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Role of Neurotrophins on Postnatal Neurogenesis in the Thalamus: Prenatal Exposure to Ethanol

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Abstract

A second wave of neuronal generation occurs in the ventrobasal nucleus of the rat thalamus (VB) during the first three postnatal weeks. The present study tested the hypotheses (1) that postnatal neurogenesis in the VB is neurotrophin-regulated and (2) that ethanol-induced changes in this proliferation are mediated by neurotrophins. The first studies examined the effects of neurotrophins on the numbers of cycling cells in *ex vivo* preparations of the VB from three-day-old rats. The proportion of cycling (Ki-67-positive) VB cells was higher in cultured thalamic slices treated with neurotrophins than in controls. Interestingly, this increase occurred with nerve growth factor (NGF) alone or with a combination of NGF and brain-derived neurotrophic factor (BDNF), but not with BDNF alone. Based on these data, the VBs from young offspring of pregnant rats fed an ethanol-containing or an isocaloric non-alcoholic liquid diet were examined between postnatal day (P) 1 and P31. Studies used enzyme-linked immunosorbent assays and immunoblots to explore the effects of ethanol on the expression of neurotrophins, their receptors, and representative signaling proteins. Ethanol altered the expression of neurotrophins and receptors throughout the first postnatal month. Expression of NGF increased, but there was no change in the expression of BDNF. The high affinity receptors (TrkA and TrkB) were unchanged but ethanol decreased expression of the low affinity receptor, p75. One downstream signaling protein, extracellular signal-regulated kinase (ERK), decreased but Akt expression was unchanged. Thus, postnatal cell proliferation in the VB of young rat pups is neurotrophin-responsive and is affected by ethanol.

Keywords

brain-derived neurotrophic factor; development; fetal alcohol syndrome; nerve growth factor; proliferation; stem cells

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Introduction

A pathognomonic feature of fetal alcohol syndrome is intrauterine growth retardation. This is typified by microencephaly. In this regard, one sentinel target of gestational ethanol exposure is the trigeminal-somatosensory system. The trigeminal-somatosensory system is represented at three levels of the central neuraxis: the brainstem, thalamus, and cortex. The numbers of neurons in the mature brainstem and cortex are permanently reduced by prenatal exposure to ethanol (e.g., Miller and Muller, 1989; Miller and Potempa, 1990; Miller, 1995; 1999; Mooney and Miller 2007c). This is mirrored by reductions in the cortical representations in the somatosensory map (e.g., Margret et al., 2005; Powrozek and Zhou, 2005; Chappell et al., 2007). In contrast, prenatal ethanol exposure does not alter the number of neurons in the somatosensory thalamus, i.e., the ventrobasal nucleus (VB; Mooney and Miller, 1999; 2010). There is also a lack of an effect of early exposure to ethanol on the motor thalamus in the ventrolateral nucleus (Livy et al., 2001). The changes in the somatosensory system lead to disruptions in the matching of neuronal numbers among the components of the system that apparently underlie functional deficits (Miller and Dow-Edwards, 1993; Xie et al., 2010).

The VB is distinctive within the developing CNS; it is one of the few structures that experiences postnatal neuronogenesis (Mooney and Miller, 2007b). It is unique in that this occurs *in situ* and not within a distinct generative population. This postnatal neuronogenesis is altered by prenatal exposure to ethanol; the cell cycle is shorter thereby increasing the numbers of cells generated (Mooney and Miller, 2010).

Key regulators of system matching are growth factors, specifically neurotrophins (e.g., Bibel and Barde, 2000; Reichardt, 2006). Two neurotrophins, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are important for neuronal development and survival. Under- or over-expression of these proteins can directly affect the number of viable neurons. For example, in mice that over-express NGF, twice the normal numbers of trigeminal ganglion cells survive (Jhaveri et al., 1996). BDNF overexpression alters mouse behavior and the electrophysiological responses of cells (Croll et al., 1999; Cunha et al., 2009). In contrast, mice lacking BDNF have deficits in neuronal number in the trigeminal ganglia and reductions in cholinergic forebrain neurons (Ernfors et al., 1994; Ward and Hagg, 2000).

Neurotrophins are important for the postnatal production and survival of neurons (Balu and Lucki, 2009). This effect may be site-specific. NGF is mitogenic for cells in the external granule layer of the cerebellum (Charrasse et al., 1992; Muller et al., 1994), and although it plays a role in the survival of newly generated hippocampal neurons, it does not appear to have a direct effect on cell proliferation (Frielingsdorf et al., 2007). In contrast, the availability of BDNF defines progenitor cell generation; increasing the amount of BDNF increases cell proliferation (Pencea et al., 2001; Scharfman et al., 2005), whereas reductions in the amount of BDNF decreases hippocampal neurogenesis (Lee et al., 2002). This action of BDNF is mediated through p75, the low affinity neurotrophin receptor. Mice that do not express p75 have fewer hippocampal stem cells, a smaller granule cell layer, and behavioral alterations (Young et al., 2007; Catts et al., 2008). BDNF also appear to be mitogenic for cells in the developing rat cerebellum (Koibuchi et al., 1999).

