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Environmental Enrichment Alters Neurotrophin Levels After Fetal Alcohol Exposure in Rats

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Abstract

Background—Prenatal alcohol exposure causes abnormal brain development, leading to behavioral deficits, some of which can be ameliorated by environmental enrichment. As both environmental enrichment and prenatal alcohol exposure can individually alter neurotrophin expression, we studied the interaction of prenatal alcohol and postweaning environmental enrichment on brain neurotrophin levels in rats.

Methods—Pregnant rats received alcohol by gavage, 0, 4, or 6 g / kg / d (Zero, Low, or High groups), or no treatment (Naïve group), on gestational days 8 to 20. After weaning on postnatal day 21, offspring were housed for 6 weeks in Isolated, Social, or Enriched conditions. Levels of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) were then measured in frontal cortex, occipital cortex, hippocampus, and cerebellar vermis.

Results—Prenatal alcohol exposure increased NGF levels in frontal cortex (High-dose group) and cerebellar vermis (High- and Low-dose groups); increased BDNF in frontal cortex, occipital cortex and hippocampus (Low-dose groups), and increased NT-3 in hippocampus and cerebellar vermis (High-dose). Environmental enrichment resulted in lower NGF, BDNF, and NT-3 levels in occipital cortex and lower NGF in frontal cortex. The only significant interaction between prenatal alcohol treatment and environment was in cerebellar vermis where NT-3 levels were higher for enriched animals after prenatal alcohol exposure, but not for animals housed under Isolated or Social conditions.

Conclusions—Both prenatal alcohol exposure and postweaning housing conditions alter brain neurotrophin levels, but the effects appear to be largely independent. Although environmental enrichment can improve functional outcomes, these results do not provide strong support for the hypothesis that rearing in a complex environment ameliorates prenatal alcohol effects on brain neurotrophin levels in rats.

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Keywords

Fetal Alcohol Syndrome; Nerve Growth Factor; Brain-Derived Neurotrophic Factor; Neurotrophin-3; Environmental Enrichment

Exposure of the fetus to alcohol can result in fetal alcohol spectrum disorders (FASD), which can include growth restriction, facial dysmorphologies, and always disturbances in the structure and/ or function of the central nervous system (CNS) (Bertrand et al., 2004; Riley and McGee, 2005). The full expression of these effects defines the fetal alcohol syndrome (FAS) (Jones and Smith, 1973). CNS abnormalities include decreased cranial size at birth, microcephaly, corpus callosum dysgenesis, and cerebellar hypoplasia (Cortese et al., 2006; Riley et al., 2004). Behavioral or cognitive abnormalities include impulsiveness, poor social perception, problems with abstract thinking, and deficits in memory, attention, and/ or judgment (Rasmussen, 2005). The underlying cause(s) and mechanisms of such diverse prenatal alcohol-related CNS anomalies are not completely understood (Abel and Hannigan, 1995). However, several useful and valid animal models produce effects on brain development and behavior that are similar to those seen in FASDs (Driscoll et al., 1990; Hannigan, 1996). Research with these models has demonstrated that prenatal alcohol exposure can alter brain neurotrophin levels in rats, changes that may represent 1 important mechanism through which prenatal alcohol exposure impairs brain development (Angelucci et al., 1999; Climent et al., 2002; Heaton et al., 1999, 2003b).

Neurotrophins are a family of peptides that influence the growth, development, and functional plasticity of the brain

. This includes synapse formation and remodeling, neurite outgrowth, establishment of neuronal connections, and the neural mechanisms of plasticity in both learning and response to stress and injury (Blum and Konnerth, 2005; Dreyfus, 1998; McAllister et al., 1999; Segal, 2003; Sofroniew et al., 2001). Three key neurotrophins are nerve growth factor (NGF), neurotrophin-3 (NT-3), and brain-derived neurotrophic factor (BDNF), each of which acts via specific tyrosine kinase receptors (i.e., Trk receptors). The observation that prenatal alcohol exposure can alter neurotrophin levels and/ or function suggests that this may be an important mechanism by which alcohol perturbs brain development and leads to enduring behavioral abnormalities in FASD. If this hypothesis is correct, then strategies to normalize neurotrophin levels or activity early in development—or perhaps even later in life —may ameliorate the effects of prenatal alcohol on CNS development and function. Evidence supporting this hypothesis includes the demonstration that transgenic mice over expressing NGF show reduced effects of prenatal alcohol exposure on brain development (Heaton et al., 2000a). In addition, in vitro studies have shown that treatment with BDNF or NGF can reduce alcohol-induced cell injury in cultured septal, hippocampal, and Purkinje neurons (Heaton et al., 1999; Mitchell et al., 1999).

Environmental enrichment has also been shown to influence brain neurotrophin levels (Ickes et al., 2000; Mohammed et al., 1993), as well as to improve motor and cognitive functions in rats exposed prenatally to alcohol (Berman et al., 1996; Hannigan et al., 1993, 2007).

Therefore, we hypothesized that environmental enrichment would alter brain neurotrophin levels, possibly ameliorating the effects of prenatal alcohol exposure on neurotrophin levels.

MATERIALS AND METHODS

Subjects

Timed-pregnant Sprague–Dawley rats arrived on gestational day 4 (GD4) (Charles River, Portage, MI). Rats were weighed and randomly assigned to 1 of 4 prenatal treatment groups, counterbalancing for initial body weight on GD4. The 4 treatment groups were Zero (0 g / kg / d), Low (4 g / kg / d), or High (6 g / kg / d) prenatal alcohol treatment groups, and a group of Naïve, untreated dams. The Zero and Naïve groups were not exposed to alcohol and were used to control for potential nonspecific effects of the mild prenatal stress due to the intragastric intubation procedure. Dams were weighed every other day from GD8 through GD20. Naïve rats were weighed but were otherwise undisturbed. All procedures had prior approval of the Wayne State University Animal Investigation Committee (IACUC). Numbers of offspring of each gender in each treatment group are shown in Table 1.

