Published in final edited form as:

Cerebellum. 2015 August ; 14(4): 413–420. doi:10.1007/s12311-015-0691-7.

CHOLINE AMELIORATES DEFICITS IN BALANCE CAUSED BY ACUTE NEONATAL ETHANOL EXPOSURE

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Abstract

Fetal alcohol spectrum disorder (FASD) is estimated to occur in 1% of all live births. The developing cerebellum is vulnerable to the toxic effects of alcohol. People with FASD have cerebellar hypoplasia and developmental deficits associated with cerebellar injury. Choline is an essential nutrient but many diets in the USA are choline deficient. In rats, choline given with or following alcohol exposure reduces many alcohol-induced neurobehavioral deficits, but not those associated with cerebellar function. Our objective was to determine if choline supplementation prior to alcohol exposure would ameliorate the impact of ethanol on a cerebellar-associated behavioral test in mice. Pregnant C57Bl6/J mice were maintained on a choline deficient diet from embryonic day 4.5. On postnatal day 1 (P1), pups were assigned to one of 8 treatment groups: choline (C) or saline (S) pre-treatment from P1-5, ethanol (6 g/kg) or Intralipid® on P5, C or S post-treatment from P6-20. On P30, balance and coordination were tested using the dowel crossing test. Overall, there was a significant effect of treatment and females crossed longer distances than males. Ethanol exposure significantly reduced the total distance crossed. Choline pre-treatment increased the distance crossed by males, and both pre- and post-treatment with choline significantly increased total distance crossed for females and males. There was no effect of choline on Intralipid®-exposed animals. This is the first study to show that choline ameliorates ethanol-induced effects on balance and coordination when given before ethanol exposure. Choline fortification of common foodstuffs may reduce the effects of alcohol.

Keywords

fetal alcohol syndrome; mouse; cerebellum; choline; brain development

INTRODUCTION

Fetal Alcohol Spectrum Disorder (FASD) covers a range of physical and developmental anomalies that result from exposure to ethanol during pregnancy. FASD is suspected to affect 1% of all live births (1). Fetal Alcohol Syndrome (FAS), the most severe manifestation of FASD, is the leading known cause of mental retardation in the western

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Conflict of Interest: All authors are employees of the University of Maryland, Baltimore; SMM received financial support to attend the 2015 Children's Environmental Health Network meeting; CFB has received grant funding from Gerber. There are no conflicts of interest.

world (2). The incidence of FAS is conservatively estimated at 0.97 per 1000 births (2) and may be as high as 43 per 1000 live births to heavily drinking pregnant women (3). Abnormalities in the cerebellum were first noted by Jones and Smith from the autopsy of a child with FAS (4). Reduced cerebellar volumes in FASD patients have since been noted in multiple studies (5-9). Along with these cerebellar structural defects, cerebellar function is also perturbed by prenatal ethanol exposure. Children and adults with FASD have cerebellar dysfunction; including alterations in gait, postural balance, motor coordination and eyeblink conditioning (10-12).

Animal work confirms the findings in humans. Cerebellar Purkinje cells are particularly vulnerable to ethanol-induced death or damage during the postnatal period in the rat (13-15), and the numbers of both cerebellar Purkinje cells and cerebellar granule neurons are significantly reduced (16). The developing cerebellum appears especially vulnerable to a single dose of alcohol around postnatal day (P) 5 with parallel losses in Purkinje and granule neurons (17; 18). Cerebellar dependent behavioral changes are also seen following ethanol exposure during the postnatal period including deficits on parallel bars, eyeblink conditioning and dowel crossing (19-22).

Choline, lecithin (phosphatidylcholine) and citicholine (cytidine diphosphate-choline) have been investigated as cerebroactive drugs since the early 1980's (23). The effect of choline on ameliorating the effects of developmental alcohol exposure was first described by Thomas et al. (24). Choline ameliorates some behavioral effects of ethanol when it's given either concurrently or after the ethanol exposure; for example, it can improve discrimination learning, hyperactivity, and trace eyeblink conditioning (19; 24-28). However, this treatment does not have an effect at mitigating the effects of ethanol on two cerebellar-dependent behaviors; delay eyeblink conditioning and motor coordination (19; 29).

Choline can also protect against structural and biochemical deficits caused by ethanol exposure. In an *in vitro* model of cerebellar granule neurons, ethanol decreases L1 cell adhesion molecule (L1)-dependent neurite outgrowth and downstream signaling (30-32). Supplementation with choline prior to ethanol exposure shows improved neurite outgrowth and signaling in the presence of ethanol (30). We conducted the current study to determine if choline supplementation prior to an acute exposure to ethanol on P5 would reduce the effects of ethanol on the dowel crossing test, a test of balance and coordination that relies heavily on normal cerebellar function in mice (21).