Developmental exposure to ethanol alters the expression of neurotrophins and their receptors (e.g., Seabold et al., 1998; Heaton et al., 1999; 2000; Light et al., 2001; Climent et al., 2002; Heaton et al., 2003a; 2003b; Miller et al., 2003). NGF expression in cortex and striatum is increased by either prenatal or postnatal exposure to ethanol (Heaton et al., 2000; 2003a; 2003b). Prenatal exposure to ethanol reduces cortical BDNF expression during the first two

postnatal weeks (Climent et al., 2002), whereas early postnatal exposure to ethanol increases cortical BDNF content (Heaton et al., 2003b). In addition to altering expression of neurotrophin ligands and receptors, exposure to ethanol alters the expression of downstream signaling proteins. Ethanol inhibits endogenous extracellular signal-regulated kinase (ERK) expression (Kalluri and Ticku, 2002) and the phosphatidylinositol-3-kinase (PI3K) pathway (de la Monte and Wands, 2002; Li et al., 2004).

The present study addresses the role of neurotrophins in the postnatal cell proliferation that occurs in the VB. The cell proliferation and neuronal survival that underlie the *in situ* postnatal neurogenesis are affected by prenatal exposure to ethanol (Mooney and Miller, 2010). We tested the hypotheses that the postnatal generation of VB neurons is regulated by neurotrophins and that these changes are responsible for the effects of prenatal exposure to ethanol on the postnatal proliferation in the VB.

Methods

Subjects

Animals—Pregnant Long-Evans rats from Harlan (Somerville, NJ) were obtained on gestational day (G) 4. G1 was defined as the first day a sperm-positive plug was found. Animals were maintained in a temperature and humidity controlled facility that was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All procedures were approved by the Committee for the Humane Use of Animals at Upstate Medical University and the Institutional Animal Care and Use Committee at the Syracuse Veterans Affairs Medical Center and were in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Rats were segregated into weight-matched pairs on G5. One rat from each pair was assigned to a control group and the other was assigned to the ethanol-treated group. Rats in the ethanol-treated group were fed a liquid diet (Research Diets, New Brunswick NJ) containing 6.7% v/v ethanol (Et) *ad libitum* from G11 through G21. Animals were weaned onto the diet between G6 and G10 (e.g., Mooney and Miller, 1999; 2001; 2010). On G6 and G7 animals received a diet containing 2.2% (v/v) ethanol, and on G8–G10 the diet contained 4.5% (v/v) ethanol. Control animals were pair-fed an isocaloric, isonutritive liquid diet with no ethanol (Ct). Fresh diet was presented each day at the end of the light cycle in order to synchronize the feeding patterns for all animals (Miller, 1992). This paradigm typically results in mean peak blood ethanol concentrations of ~150 mg/dl in Et-fed dams 2–4 hr into the dark cycle (e.g., Miller, 1987; 1992; Vavrousek-Jakuba et al., 1991; Youngentob et al., 2007). It should be noted that blood ethanol concentrations (BECs) were not determined in the present study because such measures have been performed in various other studies using the same paradigm (e.g., Miller, 1987; Vavrousek-Jakuba et al., 1991; Youngentob et al., 2007) and to minimize the use of animals as mandated by our CHUA and IACUC. The mean consumption of diet by the Et-fed rats was 279 ± 31 ml/kg/day between G12 and G21 in the present study and 267 ± 16 ml/kg/day in previous studies (see Miller, 1987; 1988; 1992).

Within 24 hr of birth, litters were culled to ten pups. All experimental animals were surrogate-fostered by rats fed chow and water *ad libitum* during the pregnancy. Brains were collected from pups throughout the first postnatal month (on postnatal day (P) 0 (within 24 hr of birth), P3, P6, P12, P21, and P31). Only one pup per litter per treatment group was used for any given experiment at any given age. For anatomical assays, animals were deeply anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine) prior to undergoing transcardial perfusion with 4.0% paraformaldehyde in 0.10 M phosphate buffer (PB). Brains were removed, post-fixed in buffered paraformaldehyde for 4 hr at room temperature, and stored in 30% sucrose in PB at 4°C. For biochemical assays, animals were anesthetized as above

and decapitated. Brains were removed, frozen rapidly on dry ice, and stored at -80°C until use.

Organotypic slice cultures—Three-day-old Long-Evans rat pups from dams fed chow and water *ad libitum* were used for generating organotypic slice cultures (Mooney and Miller, 2003; 2007a). Animals were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine) and decapitated. Their brains were removed and rinsed in Krebs's buffer (126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄·H₂O, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 11 mM glucose, 25 mM NaHCO₃, 10 mM HEPES). Coronal slices, 400 µm thick, throughout the forebrain were made using a McIlwain Tissue Chopper (Mickle Lab Engineering, Gomshall UK) and collected into fresh Krebs's buffer.

