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Fetal alcohol spectrum disorder-associated depression: Evidence for reductions in the levels of brain-derived neurotrophic factor in a mouse model

Kevin K. Caldwell [a,*], S. Sheema [a], Rodrigo D. Paz [a,b], Sabrina L. Samudio-Ruiz [a], Mary H. Laughlin [c], Nathan E. Spence [c], Michael J. Roehlk [c], Sara N. Alcon [a], Andrea M. Allan [a]

[a] Department of Neurosciences, School of Medicine, University of New Mexico, Albuquerque, New Mexico, 87131, USA
[b] Departamento de Psiquiatría y Neurociencias, Universidad Diego Portales, Santiago, Chile
[c] School of Medicine, University of New Mexico, Albuquerque, New Mexico, 87131, USA

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ABSTRACT

Prenatal ethanol exposure is associated with an increased incidence of depressive disorders in patient populations. However, the mechanisms that link prenatal ethanol exposure and depression are unknown. Several recent studies have implicated reduced brain-derived neurotrophic factor (BDNF) levels in the hippocampal formation and frontal cortex as important contributors to the etiology of depression. In the present studies, we sought to determine whether prenatal ethanol exposure is associated with behaviors that model depression, as well as with reduced BDNF levels in the hippocampal formation and/or medial frontal cortex, in a mouse model of fetal alcohol spectrum disorder (FASD). Compared to control adult mice, prenatal ethanol-exposed adult mice displayed increased learned helplessness behavior and increased immobility in the Porsolt forced swim test. Prenatal ethanol exposure was associated with decreased BDNF protein levels in the medial frontal cortex, but not the hippocampal formation, while total BDNF mRNA and BDNF transcripts containing exons III, IV or VI were reduced in both the medial frontal cortex and the hippocampal formation of prenatal ethanol-exposed mice. These results identify reduced BDNF levels in the medial frontal cortex and hippocampal formation as potential mediators of depressive disorders associated with FASD.

1. Introduction

Prenatal ethanol exposure has been shown to be associated with a spectrum of neurochemical, physiologic, and behavioral dysfunctions that affect various aspects of an individual's life. The range of clinical abnormalities that are associated with prenatal ethanol exposure is termed fetal alcohol spectrum disorder (FASD). The incidence of FASD is estimated to be as high as 1 in 100 births (Sampson et al., 1997). Although the most common manifestation of FASD is a reduction in cognitive abilities (Sampson et al., 1997; Streissguth and O'Malley, 2000), FASD is also comorbid with various psychiatric illnesses, including depression (Famy et al., 1998; O'Connor and Kasari, 2000; O'Connor et al., 2002; O'Connor and Paley, 2006; Roebuck et al., 1999). Several genetic and biochemical abnormalities have been shown to be associated with depression (Berton and Nestler, 2006; Duman and Monteggia, 2006; Hasler et al., 2007; Jans et al., 2007; Levinson, 2006); however, none of these has been implicated as a mechanism linking prenatal ethanol exposure and depressive disorders. Therefore, we sought to determine whether depressive-like behaviors are exhibited by a mouse model of FASD and, if so, to investigate the relationship between these behaviors and a mechanism that has been proposed to contribute to depressive behavior, reductions in hippocampal formation and medial frontal cortical brain-derived neurotrophic factor (BDNF) levels.

Several lines of evidence indicate that reduced BDNF levels in the prefrontal cortex, hippocampus, or both play an important role in depression. BDNF expression is diminished in postmortem hippocampus and prefrontal cortex collected from suicide cases with untreated depression compared to patients who were being treated with antidepressants at the time of suicide (Chen et al., 2001; Karege et al., 2005). Experimental paradigms, such as learned helplessness and the forced swim task, which induce behaviors modeling depression in laboratory animals, have been shown to cause reductions in BDNF protein and mRNA levels in the frontal cortex and/or hippocampus (Itoh et al., 2004; Russo-Neustadt et al., 2001; Song et al., 2006; Takeda et al., 2006). Additionally, elevated BDNF protein levels have been shown to block depressive-like behaviors in these same paradigms (Shirayama et al., 2002; Siuciak et al., 1997). Finally, long-term antidepressant therapy has been shown to increase BDNF levels in the hippocampus (Nibuya et al., 1995; Okamoto et al., 2003; Dwivedi et al., 2006) and frontal cortex (Altar et al., 2003; Okamoto et al., 2003). These results, combined with the observation

* Corresponding author. Department of Neurosciences, MSC 08 4740, 1 University of New Mexico, Albuquerque, NM 87131, USA. Tel.: +1 505 272 0033; fax: +1 505 272 8082.
E-mail address: KCaldwell@salud.unm.edu (K.K. Caldwell).

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that therapeutic responses to antidepressants generally require chronic administration of the drugs, have led to the "neurotrophic hypothesis" of depression (reviewed in Duman and Monteggia, 2006).

Prenatal exposure to ethanol has been reported to either decrease or have no effect on BDNF protein levels measured in the postnatal rat brain. Heaton et al. (2000) reported that prenatal ethanol exposure did not affect BDNF protein levels measured in hippocampal formation or the cortex/striatum on postnatal day 1. Feng et al. (2005) found that prenatal ethanol exposure reduced BDNF protein levels in postnatal days 7 and 8 cortex and hippocampal formation. Similarly, Climent et al. (2002) reported that prenatal ethanol exposure reduced BDNF protein levels in the cortex, when measured on postnatal days 5–21, while pups were being nursed by ethanol-consuming moms; however, levels were not different from controls on postnatal day 35 after weaning. We are unaware of any published studies on the effects of prenatal ethanol exposure on BDNF levels in the brains of adult rodents.

The studies described herein demonstrate that adult mice that were exposed to ethanol throughout gestation display depressive-like behaviors, manifested as augmented learned helplessness and immobility in the forced swim test. In addition, we found that prenatal exposure to ethanol was associated with reduced levels of BDNF protein, as well as mRNA, in the frontal cortex and reduced BDNF mRNA levels in the hippocampal formation. These studies demonstrate that a mouse model of FASD displays depressive-like behaviors that share similarities with clinical depression exhibited by diagnosed FASD patients and, thus, should serve as a useful tool for the identification of neurochemical alterations that underlie this disorder.

2. Materials and methods

2.1. Animals

All animal procedures employed in these studies were approved by the University of New Mexico Institutional Animal Care and Use Committee. To reduce possible litter effects, only one mouse was used from each litter for each of the assays conducted, thus the unit of analysis was litter. The offspring from 19 ethanol-consuming and 16 saccharin-consuming dams were used in these studies.

2.2. Prenatal ethanol exposure paradigm

The procedures employed for prenatal ethanol exposure were essentially as described in Allan et al. (2003). Briefly, female C57BL/6J mice (The Jackson Laboratory; Bar Harbor, ME) were offered 22 h free access to either 0.066% saccharin or water for 1 week. Following this period, ethanol was introduced to the saccharin tube for the ethanol group, while the control group continued to have access to saccharin alone. The ethanol concentration was increased from 0% to 5% in a stepwise fashion over 5 days. After 2 weeks of stable drinking, females were placed on breeder chow and a male was introduced into the female's cage. Once the female was determined to be pregnant, the male was removed, nesting material was added to the cage, and the female continued to consume ethanol or saccharin. Ethanol consumption was measured every other day and the g ethanol consumed/kg body weight/day were calculated for each dam. Within 1 day of birth, the ethanol and the saccharin concentrations were reduced by one-half every 2 days until the mice were consuming water postpartum. We have found that this procedure yields maternal care behaviors that are indistinguishable between saccharin- and ethanol-consuming moms (Allan et al., 2003) and, thus, avoids the introduction of the confound of poor maternal care that can occur with postpartum maternal consumption of ethanol or sudden ethanol withdrawal. Pups were weaned at 23–25 days and maintained in same-sex and litter-mate cages, 3–4 mice per cage, with free access to water and chow until adulthood.

