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Adenylyl cyclases 1 and 8 mediate select striatal-dependent behaviors and sensitivity to ethanol stimulation in the adolescent period following acute neonatal ethanol exposure

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Abstract

Neonatal alcohol exposure in rodents causes dramatic neurodegenerative effects throughout the developing nervous system, particularly in the striatum, acutely after exposure. These acute neurodegenerative effects are augmented in mice lacking adenylyl cyclases 1 and 8 (AC1/8) as neonatal mice with a genetic deletion of both AC isoforms (DKO) have increased vulnerability to ethanol-induced striatal neurotoxicity compared to wild type (WT) controls. While neonatal ethanol exposure is known to negatively impact cognitive behaviors, such as executive functioning and working memory in adolescent and adult animals, the threshold of ethanol exposure required to impinge upon developmental behaviors in mice has not been extensively examined. Therefore, the purpose of this study was to determine the behavioral effects of neonatal ethanol exposure using various striatal-dependent developmental benchmarks and to assess the impact of AC1/8 deletion on this developmental progression. WT and DKO mice were treated with 2.5 g/kg ethanol or saline on postnatal day (P)6 and later subjected to the wire suspension, negative geotaxis, postural reflex, grid hang, tail suspension and accelerating rotarod tests at various time points. At P30, mice were evaluated for their hypnotic responses to 4.0 g/kg ethanol by using the loss of righting reflex assay and ethanol-induced stimulation of locomotor activity after 2.0 g/kg ethanol.

Ethanol exposure significantly impaired DKO performance in the negative geotaxis test while genetic deletion of AC1/8 alone increased grid hang time and decreased immobility time in the tail suspension test with a concomitant increase in hindlimb clasping behavior. Locomotor stimulation was significantly increased in animals that received ethanol as neonates, peaking significantly in ethanol-treated DKO mice compared to ethanol-treated WT controls, while sedation duration following high-dose ethanol challenge was unaffected.

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These data indicate that the maturational parameters examined in the current study may not be sensitive enough to detect effects of a single ethanol exposure during the brain growth spurt period. Genetic deletion of AC1/8 reveals a role for these cyclases in attenuating ethanol-induced behavioral effects in the neonatally-exposed adolescent.

Keywords

Fetal Alcohol Spectrum Disorders (FASD); Adenylyl Cyclase; Ethanol; Behavior; Striatum

1. Introduction

Exposure of a developing fetus to alcohol can result in a wide range of both physical and mental impairments, termed fetal alcohol spectrum disorders (FASD), that include craniofacial abnormalities, growth deficits, mental retardation, and behavioral alterations. [1–11]. Numerous human imaging studies have demonstrated overall reductions in brain volume, with distinct vulnerability in the cerebellum, corpus callosum, cortex, hippocampus, and basal ganglia, (reviewed in [12]). These reductions in brain volume have been recapitulated and further investigated using animal models of FASD. These studies demonstrate pronounced and wide-spread cell death following neonatal alcohol exposure in similar brain regions to those affected in human studies [13–22], with the striatum showing heightened sensitivity to neurodegeneration after a single exposure to 2.5 or 5.0 g/kg ethanol [22, 23].

The cortico-striatal damage observed in children with FASD is often associated with deficits in various cognitive behaviors [24, 25]. Many studies have also demonstrated motor dysfunction including weak grasp, balance and gait impairments, delayed reaction time and deficiencies in coordination, which involve both the cerebellum and the basal ganglia (reviewed in [7]). In the current study, we performed a battery of tests to determine if a single neonatal ethanol exposure (2.5 g/kg), which has been shown to result in significant cell death in the striatum, could affect various striatal-dependent behaviors. First, we assessed developmental milestones, such as the postural reflex, negative geotaxis, and wire suspension tests. Additionally, we performed the grid hang test which is sensitive to reductions in striatal dopamine and its metabolites, a condition that is known to occur in a model of Parkinson's disease and rat models of FASD [26–29]. The tail suspension test was used to examine the presence of hindlimb claspings, which can result from ethanol-induced striatal excitotoxicity in neonates [30], a phenotype similar to that exhibited in models of Huntington's disease, in which high levels of medium spiny neuron depletion in the striatum has been reported [31]. Finally, the effects of neonatal ethanol exposure on locomotor activation by low-dose ethanol and sedation by high-dose ethanol during adolescence were also evaluated.