In slices containing the VB, the cortex was removed and the remaining diencephalic tissue was placed on Millipore culture inserts (PICMORG; Millipore, Bedford MA). Tissue was cultured (37°C, 6% CO₂) in Minimal Essential Medium (MEM; GibcoBRL, Grand Island NY) supplemented with 25 mM HEPES, 100 mM dextrose, 25 mM KCl, 100 µM penicillin/streptomycin (Gibco), and 12.5 µM fungizone (Gibco)). After a 2 hr acclimatization period, slices were treated with BDNF (0, 10, 50, or 100 ng/ml; 203702 EMD Biosciences, La Jolla CA) for a short (2 hr) or long (24 hr) time. Other slices were treated with NGF (0, 20, 40, or 80 ng/ml; N1408; St Louis MO) or with BDNF (50 ng/ml) and NGF (0, 20, 40, or 80 ng/ml) for 4 hr. Three or four slices were isolated for each treatment group. Each slice in any given group was from a different litter. After treatment, slices were fixed in 4.0% paraformaldehyde at room temperature for 30 min, then stored in 15% sucrose in PB at 4°C.

Anatomical assays

Immunohistochemistry: Sections (12 µm thick) from slices were used for Ki-67 immunolabeling, and 12 µm thick coronal cryosections from brains of six-day-old animals were used for immunohistochemical localization of NGF and BDNF.

Non-specific binding of the antibody was blocked by incubating sections in PB supplemented with 0.10% Triton X100 (TPB), 4.0% goat serum, and 1.0% bovine serum albumin. Primary antibodies (Table I) in TPB were applied to the sections for 2 hr. After a PB rinse, tissue was incubated in the relevant biotinylated secondary antibody (1:200; Vector, Burlingame CA) for 1 hr. Slides were rinsed in PB, incubated in streptavidin-horseradish peroxidase (Vectastain Elite kit; Vector), washed in PB, and exposed to the chromagen 3,3'-diaminobenzidine (DAB; Vector) or alkaline phosphatase (analysis of TrkB only). Sections immunolabeled for NGF, BDNF, or Ki-67 were lightly counterstained with cresyl violet, and those immunolabeled for phosphorylated Trk (pTrk) or TrkA were counterstained with methyl green. Additional sections were processed without either the primary or secondary antibody. In each case, no labeling was detected.

All sections were dehydrated through solutions with increasing concentrations of alcohol and cleared in xylene before being coverslipped.

Cell counting: All cell counts were performed within the VB. The VB can be readily identified using cytoarchitectonic criteria (Paxinos and Watson, 1982). It has a lateral border with the acellular thalamic radiation and a ventral border with the acellular medial lemniscus. Medially, it is bordered by the posterior thalamus which is composed of smaller cells.

For slice preparations, a series of every 5th section was labeled for Ki-67 and analyzed. All sections were used (n = 4-5). Two counting frames (80 µm × 80 µm) were applied to each

section and all cells within the frame (excluding endothelial cells) were counted and designated labeled or unlabeled. A minimum of 100 cells was counted for each slice.

For whole brains of six-day-old animals, a series of every 5th section was immunolabeled for NGF and a second series for BDNF. All sections (n = 5-7 per brain) were used in the tallies. One counting frame (80 μ m $\times$ 80 μ m) was applied to each section and all cells within the frame (excluding endothelial cells) were counted and designated as labeled or unlabeled. Between 100 and 200 cells were counted for each brain.

A labeling index (LI) for NGF, BDNF, or Ki-67 immunolabeling was determined. In each case, the LI was calculated by multiplying the quotient of the number of labeled cells and the sum of the numbers of labeled and unlabeled cells by 100%. Immunopositive cells were identified as being brown (for DAB) and blue/purple (for alkaline phosphatase) (solid arrows in Fig. 2). Unlabeled cells appeared purple (labeled with cresyl violet) or green (labeled with methyl green) (open arrowheads in Fig. 2).

Biochemical assays: For neonates (on P0 and P3), the dorsal thalamus was isolated by removing the cerebral cortex, then making coronal cuts anterior to the colliculi and posterior to the caudoputamen. At older ages, VB tissue was collected. Brains were cut into 1 mm thick slabs, then a Micron Biopsy Punch (Harvard Apparatus, Holliston MA) was used to take a plug of tissue (2.0 mm in diameter) from the VB. The VB could be distinguished based on its characteristic hue in the fresh tissue; it was lighter than surrounding tissue. Four or five punches were taken from each hemisphere. Tissue from the left hemisphere was used for enzyme-linked immunosorbent assay (ELISA) and tissue from the right hemisphere was used for immunoblotting. Dissected tissue was stored at -80°C until it was used.