2.3. Measurement of blood ethanol concentrations

Maternal blood ethanol concentrations were determined as previously described (Allan et al., 2003). Blood samples were collected from the saphenous vein of mice that were approximately 14 days pregnant during the middle of the drinking period. These measures were performed on a separate group of dams that were drinking at a rate similar to that of the dams whose offspring were used in the present studies.

2.4. Behavioral testing procedures

Mice were maintained on a 12 h light:12 h dark cycle (lights on 7:00 AM). Behavioral testing was conducted between 9 AM and 3 PM. All studies were performed using female mice between 60 and 90 days of age at the time of testing. Animals were used in only one behavioral test.

2.5. Learned helplessness

The apparatus for the learned helplessness task consisted of a Coulbourn™ Habitest© shuttlebox with a stainless steel grid floor for administration of the footshock. The apparatus was located within a sound-attenuated chamber. 70% isopropanol was used to clean the walls and floor after the removal of each mouse from the shuttlebox.

The method for assessing learned helplessness was modified from Caldarone et al. (2004). On Day 1, animals received 20 uncontrollable and unpredictable footshocks (0.5 mA, 2 s duration); the probability for delivery of the footshock was assigned at 0.5 every 15 s. The animal was removed 30 s after the delivery of the last shock and returned to its home cage. We note that, although many published procedures for the production of learned helplessness employ more than 100 inescapable footshocks to an animal (Caldarone et al., 2000; Caldarone et al., 2004; MacQueen et al., 2001; Newton et al., 2002; Ridder et al., 2005; Schulte-Herbrüggen et al., 2006), we were able to identify increased levels of learned helplessness in prenatal ethanol-exposed mice with the use of this milder procedure. On the testing day (Day 2), animals experienced 28 test trials with an intertrial interval of 30 s. For the first five trials, a footshock (0.5 mA) and auditory cue (80 dB, 6 Hz clicker) both began at the start of the trial; after a 1 s delay, the guillotine door separating the two chambers of the shuttlebox opened. The last 23 trials initiated with the tone and the door rising, and the onset of the footshock was delayed for 3 s. Sensors in each compartment of the shuttlebox turned off the footshock and tone and closed the guillotine door, if the animal escaped through the raised door. If an escape was not made within 24 s from the initiation of the trial, the shock and tone terminated and the door closed. The subject's response in each trial was scored as an "escape", if it fled through the door to the non-shock side of the chamber within the 24 s test session, or as a "failure to escape", if the animal remained in the side of the apparatus in which the shock was being delivered. In addition, latency for the mouse to escape through the door was measured as the time from the door rising to the time it closed.

2.6. Forced swim test

Mice were placed in a 30 cm diameter, 46 cm tall cylinder of water (22–25 °C, depth 26 cm) for 3 min, as described previously (Porsolt et al., 1977; Sunal et al., 1994). Six behaviors were assessed every 5 s during the last 2 min of the session, as described by Schramm et al. (2001): floating, twitching, kicking, swimming, climbing, and

thrashing. The combination of floating, twitching and kicking behaviors was used as an index of immobility, with the remaining behaviors representing escape-directed behavior.

2.7. Basal open field activity

Mice were placed in a dim red light illuminated open field apparatus (102.5×102.5×47.5 cm; Opto-Varimex, Columbus Instruments, Columbus, OH) for 15 min on two consecutive days. The first day was used for acclimation to the task. The open field apparatus was equipped with a series of photo-beams (15×15). Open field locomotor (horizontal) activity was measured as the number of photo beam interruptions recorded on the second day.

2.8. General information related to animals used in the biochemical and molecular studies

Mice were maintained on the same light:dark cycle and were of the same age range as used for the behavioral studies. All studies were performed using female mice that were euthanized between 9 AM and 3 PM. Additionally, none of the animals used in the behavioral studies were used for the biochemical and molecular analyses, which were performed on naïve mice.

2.9. Determination of BDNF protein levels

BDNF protein levels were determined in 96-well ELISA plates (Chemicon, Temecula, CA), as described by the manufacturer.

2.10. Determination of proBDNF levels

The hippocampal formation and medial frontal cortex were isolated, then homogenized in 0.5 mL ice-cold homogenization buffer (Buckley et al., 2004) and centrifuged (1000 $\times g_{max}$, 6 min, 4 °C). The supernatant was decanted and the pellet resuspended, homogenized and centrifuged as before. The supernatant from the second centrifugation step was combined with the initial supernatant and frozen in aliquots at −80 °C until use. Samples (30 μg medial frontal cortex; 70 μg hippocampal formation) were thawed, diluted in 4× SDS-PAGE Sample Buffer containing NuPAGE Reducing Agent (Invitrogen Corporation, Carlsbad, CA) and separated on 12% NuPAGE Bis–Tris Polyacrylamide Gels (Invitrogen Corporation) using MOPS SDS Running Buffer containing NuPAGE Antioxidant (Invitrogen Corporation). Proteins were transferred to PVDF (Bio-Rad Laboratories, Hercules, CA) membranes in NuPAGE Transfer Buffer (Invitrogen Corporation) containing NuPAGE Antioxidant and methanol. The membranes were blocked with 3% (w/v) BSA in TBST [150 mM NaCl, 20 mM Tris (pH 7.5), 0.05% (v/v) Tween 20] for 60 min at room temperature, then incubated overnight with affinity purified anti-proBDNF rabbit antibody (Chemicon #AB5613P; 0.25 μg/mL) in BSA/TBST at 4 °C. The membrane was washed 4×5 min with TBST and incubated with HRP-conjugated secondary antibody (HRP-Goat-Anti-Mouse-Anti-IgG (H+L); Jackson ImmunoResearch) in BSA/TBST for 90 min at room temp. The membrane was washed 4×5 min with TBST and immunoreactivity detected using a Supersignal West Pico Chemiluminescent Kit (Pierce Biotechnology Inc). Luminescence was detected with film, digitized and quantified. Arbitrary units of immunoreactivity were assigned based on the measured object density divided by the amount of protein loaded onto the gel.

2.11. mRNA isolation

mRNA was isolated using the Oligotex Direct mRNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The mRNA concentration was determined (OD 260 nm) using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Purified mRNA was stored at −80 °C.

2.12. cDNA synthesis

Reverse transcription reactions were performed in 20 μL following the manufacturer's protocol (Invitrogen Corporation). Briefly, a 10 μL reaction mixture containing 10 ng mRNA, 500 ng oligo dT, 1 mM each dNTP was heated at 65 °C for 5 min and chilled on ice for 5 min, followed by addition of 1 μL (200 units) M-MLV reverse transcriptase, 1 μL RNaseOUT (40 units/μL), 2 μL DTT (0.1 M) and sterile RNAase free water to 20 μL. The whole reaction mixture was incubated at 37 °C for 50 min, then 75 °C for 10 min. The synthesized cDNA was stored at −20 °C until used. The suitability of the cDNA for PCR was determined using 2 μL of cDNA added to a 40 μL PCR. A control reaction containing 2 μl of purified mRNA was run without reverse transcription to test for presence of DNA contamination in the purified mRNA samples. The PCR was analyzed on a 2% (w/v) agarose gel.

2.13. Primers

Oligonucleotide sequences (Table 1) specific to mouse BDNF exons I–IV, VI and IX, and to β actin, cyclophilin A, hypoxanthine phosphoribosyltransferase 1 (HPRT1), and 18S rRNA were designed using Primer Express software (Applied Biosystems, Foster City, CA). The primers used for BDNF exons I, II, IV, and VI were based on sequences reported by Tsankova et al. (2006). The specificity of each primer pair was confirmed by the identification of a single PCR product of predicted size on agarose gels followed by sequencing of the excised band (sequencing performed by the UNM Proteomic and Molecular Biology Core facility).