To further examine mechanisms that might underlie striatal-related behavioral deficits resulting from neonatal ethanol exposure, we evaluated mice lacking the calcium/calmodulin-stimulated adenylyl cyclases (ACs) 1 and 8 (DKO). These mice demonstrate increased vulnerability to ethanol-induced neurotoxicity in the developing striatum, which is

associated with a decrease in the phosphorylation of the pro-survival proteins insulin-receptor substrate-1, extracellular related kinase, and Akt, compared to wild type (WT) controls [23]. Therefore, these animals are ideal to study the threshold in neurodegeneration required for behavioral effects to become evident. Similarly, these mice demonstrate increased sensitivity to high-dose ethanol sedation and resistance to stimulation by low-dose ethanol, substantiating a role for AC1 and 8 in mediating ethanol-induced behaviors [32–34].

2. Methods

2.1 Animals

All mice were backcrossed a minimum of ten generations to WT C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME). To generate mice for these experiments, homozygous mutants, DKO, and WT mice were bred in-house. Mice were maintained on a 12-h light/dark schedule with *ad libitum* access to food and water. All mouse protocols were in accordance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at Wayne State University.

2.2 Ethanol Treatment

A single dose of ethanol (2.5 g/kg), prepared as a 20% (v/v) solution using 100% ethanol (Decon Laboratories, King of Prussia, PA) in 0.9% saline was injected subcutaneously into male WT and DKO pups [23]. Corresponding volumes of saline were administered to littermate male pups as controls. Only animals weighing 2.5–3.0 g between postnatal day (P)5 and P7 were used in these experiments. Ethanol exposure during P5–P7 represents maternal exposure to ethanol during the third trimester in humans. We have previously demonstrated that this treatment results in blood alcohol concentrations (BAC) of 276 ± 5 mg/dL in WT pups and 262 ± 9 mg/dL in DKO pups 15 min after injection [35]. Only 1 mouse per condition was used from each litter. One cohort of animals were tested for the postural reflex, negative geotaxis, wire suspension, grid hang, and tail suspension tests were treated with ethanol or saline at P6 only to maintain equal time between treatment and behavioral testing, which began at P7. Two additional groups of animals, used for rotarod and loss of righting reflex tests and locomotor activity monitoring, were treated with a single injection of ethanol or saline during P5–P7 (Table 1). All experimental pups were placed on a heating pad at 31°C [22] away from dams until the ethanol pups regained consciousness (~2 h), at which time all pups were returned to their dams. All pups were weaned at P21.

2.3 Behavioral Tests

Saline and ethanol treated animals at P7, P10, P14, P21 and P30 were weighed and subjected to the wire suspension, negative geotaxis, and postural reflex tests. Additionally at P14, P21 and P30 mice were tested in the grid hang and tail suspension tests. All tests were separated by at least 10 min. Independent groups of mice were also tested at P30 for accelerating rotarod performance and loss of righting reflex, or ethanol-induced locomotor activity (Table 1).

2.3.1 Postural Reflex—Each mouse was placed on 4 paws in a 12 × 8 cm box, which was quickly moved in 2 dimensions (up/down, left/right). The animal was monitored for splaying of feet, and scored as follows: 0 = no splaying present, 1 = splaying present.

2.3.2 Wire Suspension (WS)—Each mouse was hung from a 3 mm wire from its forepaws. The ability to grasp the wire (0 = not present, 1 = present), and the time for which the wire was held (max = 30 s) was recorded. Each mouse must hold the wire for at least 1 s to be included in the time analysis.

2.3.3 Negative Geotaxis—Each animal was placed with its head facing downward on a 28 × 36 cm wire grid with 0.5 cm² openings that was set at a 45° angle. The behavior of the animal was recorded for 30 s and scored as follows: 0 = turns and climbs, 1 = turns and freezes, 2 = moves, but fails to turn, 3 = does not move. The latency to turn and initiate climbing was also recorded for all animals that received a score of 0.

2.3.4 Grid Hang—Each mouse was placed on the center of a 0.5 cm² grid at the bottom of a 28 × 36 cm box. The mouse was supported by the experimenter until it grabbed the grid with both its fore- and hindpaws. The grid was then inverted so the mouse was hanging upside-down. The duration of time that the animal remained on the grid was recorded. Only mice capable of hanging onto the grid for at least 1 s were included in analysis.

2.3.5 Tail-Suspension Test (TST)—Each animal was fastened by the tail using labeling tape (Fisher Scientific, Pittsburg, PA) to a metal rod ~30 cm above a bench top. The activity of the animal was recorded for 5 min and analyzed for total immobility time [36] and number of hindlimb clasp events (lasting for more than 1 s per event).

