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Difluoromethylornithine (DFMO) reduces deficits in isolation-induced ultrasonic vocalizations and balance following neonatal ethanol exposure in rats

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ABSTRACT

Neonatal ethanol (EtOH) exposure is associated with central nervous system dysfunction and neurotoxicity in rats. Increases in polyamine levels have been implicated as one underlying mechanism for some of EtOH's effects on the developing brain. In this study we addressed whether the inhibition of polyamine biosynthesis by α-difluoromethylornithine (DFMO) could reduce behavioral deficits induced by early EtOH exposure. Male and female rat pups received ethanol (6 g/kg/day EtOH i.g.), or isocaloric maltose (control) from postnatal days (PND) 1–8. On PND 8, animals were injected with either saline or DFMO (500 mg/kg, s.c.) immediately following the final neonatal treatment. Subjects were tested for isolation-induced ultrasonic vocalizations (USV) on PND 16; spontaneous activity in an open field apparatus on PND 20 and 21; and balance on PND 31. Animals exposed to EtOH neonatally displayed an increased latency to the first USV and reduced frequencies of USV, hyperactivity and preference for the center of the open field and poorer balance relative to controls. DFMO minimized these deficits in latency to the first USV and balance. These data provide further support that polyamines play a role in some of the functional deficits associated with EtOH exposure during early development and that reducing polyamine activity can improve outcome.

1. Introduction

Prenatal EtOH exposure disrupts central nervous system (CNS) development, leading to long-lasting cognitive and behavioral alterations (Mattson and Riley, 1998; Roebuck et al., 1998). EtOH disrupts CNS development via a variety of mechanisms, including alterations in gene expression, cell adhesion molecule interactions, and neurotrophic support (Goodlett and Horn, 2001; West et al., 1994).

One likely mechanism that contributes to fetal EtOH effects is NMDA receptor-mediated excitotoxicity, which occurs during periods of EtOH withdrawal (Hoffman and Tabakoff, 1994; Thomas and Riley, 1998). Consistent with this hypothesis, blocking NMDA receptors with the noncompetitive antagonist MK-801, during withdrawal, attenuates some of the adverse effects of EtOH on behavioral and brain development in both *in vivo* rat models of early developmental EtOH exposure (Thomas et al., 1997) and *in vitro* organotypic hippocampal cultures (Prendergast et al., 2000). Moreover, the beneficial effects of MK-801 are time-dependent (Thomas et al., 2001), indicating that NMDA receptor blockade is only effective during the withdrawal phase.

NMDA receptors comprise an assembly of subunits (an NR1 subunit plus at least one type of NR2 subunit) with a number of modulatory sites, including modulatory polyamine binding sites, which are in high levels on the NR2B subunit (Williams, 1994; Williams et al., 1994). Eliprodil inhibits NMDA receptor activity by interacting allosterically with the polyamine modulatory site on the NR2B NMDA receptor and if administered during EtOH withdrawal, reduces the severity of learning deficits associated with developmental EtOH exposure (Thomas et al., 2004a). There has been interest in the potential role of polyamines in the deleterious effects of EtOH for some time (Sessa et al., 1987; Shibley et al., 1995). Recent *in vitro* studies provide further support for the role of polyamines in EtOH withdrawal-induced neurotoxicity, since not only polyamine binding site antagonists, but also polyamine synthesis inhibitors protect against EtOH withdrawal-induced neurotoxicity (Gibson et al., 2003; Littleton et al., 2001). Accordingly, polyamines and glutamate (GLU) are released during EtOH withdrawal (Gibson et al., 2003), providing additional evidence for a role of polyamines in EtOH withdrawal-induced neurotoxicity. However, this is the first study, to the best of our knowledge, that has addressed whether inhibition of polyamine biosynthesis could reduce behavioral deficits induced by early EtOH exposure. In this study, we examined whether the administration of DFMO, which inhibits ornithine decarboxylase (ODC), the rate-limiting step in the synthesis of polyamines, reduces short and more long-term deficits induced by EtOH exposure from PND 1–8.

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2. Materials and methods

2.1. Animals

Male and female Sprague–Dawley rats were bred in the Psychology Department Animal Facility at the University of Kentucky. Birth was considered postnatal day (PND) 0. On PND 1, litters were weighed and randomly culled to 10 pups, keeping 5 males and 5 females whenever possible. The animals were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.) with *ad libitum* access to water and standard laboratory rat chow in a nursery that was temperature and humidity controlled. All experimental procedures were conducted between 9:00 and 16:00 h. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Neonatal drug administration

On PND 1, rat pups were randomly assigned to each of the six treatment groups (when possible) in such a way that each litter contributed no more than one animal for each treatment group to preclude potential litter effects (Abbey and Howard, 1973). From PND 1 to PND 8, one male and one female from each litter were given 3 g/kg EtOH (in a milk formula (West et al., 1984)) or isocaloric maltose (isocaloric control group, milk formula plus maltose) by gavage (0.0278 ml/g bw, bid, at 10:00 and 14:00) with polyethylene PE-10 tubing (Clay Adams), as previously described (Goodlett et al., 1998) resulting in 6 g/kg/day of EtOH. A non-intubated control group was also included. This neonatal EtOH exposure model is used to study a period of CNS development that overlaps the 3rd trimester "brain growth spurt" observed in human pregnancy (Dobbing and Sands, 1979).