2.14. Semi-quantitative determination of transcript levels by Real Time-PCR

Real time PCR was conducted in a Gene Amp 7700 sequence detection system (Applied Biosystems) using a 96-well plate (MicroAmp™ Fast Optical 96-well Reaction plates and MicroAmp™ Optical Adhesive Film, both from Applied Biosystems). Reactions were performed in triplicate. Each 20 μL reaction contained forward and reverse primers (100 nM each, final concentration), 10 μL Power Sybr® Green PCR Master Mix reaction buffer (Applied Biosystems) and 2 μL cDNA. Following one initial step of 95 °C for 10 min, the cycling parameters were 95 °C for 15 s, 60 °C for 1 min, and 40 cycles. The PCR products were monitored by measuring the increase in fluorescence caused by binding of Sybr® Green Dye to the double-stranded DNA; Ct (cycle threshold) values were calculated by SDS software v1.9 (Applied Biosystems). The absence of nonspecific amplification was confirmed by analyzing PCR products by 2% (w/v) agarose gel.

In studies designed to identify an internal control to be used in analyses of BDNF gene expression, we assessed the expression of several housekeeping genes (β-actin, cyclophilin A, HPRT1, and 18S rRNA) using Ct values derived from Real-Time PCR. Reactions were performed in triplicate. The relative quantification of the BDNF gene in different tissue samples was determined using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001). Initially, the ΔCt value, the

Table 1
Sequences (5′→3′) of the forward and reverse primer pairs used for PCR amplification

Target	Forward primer	Reverse primer
BDNF exon I	CCTGCATCTGTTGGGGAG	GCCTTGTCCGTGGACGTTT
BDNF exon II	GGGTTGGTATACTGGGTT	GGATGGTCATCACTCTTCTC
BDNF exon III	GGGCCGGATGCTT	CCGTGGACGTTTACTTC
BDNF exon IV	CAGAGCAGCTGCCTTGAT	GCCTTGTCCGTGGACG
BDNF exon VI	CTGCCACCACTGCCTTGG	GGATGGTCATCACTCTTC
BDNF exon IX	CAGGTGAGAAGAGTGATGACC	ATTCACGCTCTCCAGAGTCCC
β-actin	CCATCATGAAGTGTGACGTGG	GTCCGCCTAGAAGCATTTGCG
Cyclophilin A	TGCTGGACCAAACACAAACG	GCCTTCTTTCACCTTCCCAAA
HPRT1	AAGACTTGCTCGAGATGTCATGAA	ATCCAGCAGGTCAGCAAAGAA
18S rRNA	CGGCTACCACATCCAAGGAA	CCTGTATTGTTATTTTTCGTCACTACCT

Table 2
Maternal weights and litter sizes for saccharin (SAC) control and ethanol-consuming mice

Prenatal diet/ exposure	Maternal weight at beginning of drinking (g)	Maternal weight Day 6–7 after birth (g)	Litter size
SAC	20.97 (±2.63)	29.91 (±2.22)	5.9 (±2.29)
Ethanol	21.50 (±3.23)	29.20 (±3.23)	6.3 (±1.99)

Maternal weights were determined at the beginning of the drinking paradigm, as well as throughout drinking, and on postpartum days 6–7. Litter sizes were determined on Days 6–7 after delivery. The data are from six separate breeding rounds and are expressed as mean ± SEM; SAC (n=47) and ethanol (n=50) dams.

difference between the average of the triplicate Ct values for the BDNF and the internal control β actin, for each sample was calculated ($\Delta Ct_{sample} = \Delta Ct_{BDNF} - \Delta Ct_{\beta\ actin}$). Next, the average of the ΔCt values for the saccharin control animals ($\Delta Ct_{SAC\ av}$) was determined and subtracted from each sample ($\Delta Ct_{sample} - \Delta Ct_{SAC\ av}$), giving a ΔΔCt value for each. This, in turn, was used to calculate the $2^{-\Delta\Delta Ct}$ value for each. Because 2^0 is one, the mean $2^{-\Delta\Delta Ct}$ value for the saccharin control samples was approximately one, depending on the variability in the samples, and the mean $2^{-\Delta\Delta Ct}$ value for the ethanol-exposed mouse samples was expressed relative to one.

2.15. Statistical analysis

All data were analyzed using a Student two-tailed t-test except the analyses of the regression lines and curves for the learned helplessness data, which were analyzed by ANOVA, using GraphPad Prism (version 4.0).

3. Results

3.1. Fetal alcohol exposure paradigm

The present studies were conducted using C57BL/6J mice and a voluntary two-bottle choice paradigm for prenatal ethanol exposure (Allan et al., 2003). The average consumption of ethanol by the mothers of the mice used in the present studies was 9.55 (±2.57) g ethanol/kg body weight/day. In a concurrent study, we found that the average maternal blood ethanol concentration was 0.78 mg/mL in a group of C57BL/6 dams (n=7) whose average daily consumption of ethanol was 8 g/kg body weight. Across six breeding rounds, including the four from which the animals used in the present studies were derived, we observed that there were no differences between the weight gained by saccharin control or ethanol-consuming dams, nor were there differences in litter size (Table 2).

3.2. Prenatal ethanol-exposed mice display increased learned helplessness

Learned helplessness is a long-standing test for assessing depressive-like behavior (Maier, 1984; Caldarone et al., 2000; El Yacoubi and Vaugeois, 2007; McArthur and Borsini, 2006; Nestler et al., 2002b). The learned helplessness procedure that was employed in the present studies exposed saccharin control and ethanol-exposed mice to a series of inescapable footshocks, then in a subsequent test session assessed whether the animals escaped the footshock, when given the opportunity to do so; if escape occurred, the latency to escape was also recorded. The data for the % of test subjects that escaped (Fig. 1, left) were best fit by the equation for a hyperbola, whereas the data for the escape latency (Fig. 1, right) were best fit by a straight line.

Both saccharin and prenatal ethanol-exposed animals displayed increased escape behavior across the 28 testing trials, as demonstrated by an increased % of animals that escaped (Fig. 1, left) and decreased mean escape latencies (Fig. 1, right) as testing progressed. The percentage of animals that escaped was greater for the saccharin control mice than for the prenatal ethanol-exposed mice throughout the trials. Whereas nearly all of the control animals escaped from trial 15 onward, approximately 40% of the prenatal ethanol-exposed mice failed to escape in these later trials, demonstrating learned helplessness, or depressive-like behavior. The best-fit curves for the saccharin control and prenatal ethanol-exposed data sets were significantly different from each other [$F(2,52)=44.0$, $p<0.0001$] (Fig. 1 Left). The similar slopes of the regression lines fitting the escape latency data indicate that the rate of improvement in escape behavior was similar in both groups [$F(1,445)=33.0$, $p<0.01$] (Fig. 1 Right). However, the line fitting the prenatal ethanol-exposed mouse data is elevated relative to that for the saccharin data, indicating that prenatal ethanol-exposed mice displayed increased learned helplessness behavior throughout the testing session.