2.3.6 Accelerating Rotarod—At P30, motor performance was evaluated using an accelerating Mouse Rotarod (Ugo Basile, Collegeville, PA). The rod (3 cm in diameter and 14 cm above a platform) accelerated from 3.5 rotations per minute (RPM) to 36 RPM at a constant rate over a 5 min testing interval. Latency to fall from the rotating rod or to traverse around the rod for one full rotation was recorded for each mouse.

2.3.7 Loss of Righting Reflex Assay (LORR)—One h after completing the rotarod task, mice were given an injection intraperitoneally (i.p.) of 4.0 g/kg ethanol (prepared as a 20% (v/v) solution using 100% ethanol (Decon Labs, King of Prussia, PA) in 0.9% saline), and placed on their back in a U-shaped trough after losing the ability to right themselves. Duration of LORR (sedation time) was measured from the time the animals lost the righting reflex until this reflex was regained. Sedation time is defined as the time needed to regain the ability to right onto three of four paws, three times within 30 s.

2.3.8 Locomotor Activity Testing (LMA)—At P30, locomotor activity was measured within a 46 cm × 26 cm polycarbonate cage. An automated monitoring system (Digiscan DMicro, Accuscan Instruments, Columbus, OH) consisting of 16 parallel infrared emitter/detector photocells was used to measure activity. This automated measure of activity was transformed into 1 min blocks over the duration of the session. The spontaneous locomotor activity of each animal was monitored for 10 min on days 1–4, immediately following

injection of 0.9% saline. On day 5, animals were given an i.p. injection of 2.0 g/kg ethanol (20% (v/v) solution in 0.9% saline), immediately placed in the activity monitor chambers and activity was measured for 10 min. Previous studies using DKO animals have demonstrated a significant increase in baseline locomotor activity compared to WT mice [34], therefore locomotor activity levels were analyzed as a percent of respective neonatal saline-treated controls.

2.4 Statistical Analysis

Mice that did not meet the minimum inclusion criteria (stated above for each task) were eliminated before statistical analysis was performed. Three-way repeated measures ANOVA was used for body weight, grid hang time, WS time and TST immobility time with Genotype and Treatment as the between subject factors and Age as the within subjects factor. Three-way repeated measures ANOVAs were also used for LMA activity with Genotype and Treatment as the between subjects factors, and Day or Minute as the within subjects factor. Two-way ANOVA was used for latency to fall from the rotarod and sedation time (LORR) with Genotype and Treatment as the between subject factors. A two-way ANOVA was also performed for the latency to turn in the negative geotaxis test at each time point because not all the mice could perform the task at each time point, invalidating a three-way ANOVA. Statistical analysis on the postural reflex task was not performed because all animals were scored a 1 (capable of splaying the feet) at every time point examined.

Results

3.1 Body Weight

The body weight of each animal from group 1 was determined before each day of behavioral testing and was found to significantly increase with age (Fig 1), with no significant effects for either genotype or treatment (Three-way ANOVA, $\chi^2 = 55.05$, $p < 0.001$, Greenhouse-Geisser correction $\epsilon = 0.40$ [$p < 0.001$; corrected $F(1.58, 26.9) = 737.66$]).

3.2 Postural Reflex

Neither neonatal ethanol exposure nor genotype had an effect on postural reflex as demonstrated by all animals splaying all four feet as early as one day after ethanol treatment (P7) and continuing through P30 (data not shown).

3.3 Wire Suspension

Age significantly affected the formation of this reflex, with mice not being capable of hanging onto the wire before P21, at which time approximately 80% of saline-treated WT and DKO animals successfully hung onto the wire for at least 1 s (Fig 2A). Approximately 80% of ethanol-treated DKO animals were also capable of hanging onto the wire, whereas only ~60% of WT mice neonatally exposed to ethanol gripped the wire. At P30, all the animals were capable of hanging onto the wire for at least 1 second, regardless of genotype or neonatal treatment. Although fewer ethanol-exposed WT animals successfully hung on the wire at P21, no significant effect of Genotype or Treatment on the total hang duration was demonstrated between any groups (Fig 2B). Three-way ANOVA with Age (P21 and P30) as the within subjects factor and Genotype and Treatment as the between-groups

factors resulted in a significant effect of Age on the total duration of time on the wire [$p<0.001$; $F(1, 20)=16.65$].

3.4 Grid Hang

The ability of the animals to hang from a wire grid was significantly affected by age with a significant increase in the time spent on the grid by WT mice from P14–P30 [$p<0.001$; $F(2, 40)=34.17$]. A significant interaction of Age and Genotype [$p<0.01$; $F(2, 40)=6.30$] was also observed with post-hoc analysis revealing a significant difference between WT and DKO mice at P21 ($p=0.02$). At this age, the DKO mice, regardless of neonatal treatment, were capable of hanging on the wire grid longer than WT mice (Fig 2C). No significant effect of ethanol treatment was determined.

