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NERVE GROWTH FACTOR NEUROPROTECTION OF ETHANOL-INDUCED NEURONAL DEATH IN RAT CEREBRAL CORTEX IS AGE-DEPENDENT

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Abstract

Organotypic cultures of rat cortex were used to test the hypotheses that nerve growth factor (NGF) is neuroprotective for immature cortical neurons and that ethanol abolishes this neuroprotection in a developmental stage-dependent manner. Samples were obtained on gestational day (G) 16 or postnatal day (P) 3 and cultured with ethanol (0 or 400 mg/dl) and NGF (0 or 30 ng/ml) for 72 hours. Dying neurons were identified as exhibiting terminal nick-end labeling, immunoreactivity for activated caspase 3, or condensed nuclear chromatin. Two cortical compartments were examined in fetal tissue; a superficial, cell-sparse marginal zone (MZ) and a cell-dense cortical plate (CP). At P3, the CP was subdivided into a cell-dense upper cortical plate (UCP) and a less densely packed lower cortical plate (LCP). Neuronal death in the MZ was affected by neither NGF nor ethanol at both ages. In the fetal CP, NGF did not affect the incidence of cell death, but ethanol increased it. Treatment with NGF caused an upregulation of the expression of *Neg*, a gene known to be affected by NGF and ethanol. NGF did not ameliorate the ethanol-induced death. In pups, ethanol increased the amount of death in the LCP. NGF did protect against this death. Neither ethanol nor NGF altered the incidence of cell death in the UCP. The laminar-dependent neuroprotection did not correlate with expression of NGF receptors or *Neg*. Thus, NGF can be protective against the neurotoxic effect of ethanol in the neonatal brain. This effect is site-selective and time-dependent and it targets post-migratory, differentiating neurons.

Keywords

fetal alcohol syndrome; ethanol; neurotrophin; apoptosis; p75; trk

Naturally occurring neuronal death (NND) in the developing mammalian CNS is a well-orchestrated phenomenon. Neurons are overproduced and then a portion of them dies (Oppenheim, 1991; Burek and Oppenheim, 1996; Mooney and Henderson, 2006). Cell death is common among proliferating neuronal progenitors in the ventricular and subventricular zones (Blaschke et al., 1996; Thomiadou et al., 1997; Rehen and Chun, 2006) and among post-mitotic, post-migratory neurons in the cortical plate (e.g., Al-Ghoul and Miller, 1989; Ferrer et al., 1990; Finlay and Slattery, 1983; Miller, 1995; Spreafico et al., 1995; Mooney and

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Henderson, 2006). In the rat cortex, NOND peaks during the first postnatal week. The frequency of this death depends upon the time of origin of the neuron and upon its laminar location (e.g., Al-Ghoul and Miller, 1989; Wood et al., 1992; Miller, 1995).

NOND in the mammalian CNS is regulated by epigenetic factors, including neurotrophins. Among the neurotrophins, nerve growth factor (NGF) uniquely prevents the death of primary cultured, fetal cortical neurons (Seabold et al., 1998; Miller et al., 2003). NGF and its receptors, p75 and trkA, are expressed by developing (Cirulli et al., 1997; Conner and Varon, 1997; Dohrman et al., 1997; Seabold et al., 1998; Kwon et al., 2005) and mature cortical neurons (Conner et al., 1992; Pitts and Miller, 1995; 2000; Jacobs and Miller, 1999; Miller, 2000; Miller and Pitts, 2000; Bruns and Miller, 2007). The binding of NGF to trkA results in phosphorylation of the receptor, followed by activation of signaling pathways that enhance cell survival and neuronal differentiation (Bibel and Barde, 2000). In contrast, signaling via p75 can activate cell survival or cell death pathways.