3.3. Prenatal ethanol-exposed mice display increased immobility in the Porsolt forced swim test

As a second test for depressive-like behavior, we assessed the behavior of prenatal ethanol-exposed and saccharin control mice in the forced swim test, originally described by Porsolt et al. (1977). In this test, an animal is placed into a container of water, with the level of

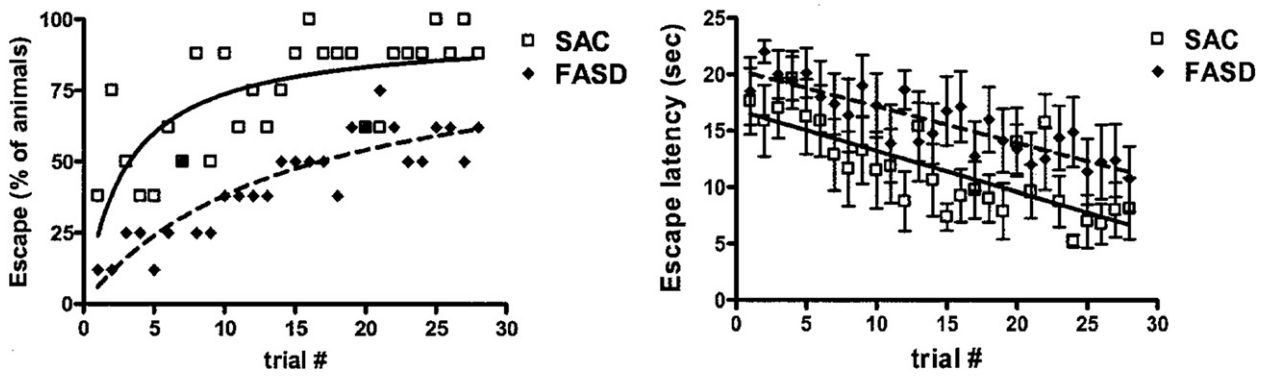

Fig. 1. Prenatal ethanol-exposed mice display increased learned helplessness. Learned helplessness behavior was assessed as described in Materials and methods section. Data are graphed as a function of trial number; saccharin control (SAC) and ethanol-exposed (fetal alcohol spectrum disorder, FASD) mice (n=8) were used in this study. Left: Percentage of animals that escaped. The data were fit to the equation of a hyperbola using GraphPad Prism (version 4.0); the best-fit curves are shown. The curves fitting the saccharin control and prenatal ethanol-exposed data sets were significantly different from each other [$F(2,52)=44.0$, $p<0.0001$]. Right: Mean+SEM escape latencies Comparisons of different models [polynomial first order (straight line), one phase exponential decay curve, two phase exponential decay curve, hyperbola (one site binding)] using GraphPad Prism (version 4.0) found that both data sets were best fit by a polynomial first order (straight line). Linear regression analyses of the two data sets revealed that the slopes of the two lines were not different but the intercepts were significantly different [$F(1,445)=33.0$, $p<0.01$].

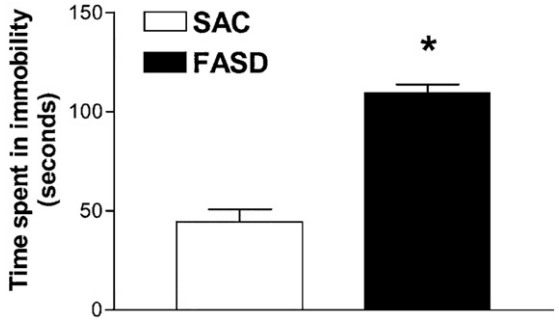

Fig. 2. Prenatal ethanol-exposed mice display increased immobility in the forced swim test. Time spent in immobile behaviors, (floating, twitching, kicking) in seconds for saccharin (SAC) control (n=8) and prenatal ethanol-exposed (fetal alcohol spectrum disorder, FASD) (1=8) mice during the last 2 min of the 3 min of the swim task. $t(14)$= 8.41, * indicates $p<0.01$.

water sufficiently high to prevent the animal from touching the bottom of the container without submerging its body. Initially, the test subject exhibits gross motor movements (swimming, climbing, and thrashing) that are indicative of escape behavior, then eventually becomes relatively immobile, either floating or making only small motor movements (twitching and kicking), which are indicative of a state of despair (Porsolt et al., 1977). Fig. 2 shows that, compared to saccharin control mice, mice exposed to ethanol prenatally displayed increased immobility (i.e., increased depressive behavior) in the forced swim test ($t(14)=8.41$, $p<0.0001$).

3.4. Prenatal ethanol-exposed and saccharin control mice do not differ in locomotor activity

Behavioral responses in both the learned helplessness and forced swim tests are dependent, in part, on an animal's motor skills; therefore, the observed depressive-like behaviors of the prenatal ethanol-exposed mice in these tests could, at least in part, be due to reduced locomotor activity in these animals, compared to saccharin control mice. To address this issue, we assessed locomotor activity in the two groups of mice under non-anxiogenic conditions (i.e., dim light illumination). We found no difference between the locomotor activity of prenatal ethanol-exposed and saccharin control mice (Fig. 3).

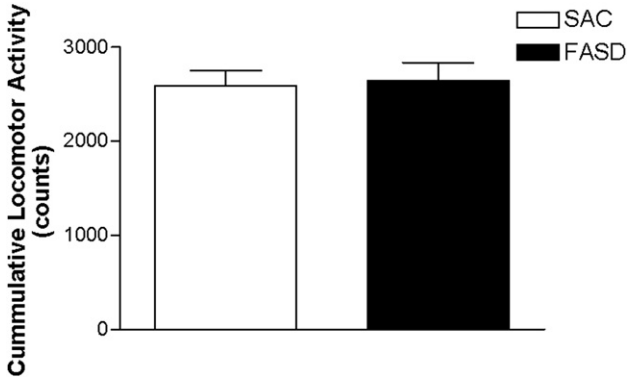

Fig. 3. Prenatal ethanol-exposed and saccharin control mice do not differ in locomotor activity. Locomotor activity, measured as counts (beam interruptions), over a 15-min period was measured in saccharin (SAC) control (n=8) and prenatal ethanol-exposed (fetal alcohol spectrum disorder, FASD) (n=9) mice, as described in Materials and methods.

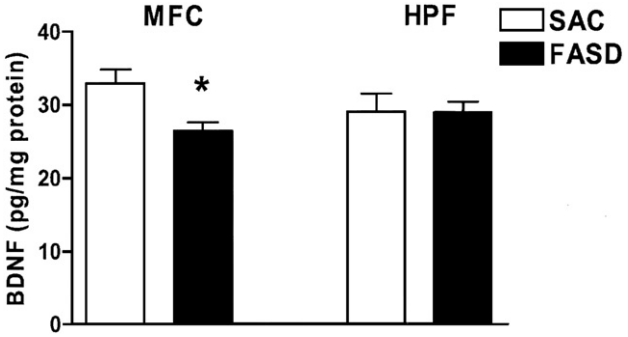

Fig. 4. Prenatal ethanol exposure is associated with reduced BDNF levels in the medial frontal cortex, but not hippocampal formation, of mice. Homogenates of the medial frontal cortex (MFC) and hippocampal formation (HPF) were prepared from adult saccharin (SAC) (n=8) and prenatal ethanol-exposed (fetal alcohol spectrum disorder, FASD) (n=7) mice and BDNF levels were quantified by ELISA. Data were expressed relative to the wet weight of the tissue sample. Compared to BDNF levels present in the medial frontal cortex of saccharin control mice, BDNF levels were significantly reduced in the medial frontal cortex of prenatal ethanol-exposed mice: $t(13)=2.7$, * indicates $p<0.02$.

3.5. Basal BDNF protein levels are reduced in the medial frontal cortex, but not the hippocampal formation, of ethanol-exposed mice

Elevated learned helplessness susceptibility has been correlated with reduced basal BDNF levels in the frontal cortex and hippocampal formation (Ridder et al., 2005); further, infusions of BDNF into the hippocampus (Shirayama et al., 2002), midbrain (Siuciak et al., 1997) or cerebral ventricles (Hoshaw et al., 2005) produce antidepressant effects when animals are subsequently tested in the learned helplessness (Shirayama et al., 2002; Siuciak et al., 1997) or forced swim (Hoshaw et al., 2005; Shirayama et al., 2002; Siuciak et al., 1997) tests. Thus, we sought to determine whether the observed increases in learned helplessness behavior and immobility in the forced swim test of prenatal ethanol-exposed mice were accompanied by decreased BDNF protein in the medial frontal cortex and/or hippocampal formation. Fig. 4 shows that, compared to saccharin control mice, BDNF levels were reduced in the prenatal ethanol-exposed mouse frontal cortex ($t(13)=2.7$, $p<0.02$), but not in the hippocampal formation.