3.5 Negative Geotaxis

The percentage of animals capable of turning and climbing up the wire grid (score of 0) increased with age from P7–P14 with no major variations between groups (Fig 3A). At P21 and P30, all animals were capable of climbing the grid (data not shown). The time required for each animal to turn and begin to climb the grid was recorded, however three-way ANOVA analysis was not possible due to a portion of animals not performing the task at each time point. Therefore, all time points were analyzed for the latency to turn using independent two-way ANOVAs. At P7, although a similar percentage of animals were capable of turning and climbing, ethanol-treated DKO animals required significantly more time to turn and climb the grid than all other groups [$p=0.04$; $F(1, 6)=6.34$] (Fig 3B). All groups demonstrated improvement in the time required for turning as demonstrated by a reduction in time to turning at P30 and no significant effects were determined at any other time point.

3.6 TST

The total amount of time spent immobile in the TST significantly increased with age [$p<0.001$; $F(2, 42)=27.60$] (Fig 4A). A significant main effect of Genotype [$p<0.001$; $F(1, 21)=34.59$] and a significant interaction of Genotype $\times$ Age [$p<0.001$; $F(2, 42)=16.53$] were determined, with the DKO mice spending significantly less time immobile compared to the WT mice at both P21 ($p<0.001$) and P30 ($p<0.001$), regardless of neonatal treatment. A significant main effect of Treatment was also demonstrated, with neonatal ethanol exposure significantly decreasing the total immobility time across both genotypes combined [$p<0.01$; $F(1, 21)=8.40$]. The presence of hindlimb claspings was also observed during the TST with DKO mice having significantly more claspings events than WT mice [$p=0.02$; $F(1, 25)=5.69$] (Fig 4B). Interestingly, only ethanol-exposed WT animals demonstrated hindlimb claspings, whereas DKO mice displayed this behavior regardless of neonatal treatment.

3.7 Rotarod

Neither exposure to 2.5 g/kg ethanol [$p=0.82$; $F(1, 29)=0.05$] nor genetic deletion of AC 1/8 [$p=0.56$; $F(1, 29)=0.34$] had any effect on the total time spent on the accelerating rotarod (data not shown).

3.8 LORR

No effect of ethanol treatment was demonstrated in the LORR assay at P30 in either genotype (data not shown). AC1/8 knockout mice trended toward having a greater sedation time after 4.0 g/kg ethanol than WT mice, regardless of neonatal treatment, but this difference failed to reach statistical significance [$p=0.059$; $F(12, 23)=59.31$].

3.9 LMA

WT mice neonatally exposed to ethanol demonstrated a slight, non-significant increase in locomotor activity following saline injection on day 1 compared to neonatal saline-treated control mice (Fig 5A). No significant differences were observed on days 2–4 (data not shown). On day 5, acute exposure to 2.0 g/kg ethanol modestly, but significantly, increased locomotor activity collectively in both genotypes when normalized to neonatal saline-treated controls (Fig 5B; effect of neonatal Treatment [$p=0.008$; $F(1, 50)=7.65$]). Figure 5C demonstrates the time course following ethanol injection on day 5. Three-way ANOVA analysis (Minute as the within-subjects factor and Genotype and neonatal Treatment as between-subjects factors) revealed a significant main effect of Genotype [$p=0.05$; $F(1, 50)=3.93$], neonatal Treatment [$p<0.01$; $F(1, 50)=7.65$] and an interaction of Genotype and Treatment [$p=0.05$; $F(1, 50)=3.93$], with DKO mice exposed to ethanol as neonates having a greater increase in locomotor activity that peaks 5 min after ethanol injection, compared to ethanol-exposed WT mice. No effect of Minute or Minute interactions were found using the Greenhouse-Geisser correction ($\chi^2 = 189.06$, $p<0.001$, $\varepsilon = 0.52$).