Enzyme-linked immunosorbent assays (ELISAs): ELISAs were used to determine the expression of NGF and BDNF (Miller and Mooney, 2004) in the thalamus. Tissue was weighed, homogenized in ELISA lysis buffer (150 mM NaCl, 20 mM Tris-HCl, 1.0% NP-40, and 10% glycerol) with Complete protease inhibitors (Roche, Indianapolis IN), and centrifuged. Supernatant was removed and the pellet fraction was resuspended in lysis buffer. After acid-treatment, protein content was determined using a Bradford assay kit (BioRad, Hercules CA). Samples were processed using an ELISA kit (G7631 for NGF and G7611 for BDNF; Promega, Madison WI). In addition, standard curves were generated using known amounts of NGF or BDNF.

Neurotrophin expression in the supernatant and pellet was calculated separately by interpolation using the standard curve. Total neurotrophin content was determined as the sum of the amount in the supernatant and pellet fractions. All samples were run in triplicate and the mean values ($\pm$ standard errors of the means) were reported.

Immunoblots: Quantitative analysis of immunoblots was used to determine the expression of high (TrkA and TrkB) and low (p75) affinity neurotrophin receptors, phosphorylated Trk (pTrk), extracellular signal-regulated kinase 1/2 (ERK 1/2), phosphorylated ERK (pERK), Akt, and pAkt (Mooney and Miller, 2000; 2001). Tissue was homogenized in immunoblot lysis buffer (20 mM Tris buffer containing 0.10% sodium dodecylsulphate, 1.0% Nonidet P-40, 0.50% sodium deoxycholate, 1.0 mM EDTA, 12.5 mM sodium fluoride, 2.0 mM sodium orthovanadate, and one Complete protease inhibitor tablet per 10 ml (Roche, Indianapolis IN)). After centrifugation, the supernatant was collected, and protein content was determined (Bradford assay kit; BioRad, Hercules CA). Samples of equal protein content (40 μ g) were loaded into lanes of a 7.5% polyacrylamide gel, separated by electrophoresis (160 mA, 2.5 hr), and transferred to a nitrocellulose blot (30 V, overnight at 4°C). In addition, molecular weight markers (Kaleidoscope prestained standards, BioRad)

and triplicate internal standards were loaded onto the gel. Internal standards, pooled homogenates from the whole brains of 12-day-old rat pups, were used to normalize immunosignals among blots (Mooney and Miller 2000; 2001).

Blots were immunolabeled using a standard procedure (Mooney and Miller 2000; 2001). After blocking non-specific immunolabeling by washing in 5.0% non-fat milk mixed in a solution of 0.10% Tween-20 in 0.010 M phosphate buffered saline (TwPBS), blots were incubated for one hour primary antibody (Table I).

Excess antibody was removed by washing the blots with TwPBS and then incubating them in a solution of horseradish peroxidase-linked anti-mouse IgG (1:1000, NA-931, Amersham, Arlington Heights IL) or anti-rabbit IgG (1:1000, NA-934, Amersham) for 30 min. The immunolabeled proteins were detected using an enhanced chemiluminescence system (Amersham) and imaged on Kodak X-OMAT film (Kodak, Rochester NY). All blots were stripped and labeled for actin or tubulin to assess loading; expression of actin or β -tubulin was unaffected by ethanol. Densitometric analyses were performed on the X-ray films using a Kodak ImageStation 4400.

Statistical analyses: Quantitative data were analyzed using one- or two-way analysis of variance (ANOVA) factoring in age and treatment. Where a significant difference was detected ($p < 0.05$), *post-hoc* Tukey B tests were performed. All tests were performed using SigmaStat software (Systat, San Jose CA).

Results

Ex vivo studies of the effects of neurotrophins on the frequency of proliferating cells

To examine the role of neurotrophins on postnatal neurogenesis in the VB, slice cultures were treated with different concentrations and combinations of neurotrophins. Altering BDNF concentration did not affect the proportion of Ki-67-positive (cycling) cells in the VB (Fig. 1 left) either in the short- or long-term.

Following short-term exposure to NGF, there was a significant ($F_{6,15} = 13.082$, $p < 0.05$) increase in the proportion of cycling cells (Fig. 1 right). This effect was concentration-dependent and occurred in the absence of BDNF. The proportion of cycling cells was significantly ($p < 0.05$) greater in slices treated with a high concentration of NGF (80 ng/ml medium) than in control or slices treated with 20 or 40 ng/ml NGF.

The effects of the neurotrophins were synergistic. That is, slices treated with 40 ng/ml NGF in the presence of 50 ng/ml BDNF had significantly ($p < 0.05$) more cycling cells than did slices treated with the same amount of NGF but in the absence of BDNF.

In vivo effects of ethanol on neurotrophin systems

Anatomical studies—NGF and BDNF were expressed by cells in the developing thalamus (Fig. 2B-E). Immunohistochemical labeling of six-day-old rat brains showed that the mean proportion of VB cells positive for NGF ($\pm$ standard error of the mean) and BDNF was above 80% (Fig. 3). Prenatal exposure to ethanol did not have a significant effect on the frequency of labeling for either neurotrophin.

Immunochemical studies—ELISAs were used to determine the effects of ethanol on NGF and BDNF content in the developing thalamus. These data corroborated and extended the anatomical studies by showing that both neurotrophins were expressed in the VB at all ages examined.