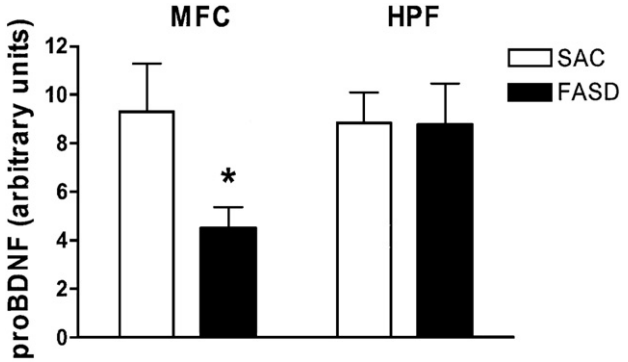

Fig. 5. Prenatal ethanol exposure is associated with reduced proBDNF levels in the medial frontal cortex, but not the hippocampal formation. Postnuclear (1000 g_{max}, 6 min) fraction were prepared from the medical frontal cortex (MFC) and hippocampal formation (HPF) of adult saccharin (SAC; n=6 for HPF; n=8 for MFC) and prenatal ethanol-exposed (fetal alchol spectrum disorder, FASD) (n=6 for HPF; n=11 for MFC) mice. proBDNF levels were analyzed by immunoblottig (see Methods). Data are presented as arbitrary units (defined as the adjusted volume measurement obtained from the immunoblot images divided by the amount of protein loaded onto the gel). Compared to proBDNF levels in the medial frontal cortex of saccharin control mice, proBDNF levels were significantly reduced in the medial frontal cortex of prenatal ethanol-exposed mice: $t(17)=2.44$, * indicates $p<0.02$.

3.6. Prenatal alcohol exposure reduces proBDNF levels in the medial frontal cortex

BDNF, like other neurotrophins, is synthesized in a precursor form, termed proBDNF (Lu, 2003), a 32-kDa protein that is cleaved to the 14-kDa mature BDNF (Mowla et al., 2001). BDNF is formed extracellularly, by the action of plasmin (Pang et al., 2004), and intracellularly, both in the trans-Golgi network by furin and in secretory granules by convertases, including PACE4 and PACE5/6-B (Mowla et al., 2001; Seidah et al., 1996).

In order to begin addressing the mechanism(s) that underlies the observed decrease in BDNF protein in the medial frontal cortex, we analyzed proBDNF protein levels in this brain region, as well as in the hippocampal formation (Fig. 5). Immunoblotting data showed that anti-proBDNF immunoreactivity was decreased in the medial frontal cortex ($t(17)=2.44$, $p<0.02$), but not the hippocampal formation, of mice exposed prenatally to ethanol, thus paralleling the data for BDNF protein levels.

3.7. Prenatal alcohol exposure is associated with reduced BDNF mRNA transcript levels in the medial frontal cortex and hippocampal formation

We sought to determine whether reduced BDNF mRNA levels may contribute to the observed decrease in medial frontal cortical proBDNF and BDNF protein. For these studies, it was necessary to identify a suitable internal standard for semi-quantitative (relative) Real-Time PCR analyses. Commonly used internal controls include β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin A/B, HPRT1, and 18S rRNA. Several reports have found effects of experimental manipulations on the expression of these genes (e.g., Maier et al., 1999; Mogal and Abdulkadir, 2006; Verma and Shapiro, 2006; Tanic et al., 2007; Nishimura et al., 2008). Prenatal ethanol exposure has been reported to alter β-actin expression in embryos (Green et al., 2007) and neonatal cerebellum (Zoeller et al., 1994), but not neonatal or adolescent intestine (Bhalla et al., 2004). We compared cyclophilin A, HPRT1, 18S rRNA, and β-actin expression in saccharin control and prenatal ethanol-exposed adult hippocampal formation and medial frontal cortex (Fig. 6). Prenatal exposure to ethanol did not alter the levels of β-actin or HPRT1 in either brain region, whereas it was associated with significantly increased 18S rRNA levels in the hippocampal formation ($t(14)=3.64$, $p<0.01$) and reduced cyclophilin A levels in the medial frontal cortex ($t(14)=5.16$, $p<0.01$). Based on these data, we chose to use β-actin as an internal control during analyses of BDNF transcript expression.

The mouse BDNF gene consists of multiple noncoding exons upstream of a common coding exon (Fig. 7A). Until recently, most studies had identified six exons in the mouse gene, whereas Liu et al. (2006) identified eight exons and Aid et al. (2007) identified nine exons in the murine BDNF gene. We chose to follow the nomenclature of Aid et al. (2007). Exons are designated using Roman numerals, according to their relative position, with I being the 5′-most exon. Exons I–IXa are noncoding exons, while exon IX contains the open reading frame that encodes proBDNF. In order to measure individual BDNF mRNA transcripts, we used PCR primer pairs consisting of a forward primer specific for the upstream, non-coding exons I–VI, excluding exon V, and a reverse primer specific for the coding exon IX. Fig. 7B shows that, in both the medial frontal cortex and hippocampal formation, prenatal ethanol exposure was associated with

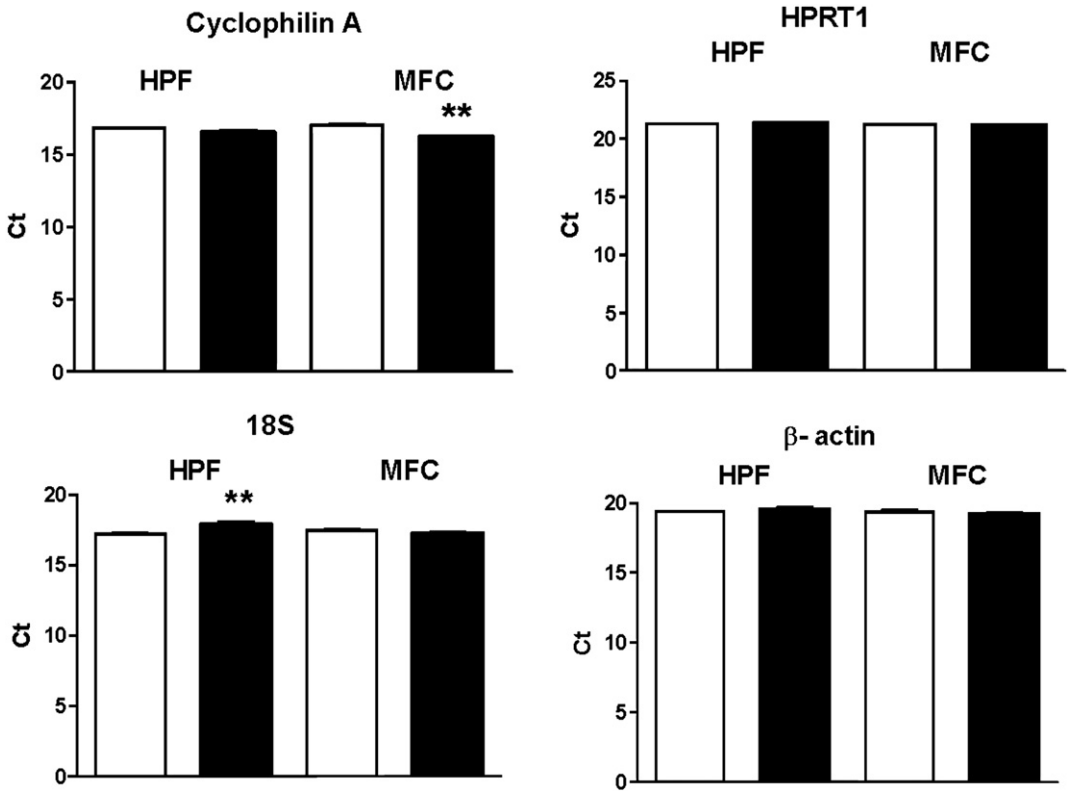

Fig. 6. Expression of housekeeping genes, cyclophilin A, HPRT1, 18S rRNA, and β-actin, in saccharin control and prenatal ethanol-exposed adult mouse hippocampal formation and medial frontal cortex. Levels of mRNA transcripts for cyclophilin A, HPRT1, 18S rRNA, and β-actin were determined as described in Materials and methods. Cycle threshold (Ct) values were determined in triplicate for prenatal ethanol-exposed (fetal alcohol spectrum disorder, FASD) ($n=8$) and saccharin (SAC) control ($n=8$) samples in both the hippocampal formation (HPF) and medial frontal cortex (MFC). Statistical significance was determined using a two-tailed *t*-test: cyclophilin A SAC vs. FASD in the MFC, $t(14)=5.16$; 18S rRNA SAC vs. FASD in the HPF, $t(14)=3.64$. ** designates $p<0.01$.

