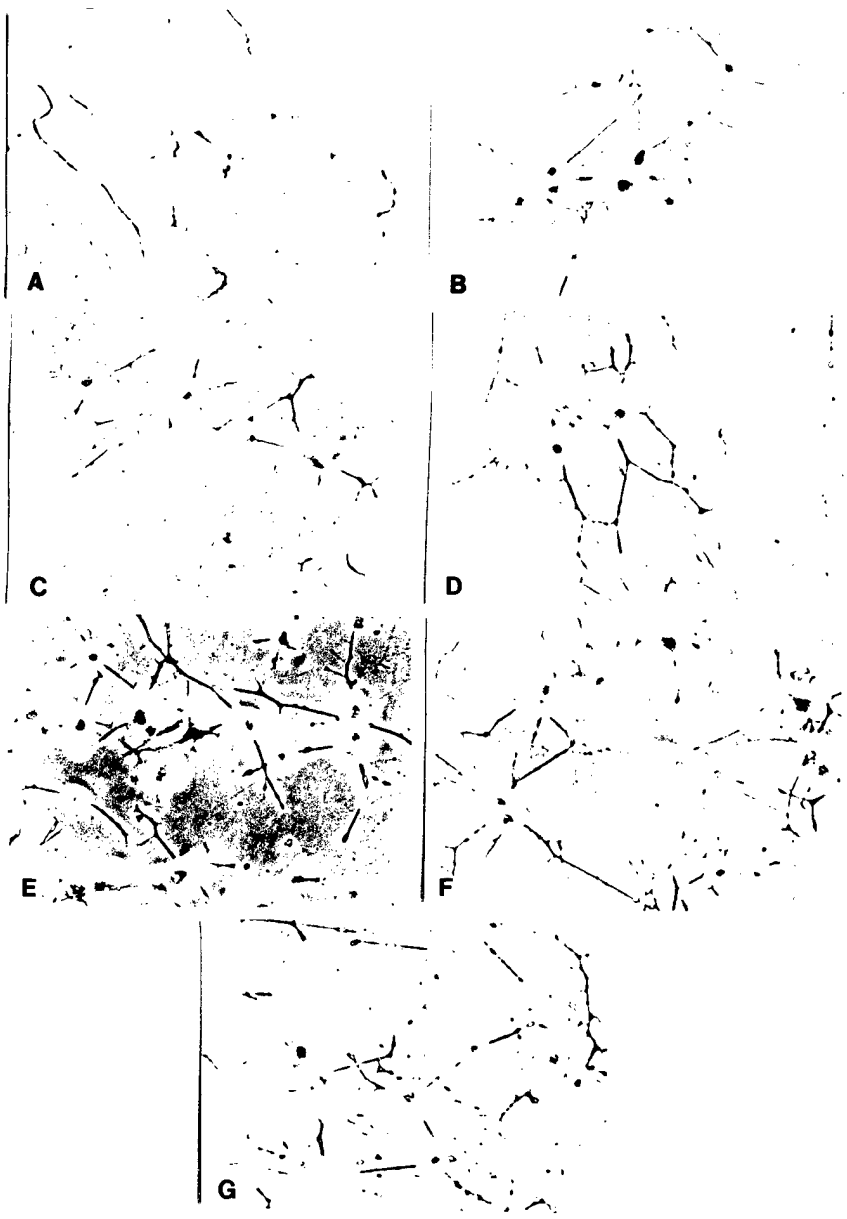

Figure 44. Effect of simultaneous treatment with ethanol and NGF on NGF induced neurite extension. PC12 cells were simultaneously treated with varying concentrations of ethanol (0.1 - 0.8 % w/v) and stimulated with 50 ηg/ml NGF for 72 hours. Treatments: a) none, b) none, c) 0.1%, d) 0.2%, e) 0.4%, f) 0.6%, g) 0.8%, b - g also contained 50 ηg/ml NGF.