METHODS

Animals; prenatal treatment

C57Bl6/J mice were maintained in an AAALAC accredited facility at the University of Maryland School of Medicine. Rooms were temperature-controlled (22°C) with a 12-hour light/dark cycle (lights on 07:00 to 19:00). All procedures were performed with prior approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland, Baltimore and were in accordance with guidelines for animal care established by the National Institutes of Health.

Mice were pair housed, one female and one male. Females were checked daily for signs of a sperm-positive plug. The first morning a plug was seen was designated embryonic day (E) 0.5. From E4.5 until pups were weaned on postnatal day (P) 21, dams were given a choline-deficient pellet diet (518753, Dyets, Bethlehem PA). After weaning, dams were put back on normal diet for one week before another cycle of mating. Pups were kept on the choline-deficient pellet diet until the test day.

Postnatal Animals; pre-exposure treatment

Within two days of birth, pups were pseudorandomly assigned to one of eight groups such that a maximum of one male and one female from any litter was assigned to a single group. Postnatal treatments were a 2 (pre-exposure) $\times$ 2 (exposure) $\times$ 2 (post-exposure) design: treatments prior to ethanol administration were saline (10 μ L of normal saline (0.09% sodium chloride)) or choline (10 μ L of 18.8 mg/ml choline chloride; C7527, Sigma, St Louis MO) given subcutaneously once per day from P1 – 5 (day of birth=P0) at 9 AM.

Postnatal Animals; exposure

Exposure was ethanol (6.0 g/kg) or Intralipid® (I-141, Sigma) given in two divided doses two hours apart via intragastric gavage on P5, 1 h after the choline or saline injection. Ethanol solution was prepared to a final concentration of 13.6% (v/v) in Intralipid® and the ethanol-free solution was made isocaloric by adding 202 mg maltose–dextrin to Intralipid®. All pups were removed from the dam and placed on a 37°C heating pad for 10 min while the whole litter was gavaged. Each pup was gavaged with a sterile Clay Adams 7401 tube connected to a Hamilton syringe. The tip of the tube was dipped in corn oil (Sigma) for lubrication, then the tube was gently guided down the esophagus into the stomach and the feeding solution was slowly injected. Pups were returned to the dam immediately after the last animal was gavaged. This process was repeated for the second dose of ethanol. It took approximately 10 min to gavage each litter. 100% of animals survived the day of ethanol administration.

Postnatal Animals: post-exposure treatment

Treatments were saline or choline chloride as above given once per day from P6 – 20.

Blood alcohol concentrations

Four animals were gavaged with ethanol as above, and were euthanized at 2 or 4 h after the second gavage and blood ethanol concentration was determined by Ethanol L3K (Diagnostic Chemicals Limited, PE, Canada).

Dowel crossing test

Six animals per sex per treatment group were tested on P30. They were habituated to a testing room lit with a red light for at least 1 h prior to testing. The balance apparatus consisted of a horizontally oriented dowel rod (0.635 cm diameter $\times$ 120 cm length) that was marked at 3 cm intervals and was suspended approximately 75 cm above a plastic covered cushion inside of a catch box (90 $\times$ 42 $\times$ 17 cm). A darkened escape goal box (21 $\times$ 10 $\times$ 17 cm) was affixed at one end. Prior to the first trial, subjects were placed 9 cm from the goal

box and allowed to enter and habituate to the goal box for 1 min before returning to the home cage for a 30 s inter-trial interval.

Subjects were given 5 trials to cross the dowel rod. During each trial, the animal was placed facing the goal box on the rod at the 111 cm mark start position and allowed to travel on the rod to the goal box. Each trial ended when the mouse fell or slipped and hung suspended from the dowel. For mice that reached the goal box, they were given 10 s in the goal box before being re-positioned at the start and allowed to cross again. Following 5 trials, mice were returned to the home cage. The distance traveled for each of the 5 trials was summed to give total distance traveled (cm). The total distance traveled was used as an achievement dependent measure.

Statistics

All data are reported as the mean $\pm$ standard error of the mean. Body weight data were analyzed independently for each age using a two way analysis of variance (ANOVA) with the factors sex and treatment group. For the dowel crossing data, an initial two way ANOVA was run (sex and treatment group as factors) with follow-up one way ANOVAs within sex. Data were then further broken down into four groups defined by sex and exposure on P5. One way ANOVAs were run to test the *a priori* hypothesis that choline treatments would improve outcomes in ethanol exposed animals but not in control animals. Where ANOVA identified a significant effect, *post hoc* pair-wise testing was done using the Fisher's Least Significant Difference test to minimize possibility of a Type II error ("false negative" (33)). For non-parametric data, one way ANOVA on ranks was run using the Kruskal-Wallis test, followed by *post hoc* Mann Whitney U tests. For all tests, statistical significance was set to alpha 0.05. Statistical software was SigmaPlot v.12.3 (Systat Software Inc., San Jose CA).