On PND 8, offspring were also injected with either 0.9% NaCl (saline, 5 ml/kg, s.c.) or DFMO (500 mg/kg, s.c.) immediately after the last gavage. DFMO was administered immediately after the last gavage because a single injection of 500 mg/kg DFMO markedly reduces polyamine levels in neonatal rat brain for at least 24 h (Slotkin et al., 1982) and thus polyamines would be suppressed during withdrawal at times that have previously been shown to be the most responsive to blockade of GLU or NMDAR during EtOH withdrawal (Thomas et al., 2001, 2004a). This dose has also been shown to reduce the severity of EtOH withdrawal behaviors (Davidson and Wilce, 1998). DFMO was administered only on a single day because of concern regarding the important role that polyamines play in CNS development and because repeated injections of DFMO itself can cause reductions in cerebellar size (Schweitzer et al., 1989; Sparapani et al., 1996). This resulted in six treatments groups: Intubated Control/Saline, Intubated Control/DFMO, Intubated EtOH /Saline, Intubated EtOH /DFMO and Non-intubated Control/Saline and Non-intubated Control/ DFMO. The *ns* for each treatment condition are presented in the data figures. Approximately 16–20 dams and their litters were used for this series of studies.

2.3. Isolation-induced Ultrasonic Vocalization (USV) testing

USVs were examined according to published procedures (Barron and Gilbertson, 2005). In brief, an ultrasonic bat detector (Ultra Sound Advice Model #S-25, UK-http://www.ultrasoundadvice.co.uk) set at 40 kHz with a condenser microphone (SM-1) set 21.5 cm above the test cage floor was used. The output was recorded on a SONY #WM-D8C Cassette Recorder using low noise cassette tapes. Testing was conducted on PND 16 since pilot data from our laboratory has shown that this age is sensitive to neonatal EtOH's effects on isolation induced USVs.

On PND 15, each pup was briefly removed from the litter and tail-marked in random order (for neonatal treatment condition and sex) with a permanent marker to keep the experimenter blind to treatment condition. On PND 16, the dam was removed from the home cage, placed in a holding cage and returned to the cage rack. The litter was maintained with conspecifics in the home cage placed on a heating pad. White noise was generated via small fans to mask extraneous environmental noises or noises from other littermates. Each pup was individually tested by placing the pup in the lower right corner of the test cage (21 cm×11 cm) with clean pine bedding for a 6 min test session. At the conclusion of testing, the pup was removed from the test chamber and weighed. Once all of the subjects in the litter were tested, the dam was returned to the litter. Audiotapes were subsequently played back and the latency to the first vocalization and the number of USVs were recorded. Two experimenters blind to treatment condition scored the USVs and the reliability across experimenters was at least 90%.

2.4. Open field

Beginning on PND 20, animals were individually tested in a round open field apparatus (55 cm in diameter) between the hours of 1000 and 1400 h during the light cycle. Pre-weanling animals were used in this study because previous reports have shown that neonatal EtOH exposure induces hyperactivity at this age (Gilbertson and Barron, 2005; Kelly et al., 1987; Melcer et al., 1994). Pups were separated from their dam and put on heating pads in their home cage until all pups in the litter were tested. Each subject was individually placed in a holding cage and brought into the test room for 10 min habituation prior to placement in the open field. Locomotor activity was measured by a Polytracker Video Imaging System (San Diego Instruments) interfaced with an IBM computer for 20 min daily for two consecutive days (PND 20 and 21). Testing was conducted in a darkened room with fans to provide white noise. The dependent variables recorded included total distance traveled, and the percent of distance traveled and percent of time spent in the center (31.8 cm in diameter) of the open field. The open field apparatus was cleaned with isopropyl alcohol before and after each subject. Body weights were taken one day prior to testing (PND 19). After open-field testing, offspring were weaned on PND 21 and housed with 1–2 same sex conspecifics.