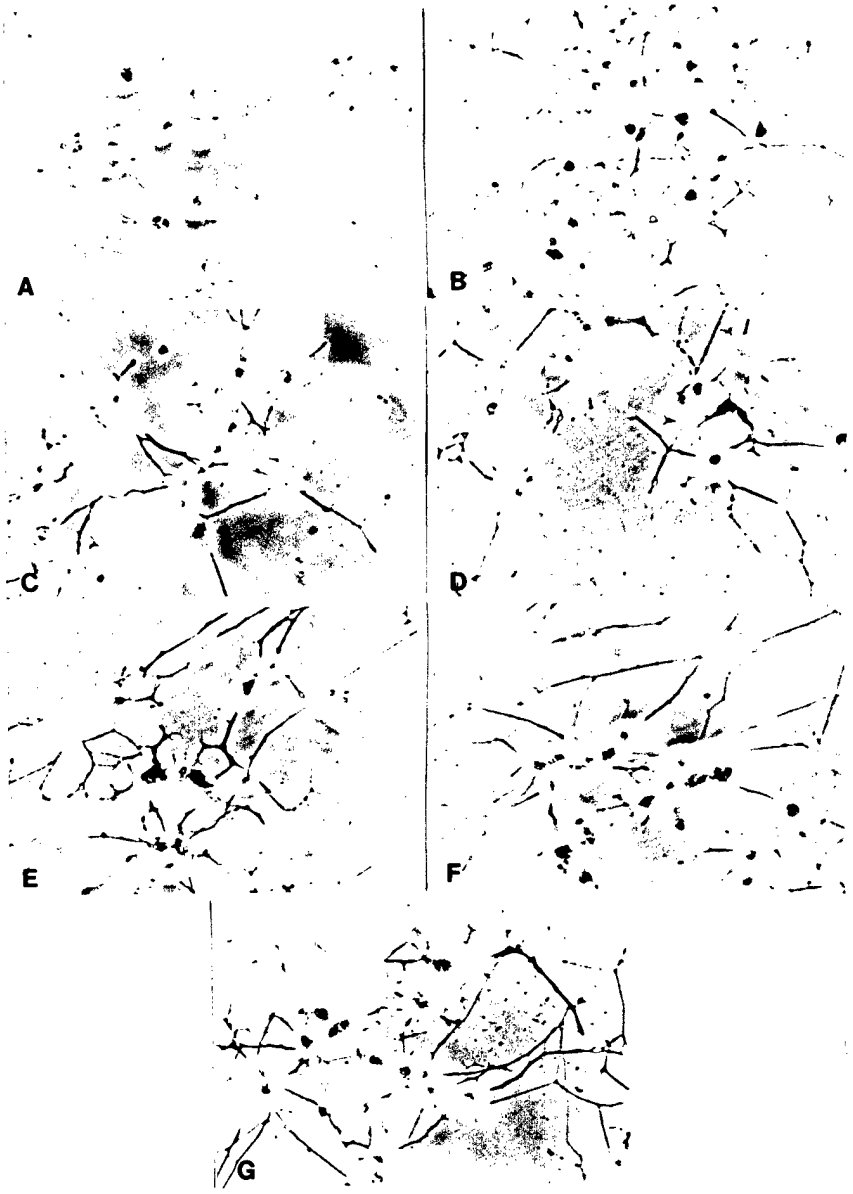

Figure 45. Effect of ethanol pretreatment prior to NGF stimulation on NGF induced neurite extension. PC12 cells were treated with varying concentrations of ethanol (0.1 - 0.8% w/v) for 24 hours prior to stimulation with 50 ηg/ml NGF for 72 hours in the continued presence of ethanol. Treatments: a) none, b) none, c) 0.1%, d) 0.2%, e) 0.4%, f) 0.6%, g) 0.8%, b - g were followed by 50 ηg/ml NGF for 72 hours.