significantly decreased levels of BDNF total mRNA (exon IX-containing transcripts; medial frontal cortex, $t(14)=3.7$, $p<0.01$; hippocampal formation, $t(14)=3.9$, $p<0.01$) and BDNF mRNA transcripts containing exon III (medial frontal cortex, $t(14)=2.8$, $p<0.02$; hippocampal formation, $t(14)=4.2$, $p<0.01$), IV (medial frontal cortex, $t(14)=8.1$, $p<0.01$; hippocampal formation, $t(14)=6.2$, $p<0.01$), or VI (medial frontal cortex, $t(14)=8.6$, $p<0.01$; hippocampal formation, $t(14)=3.6$); $p<0.01$), but not those containing exons I or II.

4. Conclusions

4.1. FASD mice display depressive-like behaviors

We have identified increased learned helplessness, as well as increased immobility in a forced swim test, in adult mice prenatally exposed to moderate levels of ethanol. Thus, using two well accepted tests for behaviors that model depression, we found that prenatal ethanol exposure is associated with depressive-like behavior in a mouse FASD model. These findings in prenatal ethanol-exposed mice confirm and extend those of Slone and Redei (2002), Wilcoxon et al. (2005), and Carneiro et al. (2005), who reported that adult rats that were exposed prenatally to ethanol displayed increased immobility in the forced swim test.

The escape response in the learned helplessness test includes a learning component; thus, it can be argued that the observed increase in escape latency and escape failures in prenatal ethanol-exposed mice is a consequence of their known learning impairments (Allan et al., 2003). However, this is refuted by our finding that the slopes of the regression lines fitting the escape latency data for the ethanol-exposed and saccharin mice were similar, indicating that the two groups learned the escape response at comparable rates. The observed increased immobility in the forced swim test, which does not involve learning, also supports our conclusion that prenatal ethanol-exposed mice display behaviors that model depression. It can also be argued that the observed increase in escape latency in the learned helplessness task and increased immobility in the forced swim task are due to reduced locomotor activity in the prenatal ethanol-exposed mice. However, these mice do not differ from saccharin control mice in their locomotor activity in an open field, further supporting the conclusion that the behaviors in the learned helplessness and forced swim tests represent depressive-like behaviors.

4.2. BDNF protein and mRNA levels are reduced in the brain of prenatal ethanol-exposed adult mice

Several hypotheses have been put forth regarding the genetic and biochemical bases of depression (Hayley et al., 2005; Jans et al., 2007; Levinson, 2006; Nestler et al., 2002a). Many of these have focused on mechanisms underlying the compromised functioning of the medial frontal cortex and hippocampal formation which is observed in depressed individuals (Berton and Nestler, 2006; Drevets, 2000; Johnstone et al., 2007; Phillips et al., 2003a,b). Consistent with the neurotrophic hypothesis of depression, we found that proBDNF and BDNF protein levels were reduced in the medial frontal cortex of prenatal ethanol-exposed mice compared to saccharin control mice; however, proBDNF and BDNF protein levels were not different in the

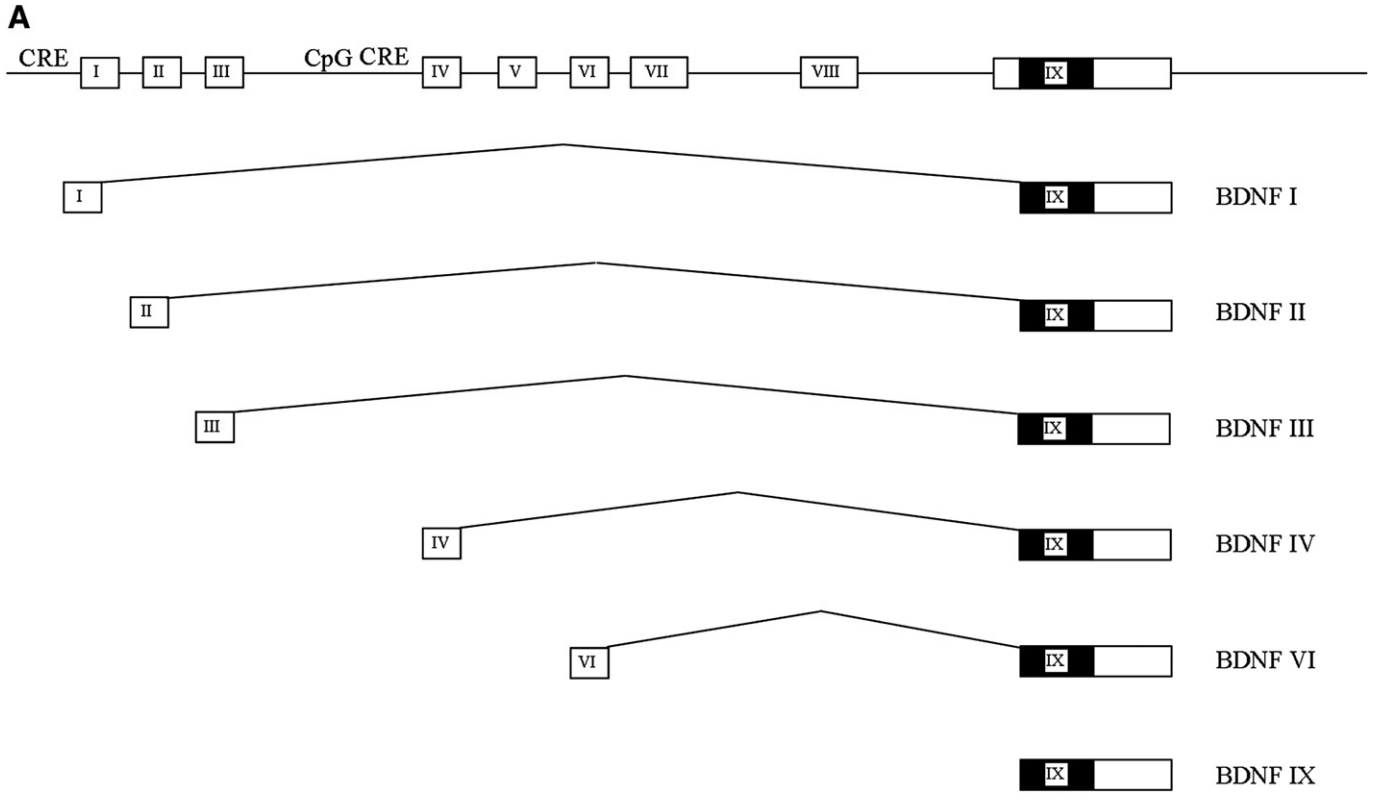

Fig. 7. A. Mouse BDNF gene structure and alternative transcripts. The upper figure, modified from Aid et al. (2007), shows the exon/intron structure of the BDNF gene with boxes representing exons and lines representing introns. The lengths (number of nucleotides) of the exons and introns are not drawn to scale. Each of the 5′ exons can be spliced together with exon IX, which contains the open reading frame (shaded) encoding proBDNF. Below the gene structure are schematic representations of the splice variants analyzed in Fig. 7B. B. Levels of BDNF total (exon IX–containing) and specific mRNA transcripts in the medial frontal cortex (MFC) and hippocampal formation (HPF) of saccharin control (SAC) and prenatal ethanol–exposed (fetal alcohol spectrum disorder, FASD) mice. Real Time PCR was performed and data were calculated as described in Materials and methods. Compared to SAC control ($n=8$), prenatal ethanol-exposed mice ($n=8$) mice had significantly reduced levels of exons III [MFC, $t(14)=2.8$; HPF, $t(14)=4.2$], IV [MFC, $t(14)=8.1$; HPF, $t(14)=6.2$], VI [MFC, $t(14)=8.6$; HPF, $t(14)=3.6$], and IX [MFC, $t(14)=3.7$; HPF, $t(14)=3.9$]. * indicates $p<0.02$; ** indicates $p<0.01$.