Prenatal Alcohol Exposure

Alcohol was administered by intragastric gavage (oral intubation) as previously described (Berman et al., 1996; Hannigan and Abel, 1996). This allowed experimenter control over dosage, maternal blood alcohol concentrations (BACs), and the pattern of alcohol administration. Dams received alcohol in 2 intubations per day, at approximately 9 and 10 AM, beginning on GD8 and continuing through GD20. Beginning intubations on GD8 provides prenatal alcohol exposure throughout much of the prenatal developmental period, while avoiding too early an exposure which can lead to increased rates of fetal loss. It also allowed pregnant rats to acclimate to the lab environment after delivery from the vendor on GD4 before alcohol exposure. The split-dose procedure modeled a binge-like alcohol exposure and yet avoided a peak maternal BACs that could be associated with increased fetal loss. The total daily doses were 0, 4, or 6 g / kg for the Zero-, Low-, and High-dose prenatal alcohol-exposure groups, respectively. The Zero- and Low-dose solutions were made isocaloric to the High-dose by the addition of sucrose. Peak maternal BACs with these procedures, assessed 30 minutes after the second intubation in another groups of dams whose offspring were not used in this study, were 180 to 220 and 250 to 280 mg/dl alcohol for the Low-dose (4 g /kg /d) and High-dose (6 mg/kg /d) groups, respectively. All animals had free access to water, except for the hour in between the 2 intubations. To limit differential food intake among the prenatal treatment groups, food was removed from all dams during the 5 hours after the first intubation, a period when the alcohol-exposed dams were intoxicated and less likely to eat than nonexposed controls. Beginning on GD20, the dams' cages were inspected twice daily for new births, which usually occurred on GD22 or GD23. The day of birth was designated postnatal day 0 (PN0). On PN1 the pups were weighed, examined for gross physical abnormalities, and the litters culled to 10 pups, 5 male and 5 female when possible.

Environmental Enrichment

Offspring remained with their birth dams until weaning on PN21. Rats in each litter were then randomly assigned to 1 of 3 housing conditions for 6 weeks (i.e., Isolated, Social or Enriched). Animals reared in the Isolated condition were housed singly in hanging steel / wire cages (24 × 18 × 18 cm) and had no direct contact with other animals, were not handled, and had nothing in the cage except ad lib access to food and water. Animals in the Social condition were housed in same-sex groups of 3 in standard polyethylene tub cages (43 × 23 × 21 cm) with contact bedding, but with no special handling or toys. Animals in the Enriched condition were housed in same-sex groups of 12 to 15 rats in large Nalgene® arenas (75 × 75 × 66 cm; Nalge Nunc Corp., Rochester, NY) with a variety of novel objects (toys) to manipulate, climb upon, and explore. Environmental enrichment included brief handling and changing of the toys every 2 to 3 days. “Handling” involved removing each rat from the arena, briefly stroking the rat, letting it move about on a table for ~3 minutes, and then returning it to the arena. “Toys” include objects such as small wire cages, jars and bottles, plastic tubes, hanging chains, ramps, ladders, etc. Rearing under these environmental conditions continued for 6 weeks, a typical and effective enrichment duration for modifying brain structure and behavior (Berman and Hannigan, 2000; Berman et al., 1996; Greenough and Chang, 1988; Hannigan et al., 1993). Only 1 or 2 rats / sex / litter were placed in each housing environment to minimize the influence of possible “litter effects” (Hannigan and Abel, 1996; Holson and Pearce, 1992).

Neurotrophin Assays

On approximately PN66 (range PN63 to 71; median PN66), rats were killed by decapitation. The brains were quickly removed and frozen in isopentane cooled to −50°C in an acetone-dry ice bath, then stored at −80°C until shipment to the University of California Davis for neurotrophin analyses. Brains were partially defrosted in a cold room and hemisected in the saggital plane. Half of each brain was immediately refrozen for possible future studies. The brain halves were selectively dissected using landmarks to locate the desired brain regions. The 4 brain regions dissected were the anterior vermis of the cerebellum, hippocampus, a portion of frontal cortex corresponding to Fr2, and a portion of occipital cortex corresponding to Oc1B and Oc2L (Zilles and Wree, 1985). These regions were selected because morphological changes have been observed there in previous studies of perinatal alcohol exposure (Berman and Hannigan, 2000; Berman et al., 1996; Maier and West, 2001; Riley et al., 2004; Sakata-Haga et al., 2001; Thomas et al., 1998). The same experimenters dissected the same regions for consistency (EAP: frontal cortex and hippocampus; RFB: occipital cortex and cerebellar vermis). Experimenters were blind to prenatal treatment, housing environment, and sex of each specimen. Tissue was homogenized on ice in 1:20 (w/v) cold homogenization buffer (100 mM Tris, 1 M NaCl, 4 mM EDTA, 0.1% NaN_3, 0.2% Triton X, 20 μM phenylmethylsulfonyl fluoride, 157 μg / ml benzamidine, and 50 μg / ml aprotinin) and centrifuged at 12,000 rpm (11,752 × g) for 15 minutes at 4°C. The supernatant was aliquoted and frozen at −80°C for later analysis.

Enzyme-linked immunosorbant assays were performed for NGF, BDNF, and NT-3 using Emax® ImmunoAssay kits (Promega, Inc., Madison, WI). This assay does not distinguish between NGF and its precursor, pro-NGF. Pro-NGF, and 2 peptides derived from pro- NGF,

LIP1, and LIP2, have recently been shown to have biological activity and may also contribute to the neuronal effects previously attributed to NGF (Dicou, 2006; Fahnestock et al., 2004). Future work may further elucidate the various effects of these related peptides. All antibodies and reagents, with the exception of those specified below, were provided in the kits. Incubation times varied with the neurotrophin being tested per kit directions. All assays were at room temperature unless otherwise specified. Samples were run in duplicate. Tissue samples were diluted 1:5 with Dulbecco's phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.35) and acidified to pH~3 with 1 N HCl. After incubating for 15 to 20 minutes, samples were neutralized with 1 N NaOH. Samples were further diluted with block and sample buffer (BSB) as needed to bring concentrations within the range of the standard curve, and 100 μl were placed in designated wells of a 96-well plate previously coated with primary neurotrophin antibody in carbonate buffer (25 mM Na_2CO_3 and 25 mM $NaHCO_3$, pH 9.7, incubated at 4°C) and then blocked with BSB. Samples and standards were incubated, rinsed 5× with Tris-buffered saline with detergent (TBST; 20 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.6), and 100 μl of the second primary antibody dilution placed in each well. After incubation, wells were rinsed 5× with TBST, and the horseradish peroxidase-conjugated secondary antibody placed in each well and incubated. Wells were rinsed 5× with TBST and the tetramethylbenzidine substrate placed in each well. After incubation, the reaction was stopped by the addition of 1 N HCl, and the absorbance read at 450 nm on a plate reader (HTS 7000 Bioassay Reader; PerkinElmer, Boston, MA).

Data Analyses

Percent maternal weight gain, gestational age at birth, number of male and female pups per litter, male:female ratio within litter, pup weight on PN1, and weaning weight on PN21 are all reported as mean ± SEM. Separate ANOVAs were used to assess the effects of prenatal alcohol exposure on maternal and litter characteristics (SPSS, Inc., Chicago, IL). Separate treatment × sex (4 × 2) ANOVAs assessed mean body weights at PN1 and PN21. Neurotrophin levels are expressed as nanograms protein per grams wet weight tissue (ng / g) and are displayed as mean ± SEM. Data files were examined for outliers, defined as values exceeding 3 SD from the mean, and these were not used in the data analysis. Separate 4 × 3 × 2 (treatment × environment × sex) ANOVAs were performed on each brain region and for each neurotrophin. When appropriate, individual post hoc group comparisons were carried using the Tukey–Kramer test which controls for the family wise error rate when multiple comparisons are carried out (Keppel, 1991). Differences were considered significant at the $p < 0.05$ level.