NGF can be neuroprotective, i.e., it can reduce the amount of cell death *in vitro* that is normally detected within the first 48 hours of plating (Seabold et al., 1998; Miller et al., 2003). Ethanol blocks this effect. In other words, NGF fails to prevent the ethanol-induced death of primary cortical neurons (Seabold et al., 1998; Miller et al., 2003; Barclay et al., 2005). Conversely, NGF is protective against ethanol-induced death in cultured septal, hippocampal (Webb et al., 1997), and cerebellar neurons (Luo et al., 1997). These results reflect (a) site-specificity, (b) differential windows of vulnerability to ethanol, and/or (c) differences in responsiveness to NGF.

The present study tests the hypothesis that the neuroprotective effect of NGF and the neurotoxic effect of ethanol coincide with critical periods of cortical development. These periods concur with those of neuronogenesis and NOND which in rat neocortex occur during the third week of gestation (Brückner et al., 1976; Miller, 1988; Uylings et al., 1990) and the first postnatal week (Ferrer et al., 1990; Miller, 1995), respectively.

EXPERIMENTAL PROCEDURES

Animals

Pregnant Sprague-Dawley rats were obtained from Taconic (Germantown, NY). Animals were housed in a temperature- and humidity-controlled facility at the Syracuse Veterans Affairs Medical Center (VAMC) that was accredited by Assessment and Accreditation of Laboratory Animal Care. All procedures were approved by the Committee for Humane Use of Animals at Upstate Medical University and the Institutional Animal Care and Use Committee at the Syracuse VAMC.

Tissue preparation

Brains were collected from fetuses on gestational day (G) 16 or from pups on postnatal day (P) 3. G1 was defined as the first day a sperm-positive plug was found and G22 was uniformly designated as P0. To collect fetal tissue on G16, pregnant dams were anesthetized (100 mg/kg ketamine and 10.0 mg/kg xylazine) and four fetuses were removed from each dam. A mid-sagittal cut was made in the fetal cranium to expose the cerebral vesicles. Each vesicle was removed by making a circumferential cut at its base. To collect tissue from pups on P3, each animal was decapitated while anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine). Brains were rapidly removed and cut into 400 μ m coronal slices using a McIlwain Tissue Chopper (Mickle Lab Engineering, Gomshall UK). At each age, tissue was randomly assigned to one of four treatment groups: untreated controls, or those exposed to NGF (30 ng/ml), ethanol

(400 mg/dl), or both NGF and ethanol. For each experiment, a total of five animals, each taken from a different litter, was assigned to each treatment group.

Tissue samples, vesicles from 16-day-old fetuses and slices from three-day-old pups, were collected in ice cold 100 mM phosphate buffer (PB) in saline (PBS) prior to transfer onto a sterile culture filter (PICMORG50, Millipore, Bedford MA). Tissue was incubated overnight (37°C, 6% CO₂) in a medium (Minimum Essential Medium (MEM; GibcoBRL, Grand Island NY), 200 mM L-glutamine, and 24% glucose) supplemented with 25% fetal bovine serum (FBS). After 24 hours, the four treatment groups were made by replacing the FBS-laced medium with a serum-free medium containing human recombinant NGF (0 or 30 ng/ml; Sigma, St Louis MO) and ethanol (0 or 400 mg/dl). This concentration of ethanol was used because it mimics the effects caused by *in vivo* exposures that result in peak blood ethanol concentrations of 120–150 mg/dl. These include changes in cell proliferation (cf. Miller and Nowakowski, 1991; Jacobs and Miller, 2001) and migration (cf. Miller, 1993; Siegenthaler and Miller, 2004). Cultures were maintained in treatment for 72 hours and fresh medium was replaced every 24 hours.

Cultures were kept in humidified chambers containing 200 ml water and 60 ml CO₂. For ethanol-treated cultures, the chambers also contained 1.5 ml ethanol. This stabilized the ethanol concentration in the medium (Luo and Miller, 1997). Samples of medium were extracted at the beginning and end of the experiment. Ethanol content was analyzed using the Analox GM7 (Analox Instruments, Lunenburg MA) and was found to remain constant over a 24 hr period.