2.5. Balance test

The balance apparatus consisted of a single elevated dowel rod (120 cm long, 1.85 cm diameter), with a darkened escape box (21×10×17 cm) on one end. The rod was raised (60 cm) above the ground, which was well padded in case of falls. The balance test was conducted on PND 31 and animals received three trials. Each animal was habituated to both the testing room and escape box for 1 min each on PND 31. This age was also chosen based on previous findings from our lab (Lewis et al., 2007a). During the first trial, the animal was placed gently on the rod, 10 cm from the escape box. Upon successfully reaching the escape box, the animal was allowed to remain in the box for 10 s, and was then returned to its home cage. If the animal did not successfully reach the escape box (either fell or more often swung from the rod), it was retrieved and placed in the escape box for 10 s, before being returned to the home cage. If the subject successfully traversed the rod, the distance on the next trial was increased by 13 cm. Each trial was separated by a 30 s intertrial interval. Subjects that were unsuccessful were retested at the same distance on the rod. The dowel rod and the escape box were cleaned with 30% isopropyl alcohol before and after each rat occupied it. The dependent measure was the distance successfully achieved on each trial (if an animal fell on any trial, the last successfully completed distance was recorded for that trial). Body weights were recorded on the day before testing (PND 30).

2.6. Blood ethanol assay

Pups from 14 additional litters were intubated twice daily on PND 1–8 for measurement of blood EtOH concentrations (BECs) to assess whether DFMO had effects on EtOH metabolism. Blood was collected

after the second EtOH intubation on PND 8 by making a 1 mm cut at the tip of the subject's tail and collecting 20 µl of blood. The BEC curve was established by collecting samples at 30, 60, 120, 240, 480 and 600 min (and 24 h) following EtOH administration. In order to reduce stress, each subject was only sampled three times with one male and one female represented in each time point. These subjects were not used for further behavioral study. Plasma was separated and frozen at −70 °F. BECs were assayed using an Analox AM 1 Analyzer (Analox Instruments).

2.7. Statistical analysis

The data were analyzed using ANOVA followed by post hoc analyses (Newman–Keuls test or *F* test for simple effect analyses) with between group factors including neonatal treatment group, sex, and repeated measures as warranted. In addition, to better directly examine the interaction of EtOH and DFMO, additional ANOVAs were conducted using EtOH and DFMO as grouping factors. For these analyses, the intubated control and the nonintubated control groups were combined into a single control group as separate ANOVAs did not reveal any differences between these control groups in any of the behavioral measures or in body weight. For ease of presentation, the data was collapsed across sex since there was no main effect or interaction with sex. There was some variation in ns across experiments due to experimenter error, equipment failure or computer malfunction although this was equally distributed across treatment condition. The n/treatment group in each experiment is presented in its respective figure with the *ns* for controls representing the pooled control group.

3. Results

3.1. Isolation-induced USVs

Fig. 1A shows the effects of DFMO and neonatal EtOH exposure on the latency to the first vocalization. Statistical analysis (two-way ANOVA) revealed that the EtOH exposed offspring took longer to vocalize and the administration of DFMO minimized this effect (significant EtOH×DFMO interaction: $F(1,98)=4.20$, $p<0.05$).

Fig. 1B shows the effect of DFMO and neonatal EtOH exposure on the frequency of USVs. Statistical analysis (two-way ANOVA with repeated measures) revealed a main effect of EtOH [$F(1,98)=13.05$, $p<0.05$] although there was no interaction with DFMO. Thus, neonatal EtOH exposure reduced the frequency of USVs across the test session relative to controls and DFMO did not eliminate this deficit.

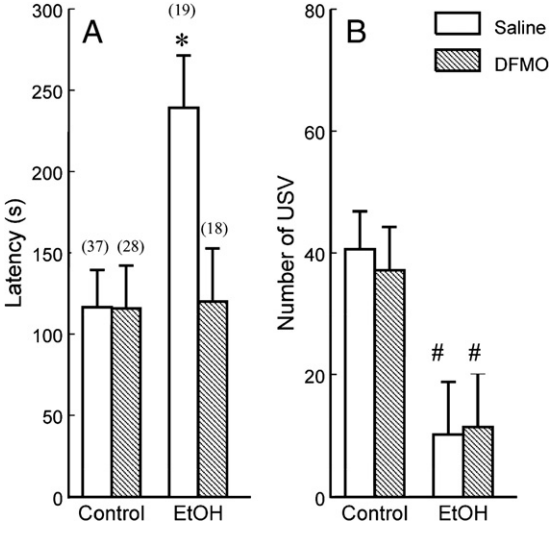

Fig. 1. DFMO minimized the increase in latency to the first vocalization as a result of neonatal ethanol (EtOH) exposure when offspring were tested at PND 16 (A), but had no effect on the EtOH -related decrease in the number of USVs (B). Data presented are mean (collapsed across sex)+SEM. The control group represents the pooled nontreated and intubated controls since these groups did not differ from each other and this pooled control was used in the 2×2 factorial design. The *ns* per treatment condition are presented in the parentheses above each bar. *differs from all other treatment groups; #differs from controls (*p*s<0.05).