Relevant comparisons among the eight treatment groups were considered to be those comparisons between groups sharing the exposure condition (i.e., within Intralipid® or ethanol groups) or if they shared the pre- and post-exposure conditions (e.g., SIC vs. SEC). Other comparisons (e.g., SIS vs. SEC) are not described.

RESULTS

Litter size ranged from four to seven pups per litter and 44% of pups were male. A two way ANOVA on body weight at P5 showed no effect of sex or treatment group and no interaction between the factors at this age (Table 1). A two way ANOVA on body weight at P30 showed a significant effect of sex at this age ($F_{1,80}=7.223$, $p=0.009$); overall males were significantly ($p<0.05$) heavier than females at P30 (16.41 ± 0.34 g and 15.28 ± 0.30 g, respectively). There was no effect of treatment on body weight at this age, nor was there an interaction between sex and treatment.

Blood Alcohol Concentration

Blood alcohol concentration was 517 ± 55 mg/dL at 2 h post exposure, and 397 ± 12 mg/dL at 4 h post exposure.

Dowel Crossing

A two way ANOVA on the total distance crossed showed that females cross significantly longer distances than males ($F_{1,80}=4.749$; $p=0.032$), and that there was a significant effect of treatment group ($F_{7,80}=13.774$; $p<0.001$). A two way ANOVA (male/female and saline/choline) within the intralipid groups has an F value of 2.365 ($p=0.085$), showing that choline did not have a significant effect on total distance crossed in the unexposed to ethanol group. Subsequent analyses were conducted on male and female animals separately. The Figure shows total distance traveled for each treatment group for each sex. Table 2 shows p values for all relevant comparisons.

Females—A one way ANOVA on ranks using all female data found a significant difference among the treatment groups ($F_{7,40}=12.093$, $p<0.001$; Table 2). Multiple differences were identified, but we only report the relevant comparisons (as defined above).

First we performed a pair wise comparison of the effect of ethanol on each of the four treatment groups (S or C pre-exposure, S or C post-exposure; Table 2). Ethanol significantly reduced total distance within each treatment group.

Next, we investigated the effect of choline on Intralipid® exposed female animals. There was no significant difference ($F_{3,20}=2.549$, $p=0.085$) among the Intralipid® exposed female groups.

Within the female treatment groups exposed to ethanol on P5, there was a significant effect of treatment ($F_{3,20}=8.831$, $p<0.001$). Choline supplementation before and after ethanol treatment significantly increased distance traveled compared to any other group. Choline supplementation prior to ethanol did not reach statistical significance ($p=0.056$). Choline supplementation post-ethanol exposure had no effect on dowel crossing.

Males—A one way ANOVA identified significant differences among the groups ($F_{7,40}=4.043$, $p=0.002$). A pairwise comparison of each treatment group for ethanol effect showed a significant effect of ethanol for two treatment groups; the choline pre-treatment, saline post-treatment group (CIS vs CES, $p=0.004$) and the saline pre-treatment, choline post-treatment group (SIC vs SEC, $p=0.010$). There was no significant effect of ethanol on the saline pre-, post-treated groups, or the choline pre-, post-treatment groups.

To investigate if choline supplementation itself had an effect on dowel crossing, we performed an ANOVA on all Intralipid® treatment groups. There was no significant difference between these groups ($F_{3,20}=0.830$, $p=0.493$).

To investigate the effect of choline supplementation on ethanol-induced reduction in total distance crossed, we performed a pairwise comparison of each treatment group in the ethanol exposed male animals (Table 2). Animals supplemented with choline both pre- and post-ethanol exposure crossed significantly longer distances than any other ethanol exposed group ($p<0.001$, $p<0.001$, $p=0.029$ respectively). The choline pre-ethanol group crossed significantly longer distances than either the unsupplemented group ($p=0.009$), or the group that received choline supplementation post-ethanol exposure ($p=0.036$). The group that

received choline only after the ethanol exposure did not significantly increase the distance traversed on the dowel rod ($p=0.522$).

DISCUSSION

The major finding of our study was the result that supplementation with choline prior to ethanol could result in significant improvements in balance and coordination as demonstrated by the dowel crossing task in mice. Animals exposed to ethanol on P5 were impaired on the dowel crossing task for total distance crossed. Choline treatment prior to ethanol exposure resulted in a significant improvement in males on the total distance crossed in the dowel crossing test. Choline treatment both before and after ethanol exposure significantly improved dowel crossing in both sexes. Choline supplementation of Intralipid® exposed animals had no effect on total distance traveled in either sex.