Labeling

Tissue was fixed in 4.0% paraformaldehyde in 100 mM PB at room temperature for four hr, then cryoprotected in 30% sucrose in PB at 4°C. Tissue was cut into a complete series of 10 µm sections. Sections were processed for terminal uridylated nick-end labeling (TUNEL; Intergen, Purchase NY), protein immunohistochemistry, and *in situ* hybridization for *Neg*.

TUNEL was used to identify cells exhibiting fragmented DNA, a characteristic feature of apoptotic cells (Gavrieli et al., 1992; Jacobs and Miller, 2001). Sections were incubated in terminal deoxynucleotidyl transferase for one hour at 37°C, rinsed in PBS and then washed in horseradish peroxidase-conjugated anti-digoxigenin for 30 min. Labeled cells were identified by a wash in a solution of 0.010% hydrogen peroxide and 0.20% 3,3'-diaminobenzidine (DAB). Sections were counterstained with methyl green and coverslipped.

Sections from neonatal animals were stained immunohistochemically for the expression of p75, trkA, phosphorylated trk (p-trk), or activated caspase 3. All steps were performed at room temperature. Non-specific labeling was quenched by a wash in 5.0% bovine serum albumin (BSA) in PB containing 0.10% Tween 20 (TPBS). Sections were incubated for one hour in a solution of primary antibody (anti-p75, 05–446, Upstate Biotech, 1:100 in TPBS), anti-trkA (a generous gift from Louis F. Reichardt, 1:200), anti-p-trk, (sc-8058; Santa Cruz, 1:100), or anti-activated caspase 3 (AF835; R&D Systems, 1:1000). Subsequently, unbound antibody was removed by a wash in 5.0% non-fat milk in TPBS. Sections were processed with a Vectastain Elite kit (Vector, Burlingame CA). Non-specific labeling was examined by omitting either the primary or the secondary antibody.

In situ hybridization was used to detect the expression of the mRNA for *Neg*. *Neg* is a gene identified in primary cultures of cortical neurons that is (a) up-regulated by treatment with NGF and (b) down-regulated by concurrent treatment with ethanol (Miller et al., 2003). Sections were incubated with a digoxigenin-labeled anti-sense probe to *Neg* for 18 hr at 55°C. Non-annealed probe was removed by a series of stringent washes, then tissue was treated with RNase to degrade any remaining probe.

Localization of the digoxigenin-labeled probe was performed using immunohistochemistry similar to that described above. After blocking, an alkaline phosphatase-conjugated anti-digoxigenin antibody was applied to the tissue for 18 hr at 4°C. Tissue was rinsed, then incubated in the chromagen nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche) for 2–4 hr at room temperature. Slides were dehydrated, cleared, and coverslipped. Two controls were performed; sections were incubated with the sense probe to *Neg*, or to a sense or anti-sense probe for actin mRNA.

Analysis

In the fetal tissue, analyses focused on two cortical segments; the marginal zone (MZ; the relatively acellular layer at the external aspect of the cortex) and the cell body dense cortical plate (CP). In the sections derived from postnatal animals, the CP was subdivided into the upper cortical plate (UCP; a cell body dense region subjacent to the MZ) and the lower cortical plate (LCP; the less densely packed region below the UCP).

The frequency of dead/dying cells was calculated as the total number of TUNEL-positive cells plus the total number of cells exhibited condensed chromatin divided by the total number of cells counted. Cell counts were generated separately for (1) TUNEL-positive cells (cells exhibiting any DAB staining), (2) cells with condensed nuclei but were DAB-negative, i.e., pyknotic cells, and (3) viable cells (cells that were TUNEL-negative and were not pyknotic). Cell density was determined using an unbiased stereological method (Gundersen and Jensen, 1987; Mooney and Miller, 2003). The number of cells in a counting frame (50 $\mu\text{m} \times 50 \mu\text{m}$) was counted. To ensure that the counts accurately represented a section, a minimum of 10 counting frames was used, each was randomly placed over the image within a compartment of the developing cortex. Using this procedure, a minimum of 100 cells was counted in each organotypic preparation.