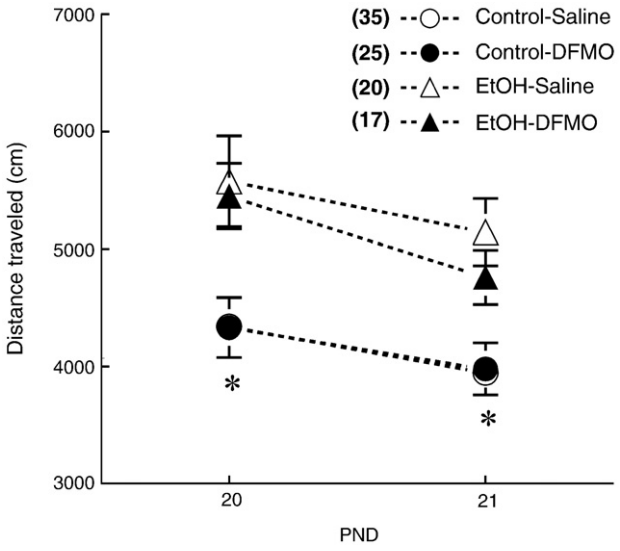

Fig. 2. Neonatal EtOH increased the total distance traveled in the open field on PND 20–21. Data presented are mean (collapsed across sex)±SEM. The control group represents the pooled nontreated and intubated controls since these groups did not differ from each other and this pooled control was used in the 2×2 factorial design. The *ns* per treatment condition are presented in the parentheses alongside the figure legends. *differs from EtOH subjects ($p<0.05$).

3.2. Open field

Fig. 2 shows the effect of neonatal DFMO and EtOH exposure on locomotor activity. The 2×2 ANOVA revealed a main effect of neonatal EtOH exposure [$F(1,93)=26.15$, $p<0.001$] and day [$F(1,93)=15.05$,

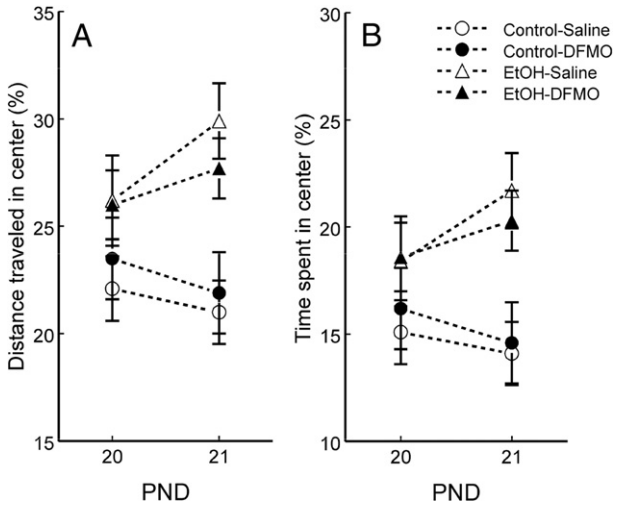

Fig. 3. Neonatal EtOH exposure increased the percent of distance traveled (A) and time spent (B) in the center 50% of the open field on PND 20–21. Data presented are mean± SEM. The control group represents the pooled nontreated and intubated controls since these groups did not differ from each other and this control was used in the 2×2 factorial design. The *ns* per treatment condition are presented in the parentheses alongside the figure legends in Fig. 2.

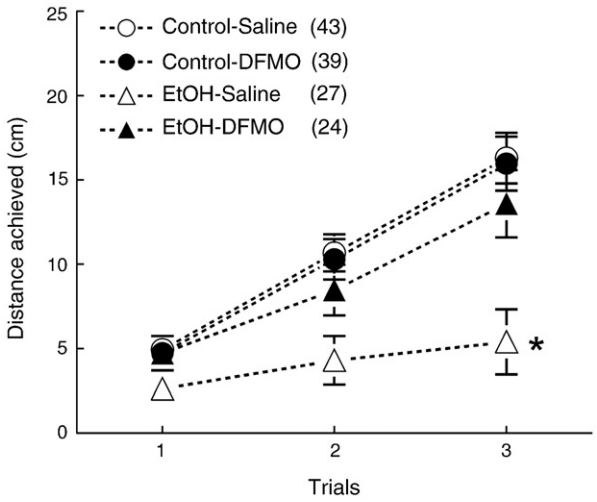

Fig. 4. DFMO on PND 8 reduced the deleterious effect of neonatal EtOH exposure on the total distance achieved in the balance test at PND 31. Data presented are mean (collapsed across sex)±SEM. The control group represents the pooled nontreated and intubated controls since these groups did not differ from each other and this pooled control was used in the 2×2 factorial design. The *ns* per treatment condition are presented in the parentheses alongside the figure legends. *differs from all other treatment groups.