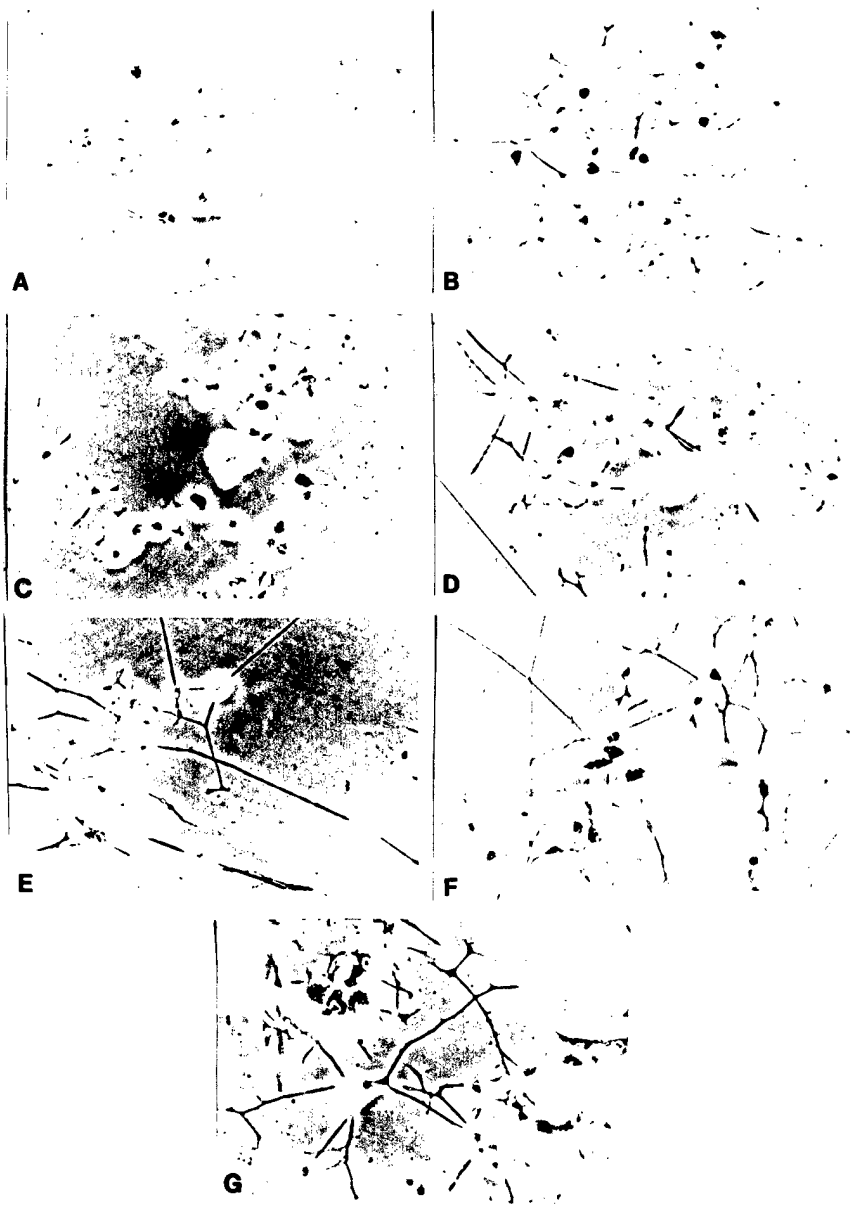

Figure 46. Effect of ethanol pretreatment prior to NGF stimulation on NGF induced neurite extension. PC12 cells were treated with varying concentrations of ethanol (0.1 - 0.8% w/v) for 72 hours prior to stimulation with 50 ηg/ml NGF for 72 hours in the continued presence of ethanol. Treatments: a) none, b) none, c) 0.1%, d) 0.2%, e) 0.4%, f) 0.6%, g) 0.8%, b - g were followed by 50 ηg/ml NGF for 72 hours.

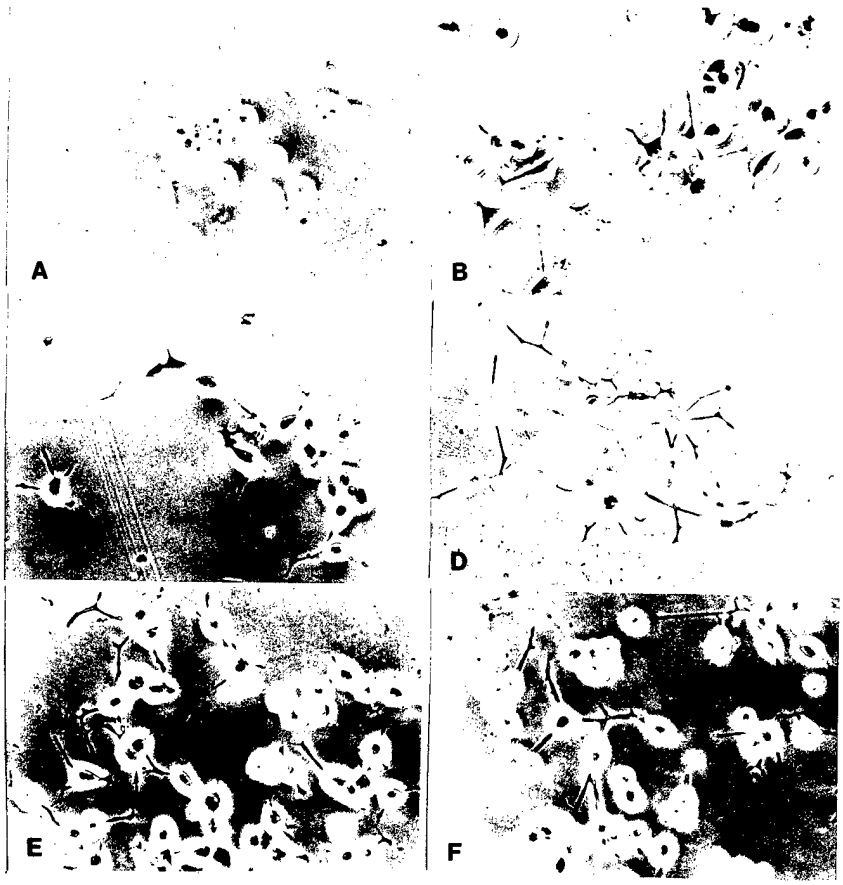

Figure 47. Effect of simultaneous treatment with varying alcohol chain length and NGF on NGF-induced neurite extension. PC12 cells were simultaneously treated with 0.4% (w/v) of varying alcohols and stimulated with 50 ηg/ml NGF for 24 hours. Treatments: a) none, b) none, c) MeOH, d) EtOH, e) PrOH, f) BuOH, b - f also contained 50 ηg/ml NGF.