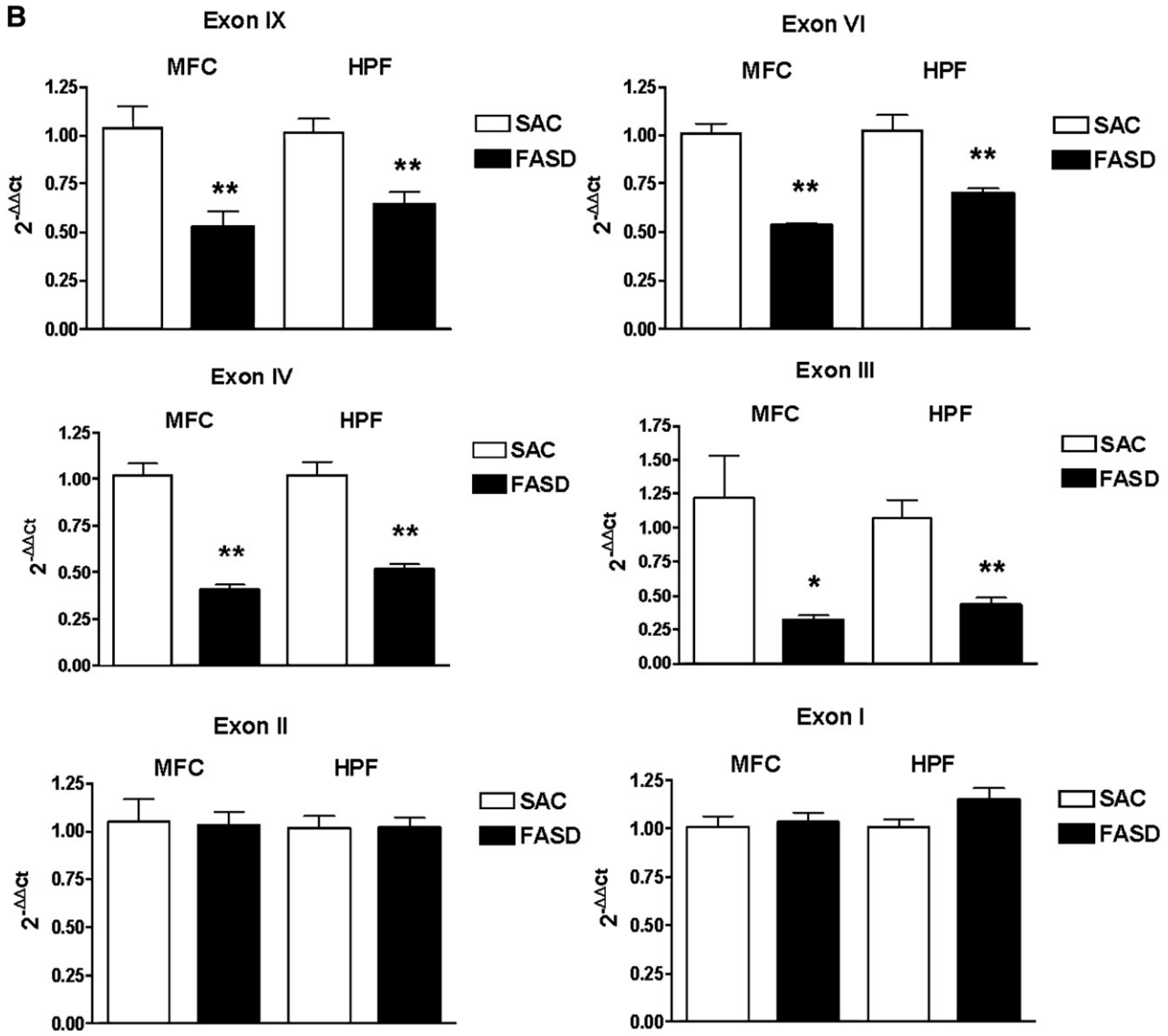

Fig. 7 (*continued*).

hippocampal formation of these same animals. We also found that prenatal ethanol exposure was associated with reduced BDNF total mRNA levels in both the medial frontal cortex and hippocampal formation. In both brain regions, the levels of exons III-, IV-, and VI-containing transcripts were decreased, while the levels of exons I-and II-containing transcripts were not different between prenatal ethanol-exposed and saccharin control mice. Thus, the observed decreases in BDNF mRNA transcript levels in prenatal ethanol-exposed mice correlated with reduced proBDNF and BDNF protein levels in the medial frontal cortex, but not in the hippocampal formation. This indicates that proBDNF and BDNF protein levels in prenatal ethanol-exposed mice are likely controlled primarily by transcription in the medial frontal cortex, but post-transcriptionally in the hippocampal formation. A similar lack of correlation between hippocampal BDNF mRNA and protein levels has been reported by Nawa et al. (1995) and Greisen et al. (2005). Studies conducted by Nawa et al. (1995) using 2-month old male Sprague–Dawley rats show that hippocampal BDNF protein levels are elevated 4 days following limbic seizures induced by electrolytic lesion of the dentate gyrus hilus. In contrast, Isackson et al. (1991), using the same procedure to induce limbic seizures in adult male Sprague–Dawley rats, reported that BDNF mRNA levels in the hippocampus were returned to baseline levels by 4 days following seizure induction. Greisen et al. (2005) reported that, compared to control animals, hippocampal formation BDNF protein levels were increased 63%, while BDNF mRNA levels were unchanged, in 3-month old male Sprague-Dawley rats that had experienced daily maternal separation on postnatal days 2 through 14. In addition to alterations in BDNF mRNA and protein metabolism, these investigators propose that the disparity between BDNF protein and mRNA levels may be accounted for by anterograde transport of BDNF protein to the hippocampus by afferent neuronal projections from other brain regions, such as the medial septal nucleus. Finally, a lack of agreement between BDNF mRNA and protein levels has been observed in the striatum and prefrontal cortex following chronic cocaine administration in rats (Fumagalli et al., 2007).