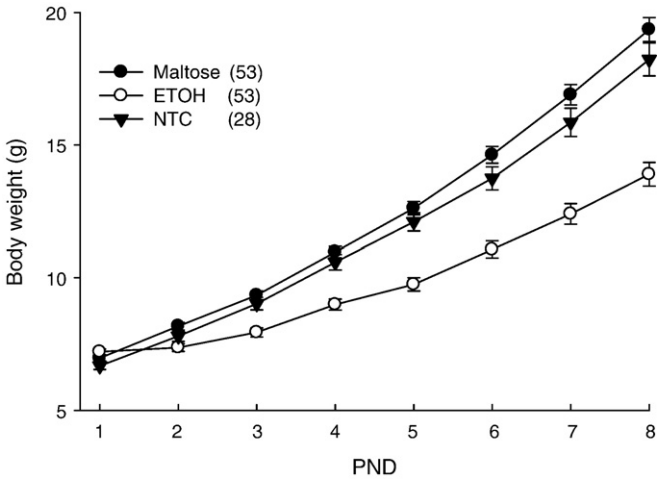

Fig. 5. Neonatal EtOH administration was associated with reduced body weights relative to controls beginning on PND 2 and continuing through PND 8. Data presented are mean (collapsed across sex)±SEM. The control group represents the pooled nontreated and intubated controls since these groups did not differ from each other and this pooled control was used in the 2×2 factorial design. The *ns* per treatment condition are presented in the parentheses alongside the figure legends. The EtOH group differed from controls from PND 2 through PND 8 (*ps*<0.05).

$p<0.001$]. Animals exposed to neonatal EtOH were more active (i.e. increased distance traveled) on both days of testing relative to animals treated with DFMO alone or controls. The addition of DFMO to the EtOH exposed group did not reduce this hyperactivity. The main effect of day was due to a reduction in locomotor activity across the two days of testing (habituation). This was displayed by all treatment groups.

Fig. 3 shows the effect of neonatal DFMO and EtOH exposure on the percent distance traveled and time in the center of the open field. Animals treated with EtOH neonatally traveled proportionally more distance (Fig 3a) and spent proportionally more time in the center (Fig. 3b) of the open field relative to control animals. DFMO did not alter or eliminate this EtOH effect. The ANOVA revealed a main effect of EtOH for distance traveled [$F(1,93)=13.16$, $p<0.001$] and the amount of time spent in the center [$F(1,93)=8.93$; $p<0.005$].

3.3. Balance test

Fig. 4 shows the effect of neonatal DFMO and EtOH exposure on performance on the single dowel task. Statistical analysis (three-way ANOVA) revealed a significant EtOH by DFMO by trial interaction [$F(2,250)=3.45$, $p<0.05$]. Post hoc analyses revealed that neonatal EtOH exposure impaired performance as measured by a reduction in the distance successfully traversed across trials compared to controls [$F(2,250)=14.49$, $p<0.01$]. DFMO administration on PND 8 minimized this effect of EtOH exposure by the third trial [$F(1,125)=5.70$, $p<0.05$; univariate two-way ANOVA followed by Student–Newman–Keuls test].

3.4. Body weights

Statistical analysis of body weight during neonatal treatment (i.e. from PND 1 to 8) with a three-way ANOVA, with neonatal treatment, DFMO and gender as fixed factors and day as a repeated measure (within-subject factor) revealed a significant neonatal treatment by day interaction [$F(14,854)=39.70$, $p<0.001$]. DFMO was included as a factor (although DFMO was not administered until after pups were weighed on PND 8) to ensure that there were no unintended baseline differences in body weight in subjects designated to receive DFMO. Subsequent univariate ANOVAs showed that pups exposed to EtOH weighed less than controls from PND 2 onwards and the controls did not differ from each other (see Fig 5). In addition, statistical analysis revealed that males weighed more than females regardless of neonatal treatment [$F(1,122)=5.16$, $p<0.05$].

The body weights recorded prior to or after behavioral testing are presented in Table 1. There were no differences between the nontreated and intubated/SAL control groups nor the DFMO controls (nontreated and intubated) across any of the ages examined, so the data were collapsed for the control groups (as was done for the behavioral analyses).

Body weights assessed on the day of USV testing (PND 16) revealed a main effect of both EtOH [$F(1,87)=50.65$, $p<0.0001$] and DFMO [$F(1,87)=4.25$, $p<0.05$] (see Table 1). These data indicate that neonatal treatment with EtOH or with DFMO resulted in reductions in body weight relative to non exposed offspring. There was no EtOH×DFMO interaction.