NGF: Two-way ANOVA showed that NGF expression varied significantly with age ($F_{5,35} = 5.957$; $p=0.01$) and treatment ($F_{1,35} = 31.145$; $p<0.01$; Fig. 4A). There was no interaction between these factors ($F_{5,35} = 0.641$; $p=0.670$). Based on one-way ANOVAs, NGF expression in Ct-treated animals was significantly ($F_{5,17} = 4.375$; $p<0.05$) higher on P0 and P3 compared with that on P31.

The temporal pattern of NGF expression varied in rats prenatally exposed to ethanol. NGF expression was stable between P0 and P6, rose significantly ($p<0.05$) between P6 and P12, then declined significantly ($p<0.05$) by P31. Based on post-hoc tests, the expression of NGF in Et-treated animals was higher than in Ct-treated animals at all ages except three-day-olds.

The results of the anatomical and biochemical studies paradoxically differed. Though the immunohistochemical data showed no change in the proportion of NGF-expressing cells, data from the ELISAs revealed an ethanol-induced increase in NGF expression. These data were justified by taking the two sets of data together. Accordingly, it appeared that the amount of NGF expressed per cell increased following prenatal ethanol exposure.

BDNF: Total BDNF content in the VB varied over the postnatal period from P0 to P31 (Fig. 4B). A two-way ANOVA showed a significant effect of age ($F_{5,35} = 6.707$; $p<0.01$), but not of treatment ($F_{1,35} = 1.893$; $p=0.182$). There were two ways to analyze the data: by collapsing the data across treatment group and by separating the data for each treatment group. Separating the data for the treatment groups allowed analysis of age-dependent patterns within that treatment and, hence, identified differences that otherwise would be overlooked.

When the data were collapsed across treatment, BDNF expression was significantly higher at P0 than P21 or P31, and at P6 vs. P21 or P31 ($p<0.01$ for all comparisons). When the data were not collapsed but analyzed within a treatment group using exploratory one-way ANOVAs, the pattern of change in BDNF expression was shown to be different between Ct- and Et-treated rats. That is, in Ct-treated animals, BDNF expression was significantly higher at P0 than P21 and at P6 compared with P21 or P30 ($p<0.05$ for all comparisons). In contrast, there were no significant differences in BDNF expression in the Et-treated rats.

Expression of neurotrophin receptors: All three neurotrophin receptors examined were expressed in the VB (Fig. 2F - H). The immunoblot data show that expression of all receptors examined (TrkA, TrkB, pTrk, and p75,) varied as a function of age (Fig. 5). Two main patterns were identified: one in which receptor expression was initially low, increased with age, and then decreased again. This pattern was apparent for TrkA and full-length TrkB (Fig. 5A and 5B). The second pattern was one in which receptor expression was high in the neonates and then waned over time (seen for truncated TrkB, pTrk, and p75; Fig. 5C – 5E).

Two bands were recognized by the antibody to TrkB, one at 95 kDa and a lower one at ~80 kDa (Fig. 5C). The upper band was analyzed. Truncated TrkB expression was significantly different by age ($F_{5,35} = 24.111$, $p < 0.001$), it was higher in younger animals, i.e., during the first two postnatal weeks, than in older animals. It also showed a treatment by age interaction ($F_{5,35} = 2.927$, $p = 0.033$) on P0.

pTrk expression differed by age ($F_{5,35} = 25.144$, $p < 0.001$) and was significantly ($p<0.05$) higher during the first two postnatal weeks than in 21- or 31-day-old animals. Additionally, there was a trend towards an ethanol-induced decrease in pTrk expression ($F_{1,35} = 4.096$, $p = 0.054$).

Expression of p75 was significantly affected by age ($F_{5,35} = 11.857$, $p < 0.001$), it was higher in animals during the first two postnatal weeks than in 21- or 31-day-old animals. It

was also significantly altered by treatment ($F_{1,35} = 4.761$, $p = 0.039$), it was lower in animals exposed to ethanol.

Expression of neurotrophin signaling proteins: ERK had two isoforms (Shaul and Seger, 2007): ERK1 was a 42 kDa protein, ERK2 was a 44 kDa protein (Fig. 6A and 6B). Expression of both isoforms remained constant over the first postnatal month, and was significantly reduced following prenatal exposure to ethanol (for ERK1 $F_{1,35} = 9.732$, $p < 0.01$; for ERK2 $F_{1,35} = 4.311$, $p < 0.01$). ERK1 expression was significantly ($p < 0.05$) less in Et-treated rats on P0 and P3 than in controls. ERK2 expression was significantly ($p < 0.05$) lower in Et-treated than in Ct-treated animals on the day of birth, but by P3 the ethanol-induced difference had disappeared.