RESULTS

Maternal and Litter Characteristics

Maternal and litter characteristics are summarized in Table 2. Dams in the High-dose prenatal alcohol exposure group gained significantly less weight between GD4 and GD20, expressed as percent weight gain, than dams in each of the other groups ($F_{3,21} = 10.20$, $p < 0.001$). The Naïve, Zero, and Low groups did not differ in percent weight gain. A prenatal alcohol effect on gestational age at birth approached significance ($F_{3,21} = 3.12$, $p = 0.052$),

suggesting a slightly longer gestation in the High alcohol group compared with the Zero group ($p = 0.059$). There were no significant effects of prenatal alcohol treatment on litter size, the number of male or female pups per litter, total pups per litter, or the litter sex ratio (all $p > 0.18$). At PN1, males weighed significantly more than females across treatment groups ($F_{1,18} = 56.46$, $p < 0.001$).

At weaning on PN21, there were significant main effects of prenatal alcohol treatment ($F_{3,228} = 5.95$, $p < 0.001$) (Table 2). Post hoc tests indicated that rats in the High-dose alcohol group weighed more than the Naïve- and Low-dose alcohol groups (both $p < 0.05$), and the Low alcohol group weighed less than the Zero alcohol group ($p < 0.05$). On PN21 males weighed significantly more than females ($F_{1,228} = 8.72$, $p < 0.01$, data not shown).

Prenatal Alcohol and Environmental Effects on Body Weight

At PN66, after completion of the differential rearing, there were significant main effects of prenatal alcohol treatment ($F_{3,228} = 2.83$, $p < 0.05$), Sex ($F_{1, 228} = 1{,}226.4$, $p < 0.0001$), and Environment ($F_{2,228} = 22.42$, $p < 0.001$) on body weight, and no significant interactions among any of these factors. Post hoc contrasts showed that the Zero (343.1 ± 11.2 g) and High-dose (348.8 ± 13.8 g) alcohol groups weighed significantly more than the Naïve (306.8 ± 10.6 g; $p < 0.05$) and Low alcohol groups (320.5 ± 9.8 g; $p < 0.05$), but the Zero- and High-dose groups did not differ significantly ($p > 0.65$). Males weighed more than females (398.4 ± 2.9 vs. 243.3 ± 2.4 g). The Enriched group (305.8 ± 8.9 g) weighed significantly less than the Isolated (337.9 ± 9.7 g) and Social groups (338.4 ± 10.1 g) (both $p < 0.0001$), possibly due to increased behavioral activity by the Enriched group in the larger housing space.

Nerve Growth Factor Protein Levels

No statistically significant interactions were found between prenatal alcohol exposure and environmental rearing conditions for the frontal cortex, hippocampus, occipital cortex, or cerebellar vermis for NGF protein levels. Therefore, Fig. 1 shows the effects of prenatal alcohol exposure on NGF protein levels collapsed across environmental rearing conditions, and Fig. 2 shows effects of rearing conditions on NGF levels collapsed across prenatal alcohol exposure. Sex differences are shown in Table 3.

As shown in Fig. 1 prenatal alcohol treatment significantly increased NGF protein levels in the frontal cortex ($F_{3,165} = 3.70$, $p < 0.05$), where the High alcohol group had significantly higher NGF levels than the Naïve and Zero alcohol groups ($p < 0.05$). NGF levels were also elevated in the cerebellar vermis ($F_{3,153} = 5.97$, $p < 0.01$), where both the Low and High alcohol groups had significantly higher NGF levels than the Naïve group ($p < 0.05$). There were no statistically significant effects of prenatal alcohol exposure in hippocampus or occipital cortex.

Figure 2 shows significant environmental rearing effects on NGF levels in the frontal ($F_{2,165} = 4.27$, $p < 0.05$) and occipital cortices ($F_{2,170} = 6.49$, $p < 0.01$). In frontal cortex, Enriched rats had lower NGF levels than Isolated rats ($p < 0.01$) and in the occipital cortex, Enriched rats had significantly lower NGF levels than both Social ($p < 0.05$) and Isolated rats ($p <$

0.01). Housing environment did not significantly affect NGF levels in the hippocampus or cerebellar vermis. As shown in Table 3, females had significantly higher NGF levels than males in frontal cortex, hippocampus, occipital cortex, and cerebellar vermis (each $p < 0.01$).

Brain-Derived Neurotrophic Factor Protein Levels

As there were no statistically significant interactions between prenatal alcohol exposure and environmental rearing conditions on BDNF levels for any of the 4 brain regions, the effects of prenatal alcohol exposure on BDNF protein levels are shown in Fig. 3 collapsed across environmental rearing conditions, and the effects of rearing on BDNF levels are shown in Fig. 4 collapsed across prenatal alcohol exposure.

As shown in Fig. 3, there were significant main effects of prenatal alcohol treatment on BDNF levels in frontal cortex ($F_{3,201} = 3.83$, $p < 0.05$), hippocampus ($F_{3,201} = 3.04$, $p < 0.05$), and occipital cortex ($F_{3,201} = 3.49$, $p < 0.05$), and this same effect approached significance in cerebellar vermis ($F_{3,186} = 2.31$, $p < 0.08$). In frontal cortex ($p < 0.01$), hippocampus ($p < 0.05$), and occipital cortex ($p < 0.05$) BDNF levels in the Low alcohol groups were higher than in the Naïve group. In addition, in the hippocampus there was also a significant treatment × gender interaction ($F_{2,201} = 2.94$, $p < 0.05$) shown as an inset in Fig. 3. Further analysis demonstrated that the High alcohol males had lower BDNF levels than the Zero ($p < 0.01$) and Low alcohol groups ($p < 0.05$), but there were no significant treatment effects in females.

As shown in Fig. 4, there was a significant effect of environment on BDNF levels in occipital cortex ($F_{2,201} = 4.02$, $p < 0.05$), with significantly lower levels in the Enriched versus Isolated groups ($p < 0.01$). There were no statistically significant effects of environmental rearing conditions on BDNF levels in frontal cortex, hippocampus, or cerebellar vermis. Females had significantly higher BDNF levels than males only in the frontal cortex ($p < 0.05$, Table 3).

Neurotrophin-3 Protein Levels

As was carried out for the NGF and BDNF analyses, prenatal alcohol effects on NT-3 are shown collapsed across environmental rearing conditions in Fig. 5, and environmental rearing effects are shown collapsed across prenatal alcohol effects in Fig. 6. A single statistically significant interaction between the effects of prenatal alcohol with environmental rearing was found for NT-3 in the cerebellar vermis and this interaction is shown separately in Fig. 7 below.