The dowel crossing test is a test of balance, motor coordination, and motor learning and is an adaptation of the beam walking test. It has been suggested that dowel crossing is more sensitive than the rotarod test at assessing motor coordination (34). Motor coordination relies on multiple brain regions including motor, vestibular, and somatosensory systems. It is also heavily dependent on the cerebellum, as evidenced by the prevalence of motor coordination deficits in cerebellar mutant mice (e.g., Bosch et al (35)). The ethanol exposure paradigm used in this study affects cells in multiple parts of the mouse brain including those described above (36-38). Regardless of which regions underlie the ethanol-induced behavioral deficit seen here, we show that choline can partially ameliorate this damage if given before ethanol exposure in males and both before and after the exposure to ethanol in both sexes. This finding is in contrast to previous work where choline was given either with or following an ethanol exposure (20; 29). Using rat pups dosed with 5.25 – 6.6 g/kg/d of ethanol, choline given either concurrently or following the ethanol exposure showed improvements in a variety of behavioral tests including activity level, spatial discrimination reversal learning, Morris water maze and trace fear conditioning (19; 20; 24; 26; 27). However, no effect on the impact of ethanol on parallel bar motor coordination or delayed eyeblink conditioning was found (20; 29). This is the first report where choline is given prior to ethanol administration, and it significantly improves balance and motor coordination.

Despite females crossing a statistically significant longer distance in the dowel crossing test, both males and females were equally affected by ethanol exposure; in both sexes the distance crossed was reduced to approximately 9% of control animals (SIS). In males, choline given before ethanol improved the distance crossed to 56% of SIS animals, and choline before and after improved performance to 93% of SIS. In females, choline prior to ethanol only improved performance to 33% of SIS, and choline before and after resulted in an improvement to 65% of SIS. It is interesting that the effect of choline appears to be different between the two sexes. Prior studies using choline to ameliorate the effects of ethanol have shown no sex difference (19; 20; 24; 26; 27). The sex difference may be due to the pretreatment strategy used here, or an effect on the cerebellum specifically. More investigation into this difference is needed.

This is the first study to show that mice exposed to ethanol in the first postnatal week show behavioral deficits that can be partly ameliorated by choline. The advantage of using mice is that further studies to elucidate the mechanism of the protective effects of choline can use genetic manipulation to achieve this goal. The blood alcohol concentrations achieved with this model are similar to those reported in both rats and mice (37; 39; 40) and are similar to our previously reported concentrations in P6 rat pups (41). Ikonomidou et al. found that neuroapoptosis was not increased in rat pups unless the blood alcohol concentration was greater than 200 mg/dL (41). Even at these blood alcohol concentrations, no mouse pup died on the day of ethanol administration when peak blood alcohol concentrations would have occurred. It is therefore even more significant that at these high levels, choline supplementation had a significant protective effect.

Choline is an essential nutrient with many roles. It is important in cellular membranes, synthesis of methionine, methylation of a wide range of molecules, and neurotransmission (42). Choline can be metabolized by 3 different pathways: 1) Choline can be phosphorylated to phosphocholine by the choline kinases (CK α , CK β), of which CK α is the most important in the developing nervous system (43; 44). Phosphocholine (PC) is the precursor for both phosphatidylcholine (PtdCho) and sphingomyelin (SM), major constituents of the plasma membrane. These choline metabolites can be modulated by dietary choline (45). 2) Choline can also be oxidized by choline dehydrogenase (CDHD) to betaine which is a precursor to methionine and is a 1-carbon donor for methylation via S-adenosylmethionine (SAMe) (46). 3) Choline can be acetylated by choline acetyltransferase (ChAT) to acetylcholine (ACh), a major neurotransmitter in the forebrain and hippocampus (47). The lack of return to control in the dowel crossing test of choline treated mice makes determining the mechanism of the effect of choline even more important so that better prevention can be obtained.

In our study, mice were placed on a choline deficient but methionine sufficient diet to model the human situation, where diets deficient in choline are common. The Recommended Dietary Allowance (RDA) for choline is 425 mg/day for women and 500 mg/day for men. Pregnant and lactating women have higher RDAs of 450 mg/d and 550 mg/d respectively. Less than 10% of the adult population attained these RDA in the National Health and Nutrition Examination Survey (NHANES) 2003-2004 (48). The mean average daily intake for adults was 302 mg/d in the 2007-2008 NHANES (49).