The data were compiled and means ($\pm$ standard errors of the means) were calculated. A comparison among multiple groups was performed using one-way analysis of variance (ANOVA). Where the ANOVA showed a significant effect, *post-hoc* testing was done using the Student-Newman-Keuls test.

RESULTS

Fetal preparations

Two distinct cortical regions (the relatively cell body-sparse MZ and the cell body-dense CP) were discerned in cultured cortical vesicles from 16-day-old fetuses (Fig. 1). Treatment with NGF or ethanol had no apparent effect on this organization. Cell packing density in the MZ was significantly ($F_{3, 19} = 6.137$; $p < 0.05$) affected by treatment (Fig. 2). Density was similar in control and NGF-treated cultures, but, as shown previously (Mooney and Miller, 2003), exposure to ethanol significantly ($p < 0.05$) increased the cell packing density in the MZ. Concurrent administration of NGF and ethanol resulted in a density similar to that seen after treatment with ethanol alone. Thus, NGF did not offset the ethanol-induced change. In contrast, the cell packing density in the CP was unaffected by treatment with NGF and/or ethanol.

The frequency of dead/dying cells, i.e., TUNEL-positive or pyknotic cells, was determined separately for the MZ and the CP. Treatment with NGF and/or ethanol did not significantly alter this frequency in the MZ (Fig. 2). On the other hand, the frequency of dead/dying cells in the CP doubled following treatment with ethanol, in the presence or absence of NGF.

The pattern of expression of activated caspase 3 immunoreactivity paralleled TUNEL labeling (data not shown). That is, immunopositive cells were apparent in both cortical layers in all

treatment groups. The frequency of labeling was higher in the CP of slices treated with ethanol regardless of the presence or absence of NGF.

p75 immunolabeling was apparent throughout the depth of the cortical wall, i.e., in the MZ and the CP. Treatment did not affect the pattern of staining. TrkA was expressed in control tissue, as well as that treated with ethanol and/or NGF (Fig. 3). Trk receptors were evenly distributed through both regions. Treatment did not appear to alter the pattern of expression of either receptor. Phosphorylated trk was also expressed throughout the developing cortical plate, in a similar pattern as the non-phosphorylated isoform. Treatment failed to alter the pattern of expression of p-trk in the cortical wall.

A low amount of *Neg* was expressed in the MZ and CP of fetal cortices (Fig. 4). *Neg* expression increased following three days of treatment with NGF, i.e., relative to the amount of *Neg* expressed in controls. In contrast, *Neg* expression was low in ethanol-treated slices (like that detected in controls) and ethanol eliminated the NGF-induced increase in *Neg* expression. The implication was that exogenous NGF activated normal neural signal transduction pathways in the organotypic model.

Neonatal slices

Three distinct cortical regions were discriminable after culturing the slices from 3-day-old pups for three days: the relatively cell body-sparse MZ, cell body-dense UCP, and less dense LCP (Figs. 1). Treatment with neither NGF nor ethanol affected this organization. The cell packing density was not significantly altered by treatment with NGF or ethanol in any of the three compartments of the cortical wall (Fig. 5).

In each of the three segments of developing cortex in controls, about 1/3 of the cells were dead/dying (Fig. 5). Neither NGF nor ethanol affected this frequency in the MZ or the UCP. In the LCP, however, an ANOVA showed a significant effect of treatment ($F_{3, 19} = 10.352$; $p < 0.001$). Exposure to ethanol significantly ($p < 0.05$) increased the frequency of dead/dying cells in the LCP. Following concurrent administration of NGF, the frequency of cell death in the LCP was similar to that in the control. Thus, NGF blocked the toxic effects of ethanol.