Expression of pERK1 was significantly altered by age ($F_{5,35} = 8.441$, $p < 0.01$; Fig. 6). It increased over the first postnatal month and was significantly higher at P21 and P31 than during the first postnatal week ($p < 0.05$). Prenatal exposure to ethanol significantly reduced pERK1 expression ($F_{1,35} = 4.561$, $p < 0.05$). pERK2 expression did not vary between P0 and P31. Despite the ethanol-induced significant ($F_{1,35} = 12.167$, $p < 0.01$) reduction in overall expression of pERK2, the *post-hoc* test did not identify differences at specific ages.

The ratio of pERK1 expression to total ERK1 expression was significantly ($F_{5,35} = 7.683$, $p < 0.01$) altered by age but not by treatment (Fig. 5G and 5H). This ratio was lower in the neonates (P0-P6) compared with older animals (P21 and P31). In contrast, the ratio of pERK2 expression to total ERK2 expression was not affected by age, but was significantly affected by treatment ($F_{1,35} = 7.399$, $p < 0.05$). Et-animals had a significantly ($p < 0.05$) lower pERK2/ERK2 ratio than did controls on P31.

The expression of Akt and pAkt was significantly ($F_{5,35} = 23.991$; $p < 0.05$ and $F_{5,35} = 26.871$; $p < 0.05$, respectively) altered with age (Fig. 7A and 7B). In controls, specific age-dependent changes were detected; Akt expression was significantly ($p < 0.05$) greater in animals younger than 12-days-old compared with 21- and 31-day-old rats. Likewise, pAkt expression was higher in younger animals. Prenatal exposure to ethanol did not significantly alter Akt or pAkt expression. The ratio of pAkt expression to total Akt expression was neither significantly affected by age nor treatment (Fig. 7).

Discussion

Role of neurotrophins

Data from the studies of organotypic slice cultures support the hypothesis that neurotrophins regulate cycling activity in the postnatal VB. NGF increases the proportion of cycling VB cells in a concentration-dependent manner. Although BDNF alone is insufficient to promote cell proliferation, combined BDNF and NGF treatment increases the numbers of proliferating cells. The intriguing finding is that in the presence of BDNF (50 ng/ml), a lower concentration of NGF (40 ng/ml medium) is as effective in maintaining the proliferating population as is treatment with a higher concentration of NGF (80 ng/ml medium) alone. Thus, a cocktail of neurotrophins is also effective at inducing cell proliferation.

The proportion of cycling cells in the cultures treated with the highest concentrations of neurotrophins is equivalent to the proportion of cycling cells seen in the VB *in vivo* (Mooney and Miller, 2010). Thus, it appears that under these conditions the neurotrophins maintain the normal population of cycling cells, but that lower concentrations of neurotrophins are unable to maintain the cycling cells and they exit the cell cycle, become quiescent, or die.

The postnatal generation of neurons in the VB *in vivo* is also affected by neurotrophins. Both NGF and BDNF are highly expressed in the VB during the period of postnatal neurogenesis (including P3 to P21) and most cells in the developing VB express neurotrophins and their receptors. The identity of the neurotrophin-positive cells was not determined in this study, however given the high percentage of positive cells in the VB, it is likely that they are expressed by both neuronal and non-neuronal cells. Interestingly, the postnatal period of neuronal generation in the VB coincides with upregulation of neurotrophin receptors. That is, both Trk (data not shown) and p75 (Crockett et al., 2000; Mooney and Miller, 2007b) are highly expressed in the neonate and are downregulated by the middle of the first postnatal month. Furthermore, the decrease in Ki-67 immunolabeling during the third postnatal week (Mooney and Miller, 2010) parallels the losses of p75 and Trk expression.

Based on the timing of expression in young control rats, it appears that both NGF and BDNF play a role in the postnatal neurogenesis. (1) NGF is stably expressed through most of the period of neuronal production; it declines by the end of the first postnatal month. (2) The increase in BDNF expression in Ct-treated animals aligns with the onset and increase in postnatal neurogenesis.

Prenatal exposure to ethanol alters the temporal change in BDNF expression. That is, in Ct-treated animals BDNF expression is significantly higher on P6 than on P21 or P31, whereas in Et-treated animals BDNF expression does not change during the first postnatal month. Given that prenatal exposure to ethanol neither diminishes the proportion of cycling cells in the VB nor negatively affects cell cycle kinetics (indeed, it shortens the length of the cell cycle; Mooney and Miller, 2010), it is unlikely that the surge in BDNF expression evident in Ct-treated rats between P0 and P6 is required for the postnatal neurogenesis in the VB.

In contrast to BDNF, prenatal ethanol exposure increases NGF expression in the VB during the period of postnatal neurogenesis. Thus, based on the coincidence of an ethanol-induced increase in NGF expression and a shortening of the cell cycle in Et-treated animals, it is possible that NGF plays a role in controlling the cell cycle in the VB.