4. Discussion

4.1 Developmental Behaviors

In this study, we evaluated the behavior of mice treated postnatally with 2.5 g/kg ethanol as a model of maternal alcohol consumption during the third trimester. Although previous studies using this model have demonstrated substantial cell death in the caudate putamen within 4 h of ethanol treatment [22, 23, 37], here we show that this single dose of ethanol is not sufficient to result in overt behavioral changes in attainment of the postural reflex, grid hang, negative geotaxis or wire suspension in WT mice. The lack of effect of neonatal ethanol exposure in WT mice in this study may be expected given that behavioral deficits in these procedures observed in models of Parkinson's disease are not evident until more than 80% of striatal dopamine has been lost [26, 38]. Previous studies evaluating developmental behaviors following early life exposure to ethanol demonstrate that rats given 6.6 g/kg/day (P4–P10) ethanol in 4 fractions over 8 h or in 12 fractions over 24 h do not result in deficits in the negative geotaxis task or in the cliff avoidance task [39], similar to our results in WT mice (Fig 3A and B). In addition, mice treated with 5.0 g/kg ethanol, divided into 2 doses, 2 h apart on P7 demonstrated no deficits in the negative geotaxis test with the grid set at a 60° angle at P21 [21]. Conversely, mouse pups from dams given free access to 10% ethanol throughout gestation and the early postpartum period (through P10; ~10–24 g/kg consumption) were significantly delayed in the negative geotaxis, cliff avoidance, and midair righting [40]. These data indicate that these behaviors demonstrate temporal sensitivity, with the vulnerable period occurring prenatally in rodents.

Although behavioral effects were not observed in ethanol-treated WT mice, the increase in cell death demonstrated previously following neonatal ethanol exposure in DKO mice [23] was hypothesized to induce behavioral deficiencies compared to WT controls. In support of this hypothesis, ethanol-exposed DKO animals demonstrated a deficiency in the negative geotaxis task, with saline-treated DKO and ethanol-treated WT animals completing this task in ~5 s at P7 and the DKO ethanol group requiring ~12 s (Fig 3B). The latency to turn decreased with age in both genotypes, however at P30 ethanol-exposed DKO mice required twice as much time as all the other groups to turn and climb. What is most surprising with this data is the fact that although the latency to turn for the ethanol-exposed DKO group was higher than WT, a higher percentage of DKO mice accomplished the task at P30 than the WT group. The discrepancy between WT and DKO animals in latency to turn may relate to the significant increase in striatal cell death compared to ethanol treated WT controls [23], indicating a possible threshold for neurodegeneration for behavioral effects. In addition, this discrepancy could be the result of a disruption in normal development caused by the lack of AC activity throughout gestational development in the DKO mice, making the ethanol exposure used here mimic the delay in negative geotaxis observed by Kleiber and colleagues following prenatal exposure in WT animals [40].

Our results also indicate no effect of neonatal ethanol treatment on grid hang (Fig 2C) or wire suspension (Fig 2B) in WT animals. DKO animals, however, displayed significantly increased hang time in the grid hang test, which is not speculated to be due to a difference in muscle strength as there was no significant genotypic difference in the wire suspension test. DKO mice also displayed significantly decreased immobility duration during TST (Fig 4), regardless of neonatal treatment. A similar decrease in immobility time was found in animals with a conventional deletion in 2 prominent cAMP response element-binding protein (CREB) isoforms or mice transfected with dominant negative CREB in the nucleus accumbens shell [41, 42], suggesting that the cAMP/PKA/CREB pathway is compromised in these mice. Consistent with this finding, mice lacking AC8 show reductions in CREB phosphorylation in the CA1 region of the hippocampus after stress [43]. Studies have suggested that CREB activity is crucial during neuronal development (reviewed in [44]) and for Brain Derived Neurotrophic Factor (BDNF) transcription [45, 46]. Interestingly, mice genetically altered to lack BDNF in the forebrain demonstrate hindlimb clasping [47], a phenotype similar to that currently demonstrated by DKO mice. A lack of effect of forebrain BDNF deletion on accelerating rotarod performance [47] further parallels behaviors exhibited by DKO mice in the present study. Further, deficiencies in forebrain BDNF confers a reduction in both striatal and cortical volume at P14, 35 and 120 [47], similar to that observed in animal models of FASD [21, 48] and in the clinically affected population [12, 49–53]. Previous studies in animal models have also shown reductions in BDNF expression after neonatal ethanol exposure in cortex [54–58] and cerebellum [59]. Although studies of gestational ethanol exposure have failed to show a change in BDNF levels in the striatum [57, 60], there may be temporal vulnerability of BDNF expression in the striatum, similar to that observed in the cerebellum [61], which warrant further investigation of postnatal ethanol exposure in both WT and DKO mice.