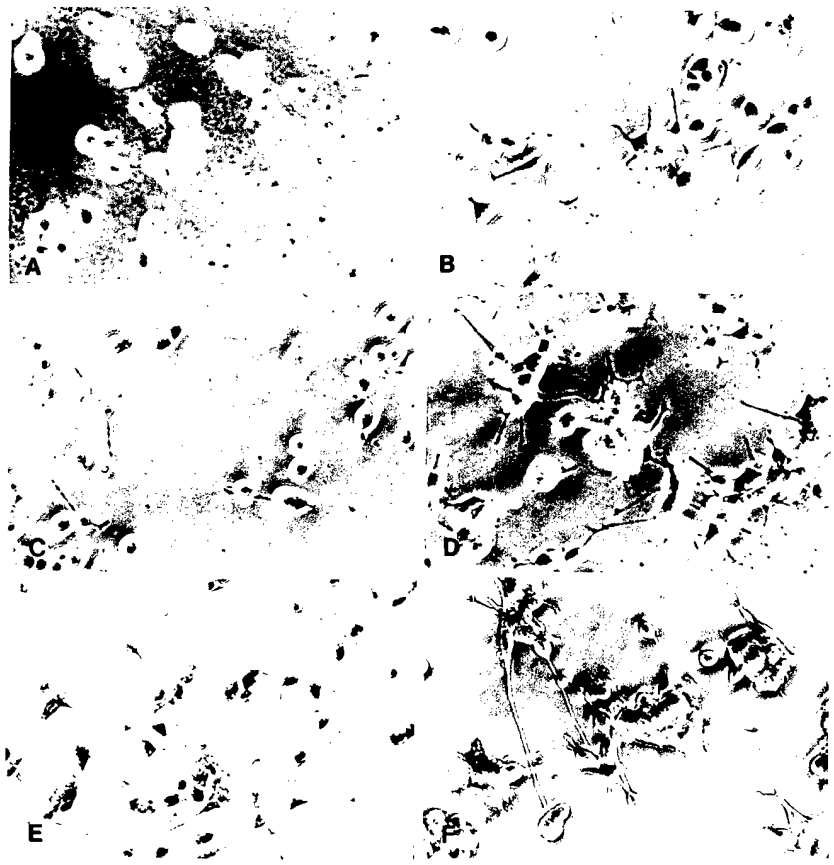

Figure 48. Effect of 24 hour pretreatment with varying alcohol chain length prior to NGF stimulation on NGF-induced neurite extension. PC12 cells were treated with 0.4% (w/v) of varying alcohols for 24 hours prior to stimulation with 50 ηg/ml NGF for 24 hours in the continued presence of alcohols. Treatments: a) none, b) none, c) MeOH, d) EtOH, e) PrOH, f) BuOH, b - g were followed by 50 ηg/ml NGF for 24 hours.

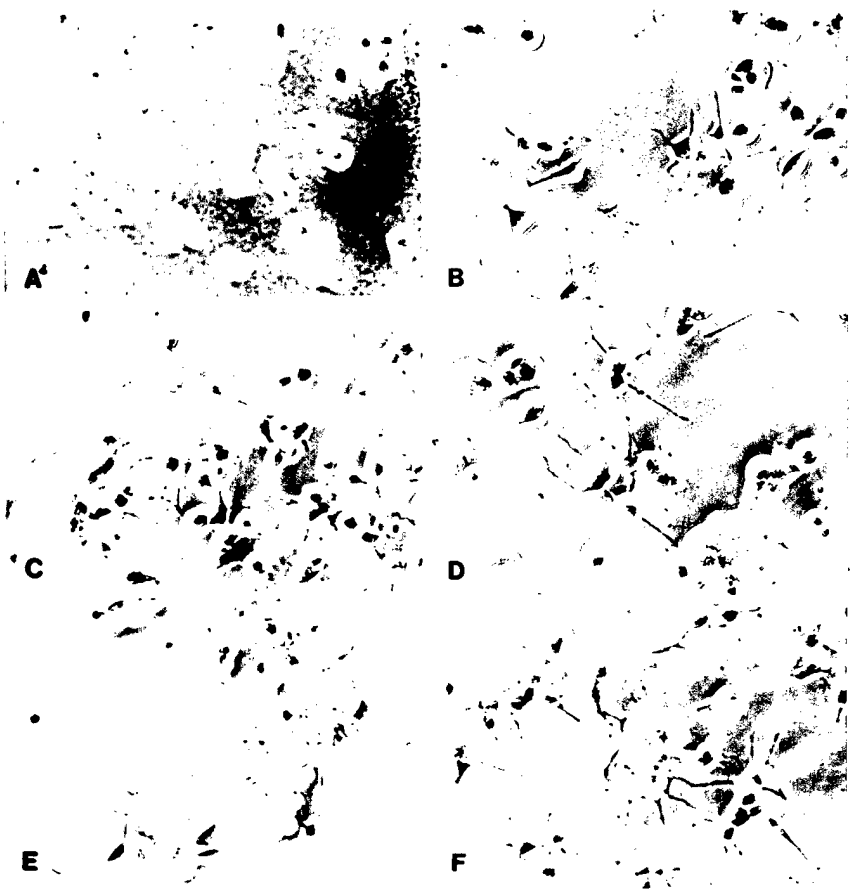

Figure 49. Effect of 72 hour pretreatment with varying alcohol chain length prior to NGF stimulation on NGF-induced neurite extension. PC12 cells were treated with 0.4% (w/v) of varying alcohols for 72 hours prior to stimulation with 50 ηg/ml NGF for 24 hours in the continued presence of alcohol. Treatments: a) none, b) none, c) MeOH, d) EtOH, e) PrOH, f) BuOH, b - g were followed by 50 ηg/ml NGF for 24 hours.