Figure 5 shows that there was a significant alcohol treatment effect on NT-3 levels in the hippocampus ($F_{3,185} = 4.03$, $p < 0.01$), where NT-3 levels were significantly elevated in the High alcohol group compared with the Naïve group ($p < 0.05$). NT-3 levels were also affected in the cerebellar vermis ($F_{3,164} = 4.41$, $p < 0.01$), where levels were elevated for the High alcohol exposed group compared with the Naïve and Zero and Low alcohol groups ($p < 0.01$).

As shown in Fig. 6, environmental enrichment significantly decreased NT-3 levels in the occipital cortex ($F_{2,173} = 5.43$, $p < 0.01$), where NT-3 levels were significantly lower in the Enriched ($p < 0.01$) and Social ($p < 0.05$) groups compared with the Isolated rats.

Figure 7 shows the statistically significant interaction in the cerebellar vermis between prenatal alcohol treatment and environmental rearing conditions ($F_{6,164} = 2.61$, $p < 0.05$). Subsequent analysis of the interaction demonstrated that NT-3 levels in the vermis were significantly increased in the Low and High alcohol groups compared with the Naïve and Zero alcohol groups ($p < 0.05$), but only for rats reared for 6 weeks under enriched environmental conditions.

Females had significantly higher NT-3 levels in the hippocampus than males ($p < 0.01$, Table 3).

DISCUSSION

Prenatal Alcohol Effects on Neurotrophins

Prenatal alcohol exposure and environmental enrichment influenced brain neurotrophin levels differently, with unexpectedly little evidence of interactions between alcohol exposure and environmental rearing conditions. The effects of prenatal alcohol exposure depended on the brain region and the neurotrophin examined. This was most clear for NGF levels in the frontal cortex (High alcohol dose) and cerebellar vermis (Low and High alcohol doses), and for NT-3 levels in the hippocampus (High alcohol dose) and cerebellar vermis (High alcohol dose) where neurotrophin levels were increased in alcohol-exposed rats compared with controls. Prenatal alcohol exposure also increased BDNF levels in the frontal cortex, hippocampus, and occipital cortex, but only in the Low alcohol exposure groups, and only when compared with the alcohol-Naïve group. While clear dose-related effects of prenatal alcohol exposure have been shown (Snell et al., 2001), prenatal alcohol effects are not always related to dose in any simple way. For example, Clausing et al. (1995) prenatally exposed rats to 1 of 2 alcohol concentrations (18 or 36% ethanol-derived calories) via a liquid diet. Spatial learning deficits were found in the lower, but not the higher alcohol exposure group. In this study, prenatal alcohol effects on BDNF levels in the hippocampus appeared to be limited to male rats. Previous studies have also reported sex-specific effects of prenatal alcohol exposure in male (Blanchard et al., 1987) and female rats (Minetti et al., 1996), possibly due to sex differences in response to alcohol dose or timing of prenatal alcohol exposure (Berman and Hannigan, 2000; Goodlett and Peterson, 1995).

Increased NGF levels in frontal cortex and vermis following prenatal alcohol exposure are consistent with Heaton et al. (2003b) who found increased NGF in cortex / striatum on PN1 following neonatal alcohol exposure via liquid diet. However, in the Heaton et al. study the increases were transient and no longer evident by PN10. In this study, the fact that NGF levels were elevated on PN66 indicates a more enduring effect of prenatal alcohol exposure. In contrast, Angelucci et al. (1999) reported decreased NGF levels in entorhinal cortex at PN60 following prenatal alcohol exposure, although surprisingly the effect was not seen on PN15 or 30. These authors suggested that either some compensatory mechanism prevented

an earlier decrease in NGF, or that prenatal alcohol affected NGF production that became evident only during a later critical developmental period.

The importance of timing for both alcohol exposure and NGF measurement is exemplified in a study by Heaton et al. (2003a) where alcohol was administered to rat pups on either PN4 or 7, followed by neurotrophin analyses at 0, 2, 12, or 24 hours after exposure. Cerebellar NGF levels were decreased by alcohol exposure on PN4 at 0 and 12 hours, but on PN7 they were increased at 0 hours and decreased at 2 hours. For the other groups, NGF levels were not affected by the neonatal alcohol exposure (Heaton et al., 2003a). Similarly varied results were observed for BDNF and NT-3 in cerebellum, and in cortex and striatum (Heaton et al., 2003a,b,c).

The present study found increased BDNF levels following the Low- but not High-dose prenatal alcohol exposure in frontal and occipital cortices and hippocampus. The reasons underlying this dose–response profile are unknown, but as described earlier the effects of prenatal alcohol exposure are not always related to dose in any simple way (Clausing et al., 1995). It is also important to consider the age at which neurotrophins are measured. Heaton et al. (2000b), found no effects of prenatal alcohol on BDNF levels at PN1, while other groups reported decreased BDNF levels in olfactory bulb at birth and at PN10 (Maier et al., 1999a); in cerebral cortex at PN5, PN14, and PN21, but not at PN35 (Climent et al., 2002); and in both cortex and hippocampus at PN7 to 8 (Feng et al., 2005). Each of these earlier studies on BDNF examined rats younger than PN66 and this age difference may contribute to the different results. Finally, we found that prenatal alcohol exposure increased NT-3 levels in hippocampus and cerebellar vermis but not in frontal or occipital cortex. Heaton et al. (2000b) found marginal increases ($p = 0.07$) in NT-3 levels in cortex / striatum after prenatal alcohol at PN1 but no effects on NT-3 in hippocampus, septum, or cerebellum.

Considered together, these studies suggest a complex time course for alcohol effects on neurotrophin levels, but they do not differentiate between the possibilities of permanent changes to neurotrophin levels or more simply to prenatal alcohol-produced neurodevelopmental delay. The rat brain shows different regional levels of NGF, BDNF, and NT-3 mRNA and protein depending on developmental age (Das et al., 2001; Dreyfus, 1998; Lauterborn et al., 1994; McAllister et al., 1999). Therefore, while it is possible that neurotrophin levels are elevated in compensation for alcohol-induced damage to the developing brains, it is also possible that altered neurotrophin levels reflect delayed brain development that is thought to occur in FAS (Nathaniel et al., 1986b; Toga et al., 2006). The present results at ~PN66 indicate that alcohol exposure early in life can have long-lasting effects on neurotrophin levels, perhaps contributing to the lasting deficits in neural plasticity that can follow prenatal alcohol exposure.