4.2 Rotarod

Similar to the results described here, previous studies of neonatal ethanol exposure have failed to show changes in rotarod performance at P18, P28 or P48 after exposure to 2.15% or 2.5% ethanol (P4–P12) [62]. Diaz *et al.* also did not observe an effect of postnatal ethanol on rotarod performance at P21–P23 after exposure to ethanol vapor for 4 h from P2–P12 [63]. Similarly, Dursun *et al.* failed to detect a significant effect of neonatal ethanol exposure in P90–P95 rats previously treated with 6 g/kg ethanol from gestational day (G) 7 to G20 [64]. However, rats exposed to 4.5 g/kg ethanol using 10.2% ethanol in 2 feedings over P4–P9 performed significantly worse on a rotarod task at approximately 405 days of age than rats exposed to 4.5 g/kg ethanol using 5.1% ethanol in 4 feedings or pair-fed controls [65]. The differences between these results is suggested to be related to the peak BAC and resulting neuronal loss, which is more pronounced in the condensed feeding paradigm [65]. These authors also speculate that the differences observed between their study and that by Meyer *et al.* [62] are due to differences in the age at testing or method of testing with the rotarod used in the Meyer *et al.* study having a 15 cm diameter rod which may not have been challenging enough to discern impairments imposed by previous ethanol exposure (7 cm diameter used in their study). In the study by Dursun *et al.*, a rotarod with a diameter of 6.5 cm was used, but the rotarod was set at 20 revolutions per minute throughout the testing procedure, whereas the others accelerated throughout the test. Our study also used an accelerating paradigm but no effects of neonatal ethanol exposure were observed, which may be due to the single, lower dose of postnatal ethanol, compared to the procedure used by Goodlett *et al.* Another important difference is the age at testing, as the rats used by Goodlett *et al.* were over a year old, while our mice were tested at P30, perhaps suggesting that deficits in motor performance on the rotarod caused by postnatal ethanol exposure require a longer time to develop.

4.3 Loss of Righting Reflex

Previous work has demonstrated that rats exposed to ethanol throughout gestation and lactation are less sensitive to the sedative effects of 4.0 g/kg ethanol at P60 [66], contrary to the LORR results here showing no effect of postnatal ethanol exposure. However, the rats used by Barbier *et al.* were exposed to higher levels of ethanol throughout their neurodevelopment, with dams consuming 8–20 g/kg ethanol (throughout gestation and lactation). These data indicate increased sensitivity to ethanol sedation in mature animals is dependent on either the timing or dose of developmental ethanol exposure. Although DKO mice were shown to be more sensitive to the neurodegenerative effects of neonatal ethanol exposure [17, 23] the degenerative effects of the ethanol dose used may not have reached the threshold required to affect ethanol-induced sedation. It should be noted that although previous studies in DKO mice have demonstrated an increase in sedation time following 4.0 g/kg ethanol [33], only a trend toward an increase in sedation was observed in the current study. One potential explanation for this discrepancy is the age at testing, as the mice used in the current study were tested at P30, while previous work was performed in adult mice (2–4 months) [33] and duration of LORR is known to increase with age [67, 68].

4.4 Locomotor Activity

LMA has been extensively studied following both pre- and post-natal ethanol treatment, although the results vary. For example, rats given 6.6 g/kg/day ethanol in 4 fractions over 8 h on P4–P10 were significantly more active in an open field at P20 than controls or rats given 6.6 g/kg/day in 12 fractions over 24 h daily from P4–P10 [39]. In a similar study, rats given 6.0 g/kg/day ethanol in 4 feedings over 4 h from P4–P10 were also significantly more active in a 30 min open field test in a novel chamber on P18 [69]. Comparable studies in mice have also shown increases in activity, for instance, pups born from mice given free access to 10% ethanol for the first 8 days of gestation were significantly more active in the first 10 min in an open field on P14 and P21 compared to water controls [70]. No significant differences in the activity during the remaining 20 min was observed [70]. Mice treated with 5.0 g/kg ethanol, divided into 2 doses, 2 h apart on P7 were significantly more active in a 1 h open field test at P70 [21].

In contrast, mouse pups from dams given free access to 10% ethanol through gestation and the early postpartum period (through P10; ~10–23 g/kg/day consumption) were significantly less active during a locomotor activity test (15 min) in a novel environment at P25, with no significant effect of general locomotor activity in their home cages over the 12 h dark phase at P34 [40]. Similarly, P80–P85 rats previously treated with 6 g/kg ethanol from G7 to G20 demonstrated a significant decrease in locomotor activity for the first 5 min in a novel open field chamber, with no significant effects for the remaining 25 min [64]. Lucchi *et al.* also demonstrated that pups born from dams given ethanol from G5 throughout pregnancy were significantly less active than control pups or pups who were exposed to ethanol during lactation [71].