The choline content of rodent chow is variable. Unlike commercial rodent food in which choline content is not controlled, we used the AIN-76A diet without added choline. AIN-76A was formulated to permit standardized studies using a nutritionally adequate diet in rats and mice and contains both added choline and methionine (50). Previous studies using this diet without added choline showed a significant reduction in phosphocholine and betaine in livers of pregnant dams (51). There was no reduction in phosphatidylcholine. The diet is adequate for methionine and hence the availability of 1-methyl donors derived from methionine. Thus, the use of this diet allowed us to more accurately control baseline levels of choline in the diet.

Our main purpose in pursuing these studies was to examine the effects of choline pre-treatment on the effects of ethanol exposure. We have previously shown that choline

supplementation of tissue culture media prior to ethanol exposure significantly ameliorates the effects of an acute ethanol exposure on L1-dependent neurite outgrowth and lipid raft-dependent signaling in cultured rat cerebellar granule neurons (30). However, previous studies on the effect of choline supplementation on rat behavior used choline supplementation with or after exposure to ethanol which did not match the experimental design of our biochemical studies (19; 20; 24-28). In addition, the reported beneficial effects of choline supplementation with or post-ethanol exposure did not include cerebellar mediated tasks (19; 29). Our results show a beneficial effect of choline supplement prior to ethanol exposure on cerebellar function. Our results support the hypothesis that improved choline intake throughout pregnancy may have significant benefits to women exposed to ethanol, whether by drinking behaviors or from the various other sources of ethanol exposure. Thus, the results of this study have important implications for interventions to prevent or ameliorate the effect of prenatal ethanol exposure on neurodevelopment.

ACKNOWLEDGMENTS

The authors thank Li Guo and Eric Ho for technical assistance.

Funding: This research was supported by the National Institute of Alcohol Abuse and Alcoholism (AA016398 to CFB; AA018693, AA022413, AA178231, and AA006916 to SMM).

REFERENCES

1. Sampson PD, Streissguth AP, Bookstein FL, Little RE, Clarren SK, Dehaene P, Hanson JW, Graham MJ. Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. *Teratology*. 1997; 56:317–326. [PubMed: 9451756]
2. Abel EL, Sokol RJ. A revised conservative estimate of the incidence of FAS and its economic impact. *Alcoholism: Clinical and Experimental Research*. 1991; 15:514–524.
3. Abel EL. An update on incidence of FAS: FAS is not an equal opportunity birth defect. *Neurotoxicol Teratol*. 1995; 17:437–443. [PubMed: 7565490]
4. Jones K, Smith D, Ulleland C, Streissguth P. Pattern of malformation in offspring of chronic alcoholic women. *Lancet*. 1973; 1:1267–1271. [PubMed: 4126070]
5. Cardenas VA, Price M, Infante MA, Moore EM, Mattson SN, Riley EP, Fein G. Automated cerebellar segmentation: Validation and application to detect smaller volumes in children prenatally exposed to alcohol. *Neuroimage Clin*. 2014; 4:295–301. [PubMed: 25061566]
6. Mattson SN, Riley EP, Jernigan TL, Garcia A, Kaneko WM, Ehlers CL, Jones KL. A decrease in the size of the basal ganglia following prenatal alcohol exposure: a preliminary report. *Neurotoxicol Teratol*. 1994; 16:283–289. [PubMed: 7935262]
7. Mattson SN, Riley EP, Jernigan TL, Ehlers CL, Delis DC, Jones KL, Stern C, Johnson KA, Hesselink JR, Bellugi U. Fetal alcohol syndrome: a case report of neuropsychological, MRI and EEG assessment of two children. *Alcohol Clin Exp Res*. 1992; 16:1001–1003. [PubMed: 1443415]
8. Archibald SL, Fennema-Notestine C, Gamst A, Riley EP, Mattson SN, Jernigan TL. Brain dysmorphology in individuals with severe prenatal alcohol exposure. *Dev Med Child Neurol*. 2001; 43:148–154. [PubMed: 11263683]
9. Sowell E, Jernigan T, Mattson S, Riley E, Sobel D, Jones K. Abnormal development of the cerebellar vermis in children prenatally exposed to alcohol: Size reduction in Lobules I through V. *Alcoholism: Clinical and Experimental Research*. 1996; 20:31–34.
10. Spottiswoode BS, Meintjes EM, Anderson AW, Molteno CD, Stanton ME, Dodge NC, Gore JC, Peterson BS, Jacobson JL, Jacobson SW. Diffusion tensor imaging of the cerebellum and eyeblink conditioning in fetal alcohol spectrum disorder. *Alcohol Clin Exp Res*. 2011; 35:2174–2183. [PubMed: 21790667]