Immunolabeling with an antibody to active caspase 3 showed a pattern of expression similar to that seen with TUNEL, i.e., cells in all three layers were immunopositive (Fig. 1). Their frequency in all three cortical segments also increased following exposure to ethanol.

NGF receptors, p75 and trkA, were present throughout the cortical plate (Fig. 6). p75 immunoexpression was apparent in the UCP and LCP, but was only rarely seen in the MZ, whereas trkA was expressed in all three cortical segments. There was no apparent effect of ethanol or NGF on expression of either receptor. Expression of p-trk was similar to that of trkA, i.e., it was apparent in all three compartments of the cortical anlage. Treatment did not appear to have an effect on its expression.

In situ hybridization showed *Neg* was present throughout the cortical wall in all four treatment groups (Fig. 6). Treatment did not alter its expression pattern.

DISCUSSION

Methodological considerations

Three methods were used in the present study to identify cells as dead/dying; TUNEL, pyknosis, and immunolabeling for activated caspase 3. Each method has advantages and limitations.

(1) TUNEL identifies cells with poly-adenylated tails found on fragmented DNA. DNA fragmentation is a feature of apoptotic cells that is associated with activation of endogenous nucleases (Wyllie, 1980). False-positive labeling may result from failure to optimize conditions (such as incorrect sample fixation or inappropriate concentrations of reagents) or may occur in tissue undergoing active RNA synthesis (Saraste, 1999).

(2) Pyknotic cells are cells with condensed or fragmented chromatin. This is a hallmark of apoptosis (Kerr et al., 1972). Unfortunately, such cells can be confused with glia that have small heterochromatic nuclei, e.g., oligodendrocytes and young astrocytes.

(3) Activated caspase 3 is an effector of cell death (e.g., Mooney and Henderson, 2006). Caspase 3 can also be activated during the cell cycle and cell differentiation (Oomann et al., 2004; 2005; 2006; Launay et al., 2005). As a death effector, activation of caspase 3 is up-stream of activation of nucleases. Thus, expression of activated caspase 3 can occur prior to, in concert with, or without TUNEL labeling.

Neither the extent of overlap between TUNEL, pyknosis, and immunolabeling for activated caspase 3 nor the time course of cell death are known in this model. In other models, depending on the structure and the insult, morphological changes indicative of cell death may be found between 6 and 24 hours after insult (e.g., McCarthy et al., 1997; Saraste and Pulkki, 2000).

Windows of vulnerability

Developing cortical neurons are susceptible to ethanol-induced death at both ages examined in the present study. Based on the incidence of markers for cell death, fetal tissue appears more vulnerable to ethanol than tissue taken from three-day-old pups. In fetal cultures, ethanol doubles the incidence of death throughout the cortical wall. Neither the NOND nor the ethanol-induced cell death in fetal cultures is mitigated by concurrent administration of NGF. This lack of protection from ethanol-induced death is similar to the non-response previously reported in primary cultures of cortical neurons taken from fetal tissue (Seabold et al., 1998; Miller et al., 2003; Barclay et al., 2005).

In contrast to the relatively uniform effect of ethanol on fetal cortical plate, tissue from neonates exhibits a region-specific response. That is, only neurons in the LCP are vulnerable to ethanol-induced death. This ethanol-induced death is completely blocked by concurrent administration of NGF. The neuroprotective effect of NGF is specific; it does not rescue cells in any of the three compartments from NOND, but it does specifically ameliorate the ethanol-induced death in the LCP. The implication of this finding is that the mechanism of the ethanol-induced death differs from that of NOND.

The neurotoxic effects of ethanol apparently target post-migratory, early differentiating neurons. The UCP, which contains neurons that are refractive to ethanol exposure, is comprised primarily of migrating neurons and neurons that have only recently begun their differentiation. In contrast, the LCP contains neurons that were born earlier and are further through their sequence of maturation. At least a subset of these LCP neurons is vulnerable to ethanol. Thus, there is a temporal component to ethanol-vulnerability which may correlate to a stage of differentiation.