Consistent with our present results in P30 mice following a single postnatal exposure to ethanol (Day 1; Fig 5A), some studies have found no effect of ethanol exposure on LMA behavior. For instance, pups from dams receiving 20 or 25% of their calories from ethanol from G5 through G18 did not display significantly different activity from controls at P12, P20, P28, or P36 in an open field paradigm [72]. In addition, exposure to 5 g/kg ethanol on P7 in 2 doses of 2.5 g/kg given 2 h apart had no effect on locomotor activity in rats examined in an open field for 35 min at P35 [73]. Likewise, mice treated with 5.0 g/kg ethanol, divided into 2 doses, 2 h apart on P7 displayed no significant effects on locomotor activity in an open field test for 1 h at P21 [21].

From these studies, it is difficult to determine which ethanol treatment is more likely to contribute to disturbances in LMA. However, Tran and colleagues endeavored to parse out these differences by treating mice from G2 through G10, G11 to G22, P2 through P10, or all three time periods. For the gestational exposures, pregnant dams were administered 4.5 g/kg/day ethanol, while pups were given 3.0 g/kg/day ethanol, both through intragastric gavage [74]. These authors only observed an increase in activity at P16 in the 2 groups exposed to ethanol during G11 to G22, whereas ethanol exposure during the postnatal period (P2–P10) alone increased activity in adult (P91) rats [74]. One major difference between this study and others is that Tran *et al.* performed the LMA testing on 2 consecutive days for both juveniles (P15–P16) and adults (P90–P91), and only observed differences on the

second day of testing in both ages. Unfortunately the variability in results of these studies, and the multitude of others examining LMA, make generalizations difficult. There are many differences between these studies, including timing, dose, and route of ethanol exposure, variations in LMA paradigms and ages at testing. Also, the stress to the animal should be taken into consideration as some of these studies use artificial rearing procedures that segregate the pup from the lactating dam for extended periods.

In addition to general LMA, activity after administration of 2.0 g/kg ethanol was examined in the present study and shown to be increased following neonatal ethanol treatment (Fig 5B). In contrast, Barbier *et al.* demonstrated no effect of ethanol exposure throughout gestation and lactation on ethanol-induced LMA at P60 in male rats [66]. The differences in results between these two studies may relate to the increased sensitivity of the DKO mice to neonatal ethanol exposure. Previous studies in adult DKO animals have shown an attenuated effect of acute ethanol exposure to stimulate LMA compared to WT animals [34]. Here, we show a significantly augmented response to acute ethanol in DKO animals treated with ethanol postnatally compared to postnatal ethanol-treated WT mice. Previous studies in animals have also demonstrated an increased propensity for ethanol intake in adolescent and adult animals neonatally exposed to ethanol (reviewed in [75]). The lower threshold for the locomotor stimulating effects of ethanol after neonatal ethanol exposure poise the DKO mice to be interesting candidates for future studies on other measures of ethanol sensitivity including voluntary consumption.

5. Conclusion

In this study, we examined the behavioral effects of postnatal ethanol exposure in WT and AC1/8 knockout mice at discrete developmental time points. Our results overall demonstrate a reduction in immobility time as well as an increase in hindlimb claspings in DKO mice regardless of postnatal ethanol exposure during the TST test, indicating a possible difference in CREB and/or BDNF activity in these mice. We also demonstrate behavioral effects of neonatal ethanol in the negative geotaxis test and the locomotor response to ethanol during adolescence in DKO mice, indicative of an altered threshold for sensitivity to ethanol's effect on these behaviors in mice lacking AC1 and 8. Future studies will explore mechanisms involving AC1/8 deletion on ethanol consumption and measures of ethanol-induced plasticity in the neonatally-exposed adolescent.

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Abbreviations

AC	Adenylyl Cyclase
ANOVA	Analysis of Variance
BAC	Blood Alcohol Concentration
BDNF	Brain Derived Neurotrophic Factor
cAMP	cyclic adenosine monophosphate
CREB	cAMP Response Element-Binding Protein
DKO	Double Knockout (adenylyl cyclases 1/8)
EtOH	Ethanol
FASD	Fetal Alcohol Spectrum Disorders
G	Gestational Day
i.p	Intraperitoneally
LMA	Locomotor Activity
LORR	Loss of Righting Reflex
P	Postnatal day
PKA	Protein Kinase A
TST	Tail Suspension Test
WS	Wire Suspension
WT	Wild Type

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Highlights

- Select striatal behaviors may be insensitive for detecting early alcohol exposure
- Adenylyl cyclase 1/8 moderate alcohol-induced activity after early alcohol exposure
- Adenylyl cyclase 1/8 loss induces behaviors suggesting impaired striatal signaling

Figure 1. Average body weight measurements through duration of behavioral testing
Body weight significantly increased with age in all groups examined with no significant effect of Genotype or ethanol (EtOH) Treatment. Mean $\pm$ SEM, $n=5-8$ mice per group.