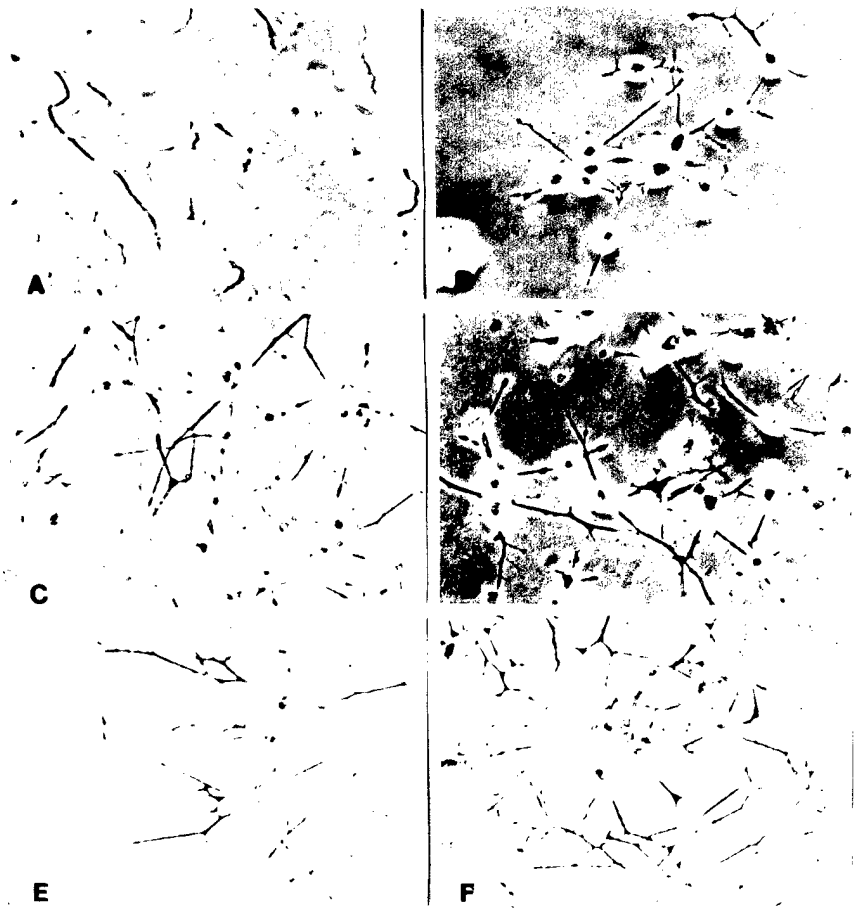

Figure 50. Effect of simultaneous treatment with varying alcohol chain length and NGF on NGF-induced neurite extension. PC12 cells were simultaneously treated with 0.4% (w/v) of varying alcohols and stimulated with 50 ηg/ml NGF for 72 hours. Treatments: a) none, b) none, c) MeOH, d) EtOH, e) PrOH, f) BuOH, b - g also contained 50 ηg/ml NGF.

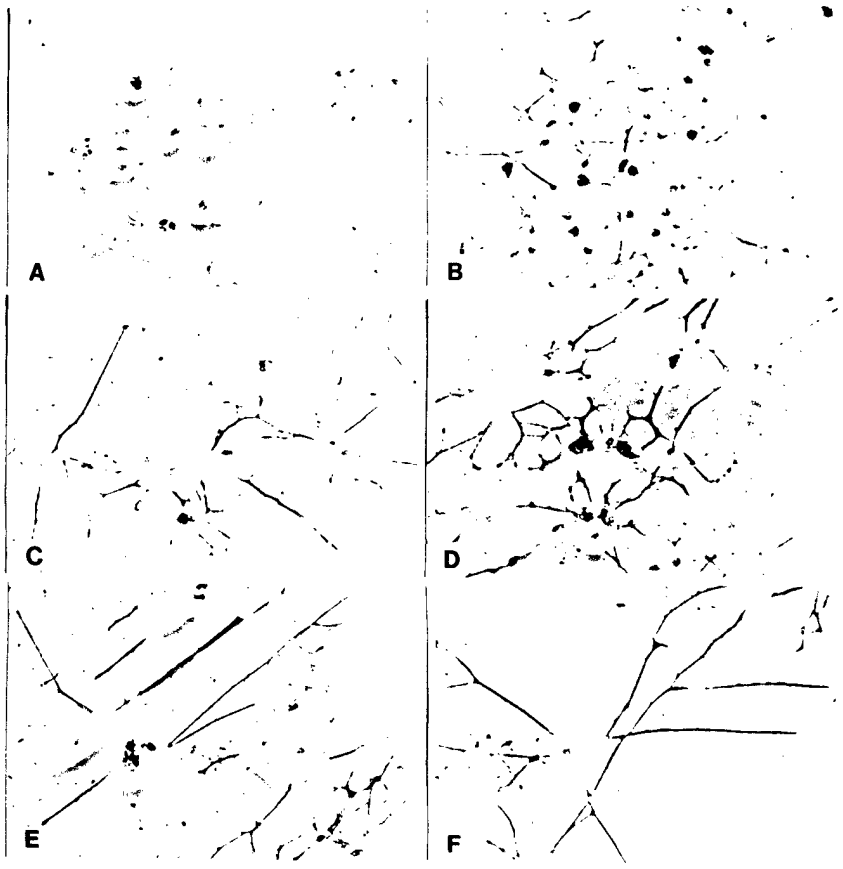

Figure 51. Effect of 24 hour pretreatment with varying alcohol chain length prior to NGF stimulation on NGF-induced neurite extension. PC12 cells were treated with 0.4% (w/v) of varying alcohols for 24 hours prior to stimulation with 50 ηg/ml NGF for 72 hours in the continued presence of alcohol. Treatments: a) none, b) none, c) MeOH, d) EtOH, e) PrOH, f) BuOH, b - g were followed by 50 ηg/ml NGF for 72 hours.