In this study, prenatal alcohol exposure elevated NGF and NT-3 levels in the cerebellar vermis at ~PN66. This is of particular interest because the cerebellum may be particularly vulnerable to the effects of early alcohol exposure. For example, perinatal alcohol exposure has been shown to decrease cerebellar weight and volume (Maier et al., 1997, 1999b; Nathaniel et al., 1986a,b; Weinberg, 1985), reduce cerebellar Purkinje and granule cell number and density (Bonthius and West, 1990, 1991; Maier and West, 2001; Maier et al.,

1999b; McGoey et al., 2003), and generally reduced expression of neurotrophin receptors in the cerebellum (Moore et al., 2004). Children with FAS also show reductions in the size of the cerebellum (Mattson et al., 1996; O'Hare et al., 2005; Sowell et al., 1996, 2001a, b, 2002). Elevated neurotrophin levels may reflect activation of compensatory neuroprotective mechanisms that may mitigate the neurotoxicity of prenatal alcohol exposure, as previously suggested (Heaton et al., 2003a). Because neurotrophins play key roles in CNS development, previously reported effects of alcohol on the development of cerebellum structure, morphology, and function may be due, at least in part, to abnormal neurotrophin levels, including NGF and NT-3, as indicated by the present results.

Neurotrophins bind to specific Trk receptors activating, among others, phospholipase C, Ras-MAP kinase, and phosphatidylinositol 3 kinase cell signaling pathways (Chao, 2003; McAllister et al., 1999). There is evidence that the levels or function of Trk receptors are altered by prenatal alcohol exposure. For example, Climent et al. (2002) reported a decrease in the ratio of truncated (TrkB-T) to full-length (TrkB-fl) receptors following prenatal alcohol exposure that would be expected to impair cell signaling by BDNF (Climent et al., 2002). Therefore, it is possible that the increased neurotophin levels found in this study could reflect a compensatory response to prenatal alcohol impairment of Trk receptor function. However, the reported effects of prenatal alcohol exposure on Trk receptors are complex and differ depending on the postnatal age of measurement, brain region and sex of the animal (Chao, 2003; Climent et al., 2002; Feng et al., 2005; Moore et al., 2004), making it difficult to generalize about possible relationships between neurotrophins levels and receptor function.

Effects of Rearing Environment on Neurotrophins Levels

The effects of 6 weeks of rearing in an enriched environment on neurotrophins levels depended on the brain region examined and the neurotrophin measured. Specifically, NGF levels were decreased in frontal cortex in Enriched compared with Isolated animals, while environmental enrichment decreased levels of all 3 neurotrophins in the occipital cortex. Neurotrophin levels did not appear to be affected by environmental rearing conditions in the hippocampus and cerebellar vermis in this study.

The decrease in neurotrophin levels in occipital cortex, as well as the absence of enrichment effects in the hippocampus, are notable because previous studies have reported that enrichment procedures can increase NGF, BDNF, and NT-3 levels, although in a brain region-dependent manner (Ickes et al., 2000; Pham et al., 1999). These earlier studies in rats used either much longer periods of enrichment (e.g., 12 months) or began enrichment procedures later in development (e.g., 50 days of age) than in this study (Ickes et al., 2000; Mohammed et al., 1993; Pham et al., 1999). This is important because the duration of enrichment has been shown to be important for effects on neurotrophin levels. For example, 30 days, but not 10 or 60 days of enrichment increased NGF levels in hippocampus (Mohammed et al., 1993). Other studies have not found changes in NGF or BDNF after 2 to 4 months of enrichment in frontal cortex, entorhinal cortex, or cerebellum (Zhu et al., 2006), in motor cortex or hippocampus (Turner and Lewis, 2003), or in NT-3 or BDNF protein levels in cortex or hippocampus (Chen et al., 2005; Hicks et al., 2002). Similarly, levels of

BDNF mRNA were unchanged in the hippocampus of rats by 34 days of environmental enrichment (Falkenberg et al., 1992). Rearing in an enriched environment, therefore, is not necessarily linked to increased neurotrophin levels, although there were enrichment-related increases in NT-3 in cerebellum after prenatal alcohol exposure as well as decreases for some conditions. The dynamic interplay between environment and neurotrophin levels is complex, being influenced by the nature and duration of enrichment, age at initiation, prenatal history, sex, the brain regions examined, and probably neuroadaptive processes not assessed in the current study, which may include alterations in Trk receptors (e.g., Ickes et al., 2000; Mohammed et al., 1993; Pham et al., 1999).

The lack of environmental enrichment effects on neurotrophin levels in the hippocampus was also unexpected. Previous research has shown morphological changes in hippocampus in response to enrichment, including increased spine density and greater dendritic branching and length (Bartesaghi and Severi, 2004; Berman et al., 1996; Fiala et al., 1978; Silva-Gomez et al., 2003), and it is well established that neurotrophins can affect synapse development and plasticity (Lu and Figurov, 1997; McAllister et al., 1999). In addition, enriched housing conditions such those used in this study afford animals increased opportunities for exercise (e.g., presence of hanging chains, ramps, and ladders), and voluntary exercise has been shown to increase BDNF levels in the hippocampus (Vaynman et al., 2004) as well as cerebellum (Klintsova et al., 2004). Therefore, it is possible that elevations in NGF, NT-3 or BDNF occur early in the enrichment period, but are not sustained and may not be evident late in enrichment when the major of structural changes in the neuropil may have already occurred.

In cerebellum, motor training in adulthood has been reported to ameliorate some prenatal alcohol-induced behavioral and morphological deficits in both male and female rats, and this plasticity has been argued to be mediated by neurotrophins (Klintsova et al., 2004). In this respect, we had hypothesized that prenatal alcohol exposure would blunt the response of the hippocampus to increased neurotrophin levels during environmental enrichment, and that this could explain the lack of an increase in dendritic spines in the hippocampus of prenatal alcohol exposed rats reared in an enriched environment for 6 weeks. However, the lack of an effect of environmental enrichment in the hippocampus on neurotrophin levels, as well as the lack of significant interactions between prenatal alcohol treatment and environmental enrichment argue against this interpretation of our earlier findings (Berman et al., 1996).

Interaction Between Prenatal Alcohol and Environmental Enrichment

We hypothesized that environmental enrichment would ameliorate prenatal alcohol effects on neurotrophin levels, and therefore expected to observe an interaction between prenatal alcohol exposure and environmental rearing conditions. However, the only significant interaction between environmental rearing conditions and prenatal alcohol exposure was for NT-3 in the cerebellar vermis. Specifically, NT-3 levels were elevated in the Low and High prenatal alcohol exposure groups compared with controls, but only in animals reared in an enriched environment. One interpretation of these results is that elevated NT-3 levels reflect a compensatory response to prenatal alcohol damage in the cerebellum that is magnified by environmental stimulation such as that provided in an enriched environment (e.g., increased

opportunity for locomotor activity). Alternatively, environmental enrichment may have lowered NT-3 levels in Naïve and Zero alcohol treatment groups, but not in the Low and High alcohol groups. However, when NT-3 levels for the Naïve and Zero alcohol groups are compared across the 3 environmental rearing conditions (i.e., Fig. 7), there were no statistically significant effects of environmental enrichment, arguing against this interpretation.