11. Jirikowic TL, McCoy SW, Lubetzky-Vilnai A, Price R, Ciol MA, Kartin D, Hsu LY, Gendler B, Astley SJ. Sensory control of balance: a comparison of children with fetal alcohol spectrum disorders to children with typical development. *J Popul Ther Clin Pharmacol*. 2013; 20:e212–28. [PubMed: 24163126]
12. Lubetzky-Vilnai A, Jirikowic TL, McCoy SW. Investigation of the Dynamic Gait Index in children: a pilot study. *Pediatr Phys Ther*. 2011; 23:268–273. [PubMed: 21829122]
13. Bauer-Moffett C, Altman J. The effect of ethanol chronically administered to preweanling rats on cerebellar development: a morphological study. *Brain Res*. 1977; 119:249–268. [PubMed: 830387]
14. Pierce DR, Williams DK, Light KE. Purkinje cell vulnerability to developmental ethanol exposure in the rat cerebellum. *Alcohol Clin Exp Res*. 1999; 23:1650–1659. [PubMed: 10549998]
15. Goodlett C, Pearlman AD, Lundahl KR. Binge neonatal alcohol intubations induce dose-dependent loss of purkinje cells. *Neurotoxicology and Teratology*. 1998; 20:285–292. [PubMed: 9638686]
16. Maier SE, Miller JA, Blackwell JM, West JR. Fetal alcohol exposure and temporal vulnerability: regional differences in cell loss as a function of the timing of binge-like alcohol exposure during brain development. *Alcohol Clin Exp Res*. 1999; 23:726–734. [PubMed: 10235310]
17. Lee Y, Rowe J, Eskue K, West JR, Maier SE. Alcohol exposure on postnatal day 5 induces Purkinje cell loss and evidence of Purkinje cell degradation in lobule I of rat cerebellum. *Alcohol*. 2008; 42:295–302. [PubMed: 18400452]
18. Hamre KM, West JR. The effects of the timing of ethanol exposure during the brain growth spurt on the number of cerebellar Purkinje and granule cell nuclear profiles. *Alcohol Clin Exp Res*. 1993; 17:610–622. [PubMed: 8333592]
19. Thomas JD, Tran TD. Choline supplementation mitigates trace, but not delay, eyeblink conditioning deficits in rats exposed to alcohol during development. *Hippocampus*. 2012; 22:619–630. [PubMed: 21542051]
20. Thomas JD, Idrus NM, Monk BR, Dominguez HD. Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats. *Birth Defects Res A Clin Mol Teratol*. 2010; 88:827–837. [PubMed: 20706995]
21. Lewis B, Wellmann KA, Kehrberg AM, Carter ML, Baldwin T, Cohen M, Barron S. Behavioral deficits and cellular damage following developmental ethanol exposure in rats are attenuated by CP-101,606, an NMDAR antagonist with unique NR2B specificity. *Pharmacol Biochem Behav*. 2012; 100:545–553. [PubMed: 22037411]
22. Rubin MA, Wellmann KA, Lewis B, Overgaauw BJ, Littleton JM, Barron S. Difluoromethylornithine (DFMO) reduces deficits in isolation-induced ultrasonic vocalizations and balance following neonatal ethanol exposure in rats. *Pharmacol Biochem Behav*. 2009; 92:44–50. [PubMed: 18992275]
23. Spagnoli A, Tognoni G. ‘Cerebroactive’ drugs. Clinical pharmacology and therapeutic role in cerebrovascular disorders. *Drugs*. 1983; 26:44–69. [PubMed: 6349963]
24. Thomas JD, La Fiette MH, Quinn VR, Riley EP. Neonatal choline supplementation ameliorates the effects of prenatal alcohol exposure on a discrimination learning task in rats. *Neurotoxicol Teratol*. 2000; 22:703–711. [PubMed: 11106863]
25. Thomas JD, Garrison M, O’Neill TM. Perinatal choline supplementation attenuates behavioral alterations associated with neonatal alcohol exposure in rats. *Neurotoxicol Teratol*. 2004; 26:35–45. [PubMed: 15001212]
26. Thomas JD, Biane JS, O’Bryan KA, O’Neill TM, Dominguez HD. Choline supplementation following third-trimester-equivalent alcohol exposure attenuates behavioral alterations in rats. *Behav Neurosci*. 2007; 121:120–130. [PubMed: 17324056]
27. Ryan SH, Williams JK, Thomas JD. Choline supplementation attenuates learning deficits associated with neonatal alcohol exposure in the rat: effects of varying the timing of choline administration. *Brain Res*. 2008; 1237:91–100. [PubMed: 18786517]
28. Thomas JD, Abou EJ, Dominguez HD. Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats. *Neurotoxicol Teratol*. 2009; 31:303–311. [PubMed: 19616089]