Body weights recorded on the day before open-field testing (PND 19) revealed a main effect of neonatal EtOH exposure [$F(1,89)=59.89$, $p<0.0001$] and sex [$F(1,89)=7.79$, $p<0.01$] (see Table 1). The EtOH exposed offspring still had reduced weights relative to controls. Additionally, as predicted, males weighed more than females.

Finally, this pattern was consistent when body weights were recorded the day before testing on the single dowel test (PND 30). The ANOVA showed with a main effect of both neonatal EtOH exposure [$F(1,125)=34.51$, $p<0.0001$] and sex [$F(1,125)=28.96$, $p<0.0001$] (see Table 1). Thus, neonatal EtOH exposure produced a persistent reduction in body weight relative to controls.

3.5. Blood ethanol levels

Blood EtOH concentrations (BECs) measured on PND 8 are presented in Fig. 6. The addition of DFMO did not alter BEC pharmacokinetics

Table 1
Mean body weight (in g)±S.E.M. as a function of neonatal treatment (PND 1–8) and sex

Group	PND 16		PND 19		PND 30	
	Male	Female	Male	Female	Male	Female
Control/SAL	41.5±1.2	40.2±1.1	54.6±1.3	51.5±1.2	104.9±2.4	94.6±2.2
Control/DFMO	38.6±1.5	38.1±1.3	53.2±1.5	50.2±1.4	103.8±2.6	93.9±2.3
EtOH/SAL	33.4±2.0*	32.4±1.5*	46.4±1.7*	41.3±1.7*	93.2±3.2*	84.4±2.7*
EtOH/DFMO	30.7±1.7*	31.2±1.6*	44.4±1.8*	41.3±1.7*	93.5±3.0*	80.4±3.3*

SAL is Saline. *Ns* are presented in Figs. 1–4. *$p<0.05$ relative to same-aged controls.

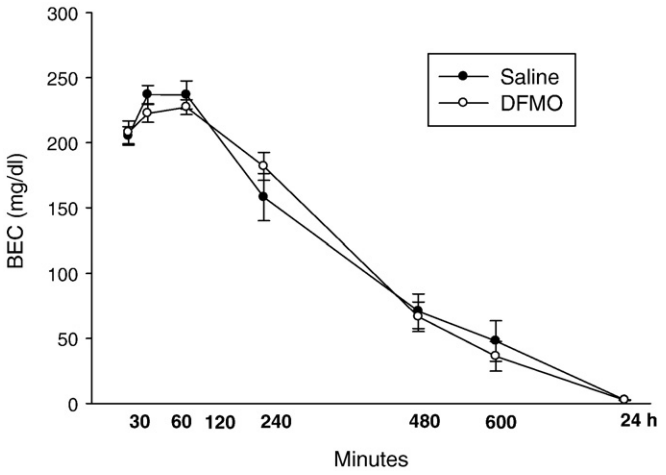

Fig. 6. Blood EtOH levels did not differ between DFMO-treated and saline control rats following EtOH intubation. EtOH (3 g/kg) was administered twice daily from PND 1–8. Blood levels were assayed on PND 8.

at any of the time points examined. The ANOVA on these data revealed no EtOH×DFMO interaction.

4. Discussion

In this study we showed that neonatal exposure to EtOH from PND 1 to 8 produced significant behavioral alterations in young rats, some of which were reduced or minimized by the administration of DFMO on PND 8. The PND 1 to 8 is a period of brain development marked by high sensitivity to EtOH (Thomas et al., 1997), to NMDA receptor-mediated excitotoxicity (Ikonomidou et al., 2000), and to polyamines (Slotkin et al., 2003). This is also a period that occurs postnatally in the rat, but overlaps a portion of the third trimester of human pregnancy in terms of CNS development (Dobbing and Sands, 1979). The findings from this study also showed that there were no differences in blood EtOH concentrations as a consequence of DFMO treatment ruling out this alternative interpretation for DFMO's effects. The behavioral impairments associated with neonatal EtOH exposure observed in the current study included increased latency to the first USV and reduced frequency of USVs at PND 16; increased locomotion and preference for the center of an open field at PND 20–21 and poorer performance in a balance paradigm at PND 31. The administration of DFMO eliminated the EtOH-related increase in latency to the first USV at PND 16 and improved balance performance on PND 31.

DFMO blocks ODC, the rate limiting step for polyamine biosynthesis. DFMO is a fairly small lipid soluble molecule that crosses the blood brain barrier and inactivates ODC molecules semi-permanently. While it reduces the capacity for polyamine synthesis quickly (Slotkin et al., 1982), it does not really alter "resting" levels of polyamines. DFMO has a significant effect when polyamine synthesis by ODC is increased such as during chronic ethanol exposure or during ethanol withdrawal (Davidson and Wilce, 1998) and the blunted response lasts until new ODC can be synthesized (maybe several days), providing "protection" during EtOH withdrawal. These results provide support for the role of polyamines in some of the behavioral teratogenic effects of neonatal EtOH exposure.