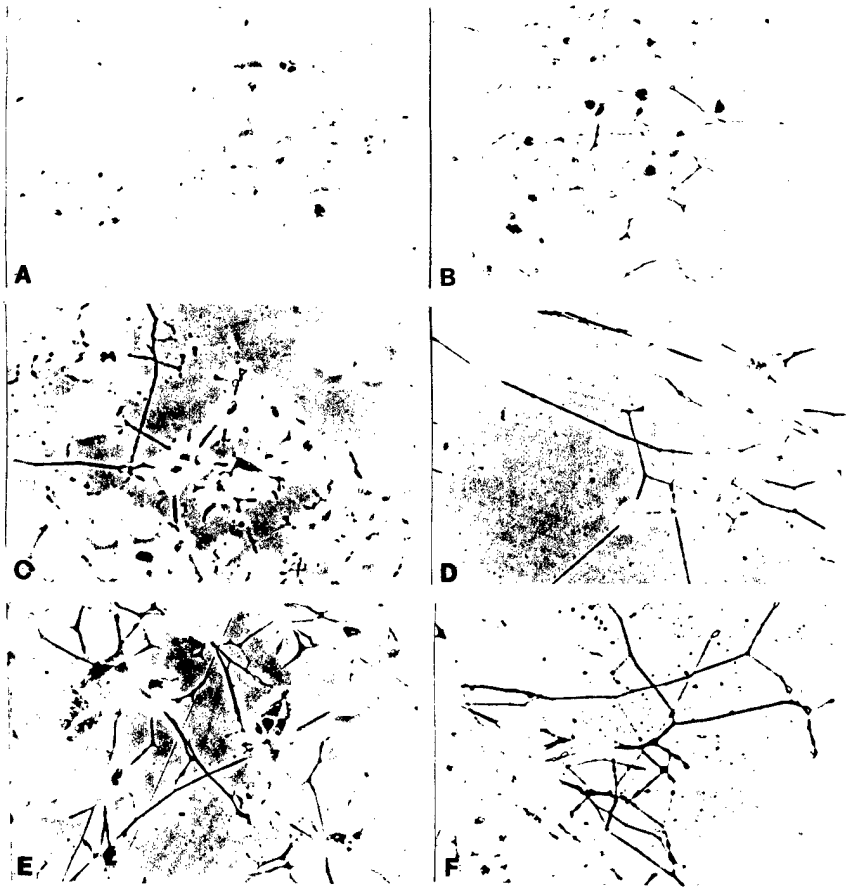

Figure 52. Effect of 72 hour pretreatment with varying alcohol chain length prior to NGF stimulation on NGF-induced neurite extension. PC12 cells were treated with 0.4% (w/v) of varying alcohols for 72 hours prior to stimulation with 50 ηg/ml NGF for 72 hours in the continued presence of alcohol. Treatments: a) none, b) none, c) MeOH, d) EtOH, e) PrOH, f) BuOH, b - g were followed by 50 ηg/ml NGF for 72 hours.

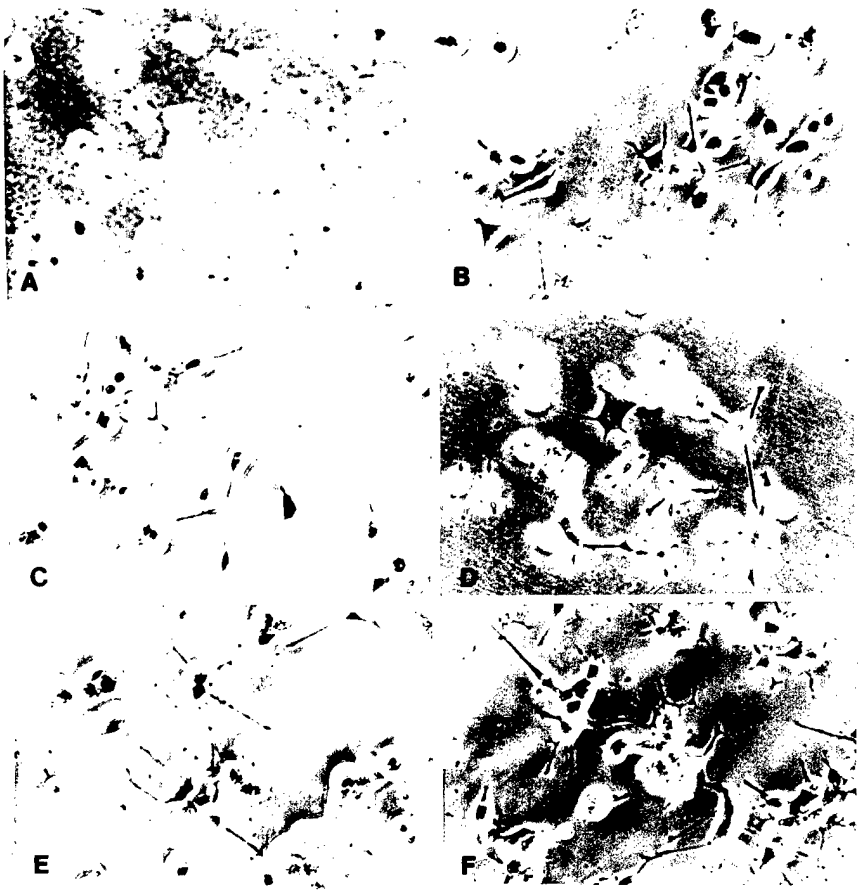

Figure 53. Effect of ethanol pretreatment time on NGF-induced neurite extension after 24 hours of NGF stimulation. PC12 cells were treated with 0.4% (w/v) ethanol for the indicated time periods and then stimulated with 50 ηg/ml NGF for 24 hours. Treatments; a) none, b) none, c) pretreatment for 12 hours, d) pretreatment for 24 hours, e) pretreatment for 48 hours, f) pretreatment for 72 hours, b - f were followed by 50 ηg/ml NGF for 24 hours.