Sex Effects on Neurotrophin Levels

Significant sex differences were found for NGF, BDNF, and NT-3. Female rats had higher NGF levels than males in all 4 brain regions examined, as well as in frontal cortex for BDNF and hippocampus for NT-3 (Table 3). Previous research has demonstrated sex differences in neurotrophin levels in rats, including higher BDNF levels in females compared with males in hippocampus (Franklin and Perrot-Sinal, 2006; Scharfman et al., 2003) and striatum (Bimonte-Nelson et al., 2008). Similarly, environmental enrichment has also been shown to affect brain morphology (Juraska, 1984; Juraska et al., 1989) and neurotrophin levels (Angelucci et al., 2000; Chen et al., 2005) differently in males and females. Therefore, sex differences in neurotrophin levels in this study could contribute to the differential susceptibility of females and males to prenatal alcohol effects on neural plasticity and learning (Goodlett and Peterson, 1995; Johnson and Goodlett, 2002; West et al., 1989).

Effects of Prenatal Alcohol Exposure and Environmental Rearing Conditions on Body Weight and Litter Characteristics

Growth restriction, prenatal and/or postnatal, is common effect of prenatal alcohol exposure in both rats and humans (Hagerman, 1999; Hannigan, 1996; Hannigan and Armant, 2000; Jones and Smith, 1973; Maier et al., 1997; Nathaniel et al., 1986b). In this study, however, the High-dose prenatal alcohol offspring weighed more than the Naïve control animals at weaning (PN21) and after 6 weeks of rearing differential environments (PN66). The rearing environment also affected offspring weight gain. Enriched animals were significantly lighter than the Isolated or Social animals on PN66. This may be due to increased behavioral activity in the larger space and presence of objects and more cohabitants with which to interact. Finally, as expected, at all 3 ages (PN1, PN21, and PN66), males were heavier than females.

Summary

Previous research has shown that prenatal and early postnatal alcohol exposure can affect brain neurotrophin levels in young rats (Angelucci et al., 1999; Climent et al., 2002; Heaton et al., 2000b, 2003b, c; Light et al., 2001; Maier et al., 1999a). Here, we demonstrate that prenatal alcohol exposure resulted in altered brain neurotrophin levels in adult rats as well. This suggests that prenatal alcohol exposure may cause long-lasting, perhaps permanent, changes in neurotrophin expression, which may contribute to the enduring reduction in neural plasticity and behavioral deficits observed in animal studies (Berman and Hannigan, 2000; Berman et al., 1996; Hannigan et al., 1993; Kelly et al., 2000; Klintsova et al., 1998) and in humans with FASDs (Connor et al., 2006; Kelly et al., 2000; Streissguth et al., 1991, 2004). We also show that the postweaning environment affected brain NGF levels in the

frontal cortex and all 3 neurotrophins in the occipital cortex, but not in the hippocampus. Finally, the interaction between environmental rearing conditions and the effect of prenatal alcohol exposure on neurotrophin levels was minimal, at least for the 4 brain regions examined. We hypothesized that environmental enrichment would ameliorate the effects of prenatal alcohol exposure on brain neurotrophin levels, but this was not borne out by our results. Rather, prenatal alcohol exposure and postweaning enrichment had largely independent affects on brain regional neurotrophin levels. That lower neurotrophin levels were found in animals housed in an enriched environment when compared with those in isolated housing points to the complexity of the brain's response to environmental stimulation.

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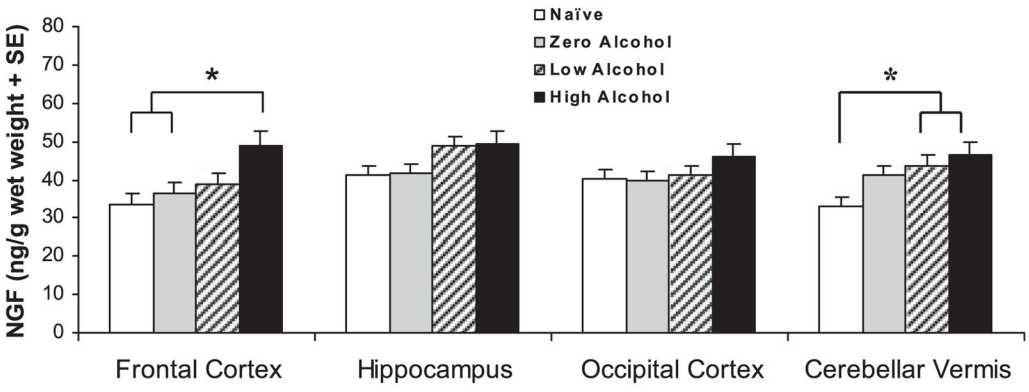

Fig. 1.

Prenatal alcohol effects on regional NGF protein levels measured at PD66; *$p < 0.05$. Data are collapsed on environment and sex to focus on prenatal alcohol effects.

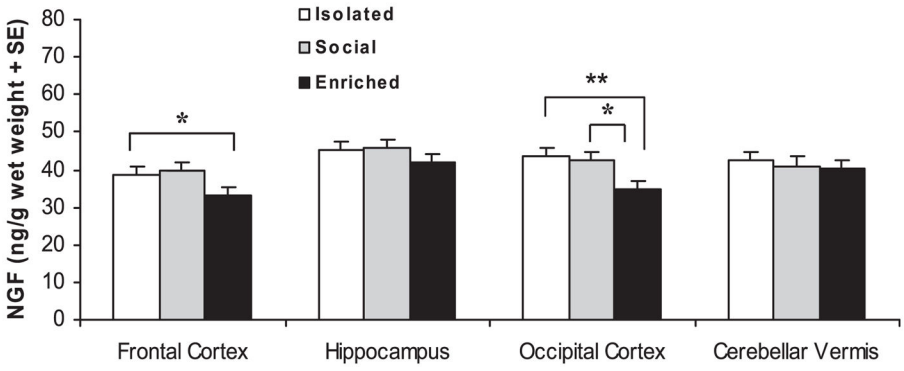

Fig. 2.

Environmental housing effects on regional NGF protein levels measured at PN66; *$p < 0.05$ and **$p < 0.01$. Data are collapsed on alcohol treatment and sex to focus on environmental housing effects.