There is a considerable body of evidence suggesting that polyamines play a major role in EtOH withdrawal-induced neurotoxicity. Data from *in vitro* studies have shown that polyamine synthesis is increased during EtOH withdrawal and that inhibition of polyamine biosynthesis via DFMO decreased hippocampal neuronal death caused by EtOH withdrawal (Gibson et al., 2003) and reduced EtOH-induced protein carbonylation (Mello et al., 2007). *In vivo* studies have shown that chronic EtOH exposure increased hippocampal ODC activity and this increase was positively correlated with physical indicators of dependence. DFMO (at the same dose used in the current study) significantly reduced these physical withdrawal signs (Davidson and Wilce, 1998) however it should be noted that we did not examine physical withdrawal signs in the current study.

The protective action of DFMO in the current study suggests that inhibition of polyamine synthesis may also reduce the damage associated with neonatal EtOH exposure and/or withdrawal *in vivo*. Of particular note, functional improvements were observed with only a single injection of DFMO on PND 8. A number of studies clearly suggest that the cerebellum is more sensitive to EtOH during the first postnatal week relative to the 2nd (Lewis et al., 2007a; Thomas et al., 1998; Goodlett et al., 1996) and the fact that DFMO was able to reduce these deficits even when administered on PND 8 is extremely interesting. It is important to note, however, that chronic DFMO has its own deleterious effects on the developing brain (Slotkin et al., 2000; Bartolome et al., 1985).

The cerebellum plays a key role in balance and coordination. The cerebellum also appears to be particularly sensitive to prenatal/neonatal EtOH exposure. In clinical studies, balance and coordination deficits have been reported in children exposed to EtOH prenatally (Kyllerman et al., 1985; Streissguth et al., 1980) and these findings have been substantiated by functional and structural imaging studies in which deficits have been observed in children with FAS (Riley and McGee, 2005; Riley et al., 2004; Roebuck et al., 1998). Rodent models have also shown that rat cerebellum appears particularly sensitive to 3rd trimester EtOH exposure with both behavioral and neuroanatomical deficits reported (Goodlett et al., 1991; Green et al., 2000; Klintsova et al., 1998, 2000; Thomas and Riley, 1998).

In rats, the first neonatal week appears to be particularly sensitive to EtOH's effects and this coincides with high expression of cerebellar NR2B subunits (Zhong et al., 1995). As stated in the introduction, the NR2B subunit displays twice the sensitivity to EtOH as other subunits (NR2C or NR2D) (Allgaier, 2002; Kuner et al., 1993; Masood et al., 1994; Mirshahi and Woodward, 1995; Sucher et al., 1996) and the NR2B subtype is also particularly sensitive to polyamine manipulations (Williams et al., 1994). Thus, EtOH exposure on PND 1–8 may have its effect on cerebellar function and structure, at least in part, due to the higher proportion of NR2B subtypes and their greater sensitivity to EtOH and to polyamines. Reducing polyamine levels via DFMO and hence reducing NMDAR activity during EtOH withdrawal could help explain the improved balance performance by EtOH/DFMO exposed offspring. This dose of DFMO also has been shown to reduce the severity of EtOH withdrawal behaviors (Davidson and Wilce, 1998). Additional support for our hypothesis includes findings that show that agmatine, which can block the polyamine site on the NMDAR (Lewis et al., 2007a) and CP 101,606 and eliprodil, both of which are NR2BR antagonists (Lewis et al., 2007b; Thomas et al., 2004a) also reduce behavioral deficits including balance following neonatal EtOH exposure.

The effects of DFMO on reducing EtOH's effects on isolation induced ultrasonic vocalizations are more complex. DFMO eliminated the EtOH-related deficit in latency to USV but had no effect on the frequency of USVs. Isolation induced USVs have been studied to assess anxiolytic agents, i.e. used as a model of stress (Iijima and Chaki, 2005) and have frequently been used to study the role of environmental factors and/or neurotoxicants that influence this species-adaptive response (Adams et al., 1983; Branchi et al., 2001). Many neurotransmitters/neurochemicals appear to play a role in USVs, including benzodiazepines (Carden and Hofer, 1990a; Insel et al., 1986), opioids (Carden and Hofer, 1991, 1990b), serotonin (Albinsson et al., 1994a,b; Hard and Engel, 1988; Winslow and Insel, 1991), dopamine (Dastur et al., 1999) and corticotropin releasing factor (Hennessy et al., 1992; Insel and Harbaugh, 1989). Isolation induced USVs elicit a variety of forms of maternal attention (Brouette-Lahlou et al., 1992; Fernandez et al., 1983) and disruption in the normal response to isolation could have significant consequences for maternal/infant interactions. We have previously

suggested that this deficit displayed by EtOH exposed offspring could have long-term consequences for the offspring (Barron and Gilbertson, 2005; Barron et al., 2000) and it is well known that impairments in maternal/offspring relationships have long-term effects on social behaviors, neuroendocrine development (Liu et al., 1997; Moore et al., 1997) and learning (Barbazanges et al., 1996; Levy et al., 2003). While DFMO did not eliminate EtOH-induced deficits, it did at least ameliorate the delayed latency to USV.