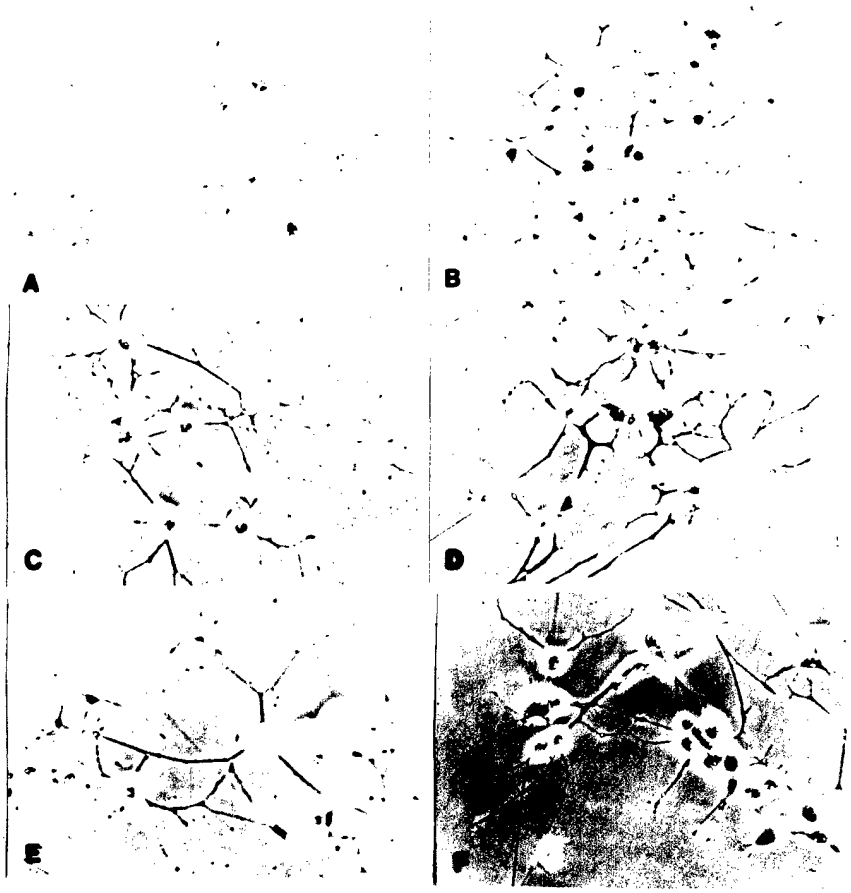

Figure 54. Effect of ethanol pretreatment time on NGF-induced neurite extension after 72 hours of NGF stimulation. PC12 cells were treated with 0.4% (w/v) ethanol for the indicated time periods and then stimulated with 50 ηg/ml NGF for 72. Treatments; a) control cells, b) none, c) pretreatment for 12 hours, d) pretreatment for 24 hours, e) pretreatment for 48 hours, f) pretreatment for 72 hours, b - f were followed by 50 ηg/ml NGF for 72 hours.