Role of neurotrophins

The specificity of NGF-induced neuroprotection in neonates may also be related to the maturational state of the cells. The spatial specificity of the neuroprotection does not correlate with the expression of receptors. That is, both p75 and trkA are present throughout the LCP and the UCP. Although there is evidence of receptor expression in the developing cortical plate, activation of downstream pathways is unknown. It is possible that a downstream effector(s)

has a temporal sequence of expression, or there is a co-factor(s) that is expressed with zonal specificity.

The ability of NGF to ameliorate cell death does not correlate with trk receptor activation, as phosphorylated trk is present throughout the laminar extent of the developing cortex and NGF only rescues cells in the LCP from ethanol-induced cell death. It is possible that signaling through p75 is important for cortical survival. The role of p75 during development is time- and area-dependent. For example, p75 can mediate cell death in specific populations, including oligodendrocytes and sympathetic neurons (e.g., Casaccia-Bonnel et al., 1996; Bamji et al., 1998). In other models, e.g., cultured hippocampal neurons or PC12 cells, p75 mediates survival (Roux et al., 2001; Bui et al., 2002; Culmsee et al., 2002). In a similar vein, primary cortical neurons exposed to ethanol show a downregulation of p75 expression that is coincident with an increased incidence of cell death (Seabold et al., 1998). In the current model, p75 apparently mediates survival as the addition of an anti-p75 antibody to the culture medium, which presumably inhibits receptor activation, results in disintegration of the slice preparation (data not shown). Likely, this is the result of widespread cell death.

The expression of *Neg*, a gene proposed to promote survival in primary cortical neurons (Miller et al., 2003), is up-regulated in fetal cultures by the addition of NGF to the medium. This occurs in the absence of any survival-promoting effect of NGF in these organotypic cultures. In contrast, in cultures from neonates, *Neg* is constitutively expressed and its expression does not appear to be altered by the addition of NGF. Thus, *Neg* expression is NGF-responsive in fetal tissue, but it does not correlate with NGF-mediated protection or survival in organotypic cultures of fetal or neonatal forebrain.

In the present study, exposure to ethanol doubled the incidence of cell death in the fetal cortical plate. Despite evidence that the concurrently administered NGF is biologically available, it is unable to protect against the ethanol-induced death. In contrast, in the neonatal tissue, only the LCP is affected by ethanol. This death is ameliorated by concurrent administration of NGF. Thus, ethanol and NGF specifically target post-migratory, differentiating neurons in the neonatal tissue. That NGF can protect against ethanol-induced cell death in the neonatal LCP but not NOND in fetal or neonatal tissue implies that the mechanism(s) by which ethanol induces cell death is different from the mechanism(s) of NOND in this model.

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ABBREVIATIONS

ANOVA, one way analysis of variance; CNS, central nervous system; CP, cortical plate; DAB, 3,3'-diaminobenzidine; FBS, fetal bovine serum; G, gestational day; LCP, lower cortical plate; mRNA, messenger ribonucleic acid; MZ, marginal zone; NGF, nerve growth factor; NOND, naturally occurring neuronal death; P, postnatal day; PB, 100mM phosphate buffer; PBS, phosphate buffered saline; p-trk, phosphorylated tyrosine receptor kinase; TPBS, phosphate buffered saline containing tween; trk, tyrosine receptor kinase; TUNEL, terminal uridylated nick-end labeling; UCP, upper cortical plate; VAMC, Veteran's Affairs Medical Center.