Signal transduction

Binding of a neurotrophin to a Trk receptor triggers receptor dimerization, the phosphorylation of receptor tyrosines, and the activation of signaling cascades. Two of the pathways initiated by receptor activation rely on (1) ERK1/2 or (2) PI3K and Akt (e.g., Datta et al., 1997; Bibel and Barde, 2000; Huang and Reichardt, 2001; 2003; Barnabé-Heider and Miller, 2003; Segal, 2003; Hetman and Gozdz, 2004). Both pathways promote cell proliferation and survival (Bonni et al., 1999; Luo and Miller, 1999a; 1999b; Sweatt, 2004; Samuels et al., 2008). NGF promotes cell proliferation *in vitro*, apparently by activating ERK 1/2 (Chambard et al., 2007; Wang et al., 2009), however, these effects are cell-type dependent. For example, NGF promotes proliferation of neural stem cells (Wang et al., 2009), but decreases proliferation of neuroblastoma and PC12 cells (Woo et al., 2004; Marampon et al., 2008). Effects on cell cycle require nuclear translocation of ERK (Brunet et al., 1999) and may be mediated by CREB (e.g., Lonze and Ginty, 2002).

Ethanol disrupts the neurotrophin system in the thalamus. Specifically, there is a significant increase in NGF expression in the VB of Et-treated animals during the first postnatal month, with no change in the expression of TrkA, and a trend towards a decrease in pTrk expression. This suggests that the ability of NGF to bind to and/or activate TrkA is hindered by ethanol; a higher concentration of NGF in Et-treated animals is necessary to achieve the same biological effect attained with treatment at a lower concentration in Ct-treated animals. Explanations for this phenomenon include that there may be an alteration in binding affinity,

effective availability of NGF, or the ability of the receptor to autophosphorylate. Differential effects within signaling pathways have been described. For example, ethanol reduces growth factor-stimulated PI3K activation in cerebellar neurons with no concomitant change in receptor or ligand expression (de la Monte and Wands, 2002).

Ethanol appears to affect the signaling pathways downstream of neurotrophin receptor activation. There is a small, but significant, decrease in the expression and activation of ERK1/2. This occurs in the face of the increased NGF expression and no change in BDNF expression. Such discordant effects are similar to the response to acute exposure to ethanol *in vivo* in cortex and hippocampus (Davis et al., 1999; Kalluri and Ticku, 2002; Tsuji et al., 2003; Chandler and Sutton, 2005) or in cultured cerebellar granule neurons exposed to ethanol (Ohrtman et al., 2006). The effect on ERK1/2 may result from (a) altered neurotrophin signaling or (b) an ethanol-induced alteration(s) of other growth factor systems that can activate ERK signaling, e.g., insulin-like growth factor in 3T3 cells (Resnicoff et al., 1993), platelet-derived growth factor in multiple cell types including cortical astrocytes (Heldin et al., 1998; Luo and Miller, 1999a), and/or transforming growth factor β in B104 cells (Luo and Miller, 1999b) or fetal rat slice cultures (Powrozek and Miller, 2009). In contrast to ERK1/2, there is no effect of ethanol exposure on expression or activation of Akt. This implies that the Ras-Raf-ERK1/2 pathway, and not the PI3K-Akt pathway, is the target of ethanol.

ERK1/2 plays a pivotal role in development, at least in the cerebral cortex. Its signaling is important for progenitor cell proliferation and cell fate decisions. A reduction (Paquin et al., 2005; Gauthier et al., 2007) or loss (Samuels et al., 2008) of ERK1/2 activation causes progenitor cells to remain undifferentiated in the presence of neurogenic stimuli, but to differentiate into glia in response to gliogenic signals. Based on the effect of ethanol on the cell cycle kinetics, as many as 50% more cells may be produced in the Et-treated animals (Mooney and Miller, 2010). Nevertheless, there is no change in the number of neurons at any of the ages examined, although there is a change in acquisition. That is, in Et-treated animals neuronal number dropped between P0 and P3, rose between P3 and P12, and was unchanged between P12 and P31. In controls, neuronal number dropped between P0 and P3, rose between P3 and P21, and then decreased again by P31. It is possible that the alteration in ERK1/2 signaling results in these newly generated cells remaining undifferentiated or becoming glia. In either case, they would not contribute to neuronal number.

The VB has a unique response to ethanol. For example, prenatal exposure to ethanol alters the structure and function of the principal sensory nucleus of the trigeminal nerve and the somatosensory cortex (Miller and Muller, 1989; Miller and Potempa, 1990; Miller and Dow-Edwards, 1993; Miller, 1995; 1999; Xie et al., 2010). In contrast, the composition and metabolic response in the thalamus appear unaffected (Miller and Dow-Edwards, 1993; Mooney and Miller, 1999; 2010). Moreover, the VB exhibits considerable postnatal neurogenesis (Mooney and Miller, 2007b) which is affected by ethanol (Mooney and Miller, 2010). The dynamics of the neurotrophin system within the thalamus and the resultant effect on cell proliferation within the VB may underlie a compensatory protection from ethanol. This likely contributes to the refractile response of the VB to ethanol. It also highlights the variability of the response of the developing nervous system in an experimental model of fetal alcohol syndrome.