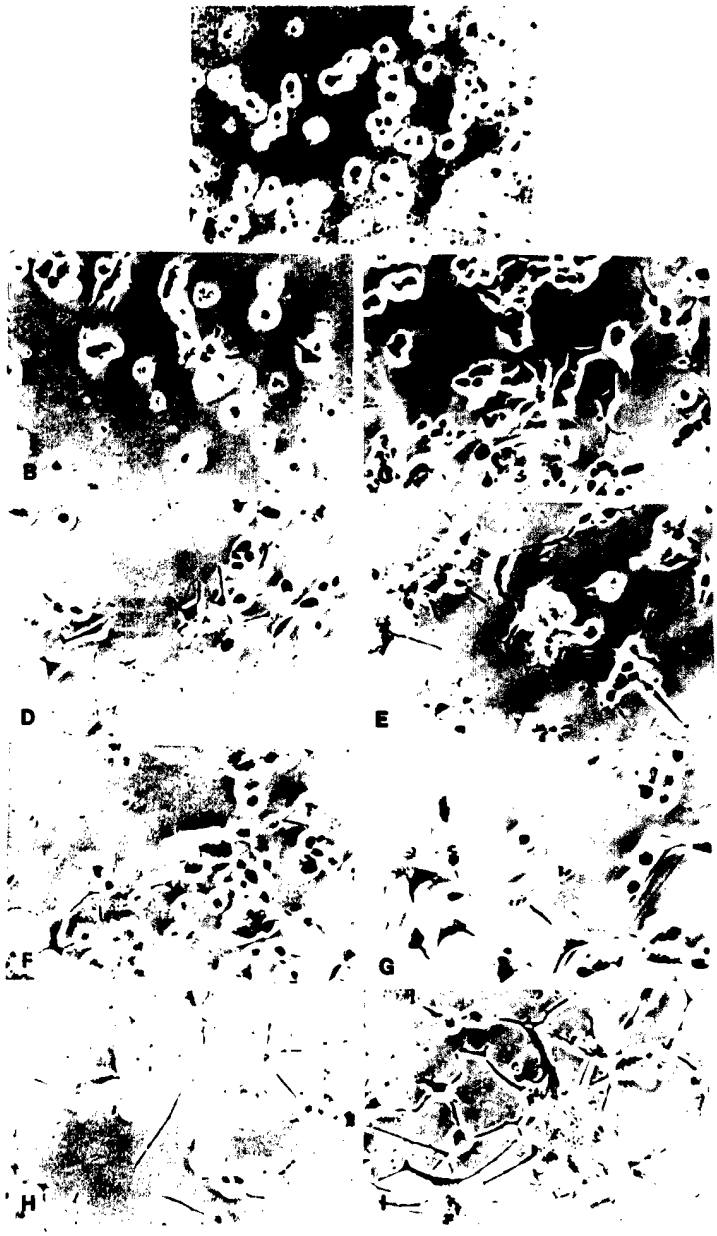

Figure 55. Effect of NGF stimulation time on NGF-induced neurite extension on cells simultaneously treated with ethanol. PC12 cells were treated simultaneously with 0.4% (w/v) ethanol and 50 ηg/ml NGF or with 50 ηg/ml NGF alone for the indicated time. Treatments: a) none, b) 12 hour NGF, c) 12 hour EtOH + NGF, d) 24 hour NGF, e) 24 hour EtOH + NGF, f) 48 hour NGF, g) 48 hour EtOH + NGF, h) 72 hour NGF, i) 72 hour EtOH + NGF.

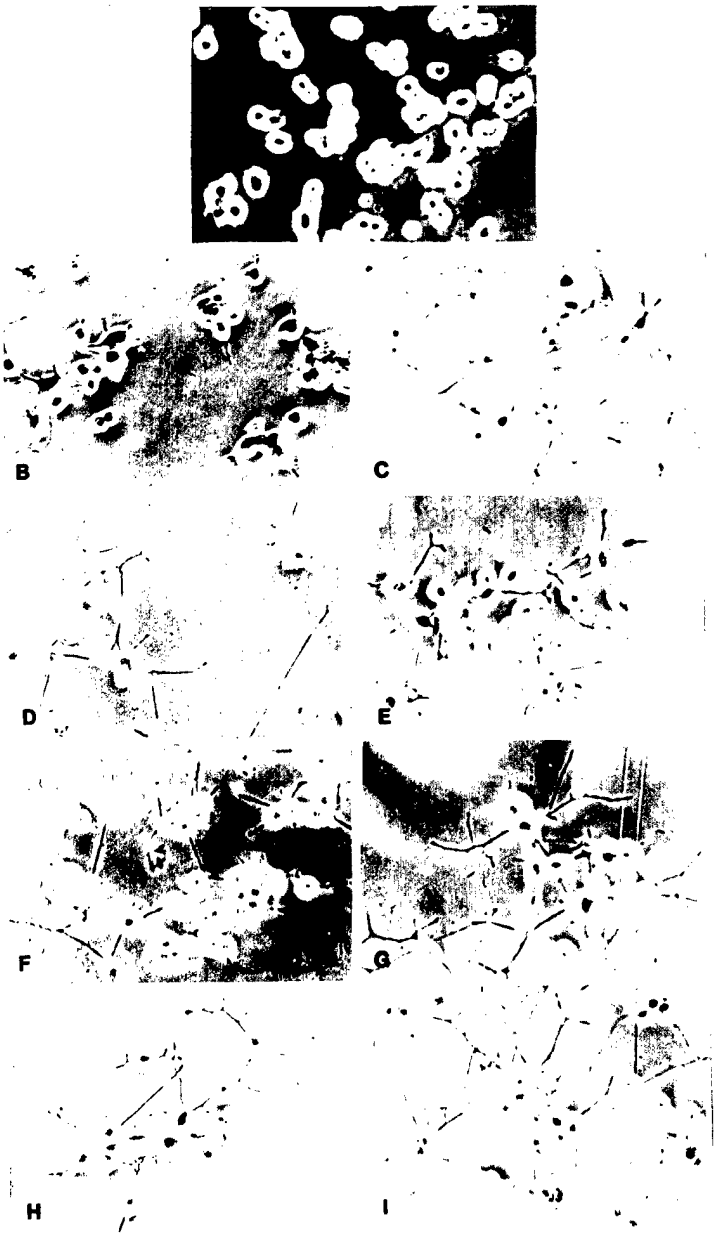

Figure 56. Effect of NGF stimulation time on NGF-induced neurite extension on cells which were pretreated with ethanol. PC12 cells were pretreated with 0.4% (w/v) ethanol for the indicated time periods and then stimulated with 50 ηg/ml NGF for the indicated time periods or with 50 ηg/ml NGF for the indicated time periods. Treatments; a) none, b) 12 hour NGF, c) 12 hour pretreatment + 12 hour NGF stimulation, d) 24 hour NGF, e) 24 hour pretreatment + 24 hour NGF stimulation, f) 48 hour NGF, g) 48 hour pretreatment + 48 hour NGF, h) 72 hour NGF, i) 72 hour pretreatment + 72 hour NGF.