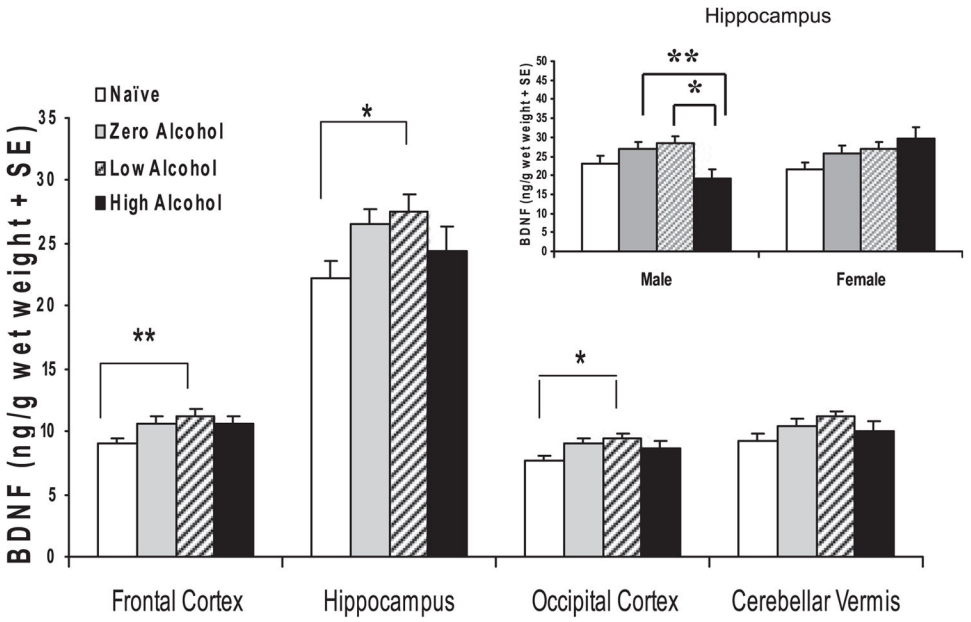

Fig. 3.

Prenatal alcohol effects on regional BDNF protein levels measured at PN66. Data are collapsed on environment and sex to focus on prenatal alcohol effects. Inset: Sex differences analyzed separately for BDNF levels in the hippocampus after prenatal alcohol treatment; *$p < 0.05$ and **$p < 0.01$).

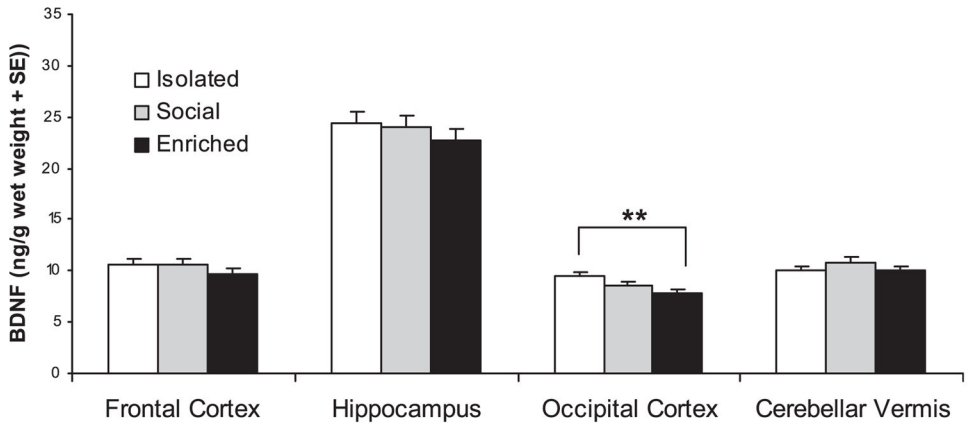

Fig. 4.

Effects of environmental housing on regional BDNF protein levels measured at PN66; $^{**}p < 0.01$. Data are collapsed on alcohol treatment and sex to focus on environmental housing effects.

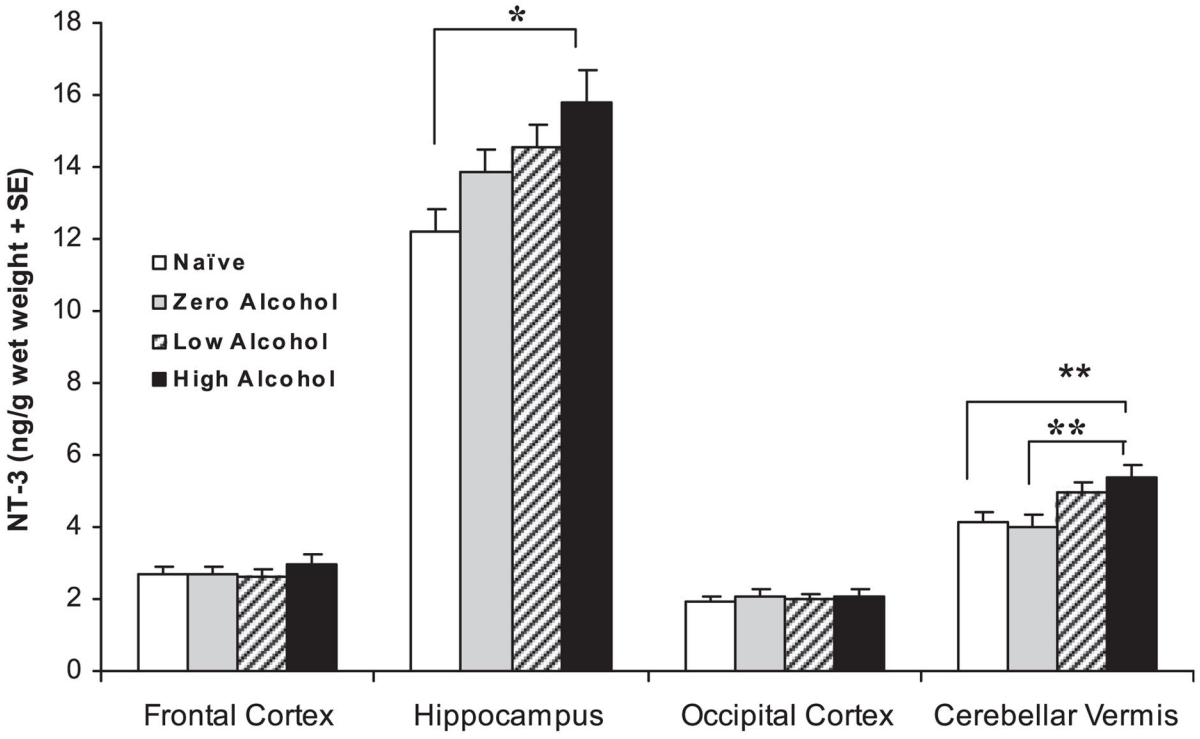

Fig. 5.

Effects of prenatal alcohol exposure on regional NT-3 protein levels measured at PN66; *$p < 0.05$ and **$p < 0.01$. Data are collapsed on environment and sex to focus on prenatal alcohol effects.