29. Thomas JD, O'Neill TM, Dominguez HD. Perinatal choline supplementation does not mitigate motor coordination deficits associated with neonatal alcohol exposure in rats. *Neurotoxicol Teratol.* 2004; 26:223–229. [PubMed: 15019955]
30. Tang N, Bamford P, Jones J, He M, Kane MA, Mooney SM, Bearer CF. Choline partially prevents the impact of ethanol on the lipid raft dependent functions of L1 cell adhesion molecule. *Alcohol Clin Exp Res.* 2014; 38:2722–2730. [PubMed: 25421509]
31. Tang N, Farah B, He M, Fox S, Malouf A, Littner Y, Bearer CF. Ethanol causes the redistribution of L1 cell adhesion molecule in lipid rafts. *J Neurochem.* 2011; 119:859–867. [PubMed: 21884525]
32. Bearer CF, Swick AR, O'Riordan MA, Cheng G. Ethanol inhibits L1-mediated neurite outgrowth in postnatal rat cerebellar granule cells. *Journal of Biological Chemistry.* 1999; 274:13264–13270. [PubMed: 10224086]
33. Carmer SG, Swanson MR. Evaluation of ten pair-wise multiple comparison procedures by Monte Carlo methods. *Journal of the American Statistical Association.* 1973; 68:66–74. Carmer SG, Swanson MR 1973. *Journal of the American Statistical Association* 68: 66-74.
34. Stanley JL, Lincoln RJ, Brown TA, McDonald LM, Dawson GR, Reynolds DS. The mouse beam walking assay offers improved sensitivity over the mouse rotarod in determining motor coordination deficits induced by benzodiazepines. *J Psychopharmacol.* 2005; 19:221–227. [PubMed: 15888506]
35. Bosch MK, Carrasquillo Y, Ransdell JL, Kanakamedala A, Ornitz DM, Nerbonne JM. Intracellular FGF14 (iFGF14) is required for spontaneous and evoked firing in cerebellar purkinje neurons and for motor coordination and balance. *J Neurosci.* 2015; 35:6752–6769. [PubMed: 25926453]
36. Young C, Straiko MM, Johnson SA, Creeley C, Olney JW. Ethanol causes and lithium prevents neuroapoptosis and suppression of pERK in the infant mouse brain. *Neurobiol Dis.* 2008; 31:355–360. [PubMed: 18595723]
37. Dikranian K, Qin YQ, Labruyere J, Nemmers B, Olney JW. Ethanol-induced neuroapoptosis in the developing rodent cerebellum and related brain stem structures. *Brain Res Dev Brain Res.* 2005; 155:1–13.
38. Yang Y, Tang Y, Xing Y, Zhao M, Bao X, Sun D, Tang X, Wu Y, Xu H, Fan X. Activation of liver X receptor is protective against ethanol-induced developmental impairment of Bergmann glia and Purkinje neurons in the mouse cerebellum. *Mol Neurobiol.* 2014; 49:176–186. [PubMed: 23900741]
39. Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovskaya V, Horster F, Tenkova T, Dikranian K, Olney JW. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science.* 2000; 287:1056–1060. [PubMed: 10669420]
40. Taranukhin AG, Taranukhina EY, Saransaari P, Podkletnova IM, Pelto-Huikko M, Oja SS. Neuroprotection by taurine in ethanol-induced apoptosis in the developing cerebellum. *J Biomed Sci.* 2010; 17(Suppl 1) S12-0127-17-S1-S12.
41. Littner Y, Tang N, He M, Bearer CF. L1 cell adhesion molecule signaling is inhibited by ethanol in vivo. *Alcohol Clin Exp Res.* 2013; 37:383–389. [PubMed: 23050935]
42. Zeisel SH, da Costa KA. Choline: an essential nutrient for public health. *Nutr Rev.* 2009; 67:615–623. [PubMed: 19906248]
43. Wu G, Sher RB, Cox GA, Vance DE. Differential expression of choline kinase isoforms in skeletal muscle explains the phenotypic variability in the rostrocaudal muscular dystrophy mouse. *Biochim Biophys Acta.* 2010; 1801:446–454. [PubMed: 20026284]
44. Gallego-Ortega D, Ramirez de Molina A, Ramos MA, Valdes-Mora F, Barderas MG, Sarmentero-Estrada J, Lacal JC. Differential role of human choline kinase alpha and beta enzymes in lipid metabolism: implications in cancer onset and treatment. *PLoS One.* 2009; 4:e7819. [PubMed: 19915674]
45. Yaqoob P, Shaikh SR. The nutritional and clinical significance of lipid rafts. *Curr Opin Clin Nutr Metab Care.* 2010; 13:156–166. [PubMed: 20010096]
46. Zeisel SH. Nutritional genomics: defining the dietary requirement and effects of choline. *J Nutr.* 2011; 141:531–534. [PubMed: 21270363]