Neonatal EtOH exposure was also associated with hyperactivity and DFMO administered on PND 8 did not eliminate this deficit. Hyperactivity following neonatal EtOH exposure is a frequently reported developmental effect of EtOH on behavior (Gilbertson and Barron, 2005; Saglam et al., 2006; Slawecki et al., 2004; Thomas et al., 2001). While the specific mechanisms by which EtOH causes hyperactivity are also not well understood, it has been argued that these deficits, at least in rodent models, may be due to hippocampal abnormalities resulting in perseveration and habituation deficits associated with prenatal/neonatal EtOH exposure (Riley et al., 1986).

In addition to hyperactivity, the EtOH-exposed offspring displayed an increased preference for the center of the open field, as measured by increased distance traveled and increased proportion of time spent in the center of the chamber relative to control animals. Rats typically display thigmotaxis and spend more time near the perimeter walls of a test chamber rather than entering or crossing into the center. Increased time spent in the center could simply be another indicator of hyperactivity or could be a reduction in the normal species-typical thigmotaxic response displayed by rats when placed in a novel open-field which might be interpreted as reduced anxiety about entering the center of the chamber. We have previously shown that a similar pattern in males treated with EtOH from PND 1 to 7 who also traveled greater distance in the center than controls on PND 19–21 in a traditional square open-field chamber (Gilbertson and Barron, 2005), but again, this paradigm does not allow us to determine if these changes in behavior are due to hyperactivity with a failure to inhibit entries into the center or some change in anxiety response to the open-field.

Clearly, DFMO did not reduce or eliminate all of the effects of neonatal EtOH exposure reported in this series of studies. This was not surprising since prenatal and neonatal EtOH exposure can cause a wide range of effects on CNS and neurotransmitter systems (Goodlett and Horn, 2001; Goodlett et al., 2005; Guerri, 1998, 2002). It is extremely unlikely that all of the functional deficits displayed by offspring exposed to EtOH are mediated by NMDAR and polyamine overactivity. Other studies in which reductions or elimination of functional deficits following early EtOH exposure provide further support for this argument as well. For example, neonatal choline supplement reduced the effects of neonatal EtOH exposure on activity and spatial learning (Thomas et al., 2004b) but had little or no effect on balance impairments (Thomas et al., 2004c).

Neonatal EtOH exposure was associated with reduced body weights and this persisted through PND 30. It is unlikely that this reduction in body weight alone contributed to the deficits observed in the current study since the DFMO/EtOH offspring had similar body weights yet performance was improved on balance and latency to the first USV. Still, EtOH did produce a persistent reduction in body weight. DFMO alone may also have exerted a mild effect on body weight with a slight but statistically significant reduction in body weight in offspring on PND 16 that was no longer observed by PND 19 (the next time that pups were weighed). Previous studies have documented that more chronic neonatal DFMO treatment can reduce body weight (Slotkin et al., 1982; Slotkin and Bartolome, 1986) but the single injection used in this study produced a transient effect at best.

In summary, DFMO administered following chronic neonatal EtOH exposure reversed some of the behavioral consequences of neonatal EtOH exposure in our rodent model. These findings suggest that compounds that reduce polyamine levels during EtOH withdrawal eliminate some of the deficits associated with neonatal EtOH exposure. It is important to note that this approach is somewhat complicated considering the important role that polyamines play in CNS development (Jasper et al., 1982; Slotkin and Bartolome, 1986) and it may be that the timing of polyamine suppression in relation to EtOH withdrawal is critical. Clearly, further work is needed to assess this potential treatment approach.

DFMO is currently used clinically as an antineoplastic therapy (due to the role of polyamines in cell growth) (Casero et al., 2005; Huang et al., 2005) and is also used in treatment of some forms of trypanosomiasis (sleeping sickness) (Chappuis et al., 2005; McCann et al., 1981) although chronic administration of DFMO can have adverse side effects (Nie et al., 2005). Additional studies are clearly needed to gauge the potential usefulness of DFMO and other polyaminergic agents in reducing fetal alcohol effects.

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