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Abbreviations

ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
Ct	isocaloric non-alcoholic liquid diet
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinase
Et	ethanol-containing diet
G	gestational day
LI	labeling index
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
P	postnatal day
p-	phosphorylated-
PB	0.10 M phosphate buffer
PI3K	phosphatidylinositol-3-kinase
sem	standard error of the mean
TPB	PB containing 0.10% Triton X100
Trk	Tropomyosin-related kinase receptor
TwPBS	0.10 M phosphate buffered saline containing 0.10% Tween-20
VB	ventrobasal nucleus of the thalamus

Figure 1. Effect of neurotrophins on organotypic slice cultures

The proportions, or labeling indices, of Ki-67-positive cells in organotypic slice cultures of the VB were determined. Left. Cultures were treated with BDNF (0, 10, 50, or 100 ng/ml) for 2 or 24 hr. Right. Other slices were treated with NGF (0, 20, 40, or 80 ng/ml) and BDNF (0 or 50 ng/ml). Each mean is based on 3 or 4 independent samples. . *, #, and & signify statistically significant differences ($p < 0.05$) relative to controls (0 ng/ml NGF and 0 ng/ml BDNF), samples treated with NGF (20 ng/ml or 40 ng/ml), and to samples treated with 50 ng/ml BDNF, respectively.

Figure 2. Labeling for neurotrophins and receptors in neonatal VB

Cells in the VB were identified as cycling by their immunoexpression of Ki-67 (solid arrowheads) (A). Ki-67-negative cells were identified by open arrowheads. Immunohistochemical labeling for nerve growth factor (NGF; B and D) and brain-derived neurotrophic factor (BDNF; C and E) in a six-day-old control-treated rat shows that both neurotrophins are expressed in the developing thalamus. High power photomicrographs show VB cells that are positive for the neurotrophin receptors, TrkA, TrkB, and pTrk (F, G, and H, respectively). Labeled and unlabeled cells are identified by solid and open arrows, respectively. Scale bars are 10 μ m (A-C and F-H) or 500 μ m (D and E).

Figure 3. Expression neurotrophins in developing VB

The proportion of NGF- and BDNF-immunopositive cells in the VB of control and ethanol-treated rat pups was determined on P6. Each bar represents the mean of three rats and the T-bars signify standard errors of the means.

Figure 4. Neurotrophin expression in developing VB

The expression of NGF (left) and BDNF (right) in the thalamus were assessed using enzyme-linked immunosorbent assays. Asterisks denote differences that was significant relative to age-matched control animals ($p < 0.05$). Solid and open circles identify data for controls and ethanol-treated rats, respectively. Each datum is the mean of three animals, each from a different litter. T-bars represent the standard errors of the means. $n = 3$. An asterisk denotes a difference that was significant relative to Ct-treated animals at the same age ($p < 0.05$).

Figure 5. Neurotrophin receptor expression in developing VB

Three neurotrophin receptors (TrkA, TrkB, and p75; A, B, and E, respectively) were expressed in the VB at all ages in the offspring of rats prenatally fed a control diet (solid circles, solid lines) or an ethanol-containing diet (open circles, dashed lines). The truncated form of TrkB (C) and phosphorylated Trk (D) were also present. $n = 3$. Notations as in Figure 4.

Figure 6. Expression of ERK in developing VB

Left. The expression of extracellular signal-regulated kinase (ERK) 2 and ERK1 and the phosphorylated forms of these isoforms (A and B, respectively) was determined in the developing thalamus of controls (Ct) and ethanol-treated rats (Et) using immunoblots. Data from the blots was quantified. Right. In addition, the ratio of expression of phosphorylated to total protein was determined for both ERK isoforms. $n = 3$. Notations as in Figure 4.

Figure 7. Akt expression

Left. The expression of Akt and its phosphorylated isoform (A and B, respectively) was determined in the developing thalamus of controls (Ct) and ethanol-treated rats (Et) using immunoblots. Data from the blots was quantified. Right. In addition, the ratio of expression of phosphorylated to total protein was determined for pAkt. $n = 3$. Notations as in Figure 4.

Table I
Antibodies used in anatomical and biochemical studies

cell type	marker	dilution	source
proliferating cells	Ki-67	1:300	RM9106-50; Lab Vision
neurotrophin ligands	NGF	1:700	AB927; Millipore
	BDNF	1:100	sc-546; Santa Cruz Biotechnology
neurotrophin receptors	TrkA	1:100	06-574; Upstate Biotechnology
	TrkB	1:100	courtesy of David R. Kaplan
	pTrk	1:500	sc-8058; Santa Cruz Biotechnology
	p75	1:200	G3231; Promega
signaling proteins	ERK1/2	1:3,000	9102; Cell Signaling Technology
	pERK1/2	1:500	9106; Cell Signaling Technology
	Akt	1:3,000	9272; Cell Signaling Technology
	pAkt	1:3,000	9275; Cell Signaling Technology
loading controls for blots	actin	1:4,000	A5316; Sigma
	β -tubulin	1:1,000	sc-9104; Santa Cruz Biotechnology