47. Monk BR, Leslie FM, Thomas JD. The effects of perinatal choline supplementation on hippocampal cholinergic development in rats exposed to alcohol during the brain growth spurt. *Hippocampus*. 2012; 22:1750–1757. [PubMed: 22431326]
48. Jensen HH, Matres-Marquez SP, Carriquiry A, Schalinske KL. Choline in the diets of the US population: NHANES, 2003-2004. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*. 2007; 21:LB46. Meeting Abstract Suppl.
49. Chester, DN.; Goldman, JD.; Ahuja, JK.; Moshfegh, AJ. Dietary intakes of choline: What we eat in America, NHANES 2007-2008. 2011. FoodSurveys Research Group Dietary Data Brief No. 9
50. <http://www.researchdiets.com/opensource-diets/stock-diets/ain-diets>
51. Zeisel SH, Mar MH, Zhou Z, da Costa KA. Pregnancy and lactation are associated with diminished concentrations of choline and its metabolites in rat liver. *J Nutr*. 1995; 125:3049–3054. [PubMed: 7500183]

Figure.

Total distance crossed in the dowel crossing test for female and male mice. Dams were placed on a choline deficient diet on embryonic day 4.5 until pups were weaned. Pups were kept on the choline deficient diet until tested. Pups were given 5 trials of traversing the dowel. The length of dowel crossed for each of the 5 trials was added to give the total distance traveled. Females crossed significantly longer distances than males for each treatment group, therefore the graphs represent the effect of treatment group within each sex. Treatment group: XYZ - X is either saline (S) or choline (C) from P1 - P5; Y is either

Intralipid® (I) or ethanol (E) on P5; Z is either saline (S) or choline (C) from P6 - P20. The bars represent the mean total distance for each treatment group ($n=6$) $\pm$ SEM. Statistical analysis is shown in Table 2.

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Table 1**Body Weights**

Group	Female Body Weight at P5 (g)	Female Body Weight at P30 (g)	Male Body Weight at P5 (g)	Male Body Weight at P30 (g)
SIS	2.33 ± 0.12	15.33 ± 0.85	2.65 ± 0.35	16.90 ± 0.81
SIC	2.81 ± 0.30	15.49 ± 0.89	2.13 ± 0.12	16.53 ± 0.54
CIS	2.62 ± 0.26	15.50 ± 0.79	2.24 ± 0.25	14.80 ± 1.20
CIC	2.38 ± 0.15	15.03 ± 0.61	2.99 ± 0.34	15.50 ± 1.00
SES	2.35 ± 0.16	15.57 ± 0.80	2.67 ± 0.19	17.42 ± 1.05
SEC	2.55 ± 0.30	15.06 ± 1.06	2.49 ± 0.22	16.76 ± 0.97
CES	2.63 ± 0.25	15.87 ± 1.01	2.30 ± 0.28	18.00 ± 0.55
CEC	2.93 ± 0.49	14.08 ± 1.20	2.60 ± 0.28	16.10 ± 0.52

Group is shown as pre-exposure / exposure on P5 / post-exposure. S, saline; C, choline; I, Intralipid®; E, ethanol. Data are the mean ± sem, n=6 per group.

Table 2

Statistical analysis for Dowel Crossing, Total distance.

Effect	Dowel crossing				
	All	ANOVA $F_{1,80}=4.749$, $p=0.032^*$ sex; $F_{7,80}=13.774$, $p<0.001$ treatment			
Females		ANOVA $F_{7,40}=12.093$; $p<0.001$			
Effect of EtOH		SIS	SIC	CIS	CIC
	SES	0.002			
	SEC		<0.001		
	CES			0.003	
	CEC				<0.001
Choline effect without EtOH (I group only)		ANOVA $F_{3,20}=2.549$; $p=0.085$			
Choline effect on EtOH (E group only)		ANOVA $F_{3,20}=8.831$; $p<0.001$			
		SES	SEC	CES	CEC
	SES				
	SEC	0.709			
	CES	0.056	0.115		
	CEC	<0.001	<0.001	0.018	
Males		ANOVA $F_{7,40}=4.043$; $p=0.002$			
Effect of EtOH		SIS	SIC	CIS	CIC
	SES	0.125			
	SEC		0.004		
	CES			0.010	
	CEC				0.071
Choline effect without EtOH (I group only)		ANOVA $F_{3,20}=0.830$; $p=0.493$			
Choline effect on EtOH (E group only)		ANOVA $F_{3,20}=11.384$; $p<0.001$			
		SES	SEC	CES	CEC
	SES				
	SEC	0.522			
	CES	0.009	0.036		
	CEC	<0.001	<0.001	0.029	

* bold indicates significant effect