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Figure 1. Appearance of fetal cerebral cortex in slice cultures

Slices were obtained from fetal rats on gestational day (G) 16 were labeled for fragmented DNA (using the TUNEL method). The marginal zone (MZ) and cortical plate (CP) were apparent in samples from all four treatment groups: those treated with nerve growth factor (NGF; 0 or 30 ng/ml) and ethanol (Et; 0 or 400 mg/dl) (upper panel). In the slices taken from animals on postnatal day (P) 3 (middle and lower panels), the CP can be subdivided into upper (UCP) and lower (LCP) compartments on the basis of cell packing density. Tissue was labeled for fragmented DNA (using the TUNEL method, middle panel) or activated caspase-3 (lower panel). Solid arrows indicate TUNEL- or active caspase-3-positive cells. Open arrows

designate cells with condensed nuclear chromatin. IZ, intermediate zone. Scale bars are 25 μm .

Figure 2. Quantification of cortical plate parameters at G16

The top three panels show the effects of nerve growth factor (NGF) and ethanol on the density of cells that exhibit terminal uridylated nick-end labeling (TUNEL) and nuclear condensations. The cell packing densities in the marginal zone (left) and cortical plate (right) of the fetal slices are shown (*bottom*).

Marginal Zone. The labeling index shows the proportion of dead/dying cells, i.e., TUNEL-positive cells and pyknotic cells. The labeling index in the MZ was unaffected by exposure to ethanol or by administration of NGF. **Bottom.** The cell packing density in the MZ of the fetal cultures was significantly higher in cultures treated with ethanol compared to untreated

cultures. NGF alone did not significantly affect cell density, and it also failed to ameliorate the ethanol-induced increase in cell packing density.

Cortical Plate. *Top*. Ethanol induced an increase in the frequency of TUNEL-positive cells, however, the total amount of cell death induced by exposure to ethanol failed to reach statistical significance. *Bottom*. Cell packing density in the CP was unaffected by exposure to ethanol or by administration of NGF.

Each value represents the mean of five cultures. T-bars are standard errors of the means. Each * denotes a significant ($p < 0.05$) difference relative to the untreated control. A # signifies a significant difference relative to the NGF-treated cultures.

Figure 3. Receptor expression at G16 in control-treated cultures

Cells immunopositive for the neurotrophin receptors, trkA, p75, and phosphorylated (p) trk, were identifiable in all three compartments of the cerebral wall; the marginal zone (MZ), cortical plate (CP) and the intermediate zone (IZ). Scale bars are 25 μm.

Figure 4. *Neg* expression at G16

In situ hybridization for *Neg* revealed no labeling in control-, ethanol-, or ethanol + NGF-treated tissue. In contrast, treatment with NGF alone resulted in up-regulation of *neg* expression. Scale bars are 25 μ m.

Figure 5. Quantification of cortical plate parameters at P3

Marginal zone. *Top.* The frequency of dead/dying cells was not significantly affected by treatment with ethanol or NGF. *Bottom.* The cell packing density in the MZ was not different among control cultures or those treated with NGF, ethanol, or ethanol and NGF.

Upper cortical plate. *Top.* The expression of indicators of cell death (TUNEL and pyknotic cells) in the UCP was not significantly affected by exposure to ethanol or treatment with NGF. *Bottom.* The density of cells in the lower cortical plate was unaffected by treatment.

Lower cortical plate. *Top.* The frequencies of TUNEL-positive cells and pyknotic cells were increased in the LCP following exposure to ethanol. Concurrent exposure to NGF ameliorated this effect. *Bottom.* Treatment did not alter the density of cells in the LCP.

Each value represents the mean of five cultures and T-bars are standard errors of the means. An *, #, and ^ denote significant ($p < 0.05$) differences relative to the untreated controls, to the NGF-treated cultures, and to the cultures treated with ethanol and NGF, respectively.

Figure 6. Expression of neurotrophin receptors and *neg* at P3

Immunohistochemical labeling of the neurotrophin receptors showed that p75-positive cells were only localized in the cortical plate, not in the marginal zone. In contrast, trkA- and phosphorylated trk-positive cells were apparent throughout the cortical wall. *In situ* hybridization for *Neg* labeled cells in all three segments of the cortical wall. Treatment with NGF did not alter *Neg* expression. Nomenclature as for Figure 5. Scale bars are 50 μ m.