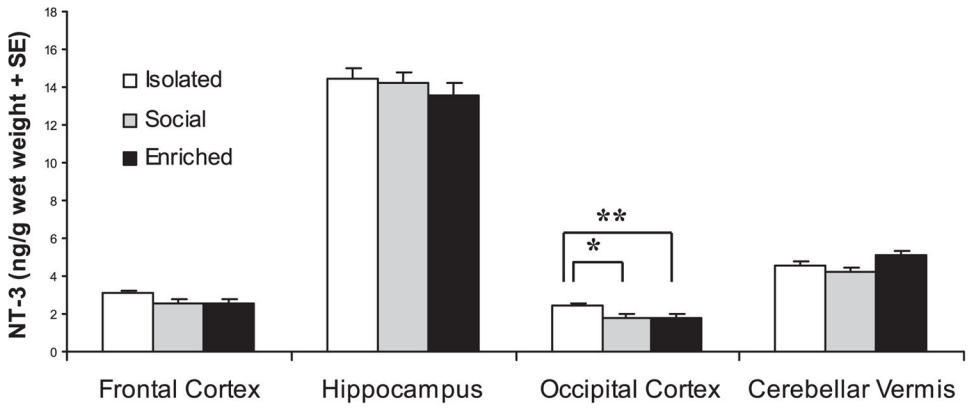

Fig. 6.

Effects of environment on regional NT-3 protein levels measured at PN66; *$p < 0.05$ and **$p < 0.01$. Data are collapsed on alcohol treatment and sex to focus on environmental housing effects.

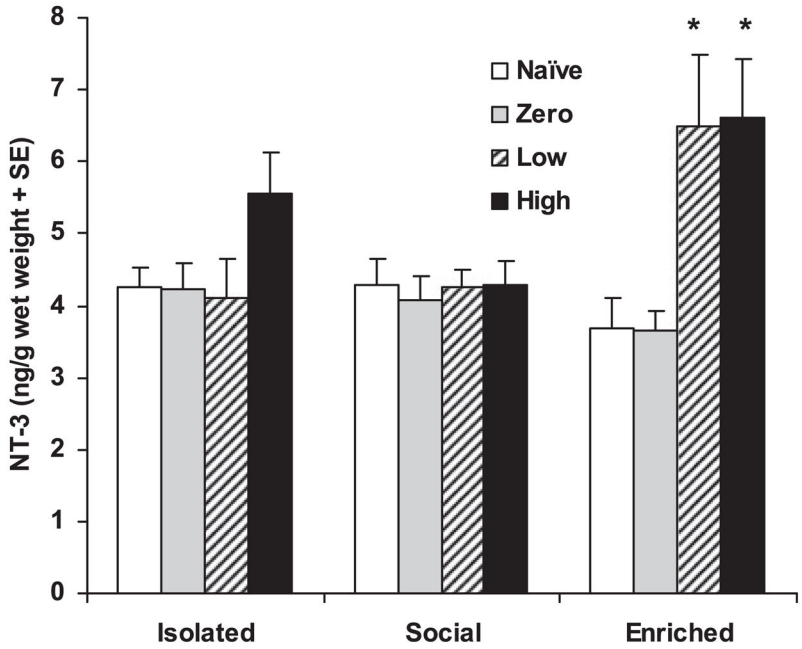

Fig. 7.

Analysis of significant interaction ($p < 0.05$) between prenatal alcohol treatment and environmental housing on NT-3 levels in cerebellar vermis. Under environmental enrichment, NT-3 levels were elevated for the High and Low alcohol groups versus the Naïve and Zero alcohol groups; *$p < 0.05$. There were no significant differences due to prenatal alcohol treatment among the Isolated and Social housed groups.

Table 1

Subjects per Treatment Group

Group	Isolated	Social	Enriched	Total
High (6 g / kg / d)	8 / 4	8 / 4	8 / 5	24 / 13
Low (4 g / kg / d)	11 / 11	13 / 9	12 / 11	36 / 31
Zero (0 g / kg / d)	13 / 9	11 / 8	12 / 8	36 / 25
Naïve (untreated)	9 / 12	9 / 12	9 / 12	27 / 36
Total	41 / 36	41 / 33	41 / 36	123 / 105

The numbers of subjects in each group (male / female). A total of 228 pups from 22 litters were used. Due to the limited amount of tissue sample obtained from each rat it was not possible to carry out enzyme-linked immunosorbant assays for each of the neurotrophins on every sample from every rat.

Table 2

Maternal and Litter Data

	Prenatal alcohol treatment group			
	Naïve	**Zero**	**Low**	**High**
Maternal weight gain (%)	61.2 ± 2.1	62.2 ± 0.5	57.7 ± 2.3	43.1 ± 5.2[a]
Gestational age at birth (days)	22.3 ± 0.2	22.2 ± 0.2	22.3 ± 0.2	23.0 ± 0.0
Total litter size	11.7 ± 0.6	10.3 ± 1.1	11.2 ± 0.5	9.5 ± 1.5
Number of males per litter	4.7 ± 0.4	6.0 ± 0.6	5.8 ± 1.0	6.0 ± 1.4
Number of females per litter	7.0 ± 0.9	4.3 ± 1.0	5.3 ± 1.1	3.5 ± 0.6
Sex ratio (M/F)	0.77 ± 0.17	2.12 ± 0.75	1.68 ± 0.61	1.97 ± 0.73
Male pup weight at PN1 (g)[b]	7.23 ± 0.21	7.38 ± 0.24	7.28 ± 0.34	7.51 ± 0.11
Female pup weight at PN1 (g)	6.89 ± 0.23	7.07 ± 0.17	6.91 ± 0.34	7.03 ± 0.16
Body weight at PN21 (g)[c]	47.7 ± 0.71	49.4 ± 0.74[d]	46.5 ± 0.69	51.1 ± 0.98[d,e]

Mean ± SEM are presented for maternal weight gain (%), various litter characteristics, and pup weights at birth and weaning.

[a] Significantly different from all other prenatal alcohol treatment groups within row ($p < 0.05$);

[b] males weighed significantly more than females on PN1 across all groups ($p < 0.001$);

[c] male and female weight combined;

[d] significantly different from the Low-dose group within row ($p < 0.05$);

[e] significantly different from the Naïve group within row ($p < 0.05$).

Table 3

Sex Differences in NGF, BDNF, and NT-3 Levels

Neurotrophin	Sex	Frontal cortex	Hippocampus	Occipital cortex	Cerebellar vermis
NGF	Males	35.1 (2.1)**	41.6 (1.8)**	36.8 (1.8)**	37.4 (2.0)**
	Females	44.0 (2.2)	48.7 (1.9)	46.6 (2.0)	47.4 (2.2)
BDNF	Males	9.7 (0.4)*	24.4 (1.0)	8.3 (0.3)	9.8 (0.4)
	Females	11.0 (0.4)	26.0 (1.1)	8.9 (0.4)	10.7 (0.4)
NT-3	Males	2.8 (0.1)	13.1 (0.5)**	2.1 (0.1)	4.8 (0.2)
	Females	2.7 (0.2)	15.0 (0.6)	2.0 (0.1)	4.4 (0.2)

Sex differences in neurotrophin levels in various brain regions in 63- to 71-day-old rats. Neurotrophin (ng / g tissue) levels are expressed as mean (SEM). Data are collapsed across alcohol exposure and environmental rearing conditions.

* Males versus females significant at $p < 0.05$ and

** males versus females significant at $p < 0.01$.

BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3.