4.3. What mechanisms may underlie the observed decreases in specific BDNF transcripts?

Chronic defeat stress has been shown to decrease the expression of exons III- and IV-containing transcripts in the hippocampal formation (Tsankova et al., 2006). Therefore, increased chronic stress levels may contribute to the observed reductions in exons III- and IV-containing transcripts in prenatal ethanol-exposed mice, at least in

the hippocampal formation. Interestingly, we have reported that prenatal ethanol-exposed female mice have higher plasma corticosterone levels than do saccharin controls (Allan et al., 2003), which could also account for the observed decrease in exon VI-containing transcripts in the medial frontal cortex and hippocampal formation, where the expression of exon VI-containing transcripts is repressed by corticosterone (Dwivedi et al., 2006; Hansson et al., 2006). The expression of BDNF exon IV-containing transcripts is controlled by the methylation of CpG dinucleotides in the promoter linked to this exon (Chen et al., 2003; Dennis and Levitt, 2005; Martinowich et al., 2003). Maier et al. (1999) reported that prenatal ethanol exposure was associated with reduced BDNF mRNA levels in the postnatal day 10 Sprague–Dawley rat olfactory bulb, and this reduction was correlated with hypermethylation of the BDNF gene. Although these investigators did not demonstrate specific reductions in exon IV-containing species or methylation of the exon IV promoter, their results suggest that the observed decrease in BDNF mRNA containing exon IV in prenatal ethanol-exposed mice may be due to increased methylation of CpG dinucleotides preceding the transcriptional initiation site for exon IV. In addition to being controlled by methylation, the promoter for exon IV, as well as the promoter associated with exon I, has a cAMP-responsive element (CRE) that binds cAMP-responsive element-binding protein (CREB; Fang et al., 2003; Ou and Gean, 2007; Shieh et al., 1998; Tabuchi et al., 2002; Tao et al., 1998). Thus, reduced levels of CREB activation in mice that were exposed to ethanol prenatally could contribute to the observed reduction in the expression of exon IV-containing transcripts in these animals. However, the absence of an observed effect of prenatal ethanol exposure on exon I-containing transcripts does not support a role for global reductions in the level of activated CREB. The expression of BDNF transcripts containing exon VI is regulated by extracellular signal-regulated protein kinase 2 (ERK2; Park et al., 2006; Takeuchi et al., 2002). We have found that receptor-dependent activation of ERK2 in hippocampal formation slices is impaired (Samudio-Ruiz, Valenzuela, Allan, and Caldwell; unpublished observation). Therefore, reduced ERK2 activation could play a role in the observed decrease in exon VI-containing transcripts in the hippocampal formation of ethanol-exposed mice. Finally, acetylated histone H4 has been shown to regulate the expression of promoter VI-containing transcripts in the medial frontal cortex (Bredy et al., 2007). We have found that global levels of acetyl (Lys5, Lys8, Lys12, Lys16) histone H4 are reduced in the medial frontal cortex, but not hippocampal formation, of prenatal ethanol-exposed mice (Caldwell and Allan, unpublished observation), implicating reduced binding of acetylated histone H4 at the promoter for BDNF exon VI as a candidate mechanism underlying the observed reduced level of exon VI-containing transcripts in the medial frontal cortex of the prenatal ethanol-exposed mice.

4.4. Evidence exists that does not support a role for BDNF in depressive-like behaviors

Although several lines of research indicate that BDNF plays an important role in depression and depressive-like behaviors, it should be noted that evidence exits that does not support this conclusion. Angelucci et al. (2000) reported that, compared to the Flinders Resistant Line (FRL), the Flinders Sensitive Line (FSL) rats, which display a variety of depressive-like behaviors, have increased basal BDNF protein levels in the frontal cortex, but not the hippocampal formation. Greisen et al. (2005) found that adult male Sprague–Dawley rats having reduced hippocampal formation, and unchanged frontal cortical, BDNF protein levels display normal behavior in the forced swim test. Hellweg et al. (2007) have reported that olfactory bulbectomy, which has been proposed as a rodent model of depression, of adult male C57BL/6N mice leads to increased BDNF protein levels in the hippocampal formation and frontal cortex. Although this study may appear to be contradictory to our findings, it is important to note that the C57BL/6J mouse strain, which we employed, and the C57BL/6N mouse strain differ in behavioral responses, such as extinction of fear-conditioned responding (Stiedl et al., 1999), and physiologic responses, such as effects of anesthetics on cardiac function (Roth et al., 2002). Thus, as Hellweg et al. (2007) have proposed, BDNF may contribute only to certain types of depressive states or only under certain condition (e.g., certain mouse strains or substrains) in laboratory models of depression. Finally, Greenwood et al. (2007) have found that learned helplessness in adult, male Fischer F344 rats does not depend on hippocampal formation BDNF. This last observation is consistent with our finding of unaltered BDNF protein in the hippocampal formation of prenatal ethanol-exposed mice compared to saccharin controls, yet FASD mice display elevated learned helplessness behavior.

4.5. Relevance to pharmacologic management of clinical depression in FASD patients

The present studies may have relevance to the choice of antidepressant for the treatment of FASD-associated depression. Chronic tranylcypromine treatment has been shown to increase BDNF protein levels in the rat frontal cortex, but not the hippocampal formation, whereas fluoxetine and desipramine did not affect medial frontal cortical BDNF protein levels (Altar et al., 2003). Dwivedi et al. (2006) reported that chronic phenelzine and desipramine increased BDNF mRNA in both the frontal cortex and hippocampal formation, while fluoxetine increased BDNF only in the hippocampal formation. Dias et al. (2003) found that chronic desipramine increased the levels of BDNF exon III-containing transcripts in the piriform cortex and inner and outer layers of the neocortex; in contrast, fluoxetine was without effect in these regions. Okamoto et al. (2003) found that chronic amitriptyline increased BDNF protein levels in the hippocampal formation and frontal cortex. Thus, drugs shown to increase BDNF mRNA or protein levels in the frontal cortex, such as tranylcypromine, amitriptyline, phenelzine, and possibly desipramine, may be effective in treating depressive symptoms in FASD patients, whereas fluoxetine may be less effective.

4.6. Summary

These studies demonstrate that prenatal ethanol-exposed female mice display depressive-like behaviors, when tested as adults. It is important to note that all of the studies described in this report were conducted in female offspring. The choice of conducting these studies in females was based, in part, on the observation that the incidence of clinical depression is higher in females than in males (Kessler et al., 1994, 2006; Weissman and Olfson 1995, Piccinelli and Wilkinson 2000, Kessler 2003; Grigoriadis and Robinson 2007). Additionally, Caldarone et al. (2000) have reported that female C57BL/6J mice are more prone to develop learned helplessness than are male C57BL/6J mice. It will be interesting to determine if a similar correlation between prenatal ethanol exposure, depression, and brain BDNF levels is found in male mice. Monteggia et al. (2007) have reported that, while conditional knockout of forebrain BDNF is associated with depressive-like behavior in female mice, male conditional knockout mice do not display increased depressive behavior.

In addition to displaying depressive-like behaviors, adult female offspring that had been exposed to ethanol prenatally had reduced BDNF mRNA and protein levels in the medial frontal cortex and BDNF mRNA levels in the hippocampal formation. These data demonstrate that depressive-like behaviors in a mouse FASD model and alterations in brain BDNF are correlated. Although they do not provide direct evidence that these reductions in BDNF are an underlying cause of the observed depressive-like behaviors, the data are consistent with the neurotrophic hypothesis of depression and indicate that the hypothesis may be extended to include FASD-associated depression. In order

to determine whether reductions in BDNF play a causative role in prenatal ethanol-associated depressive-like behaviors, future studies will need to identify the developmental stage at which prenatal ethanol exposure exerts it effects on BDNF expression and test the behavioral consequence of reversing these effects (e.g., through targeted overexpression of BDNF). The results of the present study do, however, support the use of antidepressants that have been shown to increase frontal cortical BDNF in the treatment of FASD-associated depression.

It is possible that the effects of prenatal ethanol exposure on BDNF mRNA are, at least in part, mediated by, or secondary to, changes in serotonergic neurotransmission. Several investigators have reported that developing serotonergic neurons are highly vulnerable to the damaging effects of ethanol (Rathbun and Druse, 1985; Sari and Zhou, 2004; Tajuddin, and Druse, 1993, 1999; Zhou et al., 2002, 2005). These ethanol-induced decreases in 5-HT levels and neurons are likely to have a significant cascading effect on the developing nervous system as a whole, thereby impacting BDNF. Additionally, as noted previously, several antidepressants, including serotonin-selective reuptake inhibitors (SSRIs), have been shown to modify BDNF levels in the prefrontal cortex and hippocampus. Thus, future studies will aim to determine the contribution of reduced serotonergic neurotransmission to the observed alterations in BDNF levels.

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