Figure 2. Effect of postnatal ethanol (EtOH) exposure on the wire suspension and grid hang tests (A) Postnatal ethanol exposure decreased the percentage of WT animals capable of holding onto the wire at P21, but had no significant effect on total time on the wire (B) in either genotype. (A score of 0 = unable to hang on, 1 = capable of hanging on for 1 second). Mean $\pm$ SEM, $n=4-8$ mice per group. (C) Exposure to 2.5 g/kg EtOH at P6 had no effect on the ability to hang upside down from a wire grid. However, genetic deletion of AC1/8 significantly increased the ability of mice to hang from the wire grid at P21, but not P30. Mean $\pm$ SEM, $n=5-8$ mice per group. *WT vs DKO, irrespective of treatment $p<0.05$.

Figure 3. Effect of postnatal ethanol (EtOH) exposure on the development of negative geotaxis (A) Percentage of animals displaying the negative geotaxis reflex (turning and climbing the wire grid) from P7–P14 (percent of animals with a score of 0 versus all other scores). (B) The average time required for the mice to turn and begin to climb the wire grid was significantly increase at P7 in DKO mice neonatally exposed to EtOH compared to DKO controls and WT mice neonatally exposed to EtOH. Mean $\pm$ SEM, 5–8 mice per group. *WT EtOH vs DKO EtOH, #DKO Saline vs DKO EtOH, $p < 0.05$.

Figure 4. Effect of postnatal ethanol (EtOH) exposure on total immobility time and hindlimb clasping during the tail suspension test
(A) Genetic deletion of AC1/8 significantly decreased the total immobility time at P21 and P30 compared to WT mice in the presence and absence of EtOH. *WT vs DKO, $p < 0.05$. **(B)** Saline-treated DKO mice displayed marked hindlimb clasping, which was absent in WT controls. *WT vs DKO, $p < 0.05$. Neonatal EtOH exposure increased the number of clasping events in WT mice, while decreasing this behavior in DKO mice during the TST. Mean $\pm$ SEM, $n = 5-8$ mice per group

Figure 5. Effect of postnatal ethanol (EtOH) exposure on locomotor activity following acute saline or EtOH challenge

At P30, animals exposed to neonatal (P5–7) saline or 2.5 g/kg EtOH were acclimated to locomotor chambers for 4 days following saline injections. On day 5, animals were given 2.0 g/kg EtOH i.p. before being placed in the same locomotor chambers. Activity was monitored for 10 min on each day. **(A)** On day 1, neonatal EtOH exposure marginally increased locomotor activity in WT but not DKO animals. **(B)** On Day 5, neonatal EtOH treatment significantly increased EtOH-induced locomotor activity compared to saline-

treated animals, regardless of genotype. Data represent mean ambulations $\pm$ SEM over the 10 min of testing plotted as percent of saline control. *Saline vs EtOH, $p < 0.05$. (C) EtOH-exposed DKO mice show a significant increase in locomotor activity after 2.0 g/kg EtOH exposure on day 5, compared to EtOH-exposed WT mice, peaking at 5 minutes after injection. Data represent mean ambulations $\pm$ SEM plotted as percent of saline control in 1 min bins. $n = 9-20$ mice per group. #WT vs DKO $p = 0.05$

Table 1
Summary of behavioral tasks by group, treatment age, and age at testing

The number of animals treated in each group is listed, along with the behavioral tests. X indicates the task was administered at the particular age. Timing of neonatal ethanol exposure was critical for the developmental tasks that were initiated on P7, therefore pups that were used for these studies (group 1) were injected only on P6. For the remainder of the studies (groups 2 and 3), the behavioral endpoint(s) did not occur until P30 allowing for treatment during the wider time frame, P5–P7.

Group	Age Treated	N	Behavioral Test	Ages Tested						
				P7	P10	P14	P21	P30		
1	P6	5–8	Body Weight	X	X	X	X	X		
			Postural Reflex	X	X	X	X	X		
			Wire Suspension	X	X	X	X	X		
			Negative Geotaxis	X	X	X	X	X		
			Grid Hang		X	X	X	X		
2	P5–P7	6–9	TST		X	X	X	X		
			Rotarod					X		
3	P5–P7	9–20	LORR					X		
			LMA					X		